Materials and Methods
3. MATERIALS AND METHODS

Human Immunodeficiency Virus, the causative agent in AIDS has been a challenge to medical fraternity from the time since it was first discovered in 1983. About 40 million people are living with HIV infection globally and 99% of the infected people are in South East Asia (SEA). Close monitoring of HIV infected mothers and their infants are essential in reducing the morbidity and mortality of HIV patients. Monitoring HIV disease progression and deciding the time to initiate HAART require the detection of HIV status of the infants, evaluation of hematological and immunological findings, HIV RNA viral load and drug resistance study at regular intervals.

3.1 Study subjects

The total number of subjects included in this study was 397

- 125 HIV exposed nevirapine treated infants and their 125 HIV positive nevirapine treated mothers
- 50 HIV non exposed infants and their 50 HIV negative mothers (controls)
- 47 HIV status known children (27 HIV-1 positive and 20 HIV negative).

All the study subjects were recruited from Namakkal district, Tamil Nadu, India.
3.1.1 Criteria for inclusion

3.1.1.1 HIV seropositive mothers and their infants

HIV positive mothers (n=125) who had received a single dose nevirapine at the onset of labour. They were recruited from clinics at the PMTCT centers in Namakkal District Head Quarters Hospital, Namakkal and Rasipuram Government Hospital, Rasipuram.

Infants-HIV exposed (n=125); the age range was between 15 days and 12 months and who were born to HIV seropositive mothers and who had received nevirapine syrup within 72 hours of birth.

3.1.1.2 HIV exposed Children

Children (n=47); the age range was between 2-8 years and who were born to HIV seropositive mothers and these children already confirmed their HIV status before being included as controls.

3.1.1.3 HIV seronegative mothers and their infants

Mothers (n=50); who were confirmed seronegative for HIV were selected as controls and their HIV-seronegativity was confirmed before being included as controls.

Infants - HIV non exposed (n=50); born to the above said HIV seronegative mothers were selected as controls.
All the subjects taking part in the study were from similar socio-economic backgrounds and the age of the mothers ranged between 19-34 years and the infants’ age was 15 days to 12 months.

Ethical approval for the study was obtained from Institutional Ethical committee from Tamilnadu Dr.MGR Medical University, Chennai. Written informed consent was obtained from eligible subjects who were then personally interviewed in their local language (Tamil). Details regarding socio-demographic data and status of HIV were recorded.

3.1.2 Study design

It is a cohort study for the infants born to HIV positive mothers and to identify their HIV status. If found HIV positive, the same infant was followed up to confirm its HIV positive status.

3.2 Statistical Analysis

The data were entered in Excel and analysed using SPSS ver. 20. All the variables were checked for normality. Mean and SD were estimated for those variables which follow normal distribution. Median and IQR were estimated for those variables which do not follow normal distribution. Students’ t-test was used to find the mean difference for normality distributed factors between mothers with HIV positive and mothers with HIV negative. Mann Whitney U test was used to find the median difference for non-normality distributed factors between mothers with HIV positive and mothers with HIV negative, infants with HIV positive and
HIV negative and mothers with < 350 CD4 cells and > 350 CD4 cells. A $p$-value of less than 0.05 was considered to be statistically significantly.

### 3.3 Blood collection and processing of samples

The blood samples of the infants were collected in ethylene diamine tetraacetic acid (EDTA) vacutainer tubes by venipuncture and labeled with the name and ID number (Fig.3.1).

**Figure 3.1 Blood samples collected in EDTA tubes**

The whole blood samples were spotted on to three filter papers:
1. S&S Isocode stix,
2. Protein saver card 903

3.3.1 Preparation of blood samples coated on filter papers

Figure 3.2 Whole blood spotted on S&S Isocode stix

Ten micro liters of the whole blood samples were spotted onto S&S Isocode stix (Fig. 3.2).
Fifty micro liters of the whole blood samples were spotted onto the Protein saver card 903 (Fig.3.3).

**Figure 3.4 Whole blood spotted on FTA card**

One hundred and twenty five micro liters of the whole blood was spotted onto the FTA card (Fig. 3.4)
All these whole blood spotted filter papers were allowed to dry at room temperature and then placed in individual plastic Ziploc bags with the silica desiccant and stored at -20 ºC until the processing.

3.4 Standard Operating Procedures

- DNA extraction from S&S Isocode stix (KOBZ609) (Mini et al., 2008)
- DNA extraction from Protein saver card 903 (w0716288307) (Anitha et al., 2011)
- DNA extraction from FTA card (WB120055) (Ingrid et al., 2001)
- DNA extraction from whole blood (Cat no. : MB504) (Sambrook et al., 1989)
- Amplicor® HIV-1 DNA Test, version 1.5 (Richmond et al., 1999)
- PCR Amplification using gag primers (Mini et al., 2008)
- PCR Amplification using env primers (Mehtha et al., 2010)
- PCR Amplification using pol and β-globin primers (Ingrid et al., 2001)
- Determination of Total bilirubin (Carl et al., 1996)
- Determination of AST (Bergmeyer et al., 1986b)
- Determination of ALT (Bergmeyer et al., 1986a)
- Determination of ALP (Bergmeyer, 1983)
- Determination of GGT (Henderson et al., 2001)
- Determination of Total Protein and Albumin (Doumas et al., 1981)
- Determination of CD4/CD8 counts (results generated by Flow Cytometry)
- Determination of Hemoglobin, Hematocrit, White blood cell count and Total Lymphocyte count. (results generated from K-21 cell counter)
- Quantification of HIV-1 RNA PCR using Amplicor ® HIV-1 Monitor Test (Barre-Sinoussi et al., 1983)
- Detection of Drug resistance mutation by OLA (Jacob et al., 2011)
3.4.1 DNA extraction from S&S Isocode Stix

Figure 3.5 DNA extraction from S&S Isocode stix

- The dried part (matrix) of S &S Isocode filter paper (Fig.3.5) was placed to the sterile micro centrifuge tube, 500 µl of sterile water was added into the matrix and was vortexed for three times for 5 seconds.

- The matrix was removed from the tube and placed in another tube and 100 µl of sterile water was added and centrifuged.

- This was kept in the heating block at 100° C for 15 minutes and after incubation, it was vortexed for 60 seconds. The matrix was removed and the elute containing DNA was used for PCR amplification.
3.4.2 DNA extraction from protein saver card 903

Figure 3.6 DNA extraction from Protein saver card 903

- A 3 mm punch was used to punch out from protein saver card 903 filter paper (Fig.3.6) into the screw caped tube, 500 µl of sterile water was added and vortexed for 10 seconds and centrifuged for 3 times.

- The red-tinged liquid was removed and the disc was treated with 100 µl of 10 % Chelax- 100 resin and was incubated at 56º C for 3 hours and at 100 ºC for 10 minutes.

- The disc was removed and the DNA was used for PCR amplification
3.4.3 DNA extraction from FTA card

Figure 3.7 DNA extraction from FTA card

- A 3 mm disc from FTA card filter paper (Fig.3.7) was added into the tube and 200 µl of FTA purification reagent (Cat no.: WB120204) was added and incubated for 5 minutes and the reagent in the tube was discarded and was repeated twice.

- 200 µl of TE buffer was added and incubated for 5 minutes and then the buffer was removed; the same step was repeated again.

- The disc in the tube was dried at room temperature for 1 hour and it was ready for PCR amplification.
3.5 HIV-1 DNA nested PCR using whole blood

DNA was extracted from whole blood and nested PCR amplification was performed and the final amplified products were run on the electrophoresis to check the HIV positive bands.

3.5.1 DNA extraction from whole blood

HiPurA™ Blood genomic DNA miniprep Spin kit was used for DNA extraction from the whole blood on EDTA tubes.

3.5.1.1 Principle

The DNA purification procedure using the miniprep spin column comprises of three steps viz, adsorption of DNA to the membrane, removal of residual contaminants and elution of pure genomic DNA. HiMedia’s HiElute Miniprep Spin Column format allows rapid processing of multiple samples. The columns have a high binding capacity and high quality genomic DNA is obtained from various species. The DNA obtained is compatible with downstream applications such as restriction enzyme digestion, PCR and Southern blotting.

3.5.1.2 Reagents

- Proteinase K solution (20 mg/ml) (RM2957)
- Lysis Solution (C1) (DS0010)
- Pre wash solution concentrate (DS0011)
- Elution Buffer (ET) (DS0040)
3.5.1.3 Procedure

- 20 μl of the Proteinase K solution was added into the 2 ml tubes.

- 200 μl of the whole blood was added and vortexed for 10-15 seconds to ensure thorough mixing.

- 200 μl of the Lysis Solution (C1) (DS0010) was added and this mixture was vortexed thoroughly for a few seconds to obtain a homogenous mixture and was incubated at 55°C for 10 minutes.

- After incubation 200 μl of ethanol (96-100%) was added to the lysate and was thoroughly mixed by gentle pipetting.

- The lysate from the previous step was loaded in HiElute Miniprep Spin Column (DBCA02).

- Centrifuged ≥10,000 rpm for 1 minute and the flow-through liquid in the tube was discarded. The column was placed in a new 2.0 ml collection tube.

- 500 μl of diluted Prewash Solution 1 was added into the column and centrifuged at ≥10,000 rpm for 1 minute. The flow-through liquid was discarded; the column was kept in another tube.

- 500 μl of diluted of Prewash solution 2 was added and centrifuged at 13,000 rpm for 3 minutes. Again the flow-through liquid was discarded; the column was kept in a 2 ml tube.
- 200 µl of the Elution Buffer (ET) (DS0040) was added directly into the column and incubated for 1 minute at room temperature. Centrifuged at ≥ 10,000 rpm for 1 minute and then the collected DNA in the tube was used for PCR amplification.

3.5.1.3 Amplification

The following reagents were used for PCR amplification and detection (gel electrophoresis):

10 x PCR Buffer minus Magnesium Chloride (supplied with Taq enzyme)

Taq DNA Polymerase (500 units) (Invitrogen, USA., Cat No. 11615-010)

50mM Magnesium Chloride (supplied with Taq enzyme)

dNTP mix (a mixture of dATP, dGTP, dCTP and dTTP-10mM each, Finnzymes, Finland).

Water, Nuclease –Free (Promega, P1193)

Agarose (Ultra Pure) (Invitrogen, 15510-019)

1x Tris Acetate EDTA (TAE) Buffer

Ethidium Bromide solution (10 mg/ml) (D0197, Bio Basic Inc.)

Gel loading Dye (6x) (E190 Amersco.)

Primers for gag, env, pol and β-globin (IDT, USA.)

Molecular weight DNA marker 100 bp (Cat No. 118707, Genei, India.)
3.5.1.4 Sequence of β-globin primers

GH20 : GAAGAGCCAAGGACAGGTAC
KM38 : TGGTCTCCTTTAACCTGTCTTG

Once the DNA was extracted from the samples, the presence of DNA was checked using β-globin primers and if it was not present, the DNA extraction procedure should be repeated.

3.5.1.5 β-globin PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease free water</td>
<td>83.0 µl</td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>50mM Magnesium Chloride</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>KM 38</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Fluo-GH20</td>
<td>2.0 µl</td>
</tr>
</tbody>
</table>

The reaction conditions were 94 ºC for 5 min and 35 cycles at 94 ºC for 20 seconds, 52 ºC for 20 seconds, 72 ºC for 1 minute and at the final incubation was at 72ºC for 7 minutes.

The amplified PCR products were run on 2% agarose gel containing ethidium bromide with 100 bp DNA ladder at 65-85 V for 30 minutes and to check the detection of 350-bp fragment of the human beta globin gene for the presence of
human genomic DNA in the filter papers. If the sample was found to be negative for beta globin, the DNA extraction procedure should be repeated.

3.5.1.6 Sequence of gag gene

1: 5’ TCT CTC GAC GCA GGA CTC GGC TTG CTG3’
2: 5’ TAA CAT TTG CAT GGC TGC TTG ATG TCC3’
3: 5’CTA GAA GGA GAG AGA GAT GGG TGC GAG3’
4: 5’ CTT GTG GGG TGG CTC CTT CTG ATA ATG3’

1 and 2 primers were covering the regions of P17 & P24 (partial) with the HXB2 positions 682-1392, 3 and 4 primers were covering the regions of P17&P24 (partial) with the HXB2 positions 776-1336.

3.5.1.7 Master mix preparation

First round

Nuclease free water 76.5 µl
10x PCR Buffer 10.0 µl
50mM Magnesium Chloride 1.5 µl
Taq DNA Polymerase (5U/µl) 0.5 µl
dNTP mix 10mM 0.5 µl
20 pmol/µl Gag 1 0.5 µl
20 pmol/µl Gag 2 0.5 µl
DNA template 10.0 µl
The amplification for the first round was 94 °C for 1 minute, 55 °C for 1 minute, 72 °C for 1 minute and 33 cycles at 94 °C for 15 seconds, 55 °C for 45 seconds, 72 °C for 1 minute and at the final incubation was at 72°C for 5 minutes.

Second round

Nuclease free water 81.5 µl
10x PCR Buffer 10.0 µl
50mM Magnesium Chloride 1.5 µl
Taq DNA Polymerase (5U/µl) 0.5 µl
dNTP mix 10 mM 0.5 µl
20 pmol/µl Gag 3 0.5 µl
20 pmol/µl Gag 4 0.5 µl

5 µl of first round product was used as a template for second round PCR master mix. The amplification of the second round was 94 °C for 1 minute, 65 °C for 1 minute, 72 °C for 1 minute, 33 cycles at 94 °C for 15 seconds, 65 °C for 45 seconds, 72 °C for 1 minute and at the final incubation was at 72°C for 5 minutes.

3.5.1.8 Gel electrophoresis

2 % of agarose was prepared and 2 µl of ethidium bromide was added on it, the amplified PCR products of samples, positive and negative controls were mixed with 1µl of 6x gel loading dye which was loaded on to the wells of the gel along with DNA ladder. This was run at 65-85 Volts for 30 minutes and the positive bands were visualized in UV- transilluminator. The positive results of the infant
samples were confirmed with the presence of 650 bp and compared with DNA marker and the positive controls.

3.5.1.9 Sequence of env gene

ED5 : 5' ATGGGATCAAAGCCTAAAGCCATGTG 3' (6556-6581)
ED12 : 5' AGTGCTTCCTGCTGCTCCCA AGAACCCAAG 3' (7822-7792)
ES7 : 5' TGTTAAACGACGGCCAG TCTGTAAATGGCAGTCTAGC 3' (7001-7020)
ES8 : 5' CAGGAA ACAGCTAGTACCCACTTCCATTTGT CCCTCA3' (7667-7647)

3.5.1.10 Master mix preparation

First round

Nuclease free water 35.3 µl
10x PCR Buffer 5.0 µl
50mM Magnesium Chloride 1.25 µl
dNTP mix 10 mM 0.5 µl
Taq DNA Polymerase (5U/ µl) 0.5 µl
20 pmol/ µl ED 3 0.5 µl
20 pmol/ µl ED 14 0.5 µl
DNA template 5.0 µl
The amplification for both the rounds were 94 °C for 1 minute, 72 °C for 1 minute and 33 cycles at 94 °C for 15 seconds, 55 °C for 45 seconds, 72 °C for 1 minute and at the final incubation was at 72°C for 5 minutes.

Second round

Nuclease free water                 79.5 µl
10x PCR Buffer                      10.0 µl
50mM Magnesium Chloride            2.5 µl
Taq DNA Polymerase (5U/ µl)         1.0 µl
dNTP mix 10 mM                      1.0 µl
20 pmol/µl ES 7                    2.0 µl
20 pmol/µl ES 8                    2.0 µl

2 µl of first round product was used as a template for second round PCR using ES7 & ES 8 primers.

3.5.1.11 Gel Electrophoresis

The amplified PCR products were run on 2% agarose gel containing ethidium bromide along with DNA ladder to confirm the presence of 700 bp of the positive control.
### 3.5.1.12 Sequence of pol gene

PRA : 5'CCTAGGAAAAGGGCTGTGGAAATGTGG 3' (2011-2039)

IBR1: 5'AACTTCTGTATATCATTGACAGTCCA 3' (3303-3328)

PRB : 5'ACTGAGAGACAGGCTAATTTTAGGA 3' (2068-2095)

IBR2 : 5'CAAGGAATGGAGTTCTTCTGTGATG 3' (3210-3235)

### 3.5.1.13 Master mix preparation

First round

- Nuclease free water 35.5 µl
- 10x PCR Buffer 5.0 µl
- 50mM Magnesium Chloride 1.5 µl
- dNTP mix 10mM 1.0 µl
- Taq DNA polymerase (5U/ µl) 0.5 µl
- 20 pmol/ µl IBR1 0.5 µl
- 20 pmol/ µl PRA 2.0 µl
- DNA template 5.0 µl

The amplification of both the rounds were 94 °C for 5 minutes and 35 cycles at 94 °C for 20 seconds, 55 °C for 20 seconds , 72 °C for 2 minutes and at the final incubation was at 72°C for 7 minutes.

Second round

- Nuclease free water 37.5 µl
- 10x PCR Buffer 5.0 µl
- 50mM Magnesium Chloride 1.5 µl
dNTP mix 10mM 1.0 µl  
Taq DNA polymerase (5U/µl) 0.5 µl  
20 pmol/µl IBR2 0.5 µl  
20 pmol/µl PRB 0.5 µl

Transferred 2 µl of first round product to the corresponding second round tube and placed in the thermal cycler at 94 ºC for 5 min and 35 cycles at 94 ºC for 20 seconds, 52 ºC for 20 seconds, 72 ºC for 1 minute and at the final incubation was at 72ºC for 7 minutes.

### 3.5.1.14 Gel Electrophoresis

The amplified PCR products were run on 2 % agarose gel containing ethidium bromide, 6 µl of the amplicons was added with 2 µl of the gel loading dye and DNA molecular weight marker was added along with this in the lane which were run at 80 Volts for 30 minutes to confirm the presence of 665 bp of the PCR product for pol gene and 350-bp fragment of β-globin gene amplified were visualized for the presence of DNA.

### 3.6 Amplicor® HIV-1 DNA test using whole blood

The Amplicor® HIV-1 DNA test kit was used for detection of HIV-1 DNA and the test utilizes amplification of target DNA by PCR and nucleic acid hybridization for the detection of HIV-1 DNA in human whole blood.

### 3.6.1 Reagents

Specimen preparation reagent
Controls

Amplification reagent

Detection reagents

3.6.2 DNA extraction

- The whole blood was inverted 10-15 times to mix thoroughly and 1000 µl of Blood wash solution added into 2 ml tube.
- 500µl of whole blood was added into a tube containing Blood Wash solution.
- The tubes were incubated for 5 minutes at room temperature.
- Centrifuged for 3 minutes at room temperature and the supernatant was aspirated.
- Again 1000 µl of Blood Wash solution was added into the pellet and the step was repeated.
- 200µl of working extraction reagent was added to the pellet and vortexed.
- It was incubated for 60ºC for 1 hour and 100ºC for 30 minutes after incubation the samples were vortexed gently and centrifuged at 5000 rpm for 20-30 seconds. The extracted DNA was used for PCR amplification.

3.6.3 Amplification

Master mix was prepared by adding 100 µl of HIV-1Mn2+ to the vial of HIV-1 MMX tube. 50µl of DNA was added into 50µl of maser mix in a PCR tube.
• PCR amplification was carried out in the thermal cycler at 50° C for 2 minutes, 5 cycles for 95° C for 10 seconds, 52° C for 10 seconds, 72° C for 10 seconds, 35 cycles at 90° C for 10 seconds, 55° C for 10 seconds, 72° C for 10 seconds and extension was 72° C for 15 minutes.

• After amplification immediately 100μl of denaturation solution was added.

3.6.4 Detection

• 100μl of HIV-1 hybridization solution was added into micro well plate and internal control micro well plate.

• 25μl of denatured amplicon was added into both the plates and was incubated for 1 hour at 37° C.

• Both the plates were washed 5 times using washing buffer.

• 100μl of Avidin-horseradish peroxidase was added into the plate and incubated for 15 minutes at 37° C.

• Again the plates were washed 5 times and 100μl of working substrate was added into the plates and it was incubated for 10 minutes in the dark.

• 100μl of stop solution was added and the absorbance was measured at 450nm

3.6.5 Results

HIV-1 DNA negative: O.D readings should be < 0.2

HIV-1 DNA positive: O.D readings should be > 0.8
Indeterminate: O.D readings should be within 0.2 -0.8

O.D readings of IC should be > 0.8 for both negative and positive tests.

### 3.7 Estimation of Biochemical parameters

#### 3.7.1 Sample collection

Blood was collected in plain vacutainer tubes (red top) and serum was separated.

#### 3.7.2 Determination of Total Bilirubin (Modified Jendrassic & Grof Method)

##### 3.7.2.1 Principle

Bilirubin reacts with Diazotized Sulfanilic acid in acidic medium to form Azobilirubin, a pink coloured complex whose absorbance is proportional to Bilirubin concentration.

##### 3.7.2.1 Reagents

- Diazo A
- Diazo B
- Activator
- Standard

##### 3.7.2.2 Procedure

<table>
<thead>
<tr>
<th>Reagents</th>
<th>T1</th>
<th>T2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazo A</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Diazo B</td>
<td>0.025 ml</td>
<td>------</td>
</tr>
<tr>
<td>Activator</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Dis. water</td>
<td>0.50 ml</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>Serum</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
T1 & T2 tubes were kept in dark for 5 minutes and read at 540 nm.

Total Bilirubin (mg/dl) = Abs of T1-T2/Abs of Standard x 10

3.7.3 Determination of AST (Aspartate Amino Transferase)  
(Modified IFCC-UV Kinetic Method)

3.7.3.1 Principle

AST (SGOT) catalyses the transfer of an amino group between L-Aspartate and α- keto glutarate to form oxaloacetate and glutamate. The oxaloacetate formed reacts with NADH in the presence of Malate Dehydrogenases to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGOT activity in the sample.

\[
\text{L-Aspartate} + \alpha - \text{Ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate} + \text{L- Glutamate}
\]

\[
\text{Oxalaoacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+
\]

3.7.3.2 Reagents

Reagent 1-Buffer
Reagent 2- Enzyme

3.7.3.3 Procedure

The working reagent was prepared by adding 1 ml of Reagent 1 with 10 ml of Reagent 2.
Working reagent 1.0 ml
Serum 100 µl

Mixed well and the absorbance was measured at 340 nm.

3.7.4 Determination of ALT (Alanine amino transferase)
(Modified IFCC-UV Kinetic Method)

3.7.4.1 Principle

ALT (SGPT) catalyses the transfer of an amino group between L-Alanine and α- keto glutarate to form Pyruvate and glutamate. The rate of reaction is monitored using a coupling enzyme lactate dehydrogenase. Whereby the Pyruvate formed is converted to lactate in presence of NADH. The rate of oxidation of NADH to NAD is measured as decrease in absorbance which is proportional to the SGPT activity in the serum.

\[
\text{L-Alanine} + \alpha - \text{Ketoglutarate} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{L- Glutamate} \\
\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+
\]

3.7.4.2 Reagents
Reagent 1-Buffer
Reagent 2- Enzyme
3.7.4.3 Procedure

The working reagent was prepared by adding 1 ml of Reagent 1 with 10 ml of Reagent 2.

Working reagent 1.0 ml
Serum 100 µl

Mixed well and the absorbance was measured at 340 nm.

3.7.5 Determination of Alkaline Phosphatase (ALP) (p-NPP- DEA Method)

3.7.5.1 Principle

Alkaline Phosphatase at an alkaline pH hydrolysis P NPP to form yellow coloured PNPP. The rate of PNP formation is directly proportional to the ALP activity.

P-Nitrophenyl phosphate $\xrightarrow{\text{H2O}}$ Phosphate + P- Nitrophenol.

3.7.5.2 Reagents

Reagent 1-Buffer
Reagent 2- Substrate

3.7.5.3 Procedure

The working reagent was prepared by adding 4 ml of Reagent 1 with 1.0 ml of Reagent 2.

Working reagent 1.0 ml
Serum 20 µl
Mixed well and the absorbance was measured at 405 nm.

3.7.6 Determination of G-Glutamyl transaminase (GGT)

3.7.6.1 Principle

GGT catalyzes the transfer of amino group between L-Gamma glutamyl 3-carboxy-4-nitroanilidine and Glycylglycine to form L-Gammaglutamyl Glycylglycine and 5-amino 2-nitrobenzoate. The rate of formation of 5-amino 2-nitrobenzoate is measured as an increase in absorbance which is proportional to the GGT activity in the sample.

L-Gamma glutamyl 3-carboxy-4-nitroanilide
+ Glycylglycine $\xrightarrow{GGT}$ L-Gammaglutamyl Glycylglycine
+ 5-amino 2-nitrobenzoate

3.7.6.2 Reagents

Reagent 1-Buffer
Reagent 2- Substrate

3.7.6.3 Procedure

The working reagent was prepared by adding 4 ml of Reagent 1 with 1.0 ml of Reagent 2.

Working reagent 1.0 ml
Serum 100 µl

Mixed well and the absorbance was measured at 405 nm.
3.7.7 Determination of Total Protein

3.7.7.1 Principle
Total Protein was first determined by the Kjeldahl’s method. The peptide bond of protein reacts with Copper ions in alkaline solution to form a Blue-Violet Complex. The color formed is proportional to the Protein concentration and is measured at 540 nm (520-560 nm).

3.7.7.2 Reagents
Biuret Reagent
Standard (6 gm/dl)

3.7.7.3 Procedure

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Blank</th>
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<th>Test</th>
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</thead>
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<tr>
<td>Protein reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>------</td>
<td>10 µl</td>
<td>------</td>
</tr>
<tr>
<td>Test</td>
<td>------</td>
<td>------</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Incubated for 5 minutes at room temperature and the absorbance was read at 540 nm.

Serum Protein Conc. gm/dl = \( \frac{\text{Abs of Test}}{\text{Abs of Standard}} \times \text{Conc. of Standard (6)} \)
3.7.8 Determination of Albumin

3.7.8.1 Principle

The most commonly used method for determination of Albumin is dye binding of which Bromocresol Green (BCG) is the most popular. Albumin in buffered medium binds with BCG causing proportional to the concentration in the sample.

3.7.8.2 Reagents

BCG Reagent
Standard (4 gm/dl)

3.7.8.3 Procedure

<table>
<thead>
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<th>Reagent</th>
<th>Blank</th>
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<th>Test</th>
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</thead>
<tbody>
<tr>
<td>BCG reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>------</td>
<td>10 µl</td>
<td>------</td>
</tr>
<tr>
<td>Test</td>
<td>------</td>
<td>------</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Incubated for 5 minutes at room temperature and the absorbance was read at 630 nm.

\[
\text{Serum Albumin Conc. gm/dl} = \frac{\text{Abs of Test}}{\text{Abs of Standard}} \times \text{Conc. of Standard (4)}
\]
3.8 Determination of CD4/CD8 by Flow Cytometry

3.8.1 Principle

When whole blood is added to the reagent, the fluorochrome labeled antibodies in the reagent bind specifically to the leukocyte surface antigens. During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluorescence signals, detected by the instrument, provide information about the cell’s size, internal complexity and relative fluorescence intensity. A known volume of sample is stained directly in a Trucount tube. The lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads. During analysis, the absolute number (cells /mm$^3$) of positive cells in the sample can be determined by comparing cellular events to bead events.

3.8.2 Reagents

Tri TEST CD4 FITC/CD8 PE/CD3 PerCP
Tri TEST CD4 FITC/CD8 PE/CD3 PerCP with TruCOUNT Tubes

3.8.3 Procedure

A minimum of 100µl of whole blood was used for this procedure. Anti coagulated blood stored at room temperature (20 -25ºC) must be stained within 48 hours of draw and then analysed within 6 hours of staining.

- 20µl of TriTEST CD4/CD8/CD3 reagent was added into the bottom of the Trucount tube and 50µl of well mixed whole blood was added into the
bottom of the tube. Incubated for 15 minutes in the dark at room temperature (20-25°C).

- 450µl of 1x FACS Lysing solution was added into the tube.

- This was vortexed gently and incubated for 15 minutes in the dark at room temperature (20-25°C). The sample was analysed on the flow cytometer.

### 3.9 Determination of Hemoglobin, Hematocrit, White Blood Cell and Total Lymphocyte count

All these parameters were determined by the Sysmex® KX-21 which is an automatic multi-parameter blood cell counter.

Blood collected in EDTA vacutainer tubes and these tests were performed within 24 hours.

Hemoglobin: Analysis Principle – Non-Cyanide hemoglobin analysis method.


Lymphocyte Count (WBC small cell count) Absolute count of lymphocytes (small cells) in 1µl of whole blood.
3.10 Quantification of HIV-1 RNA PCR using Amplicor ® HIV-1 Monitor Test, version 1.5

3.10.1 Principle

The viral load measurement technique is based on four major processes.

a. Specimen preparation

b. Amplification

c. Hybridization of amplified products to oligonucleotide probe.

d. Detection of probe bound amplified products.

3.10.2 Reagents

Specimen preparation reagent

Controls

Amplification reagent

Detection reagents

3.10.3 Procedure

3.10.3.1 Sample preparation

- 600µl of prepared working lysis reagent was added into the tube and 200µl of Plasma was added and vortexed for 5 seconds and incubated for 10 minutes at room temperature.

- 800µl of 100% isopropanol was added into the tube and centrifuged for 12,500 rpm for 15 minutes.
• The supernatant was removed and 1.0µl of 70% ethanol was added and then centrifuged for 5 minutes at maximum speed.

• The supernatant was removed and 400µl of HIV monitor specimen diluent was added and vortexed vigorously for 10 seconds.

100µl of HIV-I Monitor manganese solution was added to entire vial of MMX and Vortexed for 3-5 seconds.

50 µl of the master mix was added into PCR tubes and 50 µl of extracted RNA was added into tubes.

3.10.3.2 Amplification

PCR amplification was carried out in the thermal cycler at 50º C for 2 minutes and 60 ºC for 30 minutes 8 cycles for 95º C for 10 seconds, 52º C for 10 seconds, 72º C for 10 seconds, 23 cycles at 90º C for 10 seconds, 55º C for 10 seconds, 72º C for 10 seconds and extension was 72º C for 15 minutes.

3.10.3.3 Detection

• 100µl of Monitor Hybridization was added into the micro well plate and 25µl of denatured amplicon in row A and serial 5-fold dilutions from row B to F was done and incubated for 1 hour at 37ºC.

• The plate was washed for 5 times and 100 µl of Avian -HRP conjugate was added and incubated for 15 minutes.
Again the plate was washed for 5 times and 100 µl of working substrate was added and kept it for 10 minutes. 100 µl of Stop solution was added and the readings were measured at 450 nm.

HIV-1RNA copies/ml Plasma can be calculated as follows:

\[
\text{HIV - 1 RNA copies/ml} = \frac{\text{Total HIV-1 O.D. value \times input QS copies/PCR \times 40}}{\text{Total QS O.D. value}}
\]

\[
\text{Total HIV-1 O.D} = \text{O.D value - background O.D value \times dilution factor}
\]

\[
\text{Total QS O.D} = \text{QS O.D value - Background OD value \times dilution factor}
\]

3.11 Determination of Drug Resistance mutation by an Oligo nucleotide Ligation Assay (OLA)

3.11.1 Principle

The PCR products are mixed with two genotype-specific oligonucleotide probes [the mutant (mt)-specific labeled with fluorescein (F), and the wild-type (wt)-specific labeled with digoxigenin (D)] and a biotinylated oligonucleotide common to both genotypes. During ligation, the probes anneal to their complementary sequence in the PCR product, and the genotype-specific oligonucleotide becomes covalently linked to the adjacent common probe. The biotinylated ligation products are captured on streptavidin-coated microtiter wells and an ELISA is performed with alkaline phosphatase (AP) labeled anti-
fluorescein antibodies and horseradish peroxidase (POD) labeled anti-digoxigenin antibodies. Sequential addition of the AP and the POD substrates allow for detection of both genotypes in a single well.

**Figure 3.8 Flow chart of OLA**

![Flow chart of OLA](image)

### 3.11.2 Assay Components

1) Primers used for nested amplification of HIV-1 pol from patient specimens:

**First round primers**

- **PRA:** 5’-CCTAGGAAAAAAGGGCTTGGAAATGTGG (2011-2039*)
- **IBR1:** 5’-AACTTCTGTATATCATTGACAG (3303-3328*)

**Second round primers**

- **PRB:** 5’-ACTGAGAGACAGGCTAATTTTTTAGGGA (2068-2095*)
IBR2 : 5’-CAAAGGAATGGAGGTTCTTTCTGATG (3210-3235*)

2) Primers used for amplification of reference plasmids:

For K103 and Y181 plasmid controls

RT4C : 5’-TGTCAGGATGGAGTTCATACC
RT3C : 5’-GATGGCCCAAAGGTTAAACA

3) Control used for PCR sensitivity

DNA from 8E5 cells, which contain one copy of proviral HIV-1 DNA per cell, diluted in DNA from uninfected cells at a concentration of 10 copies/0.24µg total DNA/µl.

4) Reference plasmids

HIV-1 mutant and wild-type controls for the OLA were obtained by cloning PCR amplified viral isolates containing the drug-resistance mutations of interest into the PCR 2.1-TOPO vector (Invitrogen Corporation, Carlsbad, CA).

3.11.3 Amplification of Reference Plasmids

For K103 and Y181 Reference Plasmid Controls

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>27.5 µl</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>2mM dNTPs mix</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>20pmol/µl RT4C</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>20pmol/ µl RT3C</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>TAQ Polymerase (5 U/µl)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>40.0 µl</td>
</tr>
</tbody>
</table>
40 µl of the master mix was added into PCR tube and 10 µl of the reference plasmid was added and pol second round program was used for amplification.

3.11.4 Gel Electrophoresis

The amplification product was run on 2% agarose gel. 5 µl of amplified PCR products and 1ul of loading dye was loaded in the lane along with the DNA molecular weight markers and the bands were visualized at 300 bp.

3.11.5 Oligonucleotide Ligation Assay

Preparation of ligation mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Ligase Buffer</td>
<td>240 µl</td>
</tr>
<tr>
<td>10mM NAD</td>
<td>240 µl</td>
</tr>
<tr>
<td>1M KCl</td>
<td>30 µl</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>690 µl</td>
</tr>
<tr>
<td>Wild-type oligo</td>
<td>4 µl</td>
</tr>
<tr>
<td>Mutant oligo</td>
<td>4 µl</td>
</tr>
<tr>
<td>Common oligo</td>
<td>4 µl</td>
</tr>
<tr>
<td>Ampligase DNA ligase</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

- 2 µl of amplified pol PCR products were added into 2 wells of the plate along with negative and mutant controls.
- 0.1 % of Triton-X 100 was added into the same wells.
- 10 µl of the above prepared ligation mix was added into all the wells.

The reaction conditions were 93°C for 30 seconds, 37° C for 4 minutes for 10 cycles.
After completion of the ligation 10 µl of 0.1 M EDTA/0.1 % Triton X- 100 was added into the wells.

3.11.6 Capture on ELISA plate

- All the contents from the ligation plate were transferred into streptavidin coated plate and it was kept for 1 hour.
- The plate was washed with 1 X NAOH solution and then washed with 1X Tris wash and dried completely.
- 50µl of diluted antibodies were added into the wells and kept at room temperature for 30 minutes.
- The plate was washed 6 times with 1X Tris wash

3.11.7 Amplification of reaction

1. Detection of mutant type

25µl of the GIBCO substrate was added into the plate and kept it for 10 minutes and it was read at 490nm.

2. Detection of wild type

- The plate was washed 6 times with 1X Tris wash and 50 µl of TMB substrate was added and kept it for 10 minutes.
- 50 µl of 0.3M H₂SO₄ was added into all the wells and it was read at 450 nm.
3.12 FLOW CHART OF LAB INVESTIGATIONS FOR THE PRESENT STUDY

Lab Investigations

DNA PCR
- 3 Filter papers
- Whole blood

Biochemical
- Liver marker enzymes, total bilirubin, protein, albumin

Hematological & Immunological
- Hb, HCT, WBC, TLC
- CD4, CD8

RNA PCR & OLA
- Viral load & Drug Resistance

Calculated Values
- Sensitivity
- Specificity
- NPV
- PPV