The study has been carried out on the patients who were diagnosed as a case of malignancy affecting any organ in the body attending the OPD and admitted to the indoor at Lala Lajpat Rai and associated hospitals, Kanpur. Majority of the patients belong to the rural areas of Kanpur.

**CRITERIA FOR SELECTION OF CASES**

The criteria for selection of study cases were diagnosed and established cases of malignancy by standard methods. Detailed clinical history and examination of study cases were done and clinical samples were subjected for laboratory testing.

**COLLECTION OF SAMPLE**

The blood sample of study cases was collected by consent of patients. The blood was drawn by disposable syringe and needle and collected in sterilized plain vial. After an hour the blood was transferred in a centrifuge test tube and centrifuge at 1000 G for 15 seconds. The serum obtained was put into sterilized screwcap plastic vials and stored in refrigerator (2-4°C) all due precautions were taken to avoid contamination of sample at any stage.

The samples were tested for antibodies of **HIV** at Zonal blood testing centre, blood blank, Medical College, Kanpur.

**TEST FOR ANTIBODIES TO HIV**
Detection of anti-HIV antibodies is the main stay of testing for HIV and diagnosis of HIV.

**SCREENING TEST**

The commercial Kit is manufactured by J.Mitra & Co.Ltd. Microlisa ELISA tests for detection of antibodies to HIV-1 (including subgroup O&C) and HIV-2 in human serum or plasma. It takes about half an hour or so on.

Sensitivity and specificity of screening test are very much accurate about 95% or more.

**PRINCIPLE OF THE ELISA**

HIV test is an enzyme based on indirect ELISA. Recombinant proteins gp41,C terminus of gp120 and gp36 for HIV-1 and HIV-2 representing immunodominant epitopes are coated on to microtiter wells. Specimens and controls are added to the microtiter wells and incubated. Antibodies to HIV-1 and HIV-2 if presenting the specimen, will bind to the specific antigens adsorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen antibody complex present. Finally substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue color will develop in proportion to the amount of HIV-1 and or HIV-2 antibodies present in the specimens. The color reaction is stopped by the stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then
enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

**COMPONENTS IN EACH KIT**

Store all components at 4-8°C

96 test kits - 12 strips (8 well each)

**HIV** strip plates **HIV-1/HIV-2** recombinant proteins packed in a pouch with dessicant.

1. Bottle (25ml) sample diluent- buffer containing protein stabilizers and antimicrobial agents as preservative.

2. Vial (0.25ml) Enzyme conjugate concentrate (100x) – anti human IgG labeled with horseradish peroxidase with protein stabilizers.


4. Bottle (50ml) PBS with surfactant.. Dilute 1:25 with distilled water before use.

5. Vial (0.25%) TMB concentrate (100x)- To be diluted in TMB substrate diluent before use.


7. Vial (2ml) Negative control- Ready to use, normal human serum negative for
ELISA KIT

Figure 3.1
ELISA READER

Figure 3.2
**HIV-1 & HIV-2.**

8. Vial (2ml) Positive control-Ready to use, Positive for HIV antibodies containing sodium azide as preservative.


Additional material and instruments required-

- Micropipettes and disposable pipette tips.
- Timer
- ELISA reader
- ELISA washer
- Incubator 37°C
- Vortex mixer
- Glass ware
- Distilled water

**PREPARATION**

1. Preparation of samples

   (i) Use human plasma or serum as source of sample.

   (ii) Remove all the blood coagulants completely by centrifugation.

2. Preparation of Reagents

   (i) Bring the entire reagent at room temperature for 15 to 30 minutes
before use.

(ii) Take the required number of strips from sealed HIV1/2 antigen coated plate.

3. Preparation of washing solution

Make a 1:20 dilution of washing solution with distilled or ionized water to the extent of required amount (for example add 10 ml of washing solution to 190 ml of distilled water).

4. Preparation of conjugate

Make a 1:51 dilution of conjugate with diluent to the extent of required amount 10 minutes before use (for example, per strip, mix 20 µl of conjugate with 1 ml of conjugate diluent).

5. Preparation of substrate

Make a 1:101 dilution of solution with substrate buffer 5 to 10 minute before use for example, per strip, mix 20 µl of conjugate solution with 2ml of substrate buffer).

ASSAY PROCEDURE

1- Take the required numbers of strips and fix them to frame.

2- Pipette 100 µl of sample diluent in to each plate well and pipette 50 µl of negative control in to each well of 1A to 1C and 50 µl of positive control of 1D
and 1E, respectively. And then pipette 10 µl of each sample in to the remained wells. Mix it using microplate shaker at 1000 rpm for 10 sec.

3- Incubate at 37±1°C for 60 minutes after sealing the plate with cover sealer.

4- Before the last 5 to 10 minutes of 1st incubation, make a 1:51 dilution of conjugate with conjugate diluent.

5- Aspirate the content from all the wells and wash each one 5 times with 300µl of diluted washing solution (300 µl/well/times).

6- Invert the plate and tape it on absorbent paper to remove the remained washing solution. And then pipette 100 µl of prepared diluted conjugate in to each well.

7- Incubate the plate at 37± 0°C for 30 minutes after sealing it with cover sealer.

8- Before the last 5 to 10 minutes of 2nd incubation, make a 1:101 dilution of substrate solution with substrate buffer.

9- Aspirate the content from all the wells and wash each one 5 times with 300µl of diluted washing solution.

10- Invert the plate and tape it on absorbent paper to remove the remained washing solution. And then pipette 100 µl of prepared substrate in to each well and incubate at room temperature for 30 minutes.

11- Pipette 50 µl of stop solution in to each well and tap the plate gently to homogenized the coloring materials.
Read absorbance at 450 nm with in 30 minutes in ELISA READER (reference wave length at 600-650 nm) against air within 30 minutes after pipetting of stop solution.

EVALUATION OF RESULTS

ABBREVIATIONS

NC - Absorbance of negative control

NCx - Mean negative control

PC - Absorbance of positive control

PCx - Mean positive control

TEST VALIDITY

Black must be < 0.100 in case of differential filter.

NEGATIVE CONTROL ACCEPTANCE CRITERIA

NC must be < 0.150. If it is not so. The run is invalid and must be repeated.

POSITIVE CONTROL ACCEPTANCE CRITERIA

PC must be > 0.50.

CUT OFF VALUE
Absorbance

NC - 0.042 B1 Well  
- 0.040 C1 Well  
Total: 0.082 2 Wells

PC - 1.412 D1 Well  
- 1.392 E1 Well  
- 1.407 F1 Well  
Total: 4.211 3 Wells

NCx = 0.082/2 = 0.041  
PCx = 4.211/3 = 1.403

The cut off value is calculated by adding mean negative control (NCx) and mean mean positive control (PCx) as calculated above and the sum is divided by 6.

\[
\text{Cut off value} = \frac{\text{NC}_x + \text{PC}_x}{6}
\]

\[
\text{NC}_x = 0.041  \quad \text{PC}_x = 1.0403
\]

\[
\frac{0.041 + 1.403}{6} = \frac{1.444}{6} = \frac{0.240}{6}
\]

**INTERPRETATION OF RESULTS**

1- Test specimen with absorbance value less than the cut off value are non–reactive and may be considered as negative for anti HIV.

2- Test specimens with absorbance value greater than or equal to the cut off value are reactive for anti- HIV by Microlisa –HIV.

3- The O.D. for crystal clear negative samples can be in minus and the value could be in minus and the value could be in the range of (-) 0.00 to (-) 0.10.
However the minus (-) O.D. does not in any way effete the result interpretation. It rather gives better specificity.

4- Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Microlisa-HIV. Original specimen should be tested in duplicate.

5- If both duplicate retest sample absorbance value is less than cut off value, the specimen is considered non reactive.

6- If any one of the duplicate retest sample absorbance value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the criteria of Microlisa HIV. the sample consider positive shall be tested again by western blot.

CONFIRMATORY TEST

Western Blot Assay

The GENE LABS DIAGNOSTICS HIV BLOT 2.2 is qualitative enzyme immunoassay for the in vitro detection of antibodies to HIV-1 and HIV-2 in human serum and plasma. It is intended for the use as a more specific supplemental test on human serum or plasma specimens found repeatedly reactive using screening procedure such as the Enzyme Linked Immunosorbent Assay (ELISA).

In GENE LABS DIAGNOSTICS HIV BLOT 2.2 the separated specific HIV-1 viral antigens incorporated on to the strips via electrophoresis and electrotransblot procedure, combined with a specific HIV-2 synthetic peptide on the same strip allow
for further delineation of the antibody responses to specific viral proteins. Each strip also includes an internal sample addition control to minimize the risk of false negatives due to operational errors and to ensure the addition of samples.

**PRINCIPLE**

The nitrocellulose strips are incorporated with separated bound antigenic proteins from partially purified inactivated HIV-1 using electrophoretic blotting. Plus a specific HIV-2 synthetic peptide on the same strips. Individual nitrocellulose strips are incubated with diluted serum or plasma and controls. Specific antibodies to HIV-1 and HIV-2 if present in the specimens, will bind to the HIV-1 proteins and HIV-2 peptide on the strips. The strips are washed to remove unbound material. Antibodies that bind specifically to HIV protein can be visualized using series of reaction with goat anti-human IgG conjugated with alkaline phosphatase and the substrate BCIP/NBT. This method has the sensitivity to detect marginal amount of HIV specific antibodies in serum or plasma.

**KIT COMPONENTS**

1- Nitrocellulose Strips-Incorporated with HIV-1 viral lysate and specific HIV-2 envelope peptide. Keep dry and away from light.

2- Non Reactive Control- Inactivated normal human serum non reactive for Hepatitis B surface antigen (HBsAg), antibodies to HIV-1, HIV-2 & HCV. Contain sodium azide and thimerosal as preservatives.

3- Strong reactive control- Inactivated human serum with high tittered
antibodies to HIV-1 and HIV-2 and non reactive for HBsAg and anti HCV. Contain sodium azide and thimerosal as preservatives.

4- Weak reactive control- Inactivated human serum with low tittered antibodies to HIV-1 only and non reactive for HBsAg and antibodies to HIV-2 and HCV. Contain sodium azide and thimerosal as preservatives.

5- Strong Buffer Concentrate (10x) - Tris buffer with heat inactivated normal goat serum. Contains thimerosal as preservative.

6- Wash Buffer Concentrate (20x) – Tris with Tween-20. Contains thimerosal as preservative.

7- Conjugate- Goat anti human IgG conjugated with alkaline phosphatase

8- Substrate- Solution of 5-bromo-4 chloro-3indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT).

9- Blotting Powder-Fat dry milk.

10- Incubation tray -9 well each

**PREPARATION OF REAGENTS**

1- **Diluted wash buffer**- diluted 1 volume of wash buffer concentrate (20x) with 19 volume of reagent grade water. Mix well.

2- **Blotting buffer**-

(a)- Blotting buffer should be prepared fresh prior to use.

(b)- Diluted 1 volume of stock buffer concentrate (10x) with 9 volume of reagent grade water. Mix well.
(c) Add 1g of blotting powder to every 20 ml of the diluted stock buffer prepared in step 2 (b). Mix well.

3- Working Conjugate solution

(a) Prepare working conjugate solution by diluting conjugate 1:1000 in to blotting buffer for example 5µl conjugate to 5 ml blotting buffer.

(b) Working conjugate solution should be prepared fresh prior to use.

4- Substrate solution

(a) Dispense directly the required volume from the bottle. Use a clean pipette. Cap tightly after use.

ASSAY PROCEDURE

1- Using forceps, carefully remove required number of strips from the tube and place numbered side up in to each well. Include strips for strong reactive, weak reactive and non reactive controls.

2- Add 2ml of diluted wash buffer to each well.

3- Incubate the strips for at least 5 minutes at room temperature (25 ± 3 °C) on a rocking platform. Remove buffer by aspiration.

4- Add 2 ml of blotting buffer to each well followed by 20µl of patient’s sera or controls to appropriate wells.

5- Cover the tray with provided and incubates for 1 hour at room temperature (25 ± 3 °C) on a rocking platform.
6- Carefully uncovers the tray to avoid splashing or mixing of sample. Aspirate the mixture from the wells. Change aspirate tips between samples to avoid cross-contamination.

7- Wash each strip 3 times with 2 ml of diluted wash buffer allowing 5 minutes soak on the rocking platform between each wash.

8- Add 2ml of working conjugate solution to each well. Cover tray and incubate for 1 hour at room temperature (25 ± 3 °C) on the rocking platform.

9- Aspirate conjugate from the wells.

10- Add conjugate of substrate solution to each well. Cover tray and incubate for 15 minutes on the rocking platform.

11- Aspirate the substrate and rinse the strips several times with reagent grade grade grade water to stop the reaction.

12- Using forceps, gently remove strips onto paper towels. Cover with paper towels and dry.

13- Mount strips on worksheet (non absorbent white paper). Do not apply adhesive. Tape over the developed bands. Observe the bands and grade the results. For storage keep strips in the dark.

**INTERPRETATION OF RESULTS**

The presence or absence of antibodies to HIV-1 in a sample is determined by comparing each nitrocellulose strip to the assay control strips tested with the non-reactive, strong reactive and weak reactive controls.
1- It is unlikely to detect gp41 in the absence of gp160 because the gp160 is the polymeric form of gp41 and the concentration of gp160 is higher than gp41 on the HIV BLOT 2.2.

2- The p55 band is generally detected when there is strong reactivity to p24 and or p17. The bands seen as p24 and p39 are both GAG fragments and should not be interpreted as gp41 (ENV).

3- The POL bands p66, p51 and p31 are generally detected simultaneously. However the sensitivity of p66 and p31 are greater than p51.

4- HIV-2 cross reactivity is variable but typically shows reactivity with GAG and or POL antigens. However there can be cross reactivity with the gp160 band in some cases but rarely with gp41.

5- There is also high molecular weight band around 160 KD that is presumed to be a GAG–POL precursor protein. This is seen with some high tittered HIV-2 or indeterminate (GAG reactive only) sera but the band pattern is a sharp discreet band which ids different from the diffuse band of ENV gp160 (See Table 3.1).

**REACTIVE INDEX**

This index is designed to serve as an optional guideline for interpretational of results. The basis of assigning an arbitrary valve to each antigenic band lies with the relative importance of that particular diagnostic antigen in the
accepted interpretation criteria for HIV-1 seropositivity adopted by the various authorities.

Based on GENELABS recommended criteria, the presence of each band is given a value as shown:

- gp160/gp41 = 1.00
- gp10 = 1.00
- Either p24 or p55 = 0.50
- Either p31 or p51 or p66 = 0.50
- p17 = 0.25

Use the index for interpretation (See Table 3.2).
### Table – 3.1

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No viral specific bands present</td>
<td>Negative</td>
</tr>
<tr>
<td>Detection of p17 antibodies Only, no other bands</td>
<td>Negative</td>
</tr>
<tr>
<td>Detection of 2ENV (gp160/gp41, and gp120) and GAG (p17, p24, p55) or</td>
<td>HIV-1 Positive</td>
</tr>
<tr>
<td>POL (p31, p51, p66).</td>
<td></td>
</tr>
<tr>
<td>Detection of 2ENV (gp160/gp41 and gp120) and GAG (p17, p24, p55) or</td>
<td>HIV-1 Positive With</td>
</tr>
<tr>
<td>POL (p31, p51, p66) and HIV-2 specific band is visible.</td>
<td>HIV-2 indicated</td>
</tr>
<tr>
<td>Any viral specific bands present but pattern does not meet the criteria</td>
<td>INTERMINATE</td>
</tr>
<tr>
<td>HIV-2 indicated</td>
<td></td>
</tr>
<tr>
<td>Any viral specific bands present but pattern does not meet the criteria</td>
<td>INTERMINATE With HIV-2</td>
</tr>
<tr>
<td>HIV-2 INDICATED</td>
<td></td>
</tr>
</tbody>
</table>
### Table – 3.2

<table>
<thead>
<tr>
<th>Index</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum less than or equal to 0.25</td>
<td>Negative</td>
</tr>
<tr>
<td>Sum more than 0.25 but less than or equal to 2.25</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>Sum greater than 2.50</td>
<td>HIV-1 positive</td>
</tr>
</tbody>
</table>

Identify the molecular weight to each band of the test strip using strong and or non reactive control strips as a guide.

1. Assign the appropriate value to each visible diagnostic antigen using the values shown above.