MATERIAL
METHOD
MATERIAL AND METHODS

In this study hundred antenatal patients are included who were either from out patients department or admitted in M.I.B. Medical College Jhansi in ‘Bundelkhand Region’.

STUDY GROUP :- consist of hundred antenatal women including primigravida and Multigravidae with bad obstetric history.

CONTROL GROUP 50 patients are taken in this group without any bad obstetric history :-

CRITERIA OF SELECTION OF CASES :-

Patients of study group should have history in forms of:
    Repeated abortion
    Still Birth
    Congenital Malformation
    Premature Birth
History of leaking pervaginum in cases of Primigravida.
Patients were not having toxaemia, fever, any systemic illness.

Collection of Blood sample - for ELISA test 5 ml of blood was taken out from the antecubital vein under all aseptic precaution and transferred to a clear sterile test tube and was kept upright for an hour at room temperature when the serum was seperated it was poured in to a centrifuge tube and centrifugation was done at 3000 rpm for about 5
minute s the clear supernatant serum was separated and kept in small glass tube at 40 °c until use.

Hemolysed samples were discarded.

MICROELISA STRIPS FOR Quantitation of Ig G anti Toxoplasma gondii antibody.

ELITORCH TOXO Ig G is a sandwich enzyme linked immunosorbent assay for the quantitative estimation Ig G antibodies to Toxoplasma gondii in serum.

PRINCIPLE: This solid phase ELISA test consist of Microlititre strips coated with Toxoplasma antigen (Toxoantigen). If the patients serum / control has the relevant specific anti-Toxoplasma gondii antibodies they bind to Toxo antigen on solid phase. After washing the bound antibodies are sandwiched using HRP labelled antigen human Ig G conjugate resulting in formation of Toxoantigen human Ig G HRP labelled antihuman Ig G conjugate complex.

The unbound conjugate is removed by washing and the enzyme linked sandwich complex is revealed by chromogenic substrate. The intensity of the colour developed is directly proportional to the amount of Ig G Toxoplasma gondii antibodies in the serum. After stopping the reaction with stopping solution, absorbance is measured at 450 mm using an ELISA reader.
Results of patients samples are obtained by calculation / comparison using the low medium and high positive controls provided.

1. Microtitre strips (8well strips) coated with Toxoantigen (12 strips)
2. Sample dilution buffer: ready to use - 100 ml
3. Negative control: ready to use - 2 ml
4. Cut-off control (5 in/ml): ready to use - 2 ml
5. Low positive control (30 in/ml): Ready to use-2 ml
6. Medium positive control (100 in/ml): Ready to use - 2 ml
7. High positive control (200 in/ml): Ready to use -2 ml
8. Wash buffer concentrate (50 ml)
9. HRP labelled antihuman IgG conjugate - 11 ml
10. TMB solution - 1.5 ml
11. Substrate buffer - 25 ml
12. Stopping solution - 11 ml
13. Strip holder - 1

**STORAGE AND STABILITY:**
The reagents are stable up to the stated expiry date when stored at 2.8 °C.

**Preparation of working solution:**

* working wash solution
  Mix:
  Reagent 8 (wash buffer concentrate) 1 volume
  Distilled water
  The working wash solution is stable for one week at 2-8 °C

* Chromogenic substrate solution
  Mix:
Reagent 10 (TBM) 1 Volume
Reagent 11 (Substance solution) 20 volumes
Prepair only the required volume at the time of use.

* Microtitre strips coated with Toxo antigen immediately after removal of strips ..reseal the remaining strips along with the disicecant and store at 2-8 °C.

* Sample Preparation
  Mix:
  Patients serum - 10 IU/ml
  Sample dilution buffer - 1 ml
  Serum samples may be diluted at the time of use and stored at 2-8 °C before using for the day.

PRECAUTIONS:

- Reliability of the results depends on strict adherence to the procedure described in the insert.

- Do not use reagents after expiry date stated on the label.
  Do not interchange reagent vials and their caps to avoid cross contamination.
  Take the required amount of reagents from vials and close immediately after use to avoid evaporation.

Reagents 2,8,10,11 and 12 are interchangeable between lots. All other reagents are specific for individual package and must not be interchanged with other lots. No reagents of other manufactures should be used along with the kit reagent.
PROCEDURE:

Bring all reagents, samples, and controls to room temperature before use. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the form supplied with the kit. Select the required number of microtitre strips and place in a holder.

Allow one well for substrate blank and two wells for each control.

Dilute patient sera as described in sample preparation.

Do not dilute controls.

Step 1 - Leave well A1 for substrate blank. Pipette controls and samples in the following order:
- Wells B1/C1 - 100 µl Negative control.
- Wells D1/E1 - 100 µl cut-off control.
- Wells F1/G1 - 100 µl Low Positive control.
- Wells H1/A2 - 100 µl All medium positive control.
- Wells B2/C2 - 100 µl High positive control.
- Wells D2 onwards - 100 µl diluted samples.

Cover the Wells with foil or adhesive film.

Step 2: - Incubate for 30 minutes at room temperature (15-30 °C).

Step 3: - Aspirate all contents of wells into disinfecting solution (e.g. 5% sodium hypochlorite).
Wash the wells 4 times with 400 μl of working wash solution each time and aspirate off. At the end carefully remove remaining fluid by tapping the strips on tissue paper prior to next step.

Step 4: - Dispense 100 μl of HRPO labelled antihuman Ig G conjugate into all wells except the substrate blank (A1) cover wells with foil or adhesive film.

Step 5: - Incubate for 30 minutes at room temperature (15-30 °C).

Step 6: - Wash the wells 5 times as described in steps.

Step 7: - Dispense 100 μl of chromogenic substrate solution to all the well including including A1.

Step 8: - Incubate for 15 minutes at room temperature in dark.

Step 9: - Stop the enzymatic reaction by adding 100 μl of stopping solution to each well and read with in 30 minutes.

Step 10: - Read the absorbance at 450 nm (A) after setting the ELISA reader to zero using the substance blank in the first well (A1).

CALCULATION:

1. Calculate the mean absorbance reading of negative control (MNC).
2. Calculate the mean absorbance reading of cut-off control (MCC).
3. Calculate the mean absorbance reading of medium positive control (MMPC).

The test run may be considered valid provided the following criteria are met:

(I) The substrate blank in well A1 appears almost colourless to the eye.

(II) MNC = MCC

(III) MMPC = 0.750

(IV) The ratio of MMPC to MNC = 2.

**INTERPRETATION OF THE RESULTS:**

Test samples with absorbance values greater than or equal to the MCC are considered Positive for Ig G antitoxoplasma gondii antibodies. Test samples with absorbance values less than the MCC are considered negative for Ig G anti-toxoplasma gondii antibodies.

If an ELISA reader is not available a visual interpretation of the results is possible.

A specimen can be considered positive if the colour intensity of the sample well is equal to or stronger than the colour intensity in the cut-off control wells D, and E.
QUANTITATIVE ESTIMATION:

(1) Read the absorbance of controls and test samples at 450 nm against the substrate blank.

(2) Using graph paper plot the absorbance on the vertical axis and the concentration of controls on horizontal axis. The anti Toxoplasma gondii antibody in the specimen can be interpolated from the curve.

ELISA test for the other TORCH infections are done in similar manner.

Laboratory diagnosis of HIV infection:

HIV infection has a long incubation period and asymptomatic stage, many psychosocial issues and problems arise subsequent to diagnosis of HIV status, hence both false positive and false negative test results are likely to have undesirable consequence. There is neither a cure nor a vaccine available as yet for HIV infection/AIDS. All these reasons combined results in the importance of the laboratory investigation in the diagnosis of HIV infection.

OBJECTIVES OF HIV TESTING - In this study HIV testing is done in Antenatal cases which are positive for TORCH infection.

Performance characteristics of the test used criteria for selection of a diagnostic test depends on the following.

1- High level of sensitivity, specificity and predictive value.
2- Long shelf life at ambient temperature.
3- Cost effectiveness.
4- Rapidity of case of performance.

**Specimen employed for laboratory diagnosis of HIV infection.**

The various types of specimens that can be utilised for diagnosis of HIV infection and the suitable laboratory procedures are mentioned below.

<table>
<thead>
<tr>
<th>SPECIMENS</th>
<th>LABORATORY PROCEDURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood/serum/plasma</td>
<td>Anti body detection</td>
</tr>
<tr>
<td>Saliva</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>Not as good as serum</td>
</tr>
<tr>
<td>Serum/plasma</td>
<td>Antigen detection</td>
</tr>
<tr>
<td>CSF/cell culture supernate</td>
<td></td>
</tr>
<tr>
<td>Blood (PBNcells)</td>
<td>Virus isolation</td>
</tr>
<tr>
<td>Serum vaginal/</td>
<td>PCR</td>
</tr>
<tr>
<td>Cervical specimen</td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
</tr>
<tr>
<td>Less commonly</td>
<td></td>
</tr>
<tr>
<td>Saliva, urine, Breast milk</td>
<td></td>
</tr>
<tr>
<td>Tears, Amniotic fluid</td>
<td></td>
</tr>
</tbody>
</table>
Interpretation of HIV antibody assay.

(A) 'A positive test' may indicate-
   (i) Infection with HIV
   (ii) false positivity
(B) 'negative test' may indicate
   (1) Absence of infection
   (2) Failure of the test system or
   (3) Failure in detecting early (<3 months) HIV infection.

SCREENING TESTS:

(A) ELISA (Enzyme linked Immunosorbent Assay)

It is the most commonly performed test to detect HIV infection.

There are various kinds of ELISA:
   - Indirect ELISA.
   - Competitive ELISA.
   - Antigen sandwiched ELISA.
   - Antigen and antibody capture ELISA.

ELISA is also classified on the bases of the antigen utilised in the test.

1st generation: Infected cell lysates are used as the antigen (This type of ELISA is hardly a choice currently due to false positive result arising out a cross reactivity with the host cell antigen).
2nd Generation: Glycopeptides (recombinant antigens) were used as the antigen.

3rd Generation: Synthetic peptides are used as the antigen. ELISA takes up to three hours to yield results, it has major advantage being economical.

Rapid Tests: Give results within minutes. They are done utilizing commercially available kits. They include:

(i) Dot blot assays
(ii) Particulate agglutination tests (Gelatin, RBC, latex, Microbeads).
(iii) Colloidal gold tests.
(iv) Fluorometric Microparticle techniques.

Reliability of ELISA antibody assays.

ELISA reactive individuals should be confirmed by a suitable confirmatory test before declaring positive for HIV infection.

ELISA reactive blood product should be confirmed by western blot.

Clone Systems, HIV-1 and HIV-2 Test Kit

Clone Systems

HIV-1 and HIV-2 test kit is a solid phase enzyme immuno assay representing epitopes of HIV-1 synthetic HIV-2 are coated into wells of a microtiter serum or plasma samples diluted in a solution are added to these wells. If
antibodies specific for HIV-1 and/or HIV-2 are present in the sample, they will form stable complex with HIV antigen on the well. A goat antihuman IgM labeled with horseradish peroxidase is added. If the antigen antibody complex is present, the peroxidase conjugate will bind to the complex and remain in the well.

Enzyme substrate is then added; During incubation a blue color will develop in proportion to the amount of anti HIV antibody bound to the well; wells containing samples negative for anti HIV antibody are colourless.

An acid stop solution is added to each well and the colour read on the microplate reader at 450 nm.

Material supplied: -

(1) Microplate coated with HIV-1 and HIV-2 peptides  
(2) One vial negative control (1 ml human)  
(3) 1 Vial (1ml) positive control (human)  
(4) 1 Vial (20 ml) sample diluent  
(5) 1 Vial (50 ml) wash solution concentrate (10X)  
(6) 1 Vial (0.5 ml) Anti human IgM peroxidase conjugate (40X)  
(7) 1 Vial (15 ml) conjugate diluent  
(8) 1 Vial (2 ml) Tetramethyl Benzidine Reagent (TMB)  
(9) 1 Vial (20 ml) peroxidase Reagent.  
(10) 1 Vial (10 ml) stop solution (INH2 SO4)

Precautions:
- For in vitro diagnostic use only
- Do not pipette by mouth
- Do not smoke, eat or drink in areas in which specimens are handled
- Rubber or disposable gloves are worn throughout the testing procedure

* The positive control sera have been inactivated. This does not ensure the absence of viable HIV and therefore these sera should be handled as potentially biohazardous following good laboratory practice.

* All human serum components were found to be negative for hepatitis B surface antigen. This does not ensure the absence of hepatitis B virus. This kit requires the use of 1 N sulphuric oxide. Do not combine acid with waste material containing sodium oxide.

* All material used in this assay including reagents and samples should be disposed of in a manner that will inactivate human hepatitis B virus and HIV.

**Solid wastes**: Autoclaves 60 min at 121 °C.

**Liquid wastes**: Add sodium hypochloride to a final concentration of 1.0%. The waste should be allowed to stand a minimum of 30 min to inactivate the virus before disposal.

* Do not use the kit beyond its labeled expiry date.
* Bring reagent to room temperature 15 to 30 minutes before use. Room temperature for this assay is defined as 18-24°C.

* Serum or plasma samples may be stored at 2°C to 8°C or frozen at 20°C or below. Self-defrosting freezers are not recommended. Avoid multiple freeze-thaw cycles.

**Reagents Preparation**

1. Positive control - supplied in prediluted form. Do not dilute.
2. Negative control - supplied in prediluted form. Do not dilute.
3. Unknown samples diluted 1:50 with sample diluent.
4. Wash solution concentrate; dilute 1:10 with distilled or deionised water.
5. HIV-1 and HIV-2 Micropalte store at 2°C-8°C bring to room temperature before removing from pouch.
6. Peroxidase conjugate (40X liquid concentrate): Bring conjugate diluent buffer to room temperature 15 to 30 min before use. Dilute 0.3 ml conjugate concentrate in 12 ml conjugate diluent. Mix thoroughly use with in 8 hours.
7. Substrate solution: Transfer 5 ml of peroxide reagent for each 1 ml TMB. Reagent to be diluted into a clean container (alcohol rinsed followed by distilled or deionised water) allowing at least 0.1 ml substrate solution for each coated well.

Add required volume of TMB reagent to the peroxide reagent and scold gently to mix. The substrate solution should be colourless. A deep blue colour indicates that the solution has been contaminated and should be discarded.
Specimen Collection and Handling

1- Handle all blood plasma and serum as if capable of transmitting hepatitis virus and/or HIV.

2- Where possible, clear unhemolysed specimen should be used, specimens should be collected aseptically, early separation from from the dot prevent hemolysis of serum. USE OF HEAT INACTIVATED SERUM OR PLASMA IS NOT RECOMMENDED AS THIS WILL LEAD TO FALSE POSITIVE RESULT.

3- Avoid multiple freeze thaw cycles which may result in sample deterioration and cause a false positive reaction, self defrosting frezer are not recommended for storage of the samples.

4- Undiluted specimens may be stored at 2º to 8º C. If they are suitably vialled and stoppered at 20º C as below-

5- Use sample dilution with in 8 hours of preparation Bring to room temperature 15-30 min before use.

Test procedure :

Specimen may contain infections HIV or Hepatitis virus, handle as if capable of transmitting virus. Plasma or serum sample should be screened at a standard 1:50 dilution on the HIV-1 and HIV-2 microplate; initial reaction in the screening test must be retested in duplicate.
Bring all reagent to room temperature before using.

1- Into a clean test tubes uncoated microplates or other comparable container dilute 5 µl of each sample to be tested with 0.25 ml sample diluent and mix well. These methods may be performed with an automatic diluting device.

2- Remove the microplate from the refrigerator and allow to come to room temperature (approximately 10-30min). Remove the plate from its pouch. Just before use and label.

3- Dispense 0.1 ml of the sample diluent into A1 for use as a substrate blank.

4- Dispense 0.1 ml of the negative control sample into each of 3 wells and 0.1 ml of the positive control into each of 2 wells. Similarly dispense 0.1 ml of diluted samples into wells using a separate pipette for each sample.

5- Incubate at room temperature for 30 minutes.

6- Aspirate and wash the plate 5 times with 0.3 ml/well of wash solution. Automated washer should be adjusted to fill each well completely without over filling. After the final wash be sure of all the solution is removed from each well. Sharply top the plate upside down on absorbent paper to remove the last remaining fluid.

Note: Proper wash procedure is essential for good assay performance.
7- Dispense 0.1 ml of diluted peroxidase conjugate into each well of the micro plate including.

8- Incubate the plate for 30 min. At room temperature.

9- Aspirate and wash the plate times with 0.3 ml/well of wash solution, after the final wash be sure all solution is removed from each well.

10- Add 0.1 ml of freshly prepared substrate solution in to each well.

NOTE- Use of multi pipettes is recommended for manual addition of both substrate and stop solutions. Manual single well additions may affect accurate timing at this point in the procedure.

11- Incubate the substrate filled plates for 30 min. At room temperature (18-24 °C) start the timing with in 3 min. after the addition of the reagent to the first well.

12- Stop the reaction by adding 0.1 ml of stop solution in to each well in the same order used in the addition of the substrate reagent.

13- After adding the stop solution, the colour developed may be read or the plate at 450 nm. Test must be read with in 30 min.

NOTE- The reader should be blanked at against the substrate blank. Bichromatic absorbance measurements with a reference wavelength of 600-650 nm is recommended when available.
14- Record the absorbance results on a data sheet include the kit master lot number date. Operator name and any other notes about the run. If a printed copy of the absorbance reading is available. It should be attached to the data sheet.

RESULTS:

Test validity:- Three negative and two positive controls must be included on each run. The control results must be examined before the sample results can be interpreted.

Calculation of negative control mean (NCX):
Example:

<table>
<thead>
<tr>
<th>Negative control</th>
<th>Sample Number</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0.081</td>
</tr>
</tbody>
</table>

Absorbance of individual negative control values must be less than or equal to 0.15. If one value is outside this range, discard this value and recalculate the mean. If two values are outside this range the run should be repeated.

Calculation of positive control mean (PCX).
Positive control

<table>
<thead>
<tr>
<th>Positive control</th>
<th>Sample Number</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>1.536</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.551</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3.087</td>
</tr>
</tbody>
</table>

PCX = 3.087/2 = 1.544.
The positive control mean must be equal to or greater than 0.80, if the mean value is less than 0.80 the run should be repeated.

\[
\text{Cut off} = \text{NCX} + 0.15
\]

Example: NCX = 0.027 + 0.15 = 0.177.

Interpretation of sample results.

1- If the initial test result absorbance value is less than the calculated cut off value than sample is considered non reactive.

2- If the screening value of a sample is equal to or a greater than the cut off retest in duplicate using a fresh dilution of the original sample.

3- If both the retested values are less than the cutoff. The interpretation of the total testing is non reactive for HIV-1 and HIV-2 antibodies. If both result values are equal to or greater than the cut off or if one of the duplicates is equal to or greater than the cutoff and one is less than cutoff. The interpretation of the testing resulting is repetitively reactive. The sample should be considered reactive or positive of the HIV EIA test.

**WESTERN BLOT TEST**

It is an assay to the presence of HIV antibodies of IgG and IgM. Class.
Important steps are given below:

1- HIV is lysed and proteins are separated on poly acrylamide gel by electrophoresis.

2- The separated proteins are then transferred to a nitro cellulose sheet by blotting or by electrophoresis.

3- The sheet is then cut into strips provided with the commercial kits.

4- Serum sample is incubated with this strip. HIV antibodies bind to the various HIV proteins.

5- Enzyme labelled antibody conjugate is added to the strip after washing. The conjugate binds to the antigen antibody complex.

6- Substracts is added after incubation and washing.

7- This produces coloured bands at the sites where patients antibody (IgG or IgM) bind with the viral proteins or the strip.

8- The results of the test sera are compared with the result of the known positive and negative sera supplied with the kit.

The position of the bands confirms the positive and negative status of the individuals.
9- WHO criteria for interpretation of the western blot test are given in the table.

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>HIV - I</th>
<th>HIV - II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>2 env band with or without gag polbands</td>
<td>2 env band with or without gag polbands</td>
</tr>
<tr>
<td>Negative</td>
<td>No bands or presence of bands that does not correspond to the structural HIV-1 proteins.</td>
<td>No band or presence of bands that do not corresponds to the structural HIV-2 proteins.</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Other profile not considered positive or negative.</td>
<td>Other profile not considered positive or negative.</td>
</tr>
</tbody>
</table>

Presence of $p^{24} + gp.120/gp^{160}$ strongly indicates seroconversion.