MATERIAL AND METHODS
3. Material and Methods

3.1 Survey Area :-

Survey Area includes three cities namely Jhansi, Lalitpur and Orai. Jhansi is the head quarter of Bundelkhand division.

3.2 Climate :-

Climate of the area is hot and dry. Mean monthly maximum and minimum temperature usually ranges between 24.1\(^{0}\)C to 42.6\(^{0}\)C in summers and 9.2\(^{0}\)C to 39.3\(^{0}\)C in winter seasons respectively.

3.3 STUDY UNIT :-

The study unit for the purpose of this study, was an individual of different high risk groups for example- commercial sex workers, Truck drivers, Jail inmates, Police and PAC personnel and low risk groups for example students, teachers and paramedical staff.

3.4 STUDY AREA

The Present study was undertaken in the following high-risk groups for HIV/AIDS in above mentioned cities.

I. Jail inmates- jail inmates were from District jails of Jhansi, Lalitpur and orai.

II. Police Personnels- Police Personnels were from the Police Line and Thana Nawabad of Jhansi city, police line of Lalitpur and Orai city.

III. PAC personnels- They were from P.A.C. vahini 33 near Rajgarh, Jhansi.

IV. Truck drivers- They were studied on road-sides on Kanpur Jhansi road opposite M.L.B. medical college and Hospital, Jhansi and on
highway in Lalitpur

V. **Commercial sex workers**- They were studied on road side Juggi-Jhopri near railway station Jhansi, Elite crossing, Mission Compound, Gudri in Manik Chowk, villages near BHEL in Jhansi city, Juggi-Jhopri and slums in Lalitpur and Orai, as there is no well defined area for these people. They were in dispersed form. Their names were taken from Kotwali and other Police Stations and from some NGO’S.

The present study was undertaken in following low risk groups for HIV/AIDS in above mentioned cities.

1) **Students**- Graduate and postgraduate students studying in various degree colleges affiliated to Bundelkhand University and students of Bundelkhand University itself, were studied under this group.

2) **Teachers**- Teachers of various schools and colleges of Bundelkhand region in general were studied under this group.

3) **Medical Personnels**- Under this, nurses and ward boys of civil hospital, Jhansi Lalitpur and Orai M.L.B. Medical College, were studied.

3.5 **THE QUESTIONNAIRE**

The required data were collected on a pre-designed questionnaire (See, Annex 1) by direct personal interviews method. This questionnaire had five parts. Part I was designed to collect data on socio-demographic characteristics of respondents, part II had questions to assess the respondents, knowledge on HIV/AIDS part III assessed their opinion on HIV/AIDS, part IV was framed to assess sources of infection of HIV/AIDS and part V included details of investigations. Most of the questions were close with multiple choice answers.
3.6 SURVEY TECHNIQUES

The survey was carried out from 1 January 2001 to 31 October 2005 by the investigator herself, using departmental resources, particularly the help of the working technician in Microbiology department of M.L.B. Medical College. Help of lab technician was sought, at times for collection and transportation of blood samples.

During the survey, firstly the written consent was taken from each individual after detailing them about aim and procedure of the study. On an average, 20-25 minutes were spent for interviewing and in taking blood samples from each individual. Individuals were interviewed and investigated separately. An effort was made to contact maximum number of high risk/low risk individuals of these groups. After completion of interview, 5ml blood was taken by using disposable needles and syringes, later on, blood was centrifuged and serum was separated on the same day and the serum was stored at, $-20^0\text{C}$ in the Department of Microbiology of the institution. Every attempt was made to ensure the confidentiality of blood samples results.

3.7 LABORATORY TESTS INCLUDING STRATEGIES OF TESTING

LABORATORY TESTS

All the coded serum samples were subjected to HIV EIA (Lab systems) a solid phase enzyme immunoassay for detection of antibodies of HIV-1 and HIV-2 in human serum or plasma.

The repeated ELISA positive samples were further subjected to testing with rapid methods like TRIDOT, CAPPILUS, UNIGOLD™ etc.
PRINCIPLE OF ELISA TEST

The principle of HIV EIA test is based on an indirect solid-phase enzyme immunoassay with horseradish peroxidase as the marker enzyme. The assay proceeds according to the following reactions.

1) When present in patient serum HIV antibodies (>) combine with HIV peptide (◊) attached to polystyrene surface (□) of the microstrip wells.

2) Residual patient sample is removed by washing and horseradish peroxidase conjugated anti-human IgG sheep antibody (m) is added.
3) Wells are washed and a colourless enzyme substrate (H₂O₂) and chromogen Tetramethyl benzidine (TMB), a non-mutagenic chromogen for horseradish peroxidase are added. The enzyme reaction of chromogen / substrate produces a coloured end product.

4) Enzyme-chromogen/substrate reaction is terminated with acid (H₂SO₄). The colour intensity is directly related to the concentration of HIV antibodies in a patient sample.
TEST PROCEDURE

OUTLINE OF THE PROCEDURE

**STEP-I**

Add 150 μl sample diluent
add 50 μl specimens / controls
mix carefully while pipetting

Incubate 30 min., at 37°C

Wash 5x400μl / well

**STEP-II**

Add 200 μl conjugate solution

Incubate 30 min., at 37°C

Wash 5x400 μl / well

**STEP-III**

Add 200 μl substrate solution

Incubate 15 min. at room temperature in dark.

**STEP-IV**

Add 50 μl 2M H₂SO₄

Measure absorbance at 450 nm in ELISA Reader.
**QUALITY CONTROL VALUES**

<table>
<thead>
<tr>
<th>QC Samples</th>
<th>Expected Values at 450 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(In absorbance unit)</td>
</tr>
<tr>
<td>Reagent Blank</td>
<td>$\leq 0.10$</td>
</tr>
<tr>
<td>Negative Control (3a)</td>
<td>$\leq 0.15$ x)</td>
</tr>
<tr>
<td>Positive control 1 (3b)</td>
<td>$0.70 \leq Apcl &lt; 2.00$ x)</td>
</tr>
</tbody>
</table>

x) The absorbance of the reagent blank has already been subtracted from these values.

**ABBREVIATIONS**

A = Absorbance
Arb = Mean absorbance of the reagent blank.
Apcl = Mean absorbance of the positive control 1 (3b)
CO = The cut-off value in absorbance units.

When the microplate reader is blanked against the reagent blank the following formula was used for cut off.

\[
CO = 0.3 \times Apcl
\]

When the microplate reader is NOT blanked against the reagent blank, the cut-off formula is.

\[
CO = 0.3 \times (Apcl - Arb) + Arb
\]
# INTERPRETATION OF THE RESULTS

<table>
<thead>
<tr>
<th>RESULT</th>
<th>INTERPRETATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; CO</td>
<td>A negative result means that the sample tested either contains no antibodies to HIV or the antibody level is below the detection limit of the test Kit. With negative test results, when infection is suspected, it is advised to repeat the test with a new serum sample taken 2-4 weeks later.</td>
</tr>
<tr>
<td>≥ CO</td>
<td>An initially reactive test result has to be retested. Only after receiving a repeatedly reactive results, the sample may be presumed to contain antibodies to HIV. The result should be verified with a recognized confirmatory test.</td>
</tr>
</tbody>
</table>

As with other immunoassays, occasional false positive results may occur, which are in most instance non-repeatable. It is therefore recommended to retest all samples giving an initially positive result.
ELISA PLATE

The Photograph of ELISA Plate showing 5 positive samples in which 3 are of Truck Drivers of Jhansi and other 2 samples are of M.L.B. Medical College, Microbiology Department.
OBSERVATION OF ELISA PLATE

The ELISA Plate showing 96 wells. In which

A1 → Blank (0.033)
B1 → Negative Control (0.046)
C1 → Positive Control (2.435)

By photograph we can watch 5 positive cases of HIV infection in the wells D2, H1, A7, D7 and F10 shown in the yellow colour. In these positive cases of HIV infection the sample of Truck Drivers found to be positive are in the wells

A7 → (1.966)
D7 → (1.911)
F10 → (1.857)

DESCRIPTION OF ELISA PLATE WELLS

The samples of Truck Drivers are

A2 – A12
B2 – B12
C2 – C12
D2 – D12
E2 – E12
F2 – F12
G2 – G12
H2 – H12

The samples of M.L.B. Medical College, Microbiology Deptt. Jhansi
Are : - D1, D2, E1, F1, G1 and H1.
RAPID METHODS

The samples give positive results in the ELISA TEST are further subjected to RAPID METHODS for the confirmations of positive results.

The Rapid Methods used are :-

i) UNI-GOLD

ii) TRIDOT

UNIGOLD

The Trinity Biotech Uni-Gold™ HIV test is a single reagent assay for the detection of antibodies to human immunodeficiency virus type-1 and 2 in serum, plasma or whole blood.

PRINCIPLE

Recombinant proteins representing the immunodominant regions of the envelope proteins of HIV-1 and HIV-2, glycoprotein gp41, gp120 (HIV-1) and glycoprotein gp36 (HIV-2) respectively are immobilized at the test region of the nitrocellulose strip. These proteins are also linked to colloidal gold and impregnated below the test region of the device. A narrow band of the nitrocellulose membrane is also sensitized as a control region.

During testing two drops of serum, plasma or whole blood is applied to the sample port, followed by two drops of wash buffer and allowed to react. Antibodies of any immunoglobulin class, specific to the recombinant HIV-1 or HIV-2 proteins, will react with the colloidal gold complex moves chromatographically along the membrane to the test and control regions of the test device. A positive reaction is visualized by a pink/red band in the test region of the device.
A negative reaction occurs in the absence of human immunoglobulin antibodies to HIV in the analyzed specimen. Consequently no visually detectable band develops in the test region of the device. Excess conjugate forms a second pink/red in the control region of the device. The appearance of this band indicates proper performance of the reagents in the kit.

**QUALITY CONTROL**

Good Laboratory Practice necessitates the use of control specimens to ensure proper device performance at least once daily. A built in procedural control on the test device indicates that the test is functioning correctly. A pink/red band should always appear at the control window.

**TEST PROCEDURE**

1) If any reagent / sample has been in refrigerated storage, remove and allow to stand for at least 20 minutes to reach room temperature.

2) Remove the required number of Trinity Biotech Uni-Gold™ HIV test devices from their protective wrappers.

3) Label each test with the appropriate patient information.

4) Using one of the disposable pipettes supplied, fill with sample (serum/plasma/whole blood).

5) Holding the pipette over the sample port add two drops of sample (approx. 60μl) carefully.

6) Add 2 drops (approx. 60μl) of the wash reagent to sample port.

7) Allow 10 minutes for reaction to occur. The result should be read at the
end of the 10 minutes incubation time. Results are stable for at least 20 minutes after addition of sample to the device.

INTERPRETATION OF TEST RESULTS

NEGATIVE
A line in the control region only indicates a negative test result.

POSITIVE
A line of any intensity forming in the test region, plus a line forming in the control region, indicates a positive result.
UNI - GOLD™ TEST

The Photograph of Uni-Gold™ Test
(Left one → Showing 2 bands 1st in Test Region and 2nd in Control Region indicates positive reaction)
(Right one → Showing only one band in Control Region indicates negative reaction)
TRIDOT

The HIV TRI-DOT test is a visual, rapid, sensitive and accurate immunoassay for the differential detection of HIV-1 and HIV-2 antibodies in human serum or plasma using HIV-1 and HIV-2 antigens immobilized on an immunofiltration membrane. The test is a screening test for anti-HIV-1 and anti-HIV-2 and is for in vitro lab use only.

PRINCIPLE

HIV antigens are immobilized on a porous immuno-filtration membrane. Sample and solutions pass through the membrane and are absorbed into the underlying absorbent.

As the patient's sample passes through the membrane, HIV antibodies, if present, bind to the immobilized antigens.

Conjugate binds to the Fc portion of the HIV antibodies to give distinct pinkish purple DOT(s) against a white background.

TEST PROCEDURE

1) Add 3 drops of buffer solution to the center of the device.
2) Hold the dropper vertically and add 1 drop of patient's sample using the sample dropper provided (Use a separate sample dropper for each specimen to be tested).
3) Add 5 drops of Buffer Solution.
4) Add 2 drops of protein -A Conjugate directly from the conjugate vial.
5) Add 5 drop of Buffer Solution and read result.
IMPORTANT: IT IS IMPORTANT TO ALLOW EACH SOLUTION TO SOAK IN THE TEST DEVICE BEFORE ADDING THE NEXT SOLUTION.

INTERPRETATION OF RESULTS

NEGATIVE RESULT -
If only one DOT (only the control Dot) appears, the specimen is non reactive for antibodies either to HIV-1 or HIV-2. Interpret sample as non-reactive.

POSITIVE RESULT -
1) If two DOTS, one for the control and the other for HIV-1 appear, the specimen is reactive for antibodies to HIV-1.
2) If two DOTS, one for the control and the other for HIV-2 appear, the specimen is reactive for antibodies to HIV-2.
3) If all the three DOTS, one each for control, HIV-1 and HIV-2 appear, the specimen is reactive for antibodies to HIV-1 and HIV-2.

INVALID TEST -
If no DOT appears after the test is complete, either with clear background or with complete pinkish/purple background the test indicates ERROR.
TRIDOT TEST

The Photograph of Tridot Test
(Left one → Showing one round pink dot in Control Region
indicates negative reaction)
(Right one → Showing two round pink dots. 1\textsuperscript{st} in Control Region
and 2\textsuperscript{nd} in HIV-1 region, indicates positive reaction with infection of HIV-1)
3.7 SOCIO-ECONOMIC STATUS

Socio-economic status of the individual was recorded, social classification of study subjects here is based on the mean monthly per capita income, as recommended by Prasad (1961). The criteria used here, and given below is, in fact, an improvement over that of Prasad (1961). The method proposed by Kumar (1993) was used, to make classification update. We have used (2000-2001) consumer price index of Uttar Pradesh.

SOCIAL CLASSIFICATION

<table>
<thead>
<tr>
<th>Mean monthly per capita income (Rs.)</th>
<th>Social Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>5080 &amp; above</td>
<td>I</td>
</tr>
<tr>
<td>2640 – 5279</td>
<td>II</td>
</tr>
<tr>
<td>1590 – 2639</td>
<td>III</td>
</tr>
<tr>
<td>790 – 1589</td>
<td>IV</td>
</tr>
<tr>
<td>&lt; 790</td>
<td>V</td>
</tr>
</tbody>
</table>

Prasad (1970) classification was not used as it has become obsolete due to the considerable decline in the purchasing power of the rupee.

3.8 STATISTICAL ANALYSIS OF DATA

All the tables were shown in respect of numbers and percentages, statistical analysis of the data done to compute the prevalence rates of HIV amongst high risk groups and the association of various socio-demographic variables, risk factors, with the prevalence rates. The students ‘t’ test was used, to determine the P value and the show the significance of association between the two variables.

The formula used was –

\[ t = \frac{p_1 - p_2}{\sqrt{pq\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}} \]

Where,  
\( t \) = test statistics  
\( p_1 \) = prevalence of Ist group  
\( p_2 \) = prevalence of IInd group  
\( n_3 \) = Number of study subjects in Ist group  
\( n_2 \) = number of study subjects in IInd group
\[ P = \sqrt{\frac{(P_1)^2(n_1 - 1) + (P_2)^2}{n_1 + n_2 - 2}} \]

\[ q = 100 - P \]

Degree of freedom (d.f.) was calculated by –

\[ d.f. = n_1 + n_2 - 2 \]

Level of Significance : \( \alpha = 0.10; .025; .005 \)

**Critical value**: The tabulated Critical value of \( t \) at \( \alpha = 0.10; .025; .005 \) and \( n_1 + n_2 - 2 \) i.e. \( \infty \) (infinity) degrees of freedom is \( t_{0.10, \infty} = 1.282; t_{0.025, \infty} = 1.960; t_{0.005, \infty} = 2.596 \)

**Results were interpreted as**:

If \( t \) (Calculated) \( \geq t_{0.005, \infty} = 2.596 \) HIGHLY SIGNIFICANT

\( t \) (Calculated) \( \geq t_{0.025, \infty} = 1.960 \) SIGNIFICANT

\( t \) (Calculated) \( \geq t_{0.10, \infty} = 1.282 \) SIGNIFICANT

\( t \) (Calculated) \( < t_{0.10, \infty} = 1.282 \) NOT SIGNIFICANT