Chapter 4

Materials and Methods
4. MATERIALS AND METHODS

4.1 PREVALENCE OF GENITAL CHLAMYDIAL INFECTION IN THE APPARENTLY HEALTHY POPULATION OF TAMIL NADU

The prevalence study on genital chlamydial infection in the apparently healthy population of Tamil Nadu was carried out as a part of the AIDS Prevention and Control (APAC) project entitled, "Community prevalence of STDs in Tamil Nadu", funded by USAID and conducted in the Department of Microbiology, Dr.ALM PGIBMS in collaboration with Clinical Epidemiology Unit, Christian Medical college, Vellore and Meenakshi Mission Hospital & Research Centre, Madurai.

4.1.1 Population for the study

Based on a statistical study design, a representative sample size from the state of Tamil Nadu based on 1991 census data was chosen using 'Probability Proportional to Size' (PPS) cluster technique. Administrative districts of Tamil Nadu were listed and three districts were selected randomly. The selected districts were Tanjore, Ramnad and Dindigul and from each of these three districts, 30 clusters were selected using PPS method. The clusters were villages and wards from the rural and urban areas respectively based on the census data. 15 households were randomly selected
Map of Tamilnadu showing the three districts where the study was conducted

Districts of Tamilnadu

- State Capital
- State Boundary
- District Boundary

Andhra Pradesh
Karnataka
Kerala
Sri Lanka
from each of these clusters. Adults, both men and women aged 15–45 years in the elected households were registered. Informed written consent was obtained from the elected population before recruitment.

1.1.2 Sample size

The prevalence of STD in the community of Tamil Nadu was estimated to be approximately 5% (Kantharaj, 1992). The sample size was calculated with the precision of 2% and 95% Confidence Interval (CI) incorporating 10% non-responders. It was estimated that 600 subjects were needed per district and the total sample size was calculated to be 1800 subjects from the 3 districts. The clinical samples were collected from the selected subjects following a 'medical camp approach'. 90 camps (1 camp/cluster) were conducted. The study period was August 97–October 98.

4.1.3 Specimens

In each of the general medical camp, randomly selected, pre-identified study population, after informed consent were taken up for detailed clinical examination for STDs and recording in a pretested stratified proforma. Blood and urine samples collected at the campsites adopting all aseptic precautions along with urethral/endocervical swabs for the entire study were also used for this Chlamydia study. The sera were separated and aliquoted. The urine samples collected were centrifuged at 5000 rpm. After discarding the supernatant, 1 ml of concentrated urine
Plate 1: Research team starting from MMHRC, to the campsite

Plate 2: Campsite: Enrolment of participants for the study
Plate 3: Medical examination by doctors

Plate 4: Filling up of proforma for study subjects
Plate 5: Laboratory set up at campsite

Plate 6: Collection of blood sample from participant
Plate 7: Labelling of specimens
was aliquoted in sterile screw cap vials. The samples so prepared at the campsites, after proper labeling, sealing and packing were transported on liquid nitrogen or dry ice to Chennai by rail or road within 24 hrs after collection. On receipt at the Dept. of Microbiology, Dr.ALM PGIBMS, the specimens were checked for any leakage etc., and were kept frozen until tested. The sera were stored at -20°C and the urines were stored at -70°C until tested for PCR.

4.1.4 METHODOLOGIES

4.1.4.1 Serology-IgM ELISA:

The detection of IgM antibodies against *C. trachomatis* was done using a commercial kit (*C. trachomatis* IgM ELISA, Novum Diagnostics, Germany). Totally 1849 serum samples were tested.

Principle: This was a solid phase immunoassay where the microtiter strips were coated with *C. trachomatis* antigen. The patients’ serum samples were diluted and simultaneously absorbed with IgM-sample diluent containing anti-human IgG antibodies to eliminate competitive inhibition from specific IgG and to remove rheumatoid factor. Diluted patient specimens and ready to use controls were pipetted into these wells and incubated. *C. trachomatis* specific antibodies of positive specimens and controls bind to the immobilized antigens. After a washing step to remove unbound sample and control materials, horseradish peroxidase labeled anti-human IgM conjugate was added. After a second washing step to remove the unbound
Plate 8: Unloading of specimens from liquid nitrogen cylinder at the laboratory.
conjugate, the enzyme linked immune complexes formed were detected by incubating with TMB substrate and by colorimetric reading at 450 nm.

Procedure

1. 100μl of diluted serum samples and ready to use controls were pipetted into the appropriate wells of the microtiter plate. One well was left for a substrate blank.

2. The plate was covered and incubated for 1 hour at 37°C.

3. After the incubation, the contents of the wells were washed 3 times with 300μl of washing solution using an automated ELISA washer (ELX50, Biotech Instruments, USA).

4. 100μl of ready to use anti-IgM conjugate was added into all wells except the one for blank and was incubated for 30 min at RT.

5. The washing procedure was repeated as in step 3.

6. 100μl of TMB substrate solution was added into all the wells.

7. The plate was covered and incubated for 15 min at RT in the dark.

8. The reaction was stopped by adding 100μl of the stopping solution into all wells.

9. The OD of the wells was read at 450nm (ELX 800 ELISA reader, Biotech Instruments, USA) after adjusting ELISA micro plate reader to zero using substrate blank.
Calculation of the cutoff value (COV)

\[
\text{COV} = \text{MN} + 0.300
\]

Where MN is the mean negative control value.

Interpretation of the Results

The samples giving absorbance values more than 10% above COV were considered as positive. The absorbance values more than 10% below the COV were considered negative. The absorbance values from 10% above to 10% below COV were considered as equivocal and the test was repeated for those samples.

4.1.4.2 Urine PCR

A total of 1444 random urine samples were screened by PCR to analyse *C. trachomatis* positivity using the commercial Amplicor PCR kits from Roche Diagnostics.

Amplicor CT/NG multiplex PCR

Principle: The Amplicor CT/NG PCR test is based on four major processes: specimen preparation; PCR amplification of target DNA using biotinylated primers; hybridization of the amplified products to oligonucleotide probes specific to the target(s); and detection of the probe-bound amplified products by colour formation.
Specimen preparation

Epithelial cells, leukocytes and associated *C. trachomatis* and *N. gonorrhoeae* cells, collected on swabs or pelleted from urine, are treated with a detergent solution to release both the chlamydial DNA and the neisseria DNA. A second detergent solution is then added to prepare the lysed specimen for amplification.

PCR amplification

The processed specimens/controls are added to the amplification mixture in reaction tubes and are placed in a thermal cycler where PCR takes place for 40 cycles to generate ‘amplicons’ for CT/NG target DNAs and also for Internal Control (IC) DNA. The CT/NG internal control has been added to the master mix to identify processed specimens containing substances that may interfere with PCR amplification. The CT/NG internal control is a DNA plasmid with primer binding regions identical to those of the CT target sequence and a unique probe-binding region that differentiates the CT/NG internal control from target amplicon. The CT/NG internal control is introduced into each amplification reaction and is co-amplified with target DNA from the clinical specimen.

Selective amplification of target DNA from the clinical specimen is achieved in the AMPLICOR CT/NG test by the use of AmpErase (Uracil-N-Glucosylase), an enzyme catalyzes the destruction of amplified DNA containing deoxyuridine, but not natural DNA containing thymidine. The Amplicor mastermix reagent uses
Plate 9: Amplicor PCR kit

Plate 10: Preparative steps for PCR
deoxyuridine triphosphate (dUTP) in place of thymidine triphosphate (dTTP) as one of the dNTPs. When heated in the first thermal cycling step at 50°C, the contaminating amplicons if present are destroyed. AmpErase is inactive at temperatures above 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon.

**Hybridization/Detection reaction**

Following PCR amplification, the amplicon is denatured to form single-stranded DNA. Aliquots of this mixture are then added to microwell plates (MWP) containing oligonucleotide probes specific for the *C. trachomatis*, *N. gonorrhoeae* and the internal control. The detection of *C. trachomatis* amplified DNA, *N. gonorrhoeae* amplified DNA and/or IC amplified DNA can be performed at the user’s option. Following the hybridization, the MWP is washed to remove any unbound material and an avidin-horse radish peroxidase (Av-HRP) is added to each well. The Av-HRP binds to the biotin-labeled amplicon captured by the plate-bound oligonucleotide probes. The MWP is washed again and a substrate solution and the colour reaction is measured colorimetrically at 450 nm using a MWP reader.

**Procedure**

**Specimen Preparation**

1. 500μl of *urine wash buffer* was added to appropriately labeled screw capped specimen preparation tubes.
2. After freeze thawing and vortexing the urine samples, 500μl of each of the samples were added into appropriate tubes using separate aerosol barrier tips. The tubes were vortexed for 10-15 seconds and incubated for 15min at 37°C.

3. Following incubation, the tubes were centrifuged at 12,500X g for 5min.

4. The supernatants were poured off and blotted on separate sheets of tissue paper.

5. 250μl of lysis buffer was added to the pellet and mixed by vortexing.

6. The tubes were incubated for 10 min at RT.

7. 250μl of specimen diluent was added and vortexed.

8. Centrifugation was done at 12,500X g for 10 min.

9. Supernatant (50μl) was used for PCR amplification.

**Preparation of the controls**

CT and NG controls were prepared in separate screw capped tubes.

1. *Working controls* (WC) were prepared by adding 100μl CT/NG DNA to 1ml of the specimen diluent.

2. *Processed working controls* (PWC) for PCR amplification were prepared by adding 250μl of CT/NG working controls (WC) to 250μl lysis buffer and incubating the tubes for 10 min.

3. 50μl of the PWC was used for amplification reaction.
(Note: For CT detection, CT-DNA was used as the positive control and NG-DNA as negative control. For NG detection, NG-DNA served as the positive control and CT-DNA as the negative control).

**Amplification Reaction**

1. *Working master mix* for the amplification reaction was prepared by the addition of 100µl of IC DNA to the vial of master mix reagent.
2. 50µl of the *working mastermix* was aliquoted aseptically to appropriate number of reaction tubes.
3. 50µl of the processed specimens/controls were added to the appropriate tubes and the thermocycling tubes were placed in the sample block of the thermocycler.
4. The PCR consisted the following thermocycling profile:
   - 2 min 50°C
   - 5 min 95°C
   - 10 sec 91°C; 50 sec 62°C; 35 sec 72°C (35 cycles)
   - 5 min 72°C

*Post amplification step:* Using separate aerosol barrier tips, 100µl of denaturation reagent was added to the amplified product in each of the reaction tubes while in 72°C and then incubated for 10 min at RT.
Detection of the Amplified products

1. 100µl of hybridization buffer was added to each of the wells of probe coated microwell plate (MWP).

2. Using separate filter tips, 25µl of the denatured amplicons were carefully added to the appropriate wells of the MWP and mixed until the colour changed from blue to yellow.

3. The plate was covered and incubated for 1 hour at 37°C.

4. The plate was washed to remove the unbound material.

5. To each of the well, 100µl of Av-HRP conjugate was added.

6. Incubation was done for 15min at 37°C.

7. The plate was washed to remove any unbound conjugate.

8. 100µl of the substrate (TMB) was added.

9. The plate was incubated at RT in the dark for 10 min.

10. Stop reagent, 100µl was added and the OD readings were taken at 450nm.

Interpretation of Results

The samples giving OD values ≥ 0.8 were considered as positive and those with OD values less than < 0.2 as negative; Samples giving OD values ≥ 0.2, < 0.8 were considered as equivocal.
4.1.5. QUALITY CONTROL STUDY

A quality control study for the urine PCR testing was performed for 40 random urine specimens, which were already tested by polymerase chain reaction using Amplicor PCR kit at the Dept of Microbiology, Dr ALM PGIBMS, Chennai. These samples were coded and subjected for ligase chain reaction using LCx, Abbott-LCR kit at the Speciality Ranbaxy Laboratories, USA. The results were decoded and compared.
4.2 GENITAL CHLAMYDIAL INFECTIONS IN SYMPTOMATIC STD PATIENTS

4.2.1 PATIENTS AND MATERIALS

143 symptomatic patients (different categories as mentioned below), both men (n=63) and women (n=80) who attended the STD outpatient clinic at the Government General Hospital, Chennai (November 1998-August 2000) were studied.

<table>
<thead>
<tr>
<th>Clinical groups</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urethritis</td>
<td>58 (40.5)</td>
</tr>
<tr>
<td>Cervicitis</td>
<td>53 (37.1)</td>
</tr>
<tr>
<td>PID</td>
<td>22 (15.4)</td>
</tr>
<tr>
<td>Epididymitis</td>
<td>5 (3.4)</td>
</tr>
<tr>
<td>Infertility</td>
<td>5 (3.4)</td>
</tr>
</tbody>
</table>
Plate 11: A case of urethritis showing mucoid discharge

Plate 12: A case of cervicitis showing mucopurulent discharge
4.2.2 Specimens

Four types of clinical specimens were collected from the above patients:

1. *Urethral and endocervical swabs* from males and females respectively for the isolation of *C.trachomatis* in cell culture, antigen detection and PCR.

2. *Blood* for serology

3. *First void urine (FVU)* for PCR

Collection of specimens

Because chlamydiae are obligate intracellular organisms that infect the columnar epithelium, the objective of specimen collection procedures is to obtain columnar epithelial cells from the endocervix or the urethra. Specimens for *Chlamydia* tests were obtained after taking specimens for gram staining and *N.gonorrhoeae* culture. Collection procedures were followed as per CDC guidelines.

Before collection of *endocervical specimens* from the female endocervix, a large cotton swab was used to remove excess exocervical mucus, all secretions and discharge from cervical os. A sterile swab (cotton tipped swab with plastic shaft) was then inserted 1-2 cm into the endocervical canal past the squamo columnar junction and was rotated several times for 10-30 seconds against the wall of endocervical canal with sufficient pressure to dislodge cells. Without touching any vaginal surfaces, the
swab was withdrawn and placed in the transport medium for culture (0.2M sucrose phosphate buffer which contained 5% New born calf serum and antibiotics). Another swab specimen was collected in the similar fashion and a smear was prepared for DFA test. The sampling swab was rolled over 1 cm circular area on a glass slide, smear was air dried and fixed with methanol.

In collection of male urethral specimens, the patients were instructed not to urinate for 2 hours before sampling. A sterile cotton swab was gently inserted into the endourethra 2-4 cm, rotated to collect epithelial cells, withdrawn and was placed in the transport medium for culture. Another swab was collected in similar way to prepare a smear in the slide for the DFA test.

Blood was collected aseptically. First void urine samples (10-15ml) from the enrolled patients were collected in sterile containers. All the specimens were transported on ice to the laboratory and processed immediately. The specimens for culture were inoculated on the same day. Whenever there was a delay, they were stored at -85°C until processed. All aseptic precautions were taken. The sera after separation were stored at -20°C until tested. The urine samples were centrifuged and the sediment in 1ml volume were stored at -70°C until tested for PCR.

Serum samples collected from 50 apparently healthy adults (voluntary blood donors (n=15), healthy adults who visited the Dept. of Microbiology for prevaccination screening for hepatitis B (n=30), pregnant women (n=5) were used as controls for serological comparison only. The following strains were used in the
present study. *C. trachomatis* serotypes B, E, L1 and L2 were kindly provided by Dr. W.H.F. Goessens, University of Rotterdam, The Netherlands. Purified EB suspensions of *C. trachomatis* (L2), *C. pneumoniae*, *C. pittasi* and purified DNA samples of the above three strains were kindly provided by Dr. Fay Betsou, Biobanque de Picardie, University hospital, Amiens, France).

4.2.3 METHODOLOGIES

Patient details like age, sex, clinical features, symptoms, sexual history etc., were recorded in the prescribed proforma (Annexure-I). An informed consent was obtained from all the patients.

4.2.3.1 Isolation of *Chlamydia trachomatis* in cell culture

Cell culture and maintenance

McCoy cells derived from mouse connective tissue fibroblasts (National Centre for Cell Science, Department of Biotechnology, University of Poona, Pune, India) were used for the isolation of *C. trachomatis*.

(i) Requirements for the maintenance of McCoy cell line and chlamydial Isolation

1. 25 cm² TC flasks (Nunc, USA)
2. 24 well TC plates (Nunc, USA)
3. Eagle's minimum essential medium (EMEM) containing Earle's salts, non-essential amino acids (Hi Media, India)
4. Sodium bicarbonate (Hi Media, India)
5. L-glutamine (Hi Media, India)
6. New born calf serum (Sterile, Mycoplasma free: Hyclone, USA)
7. Antibiotics (Vancomycin, Gentamycin, Amphotericin B: Hi Media, India)
8. Trypsin (Sigma, USA)
9. Ethylene diamine tetra acetic acid (EDTA: Stratagene GmbH, Heidelberg, FRG)
10. Glucose (Sigma)
11. Cycloheximide (Hi Media, India)
12. Methanol (Hi Media, India)

(ii) Preparation of medium for growth and maintenance of McCoy cells

Complete growth medium- I (for 1 litre)

1. Dehydrated EMEM Base (autoclavable) - 10.3g
2. Triple distilled water - 860ml.

The medium base was dissolved in triple distilled water, pH 7.5 and autoclaved. The following ingredients (solutions sterilized by 0.2μM membrane filtration using positive pressure) were added aseptically to the autoclaved medium base.
3. Sodium bicarbonate (7.5% solution) - 29.3ml
4. L-glutamine (200mM solution) - 10.0ml
5. New born calf serum - 100ml
6. Antibiotics
   - Vancomycin (100 mg/ml) - 0.25ml (25μg/ml)
   - Gentamycin (40 mg/ml) - 0.5ml (20μg/ml)
   - Amphotericin B (10mg/ml) - 0.5ml (5μg/ml)

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

(iii) Trypsin Versene Glucose (TPVG) for 100 ml:

1. Phosphate buffered saline, pH 7.2 - 100ml
2. Trypsin - 0.1g
3. EDTA - 0.2g
4. Glucose - 0.5g

Sterilized by positive pressure filtration through 0.2μM membrane filter (Sartorius AG, Germany), aliquoted and stored at -20°C. Sterility testing was done on all the batches.
Cultivation of McCoy cells and its maintenance

Fully confluent monolayer of McCoy cell culture in 25cm² bottle (Nunc, USA) was selected, the supernatant medium was decanted aseptically and 1ml of TPVG solution was added onto the monolayer, rinsed once and poured off. Again 1 ml of the same was added, spread all over the cell sheet and allowed to remain for 1min. After decanting the TPVG solution, the bottle was kept at RT for 2-3 min and tapped gently but firmly on the sides till the cells detach from the bottle. Complete growth medium-I was added to the bottle to form a uniform suspension. The concentration of the viable cells was adjusted to 1x10⁵ cells /ml after doing a cell viability assay trypan blue exclusion test. Depending on the number of viable cells, the cell suspension was divided and distributed (passage) in an appropriate ratio (eg. 1:4 to 1:5 ratio) in fresh bottles. The bottles were incubated at 37°C till they formed monolayers.

Determination of total cell count and viable cell number

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

Procedure: A cell suspension containing approximately 2-5 x10⁵ cells/ml was prepared in EMEM. 0.5 ml of 0.4% trypan blue solution was transferred to a microcentrifuge tube. To this, 0.3ml of EMEM and 0.2ml of cell suspension (dilution factor 5) were added and mixed thoroughly. The mixture was allowed to stand for 5
Plate 13: Normal McCoy cell monolayer
The suspension was viewed in a haemocytometer and looked for viable cells. Cell enumeration was determined by following calculations.

\[
\text{Cells per ml} = \text{average count per square} \times \text{dilution factor} \times 10^4 \\
\text{Total Cells} = \text{cells per ml} \times \text{original volume of fluid from which cell sample was removed}
\]

\[
\% \text{ Cell viability} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100
\]

B. Isolation of \textit{C. trachomatis}

(i) Preparation of chlamydial transport medium

\textit{0.2M Sucrose phosphate buffer (2SP)- For 100ml}

Phosphate Buffered Saline PBS (0.02M; pH - 7.2) was prepared. For the preparation of PBS, two solutions, solutions X and Y were prepared separately in triple distilled water.

Solution - X \hspace{1cm} \text{- NaH}_{2}\text{PO}_{4} \hspace{0.5cm} 0.3120g/100ml

Solution - Y \hspace{1cm} \text{- Na}_{2}\text{HPO}_{4} \hspace{0.5cm} 0.2839g/100ml
28ml of solution-X was added to 72ml of solution-Y. This was further made up to 200ml using triple distilled water. The buffer was then sterilized by autoclaving. The following ingredients were added aseptically and mixed together.

1. Phosphate buffered saline (0.02M) - 95ml
2. Sucrose - 6.84g (0.2M)
3. New born calf serum - 5ml
4. Antibiotics
   
   Vancomycin (100mg/ml) - 100μl (100μg/ml)
   Gentamycin (40mg/ml) - 50μl (20μg/ml)
   Amphotericin B (10mg/ml) - 125μl (12.5μg/ml)

After dissolving all the ingredients completely, the medium was sterilized by positive pressure filtration through 0.2μM membrane filter (Sartorius AG, Germany), aliquotted and stored at 4°C. Sterility testing was done on all the batches.

(ii) Preparation of medium for chlamydia1 growth

Complete medium-II (for 1 litre)

1. Dehydrated EMEM base - 10.3g
2. Triple distilled water - 760ml
The medium base was dissolved in triple distilled water completely and autoclaved. To the autoclaved medium base, sterile solutions of the following ingredients (sterilized by 0.2μM membrane filtration using positive pressure) were added.

3. Sodium bicarbonate (7.5% solution) 29.3ml
4. L-Glutamine (200mM solution) 10ml
5. Glucose (4.5% solution) 100ml
6. New born calf serum 100ml
7. Antibiotics
   Vancomycin (100 mg/ml) - 0.25ml (25μg/ml)
   Gentamycin (40 mg/ml) - 0.5ml (20μg/ml)
   Amphotericin B (10 mg/ml) - 0.5ml (5μg/ml)
   Cycloheximide (10 mg/ml) - 0.1ml (1μg/ml)

All the ingredients were dissolved. The complete medium-II for chlamydial growth after preparation and sterility testing was aliquoted and stored at 4°C.

Isolation Procedure

The isolation of *C.trachomatis* was done as per the procedure described by Iwen *et al.*, (1995) with modifications.
1. The swab specimens in 0.2M sucrose phosphate buffer were vortexed with 2-3 glass beads inside the tube.

2. 0.2ml of the specimen was inoculated in quadruplicates on to 80% confluent McCoy cell monolayers grown on coverslips in 24 well tissue culture plates. A positive control (*C. trachomatis*-serovar E) and a negative control (2SP transport medium) were included in each test.

3. The inoculam was centrifuged on to the cells at 900 X g for 60 min at 35°C in a centrifuge (Rota 4R-V/Fm, Plastocrafts, Mumbai, India) having microplate rotor.

4. Subsequently, the plates were incubated for 2hrs at 37°C in 5%CO2

5. The monolayers were rinsed with serum free medium to remove the inoculum and 1ml of chlamydial growth medium (Complete medium-II containing 1µg/ml cycloheximide and 10% of New born calf serum) was added.

6. The plates were incubated for 48-72hrs

7. The medium was removed from the wells; the monolayers in the cover slips were fixed with 95% methanol, stained using Lugol’s Iodine and observed for typical intracytoplasmic inclusion bodies of *C. trachomatis* under an inverted phase contrast microscope (Diaphot, Nikon Corp, Tokyo, Japan).

8. The cultures were further confirmed by IF using species-specific monoclonal antibodies against MOMP of *C. trachomatis* (Chlamyset Antigen FA, Orion Diagnostika, Finland). The cover slips, after removing from the 24 well plates were air dried and attached with nail polish to microscopic slides. The staining was done as per the manufacturer's instructions. The slides were observed under
fluorescence microscope (Optiphot-2, Nikon Corp, Japan) for typical uniform disc shaped elementary bodies (EBs) of *C.trachomatis*.

### 4.2.3.2 Antigen Detection - Direct fluorescent Antibody (DFA) testing

The urethral/endocervical smears from the patients were subjected for antigen detection by DFA staining using a commercial kit (Chlamyset Antigen FA, Orion Diagnostika, Finland).

**Principle:** The Chlamyset Antigen FA test is used for direct detection of chlamydial elementary bodies. The test is based on the use of fluorescein labeled monoclonal antibodies (Mabs against MOMP of *C.trachomatis*), which reacts with all serovars of *C.trachomatis* and Evan's blue as a counter stain to stain the cells. The chlamydial EBs after staining, appear as bright green dots on a red stained cellular background.

**Procedure**

1. The lyophilized reagent was reconstituted by adding 1ml of solvent (PBS) to the vial and dissolving completely.
2. 30µl of reconstituted reagent was added on to the fixed specimen in the slides.
3. The slides were placed in a moist container and incubated for 15min at RT.
4. After incubation, the slides were rinsed by dipping into double distilled water several times and were air-dried.
5. 20μl of mounting fluid was added to the slide and a cover slip was set over it without air bubbles.

6. The slides were examined under fluorescence microscope using 40X for screening and 100X magnification for verification.

Interpretation of the results

The test was considered if 2 or more typical bright green, punctate round elementary bodies were seen on a red stained cellular background at screening magnification. At higher magnification, the EBs appear as evenly fluorescing sharp-edged discs. The test was read negative if the stained specimen is free of chlamydial organisms.

4.2.3.3 Serological assays for C.trachomatis

(i) IgM ELISA

The detection of IgM antibodies against C.trachomatis was done using the commercial C.trachomatis IgM ELISA kit from Novum Diagnostics, Germany). The detailed methodology is as given in the section 4.1.4.1.
(ii) Detection of IgA and IgG antibodies to *C. trachomatis*

The detection of IgA and IgG antibodies to *C. trachomatis* was done by using a commercial indirect immunoperoxidase assay kit (IPAzyme Chlamydia, Savyon diagnostics, Israel).

This indirect immunoperoxidase assay is based on the following steps: In step one, the human serum to be tested is brought in contact with the antigenic material (*C. trachomatis* infected cells). Specific antibodies, if present will attach to the antigen, forming an antigen-antibody complex. After a rinsing step to remove the unbound materials, horseradish peroxidase (HRP) conjugate of anti-human IgA (alpha chain specific) or anti-human IgG (gamma chain specific) is added. If an antigen-antibody complex is formed in the first step, the peroxidase labeled secondary antibody will bind to the antibody moiety of the complex in the step two. A positive reaction, a blue to deep blue precipitate inside the infected cells can be seen with an aid of a light microscope following the enzymatic reaction of the peroxidase moiety with hydrogen peroxide and the chromogen reactant.

Procedure

1. The required numbers of slides were taken and were placed in a slide holder tray.
2. The following dilutions were done for each patient serum sample with IPAzyme wash buffer:
1:16 dilution for detection of IgA antibodies
1:64 and 1:128 dilutions for the detection of IgG antibodies.

3. 10μl of either a control serum (for IgA or IgG) or a dilution of a patient’s serum specimen were added to the respective wells of the slide.

4. The slides were placed in a moist chamber and incubated at 37°C for 45min.

5. Following the incubation, the slides were rinsed thoroughly with a light stream of IPAzyme buffer using a wash bottle.

6. The slides were blot dried by pressing the well surface of each slide briefly and gently over the blotting paper.

7. 10μl of HRP conjugate (anti-human IgA or anti-human IgG) was added into each well.

8. The slides were again incubated at 37°C for 45min.

9. The slides were rinsed and blot dried as in the step 5 and step 6.

10. 10μl of chromogen/substrate solution was pipetted out into each well.

11. Incubation was done for 15 min at RT.

12. The slides were rinsed and blot dried as in the step 5 and step 6.

13. Four small drops of mounting medium were placed on each slide and was covered with a cover slip.

14. The slides were observed under light microscope (Optiphot-2, Nikon Corp, Japan) with blue filter using 20X magnification.
Interpretation of the results

The presence of a blue precipitate in the infected cells indicates a positive reaction. The absence of a blue precipitate in the infected cells indicates a negative reaction. The results obtained were interpreted as follows as per the kit instruction:

<table>
<thead>
<tr>
<th>Assay</th>
<th>Dilution</th>
<th>Possible Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1  2  3  4  5  6  7  8</td>
</tr>
<tr>
<td>IgG</td>
<td>1:64</td>
<td>+  +  +  +  +  -  -</td>
</tr>
<tr>
<td>IgG</td>
<td>1:128</td>
<td>+  +  -  -  ±  -  -</td>
</tr>
<tr>
<td>IgA</td>
<td>1:16</td>
<td>+  -  +  ±  -  -  - +</td>
</tr>
</tbody>
</table>

1,2 & 3 = indicative of active infection

4 = Borderline of active infection

5 and 6 = Positive

7 = Rare possibility, repeat test

8 = Negative

4.2.3.4 Anti-HIV ELISA

Anti-HIV antibodies in the patients were detected using commercial kits. The initial screening was done using HIV CheX ELISA of Xyton Diagnostics, India
and those found positive were counterchecked by another ELISA kit, Genelavia Mixt (Sanofi Pasteur, France).

i) **HIV-CheX ELISA Kit**

This is an indirect enzyme immunoassay for the detection of the various antibodies associated to HIV 1 and/or HIV 2 in the human serum or plasma. HIV-CheX is based upon the use of synthetic peptides that represent the immunodominant epitopes of envelope glycoproteins from HIV 1 and HIV 2 and of a rabbit anti-human IgG-horseradish peroxidase conjugate.

**Procedure**

1. 250μl of diluent was pipetted out to the required number of wells in the dilution plate provided.
2. 5μl each of the controls (negative and positive) and the specimens were added to the respective wells and mixed well. One well contained only diluent, which served as the blank.
3. 100μl of each of the prediluted samples and controls and blank from the dilution plate was transferred to the corresponding numbered wells in the ELISA plate using a multichannel pipette.
4. The ELISA plate was covered and incubated at RT for 30 min.
5. The plate was washed 5 times with the wash buffer.
6. 100μl of working conjugate solution was added to all the wells and the plate and incubated at RT for 30 min.
7. The plate was again washed 5 times with the wash buffer
8. 100μl of working substrate solution was added to all the wells and the plate was left undisturbed for 10 min at RT in the dark.
9. 50μl of stop solution was added to all the wells to arrest the reaction.
10. The OD of the wells was read at 450nm taking 630nm as the reference filter (ELx 800 ELISA reader, Biotech Instruments Inc., USA) and the cut-off value was calculated.

Calculation of the COV

\[ \text{COV} = \text{Mean OD of Negative Controls} + 0.15 \]

Interpretation results

Samples with OD values less than the cutoff OD value were considered as non-reactive. Any sample having an OD value greater than the cutoff OD value was considered as reactive by this test.

ii) Genelavia Mixt ELISA Kit

This is an indirect enzyme immunoassay for the detection of the various antibodies associated to the HIV 1 and/or HIV 2 in the human serum or plasma. Genelavia is based upon the use of a solid phase coated with purified antigens (GP160 recombinant protein and peptides mimicking the immunodominant epitopes of the HIV
1 and HIV 2 envelope glycoproteins) and of peroxidase labeled anti-human IgG and IgM goat antibodies.

**Procedure**

1. 80μl of the sample diluent was added to each well.
2. 20μl of negative control, positive control, cut-off control serum and the samples were added to the assigned wells.
3. The microplate was covered with adhesive film and incubated at 40°C in a water bath for 30 min.
4. The plate was washed 3 times with the wash buffer.
5. 100μl of conjugate was added to all the wells, sealed and incubated at 40°C for 30 min, in a water bath.
6. The plate was washed 3 times with the wash buffer.
7. 100 μl of the substrate solution was added to all the wells and incubated in the dark for 30 min at 18-25°C.
8. 50μl of stopping solution was added to all the wells.
9. The OD of the wells was read at 490nm (ELx 800 ELISA reader, Biotech Instruments Inc., USA) and the cut-off value was calculated.

**Calculation of COV**

The mean absorbance of the cut-off control serum (ODR4) is calculated and the cut-off value is calculated by the following formula:
\[ COV = ODR4/10 \]

Samples with absorbance values \( \geq \) the COV value is considered to be positive

4.2.3.5 Polymerase Chain Reaction

(i) Amplicor CT/NG multiplex PCR

The Amplicor CT/NG multiplex PCR kit from Roche Diagnostics was used to detect both \( C.\text{trachomatis} \) and \( N.\text{gonorrhoeae} \) simultaneously from the urine samples. Both urine and swab specimens were subjected to internal control detection provided in the Amplicor kit to identify samples having PCR inhibitory substances. The detailed methodology is as described in 4.1.4.2.

(ii) In-house PCR systems

Polymerase chain reaction was carried out to detect the presence of \( C.\text{trachomatis} \) DNA in both urethral /endocervical swab specimens and the urine samples.

Requirements

Proteinase K (AB gene, UK)

Tris HCl (Sigma, USA)
Triton-X-100 (Sigma, USA)
Phenol (Sigma, USA)
Chloroform (Sigma, USA)
Sodium Acetate (Sigma, USA)
Magnesium Chloride (Sigma, USA)
Ethanol (Amresco, soln, Ohio, USA)
Nuclease free BSA (MBI fermentas, USA)
Deoxyribonucleotide phosphates (dNTPs: Life technologies-GIBCOBRL, USA)
Taq DNA polymerase (AB gene, UK)
Hot star Taq DNA polymerase (QIAGEN, USA)

A. Plasmid-based diagnostic PCR

The plasmid PCR assay was standardized as per Vogels et al., (1995) with modifications.

Primers: Oligonucleotide primers derived from sequences of the common endogenous plasmid of \textit{C.trachomatis} and generating a species specific, 200bp amplified product with all the known \textit{C.trachomatis} serovars were used.

\begin{tabular}{ll}
Sense Primer & T1: 5' CTA GGC GTT TGT ACT CCG TCA-3' \\
Anti-Sense Primer & T2: 5' TCC TCA GAA GTT TAT GCA CT-3'
\end{tabular}
(The primers were kindly gifted by Dr. Vogels, Laboratory for Medical Microbiology, University Hospital, EZ Groningen, The Netherlands.)

i) Extraction of DNA from urethral/endocervical swab specimens.

1. 400μl of the freeze thawed, vortexed swab specimens in the 2SP medium was centrifuged at high speed at 16,000 × g for 30 min.

2. The supernatant was removed and the pellet was dissolved in 40μl of the lysis buffer containing 1mM EDTA, 1% Triton-X-100 and 50mM Tris HCl (pH 7.5) and 400 μg/ml proteinase K.

3. The suspension was incubated for 60 min at 37°C.

4. After incubation, the lysate was heated for 10 min at 95°C to inactivate proteinase K and was centrifuged briefly.

5. 5μl of the supernatant was used as the template for PCR analysis.

ii) Extraction from urine specimens

1. 500μl of urine sample was centrifuged at 12,500 × g for 10 min.

2. The pellet was washed with 1ml of PBS and the supernatant was discarded.

3. 500μl of the lysis buffer was added (1mM EDTA; 1% Triton-X-100; 50mM Tris HCl pH 8.0; Proteinase K 400μg/ml) to the pellet.

4. The suspension was vortexed for 10 sec and was incubated for 60 min at 37°C.

5. The preparation was then extracted twice with a 1:1 mixture of phenol: chloroform and the organic phase discarded.
6. The DNA was precipitated from the aqueous phase by adding twice the volume of chilled ethanol and 1/10 volume of 3M sodium acetate, keeping at -70°C for 1 hr and centrifuging at 12,500 X g.

7. The pellet was washed with 70% ethanol, dried and dissolved in 50µl of sterile distilled water and stored at -85°C before use in PCR amplification.

**PCR Reaction Mix**

50µl of the reaction mixture was constituted by adding together the following components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl (100mM, pH-8.3)</td>
<td>5µl (10mM)</td>
</tr>
<tr>
<td>KCl (500mM)</td>
<td>5µl (50mM)</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>5µl (2.5mM)</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>1µl (200µM)</td>
</tr>
<tr>
<td>Nuclease free BSA (10mg/ml)</td>
<td>0.5µl (100µg/ml)</td>
</tr>
<tr>
<td>Sense primer (10µM)</td>
<td>0.5µl (0.1µM)</td>
</tr>
<tr>
<td>Anti-Sense primer (10µM)</td>
<td>0.5µl (0.1µM)</td>
</tr>
<tr>
<td>Taq DNA Polymerase (5U/µl)</td>
<td>0.2µl (1U)</td>
</tr>
<tr>
<td>Water</td>
<td>27.3µl</td>
</tr>
<tr>
<td>Template</td>
<td>5µl</td>
</tr>
</tbody>
</table>

Note*: 10µl of the template DNA was added in the case of urine samples and accordingly the volume of water in the reaction mix was adjusted.
Thermocycling Profile

The reaction mixture was subjected to the following thermocycling profile: 94°C for 1 min (denaturation), 52°C for 2 min and 72°C for 3 min (extension) in a PCR machine (PTC-100, MJ Research, Watertown USA). The cycle was repeated 40 times. Following PCR amplification, the amplified product was electrophoresed on a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml concentration). The gel was visualized under UV transilluminator and the 200bp product was compared with molecular weight marker (100bp) ladder (MBI fermentas, Amherst, NY, USA).

Restriction digestion analysis of the 200bp PCR product

For the confirmation of the PCR product, a restriction enzyme analysis was performed. The 200bp amplified product has a specific cleavage site for the restriction enzyme HpaII. The cleavage reaction was performed in volume of 20μl reaction constituted as following:

10x Reaction Buffer (L) - 2μl
50mM MgCl2 - 1μl (2.5mM)
5U/μl Hpa II - 2μl (10U)
Sterile DD water - 3μl
Template (PCR product) - 12μl
The reaction mixture was incubated for 37°C for 2 hrs and the enzyme was inactivated at 65°C for 20 min. The digested product was separated by electrophoresis on a 3% agarose gel and stained with ethidium bromide. The DNA fragments of size 126bp and 74bp were compared with the molecular weight marker (φ174 Hae III digest, AB gene, UK).

B. MOMP-based nested PCR

The MOMP- nested PCR was used as a confirmatory assay and was done for all the swab and urine specimens. The procedure was that of Lan et al., (1994) with modifications. The primers derived from the ompl gene of C. trachomatis were used. Primers were procured from GIBCO BRL, USA. Following were the primers used for the primary PCR:

Sero 1A: 5' ATG AAA AAA CTC TTG AAA TCG G-3' (1-22)
Sero 2A: 5' TTT CTA GAT/C TTC ATT/C TTG TT -3' (1068-1049)

Reaction mix – Primary PCR

Adding together the following compounds constituted 50μl of the reaction mixture

10X Taq Buffer - 5μl (1X)
MgCl₂ (25mM) - 3μl (1.5mM)
dNTPs (10mM) - 1μl (200μM)
Sense primer (10μM) - 1μl (0.2μM)
Anti-Sense primer (10μM) - 1μl (0.2μM)
Taq DNA Polymerase (5U/μl) - 0.2μl (1U)
Water - 33.8μl
Template - 5μl

Note*: 10μl of the template DNA was added in the case of urine samples and accordingly volume of water in the reaction mix was adjusted.

**Amplification Reaction**

A 'hot start' PCR was employed by using a special Taq DNA polymerase (Hotstar taq polymerase, QAGEN, USA). This hot start method was adapted for improved sensitivity as well as the specificity of the PCR. The PCR amplification started with 15 min at 95°C (This preincubation is required for the activation of hotstart taq polymerase) and continued with 49 cycles of amplification. Each cycle consisted of 95°C for 1min (denaturation), 45°C for 3min and 72°C for 3min (extension). The amplified product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and the gel was visualized under UV light. The PCR product (1.1kb) was compared with molecular weight marker (φ174 Hae III digest, AB gene, UK).
Nested PCR (nPCR)

The primary PCR negative samples were subjected to nested PCR for additional sensitivity. The primers used for the second round of reaction were:

Sero 2A : 5' TTT CTA GAT/C TTC ATT/C TTG TT -3' (1068-1049)
PCTM3 : 5' TCC TTG CAA GCT CTG CCT GTG GGG AAT CCT -3'

(55-84)

Briefly, 1μl of the primary PCR product was pipetted with an aerosol barrier tip into a prepared PCR mixture containing the above set of primers. The amplification conditions were the same as those of the primary omp1 PCR. The nested run of PCR generated an amplified product 55bp smaller than the primary omp1 PCR product. The 1045bp nPCR product was identified by running a 10μl aliquot of the product in 1.5% gel electrophoresis alongside the molecular weight marker (φ174 Hae III digest).

4.3 GENITAL CHLAMYDIAL INFECTION IN HIV SEROPOSITIVE WOMEN

4.3.1 PATIENTS AND MATERIALS

Specimens, endocervical smears from proven HIV positive STD cases (n=35) attending YRG AIDS care center, Chennai were analysed for C.trachomatis
positivity. Age matched controls (HIV seronegative women (n=25) were also screened.

4.3.2 Methodology: Antigen detection by DFA

The endocervical smears were tested for *C. trachomatis* specific antigen using the Chlamyset antigen detection DFA kit from Orion Diagnostika, Finland. The detailed methodology is as given in the section 4.2.3.2

4.4 STATISTICAL ANALYSIS

Data analysis was performed by using statistical software SPSS version 8.0. for windows. Analysis for cluster adjustment was done using SUDAAN statistical software (Shah *et al.*, 1997). Adjustments were made for three levels of cluster effect (district, cluster and households). The 95% CIs for prevalences were estimated incorporating the cluster adjustment. Comparison between different diagnostic tests, computation of their performance values with their 95% confidence intervals, significance testing by Chi-square analysis, Mc Nemar's test etc. were done using statistical software Epi Info version 6.04 (Dean *et al.*, 1996).