CHAPTER II

MATERIALS AND METHODS

1. Chlamydial antigen and cells lines

C. trachumatis serotype L_2, in frozen culture, was provided by the Armed Forces Research Institute of Medical Science (AFRIMS). Chlamydial antigens for micro-immunofluorescent test in the or of killed whole chlamydial elementary bodies were supplied by the Washington Research Foundation, U.S.A. The antigens were combined into 3 pools of closely related serovars namely Pool 1 : type C, H, I, J , Pool 2 : type B, E, D and Pool 3 : type G, F, K . Normal Yolk Sac used as control was also supplied by this institute.

McCoy cells were also provided by AFRIMS. Cycloheximide from Sigma, U.S.A. was added in this cells culture in order to reduce cellular metabolism thereby enhancing chlamydial growth.

2. Collection of specimens

Sera and urethral discharge were obtained from two hundred males attending the Venereal Disease Clinic, Division of Infectious Disease Control, Department of Health. These patients were diagnosed as non-specifie urethritis by clinicians and confirmed by Gram stain of urethral specimen showing no gram negative diplococci with $\geq$ 5 polymorphonuclear cells per high power microscopic field (60)

Studies for chlamydial infection in each patient included cultivation of organism from urethral specimen, antibody detection (IgG, IgM, IgA) from sera and secretion utilizing the

newly developed method of rapid immunoperoxidase in comparison with the standard m-IF test.

For isolation of chlamydia, urethral specimens were collected by inserting a small cotton-tipped aluminum stick (ENT swab : Medical wire and Equipment, England) 2-4 cm. within the urethra and rotating slowly to obtain cells or cellular elements. Special care should be taken to includes cells in the specimen since chlamydial was an intracellular bacterium (1, 2, 3, 4). The swab was then put into a plastic centrifuge tube containing 1.5 ml of chlamydial transport medium (2SP transport medium, Appendix III), immediately placed on wet ice and transported to the laboratory no later than 4 hrs.

Upon receipt in the laboratory, the specimens would be stored at 4°C if they were to be inoculated on the day of receipt, otherwise at -70° C if they were to be inoculated subsequently.

For antibody detection, the secretion was collected by inserting a small cotton swab with plastic tip which absorbed a volume of 40 ul into the urethra. The swab was then put into a small test tube (12x75 mm.) containing 280 ul of PBS pH 7.2 (Appendix II) thus,diluting the secretion to 1:8. The secretions were then transported on ice to the laboratory and stored at -70° C until assay.

As for serum, 5 ml of blood was taken by venipuncture. Sera were then separated and stored at -70° C until use.

3. Cultivation of C. trachomatis from specimens.

3.1 Culture and maintenance of McCoy cells (39, 125)

McCoy cells were cultivated in culture flasks containing 5 ml of growth medium (Appendix III) for 24-48 hrs

after which confluent monolayer were visualized under an inverted microscope. Growth media was then discarded and the cells washed with 3 ml PBS pH 7.2 (Appendix II) before 3 ml of 1% trypsin was added, let stand for 30-60 second and poured off. Cells were suspended in 5 ml of growth medium after which 1 ml was transfered to a new culture flask containing 4 ml of growth medium and incubated at 37° C for 24-48 hrs. These McCoy cultures would be subpassaged every third or forth day for maintenance.

3.2 Preparation of McCoy cells for chlamydial culture (125, 126)

Five ml of McCoy cells, 72 hrs grown in a culture flask, were trypsinized as described, adjusted to a concentration of 2 x 10^5 cells/ml in growth medium after which one ml each was transfered to a 13 mm diameter flat bottom vial (Sarstedt,W. Germany) containing a 13 mm diameter glass coverslip. Incubation at 37 °C for 24 hrs resulted in confluent cell growth on the surface of the glass converslip ready for inoculation.

3.3 Isolation of C. trachomatis from clinical specimens in cycloheximide treated McCoy cells (4, 15, 26, 39, 41)

Specimens must be processed properly before inoculation. They should be thawed rapidly in 37° C waterbath if frozen, mixed 1 min with 4-5 sterile glass beads on a vortexmixer to disperse the organism into the transport solution and let stand to sediment. The supernate was then ready for cultivation of C.trachomatis. Growth medium of the 24 hrs growth McCoy cells were poured off and 0.5 ml of the specimens were added duplicately followed by centrifugation at 3000 g for 1 hr at 35° C. The supernatant fluid was removed and 1 ml of maintenance medium with or without cycloheximide (Appendix III) was added. After 72 hrs incubation at 37° C the first vial was stained with iodine and inclusion bodies examined.

If C. trachomatis was not demonstrated, the second vial will be processed for further subpassage.

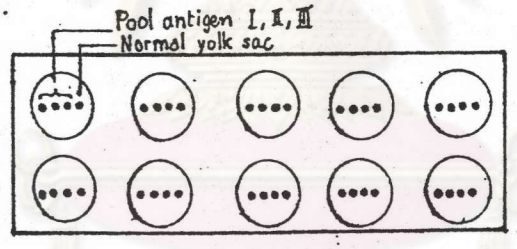
3.4 Iodine staining. (127)

The maintenance medium in inoculated vial were discarded,the cells fixed 10 min each in alcohol - formalin solution (Appendix IV) followed by absolute methanol before staining in Jone's iodine (Appendix IV) for 10 min. The coverslips were then mounted on glass slide with Jone's iodine glycerine (Appendix IV) and examined under a light microscope at 10x and 40x respectively. Positive culture was indicated when one or more inclusions was observed. (4, 26, 41)

4. Micro-immunofluorescent Test (m-IF)

4.1 Preparation of m-1F antigen slide (49, 125, 128)

Antigens were spotted with sharp pens onto wells of teflon-coated slides (Flow,North Ryde,Australia), each well containing 4 consecutive spots of 3 antigen pools and one yolk sac control.



m-IF antigen slide

Antigens were air dried for 30 min, fixed in acetone for 15 min at room temperature and stored at -70° C in a slide box in which they can keep up to a year.

4.2 Procedure of m-1F for specific IgG, IgM, or IgA antibody.

For m-1F test, frozen antigen slides were left at room temperature for 30 min before test. One drop of sera (1:8 and 1:16) or urethral secretion (1:8) was put down to cover each well on the slides. After incubation for 45 min in a moist

chamber, the slides were rinsed and agitated first in PBS pH 7.2 (Appendix II)for 10 min,then in distilled water and air dried for 30 min. Anti-human immunoglobulin (IgG, IgM or IgA) fluorescein conjugate, diluted to 1:20 in PBS pH 7.2, was applied onto each well. After incubated for 45 min, the slides were washed, air dried for 30 min and finally mounted with 1:1 dilution of glycerine and TRIS buffer pH 8 (Appendix II) before examination under a fluorescent microscope. Respective positive and negative control were included in each test. All incubation steps were carried out at 37° C in a moist chamber.

Positive results was indicated by the presence of sharply demarcated brightly fluorescing particle (Figure 3, and 4)

As for serum.1:8 and 1:6 were used for initial screening of chlamydial antibodies. Sera reactive to one or more pooled antigens will be further titrated for final positive dilutions.

5. Development of rapid immunoperoxidase test

5.1 Preparation of chlamydial inclusion antigen.

This technique utilized chlamydial inclusions in infected tissue culture as antigen.

5.1.1 Passage of C. trachomatis serotype L_2

McCoy cells were cultivated in 60 cm^3 tissue culture flask containing 5 ml of cell culture medium (Growth medium in appendix III) for 24 hrs until formation of healthy confluent cell monolayer were observed indicating cells in good condition. The growth medium was poured off and the cells incubated with 3 ml of diluted stock C. trachomatis serotype L_2 previously thawed at 37° C immediately before inoculation. After incubation at 37° C for 2-3 hrs. 7 ml of maintenance medium

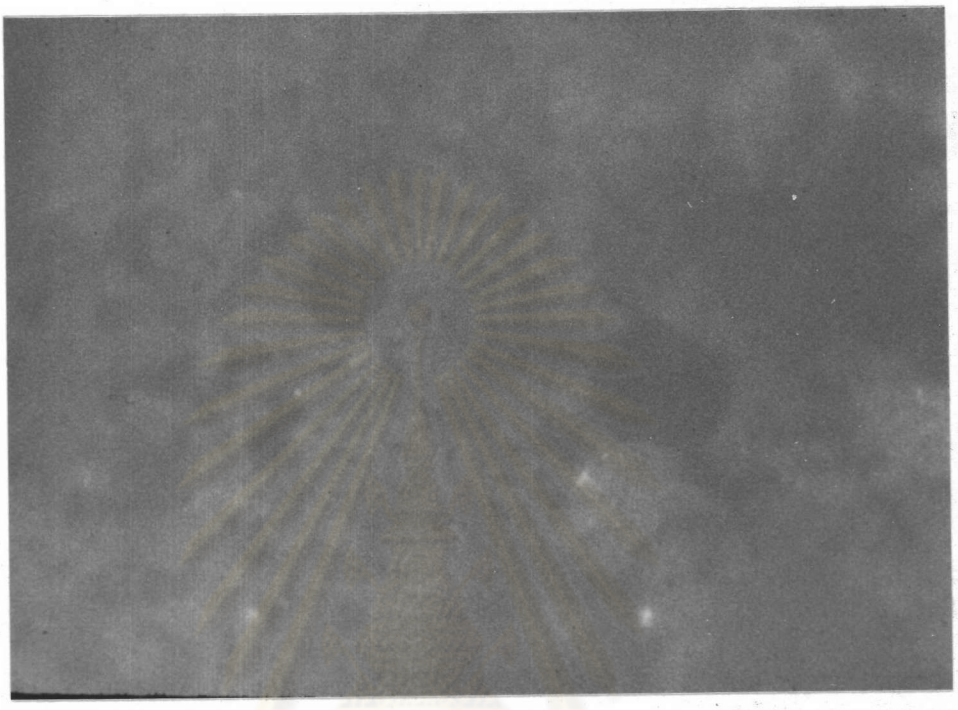


Figure 3 Negative control of m-IF test for C. trachomatis antibody (100x)

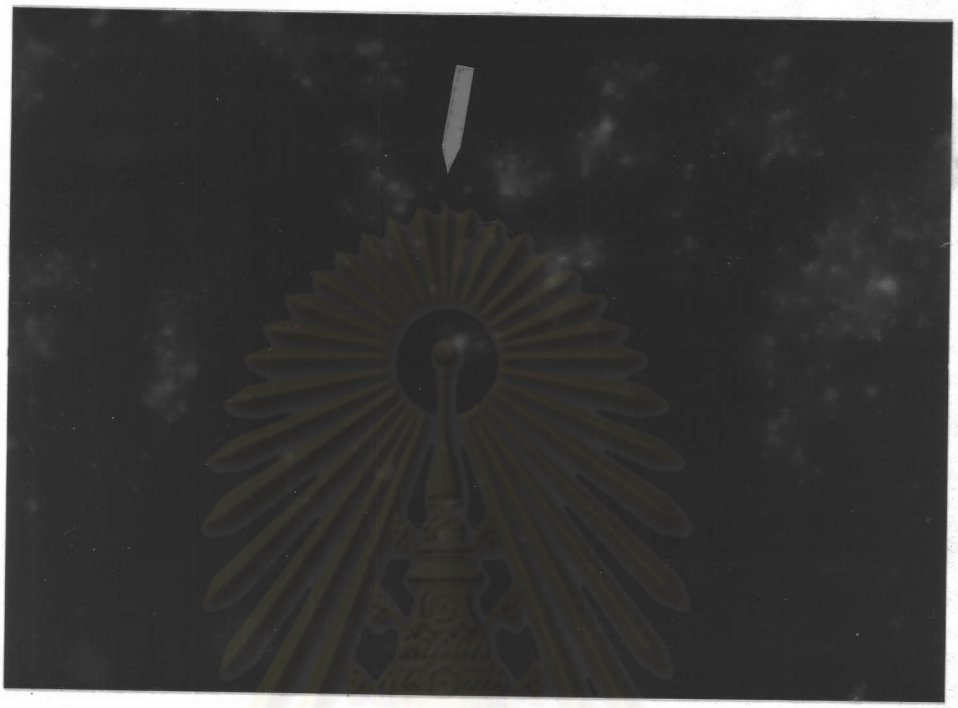


Figure 4 Positive high titer of m-IF test for *C. trachomatis* antibody(100x)

(Appendix III) was added and the incubation continued for 48-72 hrs. The amount of infected cells were checked under an inverted microscope and the culture was subpassaged until 100% infected cell culture was obtained.

For subpassage, maintenance medium was removed and the culture washed with 3 ml of PBS pH 7.2 (AppendixII), trypsinized with 3 ml of 1% trypsin solution (Appendix III) and suspended in 5 ml maintenance medium. The cell suspension was then transfered to a sterile 16x125 mm. screw capped tube containing 4-5 sterile 5 mm diameter glassbeads and mixed for 1 min on a vortex mixer upon which infected cells were disrupted and the organism released. This suspension was centrifuged at 200g for 5 min and the supernatant fluid containing C. trachomatis was then used as inoculum for further passage.

C. trachomatis could be preserved in such manner for a year or more by suspending in an equal volume of 4 sucrose phosphate solution (4SP) (Appendix III), and storing in aliquots of 1-2 ml at -70 C.

5.1.2 Coating of antigen on slide (55,56,124)

The 100% infected McCoy cells was diluted and utilized as a source of C. trachomatis for further infection. Fresh confluent monolayer of McCoy cells obtained after 24-48 hrs of cultivation in 250 ml tissue culture flasks was inoculated with 5 ml of appropriately diluted C. trachomatis and incubated at 37 C for 2-3 hrs before 15 ml of cycloheximide in maintenance medium was added without removing the previous culture fluid. Incubation was continued for 48 hrs by the time when 30-80% of infected cells were observed revealing typical intracytoplasmic inclusions under an inverted

microscope. The maintenance medium was removed and the chlamydial inclusion antigens were prepared by adding 5 ml of 1% trypsin solution for 1 min, immediately washed with PBS pH 7.2 before suspended in growth medium, the amount of which was previously determined* (table 1A,2A,Apendix V). Thirty microliters of the suspension was pipetted onto each well of the specially cleaned 10-well slides and cultivated at 37° C in a moist chamber for 24 hrs. The slides were gently rinsed with PBS pH 7.4 (Appndix II), air dried and fixed in acetone for 10 min at room temperature. The completely dried slides were then placed in a slide box and stored frozen at -70° C until use.

*Proper amount of antigen coating on glass slide was achieved by determining (1) the appropriate dilution of C. trachomatis giving rise to 30-80% infection in McCoy culture and (2) the suitable amounts of infected cells to be coated on slides. Consequently, varying dilution of C. trachomatis serotype L_2 suspension from 5.1.1 (1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:1000, 1:10,000 and 1:100,000) were inoculated on McCoy cells. The 30-80% infected culture were selected and subsequently diluted to contain varying concentration of McCoy cells ($1x10^3$, $1x10^4$, $1x10^5$, $2x10^5$ and $5x10^5$ cells/ml). Thirty microlites of the suspension was pipetted onto each well of a 10 well teflon coated slide which were then incubated for 24 hrs, washed and examined under a light microscope. Incubation was always carried out at 37° C in a moist chamber. The concentration of cells showing good distribution on slides was determined and chosen for subsequent studies.

5.2 Determination of factors affecting the rapid immuno-peroxidase assay (30, 55).

Positive control were selected from thirty non-

specific urethritis patients with positive chlamydial culture whose sera gave distinct positive result on m-IF test. Positive sera were combined in 3 sets of pooled sera each consisting of 10 sera. Negative control were obtained from 30 healthy military students whose sera were negative for chlamydial antibodies by the standard m-IF method. Three pooled negative control sera were also attained by combining ten negative sera in each set.

5.2.1 Suitable dilution of rabbit anti-human immunoglobulin peroxidase conjugate

Frozen antigen slides were thawed and washed in PBS pH 7.4 before reacting with 10 ul of control positive and control negative sera previously determinedby the standard micro-immunofluorescent test. The slides were incubated for 30 min, gently rinsed and agitated in PBS pH 7.4 for 10 min after which the slides were removed, excess liquid carefully wiped away and dried. Ten microliters of rabbit anti-human immunoglobulin peroxidase conjugate (Dako, Denmark) at various dilutions (1:20, 1:40, 1:60 and 1:80) were applied and incubated for 30 min. The slides were washed and dried as mentioned before a final 10 ul of substrate/chromogen (Appendix IV) was put on, incubated for 30 min in a moist chamber at room temperature. After washing as described, the slides were mounted in 10% PBS pH 7.4 in glycerol and examined under a light microscope at 10x or 20x. All incubation took place at 37° C in a moist chamber unless stated otherwise.

A dark blue cytoplasmic stain of C. trachomatis inclusion indicates positive reaction and the intensity of the color present in all fields was graded as follows : (Figure 5-9)

- = negative, the cells were clear, having no color staining

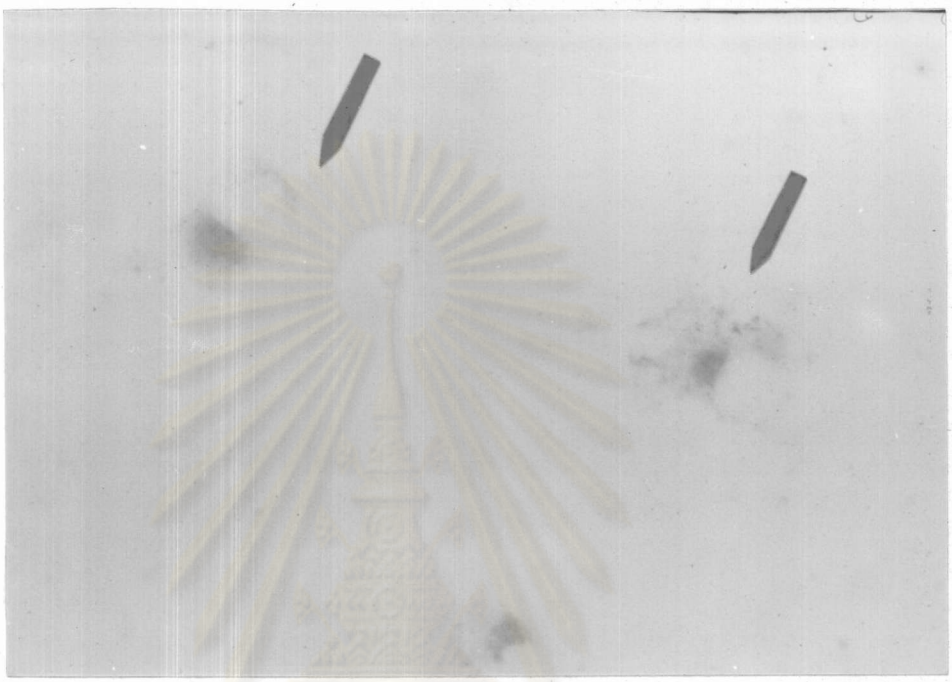


Figure 5 Negative control of IP test for C. trachomatis antibody; colorless inclusions (40x)

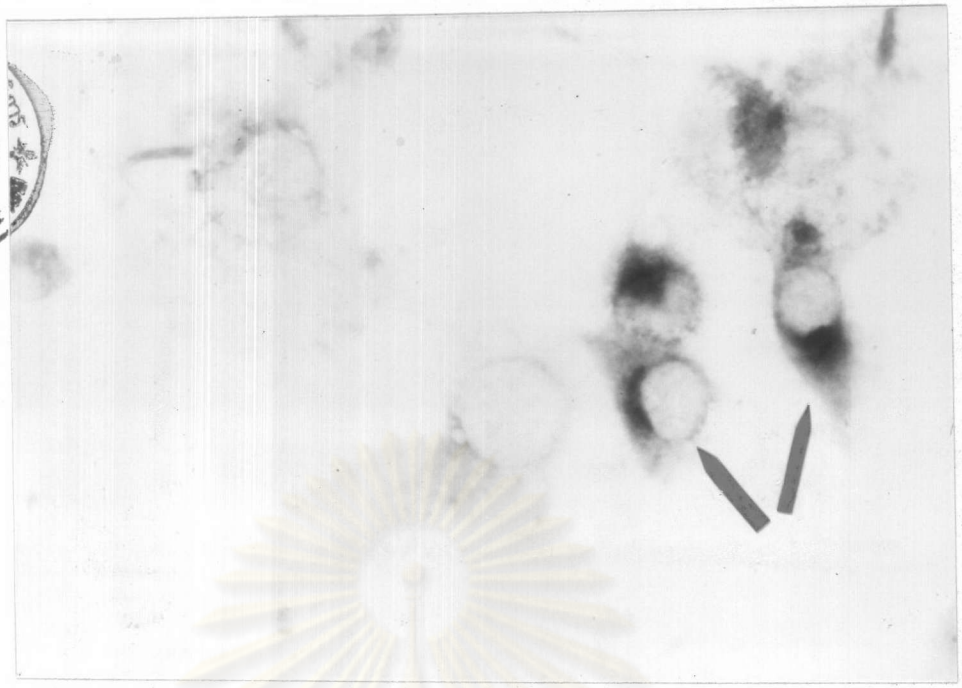


Figure 6 Positive 1^+ of IP test for C. trachomatis antibody ; light blue inclusion (40x)

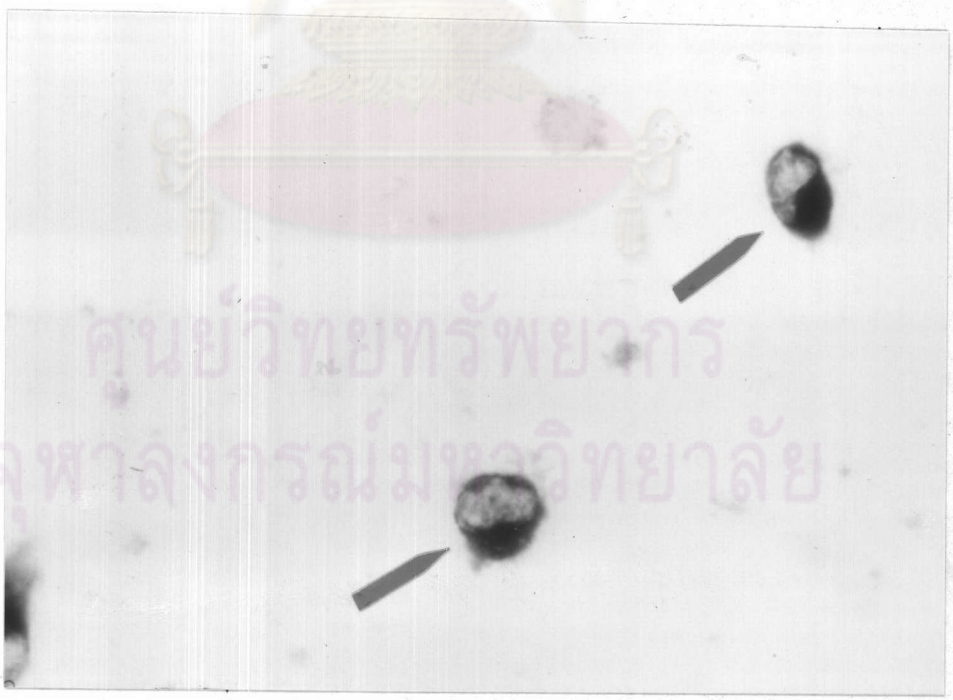


Figure 7 Positive 2^+ of IP test for C. trachomatis antibody ; blue inclusion (40x)

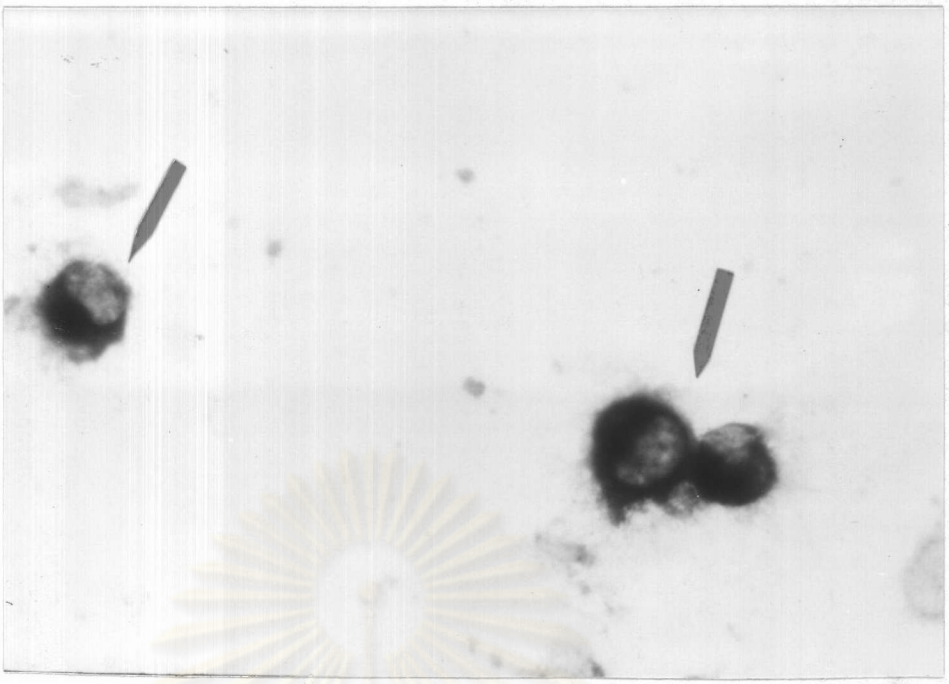


Figure 8 Positive 3^+ of IP test for C. trachomatis antibody; dark blue inclusions (40x)

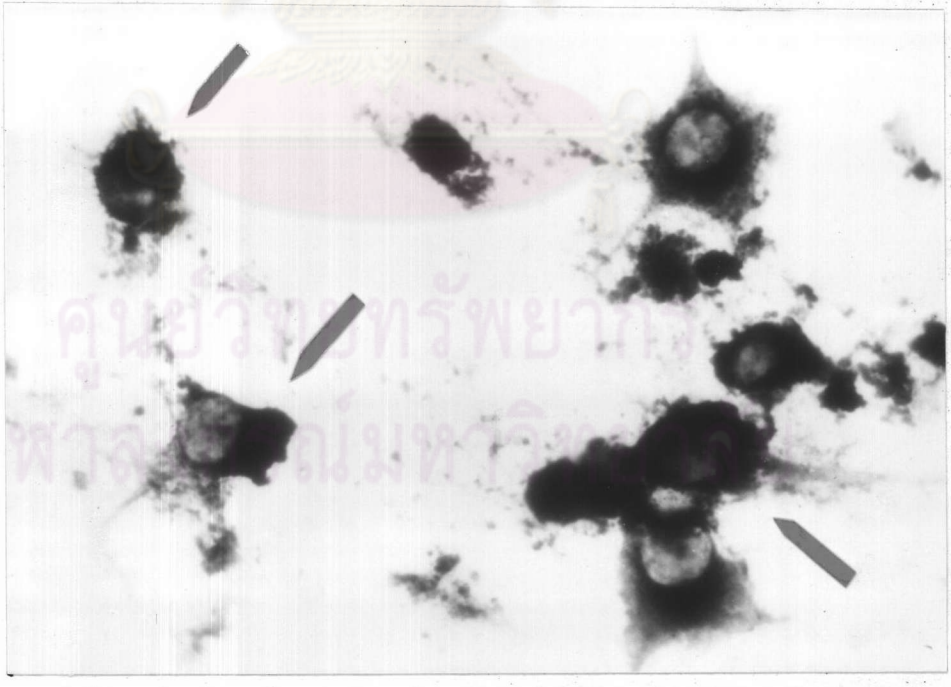


Figure 9 Positive 4^+ of IP test for C. trachomatis antibody; intense dark blue inclusions (40x)

1 = light or pale blue inclusion
2 = blue inclusion
3 = dark blue inclusion
4 = intense blue inclusion

5.2.2 Determination of time course for
a Chlamydial antigen and antibody reaction
b Enzyme-labelled antibody reaction

a. Antigen slides were allowed to react with anti-chlamydial antibodies at 37° C for a period varying from 15 to 120 min (15, 30, 60, 90 and 120 min). The slides were then processed as in routine assay using suitable dilution of rabbit anti-human Ig peroxidase conjugate previously chosen from 5.2.1 (1:20).

b. After an appropriate incubation period of chlamydial antigen and antibody reaction, 10 ul of 1:20 rabbit anti-human Ig peroxidase conjugate was added and the slides were incubated at 37° C for a period varying from 15 to 120 min (15, 30, 60, 90 and 120 min). The slides were then processed as in the routine assay.

5.2.3 Determination of optimal incubation temperature

Antigen slides in each set of four wells were allowed to react with antibodies at various temperature (room temperature RT, 37° C) for 30 min (from 5.2.2a). Ten microtiter of 1:20 anti-human Ig peroxidase conjugate was added and the slides were incubated at varying temperature (RT, 37 ° C) for 90 minutes (from 5.2.2b) after which the slides were processed as in the routine assay.

5.2.4 Determination of optimum condition for substrate/chromogen reaction

The procedure followed as above except that color development was achieved by varying incubation temperature and time for reaction with substrate/chromogen (RT for 30 min, 37° C for 15 min, 37° C for 30 min).

5.3 Shelflife and applicable storing temperature for antigen

Immediately after antigen slides were prepared, sets of 5 antigen slides were stored in -20°C and -70°C for 3 and 6 months. These frozen slides were checked for their efficacy in reacting with known positive serum by the rapid immunoperoxidase test in comparison with the standard micro-immunofluorescence (m-IF) test.

5.4 Rapid immunoperoxidase assay for specific IgG, IgM and IgA antibodies

Sera and secretion from 200 patients was assayed for chlamydial specific IgG, IgM or IgA antibodies using the procedure and all suitable condition from 5.2.

6. Avidin-biotin immunoperoxidase assay for specific IgA antibody to C. trachomatis (129,130)

Inclusion antigen slides (from 5.1) in frozen state were thawed and let dry at room temperature. Washed once in PBS pH 7.4 and airdry, 10 ul of each patient's secretion at 1:8 dilution was applied on each well. The slides were incubated at 37° C for 20 min, gently rinsed with PBS pH 7.4 from a wash bottle and agitated in a buffer bath for 10 min. After air dried, each well was covered with 10 ul of goat anti-human IgA biotin conjugate (Sigma, USA) and allowed to react at 37° C for 20 min. After washing and drying as mentioned, 10 ul of avidin-biotin

peroxidase complex (1:200, Sigma, USA) was applied, the slides were incubated at 37° C for 30 min before washing and drying as above. Finally, substrate/chromogen solution (Appendix IV) was applied, the slides incubated at room temperature for 30 min, followed by routine washing and drying. Slides were mounted in 10% PBS pH 7.4 in glycerol and examined under a light microscope 10x. A dark blue cytoplasmic stain of C. trachomatis inclusions was indicative of positive reaction, the intensity of which was graded as mentioned.

Optimum conditions of this test was determined in a similar fashion as described. In brief, the titration scheme was as follows :

6.1 Appropriate dilution of goat anti-human IgA-biotin conjugate was obtained by varying dilution from 1:10 to 1:80 (1:20, 1:40, 1:60, 1:80).

6.2 Suitable dilution of avidin-biotin peroxidase complex was acquired by titrating the complex from 1:10 to 1:80 (1:10, 1:20, 1:40, 1:60, 1:80).

6.3 Optimum incubation time was attained by varying the period of incubation as follows :

6.3.1 for chlamydial antigen and antibody reaction : from 20 to 90 min (20 min, 45 min, 60 min, 90 min).

6.3.2 for enzyme-labelled anti-human IgA-biotin conjugate reaction : from 20 to 90 minutes. (20 min, 45 min, 60 min, 90 min).

6.3.3 for avidin-biotin peroxidase reaction : from 20-90 minutes (20 min, 45 min, 60 min and 90 min).

6.4 Optimal condition for substate/chromogen reaction was

also obtained by varying temperature and time for incubation (30 min, at room temperature, 15 min at 37° C, 30 min at 37° C).

7. Statistical analysis

7.1 Two x Two contingency tables were utilized for the statistical analysis of IP test with m-IF as golden standard. The terms sensitivity, specificity, positive predictive value and negative predictive value of the tests have been used according to the following definitions.(57,58)

Sensitivity : the percentage of positive results of test in patients with positive golden standard test.

$$= \frac{\text{True Positives}}{\text{True Positives + False Negatives}}$$

Specificity : the percentage of negative results of test in patients with negative golden standard test.

$$= \frac{\text{True Negatives}}{\text{True Negatives + False Positives}}$$

Positive predictive value : the propability that a patient who had a positive test result would be positive by golden standard test.

$$= \frac{\text{True Positives}}{\text{True Positives + False Positives}}$$

Negative predictive value : the propability that a patient with a negative test result would be negative by golden standard test.

$$= \frac{\text{True Negatives}}{\text{True Negatives + False Negatives}}$$

7.2 Chi-square method was used to determine the correlation of antibodies in secretion with chlamydial infection. A "definite" diagnosis of chlamydial infection was made if C. trachomatis was present upon cultivation. In addition, patients were diagnosed as "possible" chlamydial infection if specific IgA antibody was found by standard m-IF test in secretion.(47)