CHAPTER III

MATERIALS AND METHODS

1. Study Population

The study population were pregnant women performed at antenatal clinic of Chulalongkorn Hospital from August 1984 through December 1985. At the begining, the pregnant women, total of 200, aged 20 to 30 years, and low risk for complications of pregnancy were recruited. The same pregnancy women of first trimester (12-16 weeks) and third trimester (36-40 weeks) were investigated. At the end of the study, 60 lost follow-up in the third trimester, therefore 140 were obtained the specimens twice.

First visiting in the clinic, the pregnancy women were interviewed in certain aspect. First, the information concerning the personal history such as history of STD, schooling were collected. Second, it's about the symptoms of genital infection for example,vaginal discharge, vaginal itching, dysurea, frequent urenation and vaginal bleeding within one month prior to visit, were asked.

2. Collection of Specimens (87,88)

The same doctor collected the cervical specimens by using a cotton-tipped aluminium stick (ENT swab; Medical Wire & Equipment Co. Ltd., Corsham, Wilts., England). A sterile speculum was inserted into the vagina to expose the cervical os and urethral meatus. The cervix should be cleaned with a sterile gauze. A sample then obtained by rotating the swab in the cervical canal at the squamocolumnar conjunctions. The swab was put into a centrifuge plastic tube containing 1 ml

transport medium (2SP in Appendix III), immediately place on wet ice, and shipped on ice to the laboratory on the same day as collected.

If, once a specimen was obtained, it was to be processed within 24 hr. Otherwise, the most suitable temperature are -60°C or lower.

3. Isolation and Culture Studies

3.1 Subpassage of McCoy cells in Roux-Bottle (87).

3.1.1 Check (under an inversed light stereomicroscope) that the cells have multiplied and have formed an almost confluent cell sheet, and that the cells are in good condition.

3.1.2 Pour off the cell culture medium (Appendix III) and wash the cells in PBS pH 7.2 (Appendix II).

3.1.2 Pour off the PBS and add 1% trypsin (Appendix III).

3.1.4 Pour off the trypsin and add 5 ml cell culture medium (Appendix III) per bottle (volume 2 oz.).

3.1.5 Transfer 1.0 ml cell suspension medium (in 3.1.4) to a new Roux-bottle containing 4.0 ml cell culture medium (Appendix III).

3.1.6 Incubate at 37°C.

3.1.7 Passage this McCoy cells each third or forth day of incubation at 37°C in the same way of 3.1.1-3.1.6.

3.2 Preparation of McCoy Cells Used for Chlamydial Culture(87)

3.2.1 Do in the same manner of 3.1.1 through 3.1.4.

3.2.2 Count the McCoy cells then dilute the cell to have 1.5×10^5 cells per ml.

3.2.3 Transfer 1 ml of cell suspension in 3.2.2 to a

flat bottomed plastic tube which had a 13 mm diameter coverslip.

3.2.4 Incubate cells for 1 day at 37°C.

3.3 Preparation of Clinical Specimens for Inoculating Cell Culture(85,94)

3.3.1 Thaw specimens rapidly in water-bath at 37°C if they are frozen at -60°C.

3.3.2 Agitate specimens vigorously with 4 to 5 sterile glass beads using vortex mixer about 1 min.

3.3.3 If specimens contained blood, centrifuge them for 5 min at 200 g.

The supernatant are ready for inoculation.

3.4 Culture of C. trachomatis in Cycloheximide Treated McCoy Cells

3.4.1 Check (under an inversed light stereomicroscope) that the McCoy cells in flat-bottomed plastic tube (prepared in 3.2) have formed confluent cells monolayer.

3.4.2 Remove cell culture medium (Appendix III).

3.4.3 Inoculate with 0.5 ml of specimens (prepared in 3.3).

3.4.4 Centrifuge 3000-4000 g for 1 hr at 35°C.

3.4.5 Remove inoculum and add 1 ml cell maintenance medium (Appendix III).

3.4.6 Incubate for 2-3 days at 35-37°C.

3.4.7 Stain with iodine.

3.4.8 Mount cover-slip cell side down on a slide

3.4.9 Examine by light microscopy at 100-400 magnification for presence of chlamydial inclusion bodies in the cytoplasm of McCoy cell.

3.5 Iodine Staining Technique for Detection of C. trachomatis (125)

3.5.1 Remove the cell maintenance medium (Appendix III) after incubated the specimens for 2-3 days at 35-37°C (in 3.4.6) and fix the cell with alcohol-formalin (Appendix IV) for 10 min.

3.5.2 Remove the alcohol-formalin and fix the cell again with absolute methanol for 10 min.

3.5.3 Remove the methanol and stain the cell with Jones' iodine (Appendix IV) for 10 min.

3.5.4 Mount the cover-slip with Jones' iodine glycerine (Appendix IV).

3.5.5 Examine by light microscopy at 10 x and 40 x.

4. Propagation of C. trachomatis

The remaining transport medium of clinical specimens containing positive for C. trachomatis were propagated by the following method for further performing the antimicrobial susceptibility test :-

4.1 Do the same method as Culture of C. trachomatis in Cycloheximide Treated McCoy Cells in 3.4.1 to 3.4.6 except use the maintenance medium without cycloheximide, since cycloheximide are toxic to C. trachomatis (107).

4.2 Put 4-5 sterile glass beads into the plastic vial, agitating vigorously about 1 min.

4.3 Transfer the cell debris and C. trachomatis suspension into a new sterile centrifuge plastic tube.

4.4 Centrifuge the tube for 5 min at 200 g to sediment the debris.

4.5 Use approximately 1 ml of the supernatant divided into 2 parts, each part had 0.5 ml and doing similary as 4.1.

4.6 Stain one vial with iodine and count the inclusions whether there were enough inclusions (about 5-6 inclusions/400 magnification) for seeding. In general, the specimen that had more than 1,000 inclusions/coverslip is always passage two times while the specimen that had less inclusions/coverslip must be subpassaged about 3 times.

4.7 Do the same as 4.2 to 4.4 if there were enough inclusions instead of recycling in 4.1-4.6

4.8 Add equally volume of 4SP (0.4 mol/1 sucrose phosphate butter in Appendix III) into the supernatant in 4.7; dispense 1 ml into small plastic vials, freeze at -60°C.

The method of propagation of C. trachomatis are summarized in Figure 2.

5. Antibiotic Susceptibility Test of C. trachomatis

5.1 Chlamydial Isolates

The chlamydial strains used were 30 recent isolates, 26 had been obtained from the cervix, and 4 had been obtained from infants with inclusion conjunctivitis.

5.2 Antibiotics Used

The antibiotics used in this study were tetracycline hydrochloride (Dumex Ltd.), erythromycin (Abbott Laboratories Ltd.)

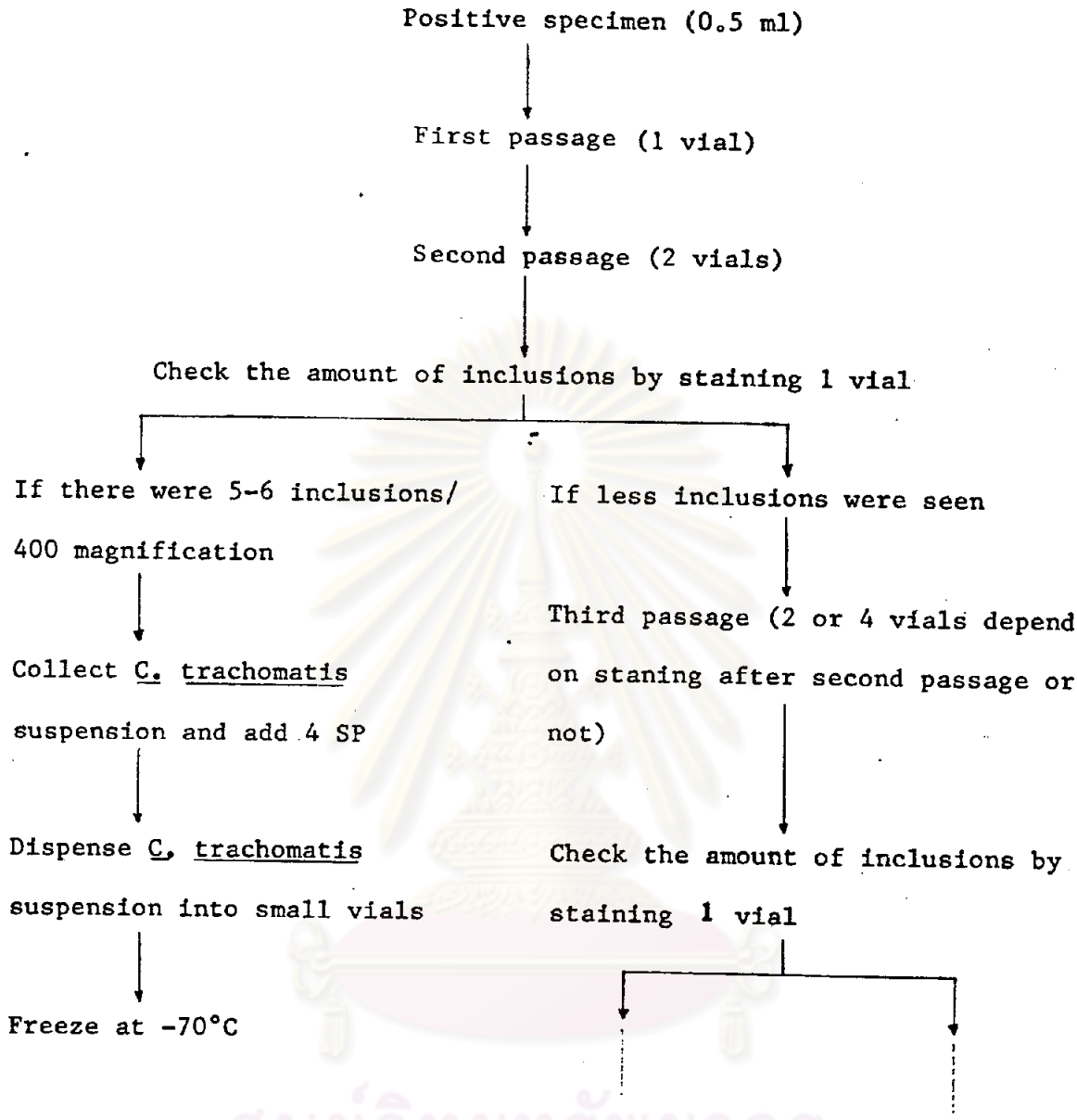

Figure 2 The method of propagation of C. trachomatis

and RU 28965 or roxithromycin (Hoechst Roussel Lab Ltd.).

5.3 Antibiotics Assay

5.3.1 Preparations of Antibiotics

5.3.1.1 Stock solution of each antibiotics = 1,000 µg/ml.

-Standard powder of tetracycline hydrochloride potency : 99.61%

Dissolve tetracycline hydrochloride powder 0.01004 g in 10 ml sterile distilled water.

-Standard powder of erythromycin potency: 935 µg/mg

Dissolve erythromycin powder 0.01070 g in 0.5 ml 95% ethyl alcohol, then add 9.5 ml sterile distilled water.

-Standard powder of RU 28965 potency : 954 µg/mg

Dissolve RU 28965 powder 0.01048 g in 0.5 ml 95% ethyl alcohol, then add 9.5 ml sterile distilled water.

Dispense these stock solution in 10 ml screw-cap sterile test tube (each tube had 1 ml), and store at -70°C.

5.3.1.2 Dilute these three antibiotics by the Table 8

5.3.2 Preparation of McCoy Cells

Do in the same manner as 3.2 except the medium used was changed to cell culture medium (Appendix III) which had no antibiotics.

Table 8 System for preparing dilutions of antibiotic*

Antibiotic solution		+ Cell maintenance medium without antibiotic	Final concentration
Volume	µg/ml	(volume)	(µg/ml)
1	1,000(stock solution)	9	100
1	100(from above)	9	10
1	10(from above)	9	1
2	1(from above)	2	0.5
1	1(from above)	3	0.25
1	1(from above)	7	0.12
2	0.12(from above)	2	0.06
1	0.12(from above)	3	0.03
1	0.12(from above)	7	0.016

* Modified from (126)

5.3.3 Preparation of Inoculum

Thaw rapidly the frozen seeding of C. trachomatis at 37°C, mix gently and divide it equally into two parts. Do both similarly as 3.4, Stain the first vial, count the inclusions to obtain approximate number. Dilute second vial with cell culture medium without antibiotics to have C. trachomatis 400 inclusions/ml.

5.3.4 Determination of MIC (Minimal Inhibitory Concentration)

5.3.4.1 Check the confluent cells monolayer in flat-bottomed plastic tube which prepared in 5.2 under an inversed light microscope.

5.3.4.2 Remove cell culture medium.

5.3.4.3 Inoculate with 0.5 ml of C. trachomatis to be tested (prepared in 5.3.3).

5.3.4.4 Centrifuge 3,000-4,000 g for 1 hr at 35°C.

5.3.4.5 Remove inoculum and replace 1 ml of cell maintenance medium containing the relevant dilution of antibiotic to be tested which prepared in 5.3.1(Positive control of C. trachomatis to be tested : Replace 1 ml of cell maintenance medium without any antibiotics)

5.3.4.6 Incubate for 2-3 days at 35-37°C.

5.3.4.7 Stain with iodine.

5.3.4.8 Mount coverslip cell-side down on a slide.

5.3.4.9 Examine by light microscope whether there were intracytoplasmic inclusions or not.

5.4 Interpretation of MIC

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic preventing the appearance of any inclusion bodies in the cell monolayer.

6. Serological Study

Blood was taken by venepuncture from 126 pregnant women in the third trimester (36-40 weeks) of pregnancy. Sera were separated and stored at -20°C. A modified micro-immunofluorescence (MIF) test was used to detect type-specific antichlamydial IgG and IgM in sera (20). The starting dilution of type specific IgG and IgM were 1 : 16 and 1 : 8 respectively (21). In the present study all sera were titrated against four pooled antigens; pool 1, C. trachomatis serotype A, B, Ba and C; pool 2 C. trachomatis serotype D, E, F, G, H, I, J and K; pool 3, C. trachomatis serotype L_1, L_2 and L_3, and pool 4, control normal yolk sac used to prepare C. trachomatis antigens.

In addition, 48 sera of normal women who never had sexual contact were included to find the lowest important titer of IgG.

6.1 Antigens

The chlamydial antigens used in this study were supported by WHO and prepared from yolk sac grown organims. The step of preparation of test antigens are in Appendix V.

It was noted that the antigens sent from WHO were chlamydial suspension in the EB form. they were processed by the following method.

6.1.1 Preparation of Test-Antigen Slide (87)

6.1.1.1 Using the sharp pen inoculate spot 3 pools antigen and 1 normal yolk sac onto the slide as the illustration (Figure 3).

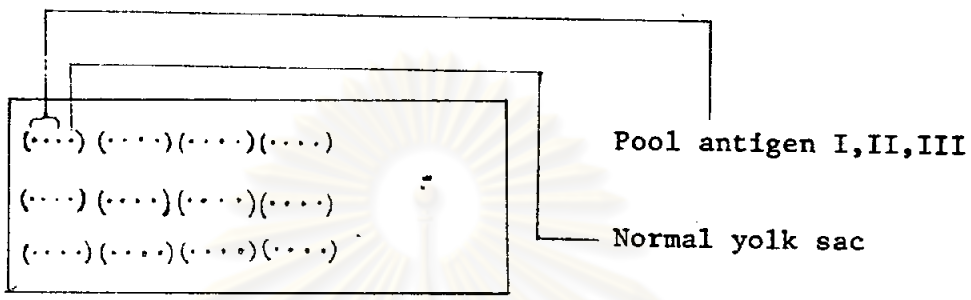

Figure 3 Test antigen slide

6.1.1.2 Let it dry for 30 min.

6.1.1.3 Fix 10 min in cold acetone.

6.1.1.4 Store -20°C in slide box until used, the antigen can be kept for 2-3 months. If the slides have been stored -70°C, they can stay for a year.

6.2 Method to Perform Micro-immunofluorescence Antibody Test for Detection of Antibodies to Chlamydia

The following method used in this study was kindly of WHO.

6.2.1 Take the slide antigen and leave it at room temperature for few min.

6.2.2 Dilute the test serum to 1:8 and 1:16 for sceening of antichlamydial IgM and IgG respectively.

6.2.3 Drop each serum dilution to cover each spot of the antigen in the same row.

6.2.4 Incubate at 37°C for 45 minutes in a moisture chamber.

6.2.5 Rinse with PBS pH 7.2 (Appendix II) and dip into PBS with magnetic stirrer for 10 min.

6.2.6 Rinse the slide with distilled water and air-dried.

6.2.7 For the detection of IgG and IgM, dilution of 1 : 20 of fluorescein isothiocyanate-labeled anti-immunoglobulin G and anti-immunoglobulin M (wellcome) will be used.

6.2.8 Drop the conjugate onto each spot.

6.2.9 Incubate at 37°C for 45 min in moisture chamber.

6.2.10 Rinse with PBS and then soak into PBS with magnetic stirrer for 10 min.

6.2.11 Rinse with distilled water and air-dried.

6.2.12 Mount the slide with 1 : 1 dilution of glycerine and TRIS buffer pH8 (Appendix II).

6.2.13 Examine under a fluorescent microscope at 10 x and then 40 x

6.2.14 The serum which screening test was positive were further two fold diluted and tested again to find the end point.

66.2.15 For each run of MIF, known positive and negative sera (which were kindly of WHO) should be included.