CHAPTER II

REVIEW OF THE LITERATURES

History

Chlamydia trachomatis was first visualized in 1907 by Halberstaedter and Prowazek, who found characteristic intracytoplasmic inclusions in the conjunctival scrapings from patients with trachoma (24). In 1911, Lidner et al found the similar organisms in opthalmia of the newborn, in urethra and cervical material from their parents. These chlamydial infections were therefore referred to as "inclusion blennorrhoeae" or "paratrachoma" (25). (inclusion conjunctivitis of the newborn caused by Chlamydia oculogenital and acquired during birth from the genital tract of the mother)

In 1930, the organisms that cause psittacosis were first isolated from affected humans and psittacine birds by Bedson et al (26). (Psittacosis is a human respiratory disease caused by Chlamydia psittaci and contracted by exposure to infectious material; usually fecal droppings from avian species.) Almost at the same time, the causative agent of lymphogranuloma venereum(LGV) was also isolated by Hellerstrom et al (27). (LGV is a sexually transmitted disease caused by C. trachomatis : serotype L_1, L_2 and L_3. It is predominantly a disease of lymphatic tissue). These isolations were done by intracerebral inoculation in mice. Because the trachoma agent is not infective for mice, it proved more difficult to recover. The first isolation by inoculating of LGV into the yolk sacs of embryonated hens' eggs was succeeded by T'ang et al,in 1957 (28). In 1959, Jones et al isolated Chlamydia trachomatis from the cervix of mother of an

infant with ophthalmia neonatorum (29). This was the first isolation of C. trachomatis other than LGV from genital tract. Then, in 1964, C. trachomatis were recovered from the urethras of men epidemiologically associated with conjunctivitis cases (30). Therefore, aetiological relationships of the organisms were proved. The organisms were referred to as "TRIC agents" to indicate their origin from either trachoma (TR) or paratrachoma, including inclusion conjunctivitis (IC).

All the early isolation studies before 1965 were performed using yolk sac isolation procedures and thus were not clinically relevant because they could take up to 6 weeks to provide definitive answers (31). A tissue culture procedure for isolation of the agents causing trachoma and inclusion conjunctivitis was first described by Gordon and Quan in 1965 (32). This technique has led to the current interest in venereally transmitted chlamydiae and the diseases that they cause in the genital tract. Large number of specimens can be screened and obtained the result of an isolation within 48 to 72 hr. Making the diagnosis clinically correct. In recent years, the clinical syndromes associated with these infections rapidly expanded.

Biology and Taxonomy

Characteristics of Chlamydiae

The chlamydiae are structurally complex microorganisms, that possing cell walls and membranes, analogous in structure to the cell walls of gram negative bacteria (33). Once considered to be large viruses because they are restricted to intracellular parasite and incapable of synthesizing ATP, so they were called "energy parasites" (1,2). They differ from viruses in having both DNA and RNA, having a discrete cell wall, and sensitive to antimicrobials or antibiotics.

They multiply within the host cell by binarry fission; therefore, from these properties, chlamydiae are bacteria.

Some of the properties of chlamydiae, compared with those of mycoplasmas, bacteria, and viruses are presented in Table 1.

Cellular morphology

Chlamydiae are non-motile, spheroidal organisms 1.5-2.0 μm in diameter upon their stage of development in a unique obligatory intracellular growth cycle (4). Electron microscopy has demonstrated a trilaminar cytoplasmic membrane indistinguishable from bacteria. Outside the cell membrane is slightly thicker trilaminar structure that represents a rudimetary cell wall of a more loosely organized peptidoglycan structure than that found in typical bacteria (34). Chlamydiae are considered gram-negative, but are stained weakly by safranin.

Genome

The genome is small, only 6.6-9.5 x 10^8 dalton, with approximately 6-8.5 x 10^5 base pairs (35). It is approximately half the size of neisserial or rickettsial DNA and less than one forth of the size of Escherichia coli DNA. Virus DNA is more smaller (bacteriophage T4 has only 2 x 10^5 pairs)(1).

Taxonomy

Order : Chlamydiales

Family : Chlamydiaceae

Genus : Chlamydia

Chlamydia trachomatis

C. psittaci

Table 1 Characteristics of chlamydiae in relation to those of mycoplasmas, bacteria and viruses*

Properties	Bacteria	Mycoplasmas	Chlamydiae	Viruses
Cell wall	rigid wall muramic acid	membrane lipid	wall muramic acid	- (protein)
Free living	+	+	-	-
Size	0.2-0.5 μm	0.25-0.5 μm	0.3-0.7 μm	23-300 nm.
Nucleic acid	DNA & RNA	DNA & RNA	DNA & RNA	DNA or RNA
Reproduction	binarry fission	budding	binary fission & budding	eclipse
Cultivation	artificial media	artificial media	cell	cell
Sensitive to interferon	-	-	+	+
Sensitive to antibitic	+	+	+	-

* Adapted from (1,2,31)

Because of a unique developmental cycle that differentiates them from all other micro-organisms, the chlamydiae have been placed in their own order, the chlamydiales (4). There is one genus chlamydia, and two species - Chlamydia trachomatis and C. psittaci (3). The terms "Chlamydia subgroup A" and "Chlamydia subgroup B" for C. trachomatis and C. psittaci introduced by Gordon and Quan (32), although still used in the literature, are abandoned to avoid confusion with the alphabettically designated C. trachomatis serotypes and serogroups.

The G-C content (G + C)% of C. trachomatis and C. psittaci are 44.4% and 41.2%, respectively (34). The DNA homology between the two species is less than 11%, as has been determined by DNA hybridization (34). Although DNA homology between C. psittaci and C. trachomatis are relatively little, they have the same unique developmental cycle, common antigens and similar biological and metabolic activity, justifying their inclusion within the same genus (36).

Chlamydia psittaci is a common pathogen of avian species and domestic mammals but only involves humans as a zoonosis (37). Some C. psittaci strains are sexually transmitted in their natural hosts, and one - the guinea pig inclusion conjunctivitis (GPIC) agent - may offer a potentially useful animal model for the study of sexually transmitted chlamydial infections and immunity (38,39). C. trachomatis seems to be a specially human pathogen (except for a few strains of rodent origin). The differentiations of the two species are on the basis of the sulfonamide resistance and failure of inclusions to stain with iodine in C. psittaci. Chlamydia trachomatis is sensitive to sulfonamides and has iodine-staining inclusions.(31,35,36) The characteristics of the two species are characterized in Table 2.

Table 2 Characteristic differentiating the two species of Chlamydia (35)

Characteristic	C. trachomatis	C. psittaci
Sulfonamide susceptibility	sensitive	resistant
Iodine staining inclusion bodies	yes	no
Morphology of micro-culture	dense	diffuse
(Guanine + Cytosine) % of DNA	44-45	41
Principal host(s)	humans	nonprimate vertibrates

C. trachomatis consists of three entities (i) the Trachoma/ Inclusion Conjunctivitis (TRIC) agent, (ii) the Lymphogranuloma venereum (LGV) groups of organisms, and (iii) the Mouse Pneumonitis (MoPn) agent (35).

Chlamydia trachomatis has 15 serotypes that can be divided into 3 major serogroups based on their infections.

(i) Serotype L_1, L_2, L_3 represent the agents causing lymphogranuloma venereum.

(ii) Serotypes A, B, Ba, and C are the agents responsible for endemic blinding trachoma.

(iii) Serotypes D, E, F, G, H, I, J and K are the sexually transmitted agents that cause inclusion conjunctivitis, newborn pneumonia, urethritis, cervicitis, epididymitis, salpingitis, acute urethral syndrome, and perinatal infections (1,5,35).

The serotype of A, B, Ba, C and D to K some times are called trachoma inclusion conjunctivitis (TRIC) agents. So in this category there are LGV and TRIC agents. LGV and TRIC differ not only in the disease they produce but in the types of cells they parasitize. Most C. trachomatis strain have a very limited host spectrum in terms of susceptible cell types. In the natural host TRIC appear to infect only squamocolumnar cells. They are not efficient in infecting macrophages and are not known to grow in polymorphonuclear leukocytes. LGV strains are more invasive and appear to be more efficient at replication in macrophages (1,5,31). The agent of lymphogranuloma venereum and trachoma inclusion conjunctivitis may be relatively easily differentiated. The former are lethal when inoculated into mice intracerebrally, whereas the latter are not. The agents causing lymphogranuloma venereum are

capable of spontaneous cell-to-cell serial transmission in tissue culture, whereas those causing trachoma-inclusion conjunctivitis will not readily grow in this manner or will require mechanical assitance (lysis of cells and centrifugation of inoculum into new cells) for good growth. The infectivity of TRIC agents is enhanced by pretreatment of the cells with diethylaminoethyl dextran and inhibited by pretreatment with neuraminidase. The LGV agents are unaffected by either treatment (1,35). The differentiation between TRIC and LGV agents are summarized in Table 3.

Growth cycle

Chlamydiae are obligatory intracellular bacteria because they lack the ability to synthesize high energy compounds such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP). These compounds, essential for metabolism and respiration, must be provided by the infected host cell, leading Moulder to coin the term "energy parasites" (40). They have a developmental cycle that is unique among bacteria.

Two main structures in the chlamydial growth cycle are recognized :-

- The extracellular form, generally referred to as an elementary body (EB), is a spherical bacterium 0.25 to 0.3 μm in diameter. It is surrounded by a rigid trilaminar cell envelope similar in composition to those of other gram-negative bacteria, except that the cell walls do not exhibit characteristic endotoxin properties. The DNA and ribosomes are condensed in the center of the organisms. It is an infectious stage (41).

- The intracellular form, referred to a reticulate body (RB), is much larger than EB and differes in many respects. It is surrounded by

Table 3 Differentiation between TRIC and LGV agents (35)

Characteristic	TRIC	LGV
Serogroups	A-K plus Ba	L_1 - L_3
Human infection	epithelial cells	invasive (to lymphnode)
Follicular conjunctivitis in monkey	yes	no
Mouse brain, lethal to infection	no	yes
Infection of Hela 229 :		
without centritugation	minimal	easy
enhance by DEAE-dextran	yes	no
Neuraminidase inhibition	yes	no
Receptor competition by killed		
TRIC	yes	no
LGV	no	no

a trilaminar envelope that is very fragile and flexible so that pleomorphism results. Ribosomes and other cytoplasmic constituents are distributed homogenously throughout the cytoplasm. It is an initial stage (41). A summary of the differing characteristics of EB and RB is shown in Table 4.

Chlamydial growth cycle (Frigure 1) are initially attachment and penetration by the close adhesion of elementary body (EB) into the susceptible host cells (42,43). The attachment process may involve specific receptor sites, which could determine naturally susceptible cell (44). The EB themselves, induce phagocytic process of the host cell. Once they penetrate the cell, they specifically inhibit phago-lysosomal fusion, so they remain within the phagocytic vesicle throughout the growth cycle.

Within 6-9 hr after ingestion, the EB enlarge, synthesize ribosomes and form RB. In this stage, they use the host cell pool of precursors to synthesize RNA, DNA and protein (45). The RB are metabolically active and multiply by binary fission. During maturation the RB lose the rigid honeycomb peptidoglycan subunit structure of the EB cell wall, so they are fragile and strictly intracellular (33). Large amounts of outer envelope bud off the RB surface and form vesicle within the immature inclusions containing chlamydial antigens, which may eventually become incorporated into host cell membrane (46).

By 20 hr after infection, some of the RB reorganize to form infectious EB. It is generally assumed the DNA in a single RB condenses and gives rise to single EB, perhaps because at the initiation of infection single EB clearly gives rise to single RB. However, electron microscopy often shows the presence of more than one site of DNA condensation within reorganizing RB, and these can devide so that two

Table 4 Characteristics of Elementary Body (EB) and Reticulate Body (RB) *

Characteristics	EB	RB
Morphology	Small, dense centered	Large, homogenous
RNA : DNA	1 : 1	3 : 1
Sonication	Resistant	Sensitive
Effect of trypsin	Resistant	Sensitive, lysis
Infectivity	+	-
Toxicity	+	-
Hemagglutinin	Present	Absent
Permeability	Slight	Marked
Envelope subunit	Present	Absent
Location	Extracellular	Intracellular
Metabolic activity	Inactive	Active
Size	0.2-0.3 μm	1.0 μm

* Adapted from (36, 41)

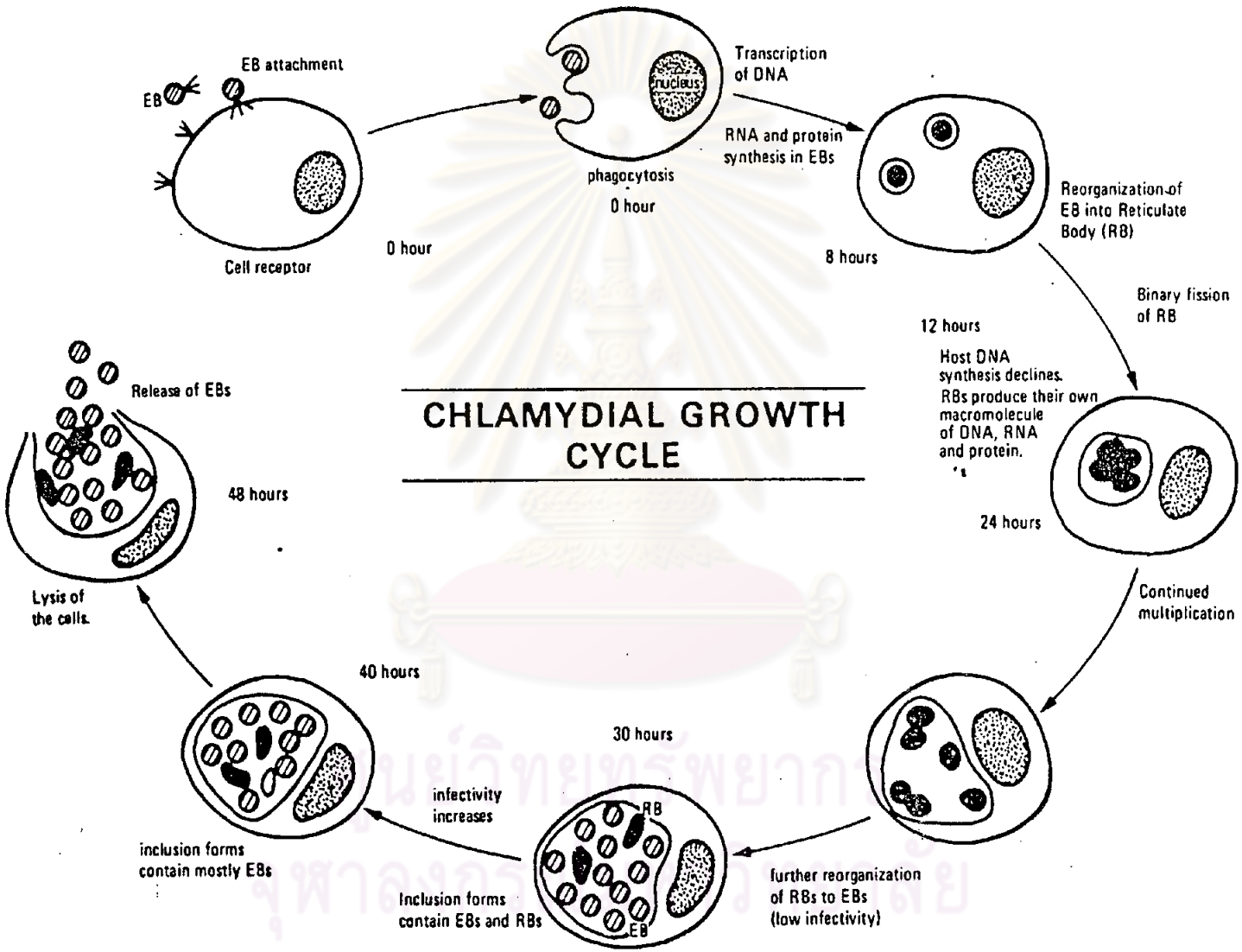

FIGURE 1. Chlamydial growth cycle. EB, elementary body; RB, reticulate body. (51).

to four EB are produced from single RB.

Beyond 18-24 hr, the numbers of EB increase and appear to predominate, although both EB and RB are found in the inclusion. This entire cycle takes place within the phagosome, which undergoes a large increase in size. At sometime between 48-72 hr, the cell ruptures and releases the infectious EB.

A clinical characteristics of infection with both C. trachomatis and C. psittaci is the tendency to produce persistent infection. At low multiplicity of infection, chlamydiae compete with the host cell for nutrients (47). The host cell is not killed and the infected cells can divide giving rise to infected progeny, but the mean generation time may be extended and the cloning efficiency reduced (48). Limitation of essential nutrients may greatly extend the chlamydial growth cycle (47), giving rise to persistently infected host cells.

The chlamydiae have cell wall to protect EB from the extracellular environment during transit from cell to cell. Synthesis of cell wall subunit is inhibited by penicillin (33) suggests a structure analogous to peptidoglycan. Furthermore, lysozyme, which cleaves the polysaccharide backbone of peptidoglycan, has been shown to reduce EB infectivity (49). However, the EB cell wall contains no more than 0.04% muramic acid which was suggested that penicillin inhibits cross linking of peptides attached to a polysaccharide that does not contain muramic acid (50).

Table 5. Serotypes and clinical spectrum of Chlamydia trachomatis infections (51)

Serotypes	Host	Infection*	Complications*
L_1, L_2, L_3	Women Men	Lymphogranuloma venereum	Vulvar/rectal carcinoma Rectal strictures
A,B,Ba,C	Men Women Children	Trachoma	Blindness
D,E,F,G, H,I,J,K	Men	Urethritis Postgonococcal urethritis Rectal Conjunctivitis Subclinical	Epididymitis Prostatitis Reiter's syndrome Sterility?
D,E,F,G, H,I,J,K	Women	Cervicitis Urethritis Subclinical Conjunctivitis	Salpingitis Perihepatitis Sterility Dysplasia? Postpartum endometritis? Prematurity? Stillbirth? Neonatal death?
D,E,F,G, H,I,J,K	Infants	Conjunctivitis Pneumonia Asymptomatic pharyngeal carriage Asymptomatic gastrointestinal tract carriage Otitis media?	

*Question mark indicates relationship not established.

Chlamydia trachomatis Infection in Man

Although C. trachomatis causes important disease in both men and women (Table 5), this thesis will emphasize on the informations relavant to chlamydial infection in the newborn.

Perinatal Infection with Chlamydia trachomatis

Several study have suggested that a substantial numbers of newborns are at risk to develop chlamydial infection (Table 6).

The infant delivered from chlamydial infected women has a 28-70% risk of acquiring the infection during passage through the birth canal (Table 6). Approximately 22-50% of exposed infants will develop conjunctivitis in 5 to 14 days of life, and 11-20% of the infants will develop pneumonia within 3 to 4 months (Table 6). In utero transmission is not known to occur,and infants delivered by cesarean section are not at risk of acquiring chlamydial infection unless there has been a premature rupture of the membranes(5, 52).

1. Conjunctivitis

Acute conjunctivitis of the new-born was initially described in 1910 (25). This mucopurulent conjunctivitis generally develop 5 to 14 days after birth (5). It is now recognized as the most common conjunctivitis in the first month of life.

The organism replicates extensively in superficial epithelial cells of the conjunctiva; causing considerable cell damage. There is an exuberant inflammatory reaction, and pseudomembranous may form. The disease often starts with a watery eye discharge which rapidly and progessively becomes very purulent. The eyelids are usually markedly swollen. The conjunctiva become very reddened and somewhat thicken throughout (5, 53).

Table 6 Prospective studies of mothers and infants for chlamydial infection

Year	Mothers positive per total	Positive infants followed				Reference
		Conjunctivitis	Pneumonitis	Culture	Seroconversion	
1977	18/142 (13%)	9/18 (50%)	ND	ND	12/18 (67%)	11
1979	30/340 (9%)	8/18 (44%)	2/18 (11%)	8/18 (11%)	3/18 (33%)	9
1979	6/322 (2%)	2/6 (33%)	1/6 (67%)	4/6 (67%)	ND	8
1979	36/900 (4%)	7/20 (35%)	4/20 (20%)	10/20 (50%)	14/20 (70%)	7
1980	23/273 (9%)	5/23 (22%)	ND	ND	ND	10
1981	240/1327 (18%)	21/95 (22%)	18/95 (19%)	27/95 (28%)	ND	12

Serological diagnosis is not helpful because of the presence of maternally transmitted antichlamydial IgG antibody and the uncertain appearance of IgM in this disease (5, 53).

2. Pneumonitis

Until 1975, it was assumed that chlamydial infection in the infant was restricted to the conjunctivitis (5).

Prospective studies have shown that conjunctivitis is not a prerequisite; and indeed, prevention of conjunctivitis by appropiate ocular prophylaxis does not prevent respiratory tract infection and pneumonia (54, 55).

Harrison et al induced chlamydial pneumonitis in two of the three baboons. All three animals developed prolonged nasopharyngitis, and seroconverted. These data support that C. trachomatis may be a causative agent for pneumonia in human infants (56).

The serum antichlamydial IgM response is an early response to primary infection. Schachter et al found the geometric mean of peak IgM titers in a small group of infants to be 1:28 in infants with conjunctivitis only, 1:448 in infants with pneumonitis and 1:48 in infants whose serum converted to specific IgG antibody but did not have clinical disease (7).

Prevention (5, 53)

There are three general approaches that can be employed as control measures. The first deal with the effort to reduced the reservoir. The routine use of tetracycline and erythromycin for the treatment of gonorrhoeae, nongonococcal urethritis, and their sexual contacts would remove chlamydial infection from the infective pool.

In addition, to prevent perinatal chlamydial infection, one can

substitute erythromycin oinment for silver nitrate in ocular prophylaxis for neonates. Since erthromycin is active against C. trachomatis and also effective in preventing gonococcal ophthalmia neonatorum. It is less irritating to the eye than the silver nitrate which is clearly ineffective in preventing chlamydial infections. It would seem appropiate to recommend that a routine shift be made to ocular prophylaxis with erythromycin oinment.

The third method would be third trimester simultaneous systemic treatment of cervically infected mothers and their sexual partners (the latter to prevent reinfection prior to delivery). The therapy must be effective for the mother and partner and non toxic to the fetus. So it involves routine testing of pregnant women for chlamydial infection and treating those found to carry the organism.

Antimicrobial Susceptibility

An essential preliminary to rational treatment of chlamydial infection is to study of the action of antimicrobial agents against chlamydia in vitro. The tecnique for determining antibiotic sensitivity which proved both simple and reproducible is cell culture method (57). Although in vitro susceptibility testing for C. trachomatis has not been rigorously standardized, studies to date have found relatively similar ranges for minimum inhibitory concentrations of individual antimicrobials against chlamydia (Table 7).

From Table 7, it is summarized that C. trachomatis is susceptible to tetracycline, macrolide (erythromycin, rosaramicin), sulfonamide and rifampicin. Tetracycline is generally considered to be the drug of choice, erythromycin is essentially equivalent to tetracycline in antimicrobial activity often used in infants and pregnancy. But it has been noted that

Table 7 Minimal inhibitory concentrations(MIC) of various antimicrobial agents against Chlamydia trachomatis

Antimicrobial agent	Range of MIC (µg/ml)	Reference
1. High activity		
Rifampicin	0.007-0.25	57-60
Tetracycline	0.008-0.06	61-63
Minocycline	0.015-0.03	59,64
Doxycycline	0.03-0.05	60,65
Oxytetracycline	0.06-0.1	57,59,65
Erythromycin	0.03- > 1	57-59,61,62,64
Chlortetracycline	0.125-1.0	58
Rosaramicin	0.015-0.25	59,61-64
2. Medium activity		
Ampicillin	0.25	58,60
Penicillin	0.01-50	57,58,60-62
Clindamycin	0.5-1.0	60-63
Mecillinam	0.25-0.5	59,60
Thiamphenicol	0.5	59
Cephaloridine	2.0	60
Sulphamethoxazole	4.0-50	59,60,65
Chloramphenicol	10	59,61
3. Low activity		
Spectinomycin	32-100	57,60
Trimethoprim	128-256	60
Cefuroxime	256	57,60
Lincomycin	512	60
Vancomycin	256	60

there are relative resistance of C. trachomatis to erythromycin in vitro (63). Sulfonamide (66) and rifampicin (67) fail to eradicate Ureaplasma urealyticum, which is the causative agents of NGU (68, 69), so they are not often used in clinical practice.

C. trachomatis is commonly persist after the gonorrhoeae has been treated (70), therefore, it is practical importance to investigate the sensitivity of antibacterial chemotherapeutic agents most likely to be used in treating sexually acquired infection. Table 7 shows that penicillin group, chloramphenicol , trimethoprim, aminoglycoside (gentamicin, etc), metronidazole, and cephalosporin (ceftriaxonem cefotaxime, etc) lack any effective in vitro activity against C. trachomatis. This suggests that these antimicrobials cannot be relied on to eradicate C. trachomatis in vivo espectially in acute PID which always use cephalosporin group.

The majority of clinical evidence regarding the effectiveness of various antimicrobials against C. trachomatis has been accumulated in men with nongonococcal urethritis (23, 65, 71-74). Two general principles have emerged from these studies:penicillin, ampicillin, and amoxicillin in single dose regimens given for treatment of gonorrhoeae usually do not eradicate concomitant chlamydial infection, but in high dose or prolonged treatment may effective (74), and seven or more days of treatment with tetracycline or macrolides eradicates C. trachomatis from nearly all men, at least determined by short term follow up (23,71, 73). Prolong used of trimetroprim-sulfadiazine and amoxicillin may eradicate C. trachomatis but do not effect Ureaplasma urealyticum (66, 72, 74).

Fewer studies have assessed the effectiveness of antimicrobial treatment of uncomplicated cervical or uretral chlamydial infection in

women (72,74-77). The data are almost the same as chlamydial treatment in men. They suggest that tetracycline or erythromycin are effective against C. trachomatis (75-77). Amoxicillin and trimethoprim-sulfadiazine also can eradicate C. trachomatis but it is not effective for other NGU unless in high dose and prolong used of them (72,74). From many studies they are showed that erythromycin or rosaramycin 500mg, four times daily, for 10-14 days have significant activity against C. trachomatis but sometimes the drugs were stopped because of severe side effects such as nausea, vomiting, abdominal cramp and/or diarrheae (71,73,75).

In recently, the activity of the newer quinoline carboxylic acid like norfloxacin , ciprofloxacin against C. trachomatis were determined, norfloxacin is intermidiate sensitive (MIC 8-16 µg/ml), but ciprofloxacin is very active against C. trachomatis (MIC 0.5-1.0 µg/ml) (78). They are active against a board spectrum of bacteria (79), and also active in vitro against Neisseria gonorrhoeae (80,81). But it is not known whether ciprofloxacin or norfloxacin is active against other sexually transmitted agents like Mycoplasma hominis and Ureaplasma urealyticum. Ofloxacin, a related compound, is active against C. trachomatis (82), M. hominis and other Mycoplasma species, but U. urealyticum was not tested (83). Rosoxacin , another quinolinecarboxylic drug, is active against C. trachomatis, N. gonorrhoeae and U. urealyticum (84).

If these studies can be extended and completed, the possibility of treating genital infections with one of these drugs as a single therapy is growing. The results of clinical trials with these drugs against C. trachomatis infections are awaited with interest. It is possible that these drugs are able to make this reservoir smaller.

Laboratory Diagnosis

The principals for diagnosing chlamydial infections are essentially the same for diagnosing any other microbial infection(85). The agent may be isolated from the patient's tissue, or demonstrated by cytologic study of clinical specimens. Serologic means may also be used to demonstrate rising antibody titers to chlamydial antigens.

1. Isolation and Culture

1.1 Collection of Specimens

As C. trachomatis is found intracellularly in epithelial cells(1,5,31), the specimens should contain many of these cells rather than the exudate, which is less often positive on culture (86).

a. Cervical Specimen

Since C. trachomatis has not grown in vaginal squamocells, and does not cause vaginitis. It causes cervicitis and seems to be a specific parasite of squamocolumnar cells which grows only within the transitional zone and the endocervix. The specimens should be obtained from the cervical canal. So cervical swab are obtained, after exposing the cervix with a speculum. The mucous in cervix should be removed by a sterile guaze, then introduced the sampling swab and rotated before withdrawal(86-88).

b. Male Urethral Specimen

Male urethral sample was taken by introducing the swab 2-3 cm up into the urethra, rather than from the urethral meatus(89), then rotated before withdrawn(87).

c. Conjunctival Specimen

Samples from the conjunctiva should be obtained with a fresh dry swab, which is rubbed firmly across the lower and upper tarsal conjunctivas and is then immediately transfered to the transport medium(86).

d. Nasophryngeal Specimen

To collect nasopharyngeal samples, the swab is introduced through the nostrils to the pharyngeal wall, before withdrawal(87). Pharyngeal samples from neonates may be positive for chlamydiae, even when cultures from the eyes are negative(54).

1.2 Sampling Swab

Sampling, particularly from the male urethra, requires the use of a thin sampling stick to avoid causing pain and mucosal lesions(87). When choosing a sampling swab, its toxic properties for chlamydiae must also be considered. In comparative studies using experimentally infected transport medium, swabs tipped with calcium alginate were more toxic to chlamydiae than other swabs tested(90,91). The least toxic type of swab was a cotton-tipped metal stick (E.N.T., Medical wire and Equipment Co., Ltd., Corsham, England)(90,91). A rayon-tipped plastic swab was also comparatively non toxic(90).

1.3 Transport Tubes

It is recommended that the use of certain plastic instead of certain glass tubes produced more chlamydial inclusions(90).

1.4 Specimen Handling and Storage

A suitable transport medium for chlamydial samples is 0.2 mol/l sucrose phosphate (2SP)(92). The formula for 2SP is given in

Appendix III. All media used for storage and transport of chlamydiae should contain antibiotics, which will inhibit contaminating micro-organisms but will not interfere with chlamydial isolation(92). Such antibiotics are streptomycin (50-100 µg/ml) or gentamicin (10-20 µg/ml), together with vancomycin (100 µg/ml) and an antifugal agent such as nystatin (2.5 µg/ml) or amphotericin B (2.5-5 µg/ml)(86,87). The formula of transport medium is given in Appendix III.

When specimens were obtained, it is to be processed within 24 hr, the swab can be expressed into the 2SP transport medium, and stored at 4°C. If the laboratory cannot process within the same day, then the specimens should be stored at -20°C or lower(91,93).

1.5 Tissue Culture Cells

Because C. trachomatis is an intracytoplasmic parasite (1,2,40), the cell that was used must have some basic biological characteristics as described below (40).

a. The parasite must gain entrance to the host cell.

b. The parasite must not be destroyed by the cell.

c. The parasite must not destroy host functions essential to parasite multiplication.

d. The parasite must multiply.

e. The parasite must be released from the host cell.

f. The parasite must survive transit to a new host cell.

There are three cell lines generally used for the culture of C. trachomatis(86,87,94). The first one is McCoy cell which are believed to have originated from human synovial cells, but have

apparently been contaminated with cells of a mouse karyotypes similar to L-929 cells(95). They were originally used as normal replicating cells by Gordon et al, 1963 , for growth of chlamydiae(96). McCoy cells which are used in laboratories all over the world are mouse fibroblast (95). The second is Hela 229 cells which originated from a human cervical cancer(97), and have been used since 1966(98). The latter is BHK-21 cells which were derived from baby hamster kidney(99). Among these cell lines, McCoy cell is the most widely used cell line for the isolation of C. trachomatis(94).

1.6 Tissue Culture Media

Eagle minimum essential mediasm(100), medium 199(101), and RPMI-1640(87,88) are all used for culture of C, trachomatis. It is recommended that RPMI-1640 is the most suitable basic medium, for using McCoy cell culture(87,88). The composition of a complete cell culture medium is shown in Appendix IV.

1.7 Pre-treatment of Tissue Culture Cells

a. Irradiation

Irradiated cell may be used in tissue culture for isolation of chlamydiae(32,95). The cells should be exposed to 4000 to 6000 rads, 6 to 10 days before being used for specimen inoculation(87, 96). After irradiation, the cells become nonreplicating giant cells.

b. IUdR (5-iodo-2-deoxyuridine)

Three days before the McCoy cells are inoculated with clinical specimen, IUdR is added to the cell culture medium in order to increase the susceptibility of McCoy cells to C. trachomatis infection(102).

c. Cytochalasin B

Cytochalasin B is another cytostatic drug that has been recommended for pretreatment of cells to be used for chlamydial isolation(103). They used cytochalasin B in a similar way to IUdR for treatment of McCoy cells.

d. Cycloheximide

In 1977, Ripa and Mardh proposed a simpler way to treat McCoy cell by using cycloheximide, a glutaramide antibiotic, which reduces the metabolic activity of eukaryotic cells by inhibiting the deoxyribonucleic acid (DNA) and protein synthesis (104). This renders pretreatment unnecessary (104). In addition, this substance does not affect prokaryotic cells such as chlamydiae (104).

In comparisons between vasious McCoy cell treatment procedures, more inclusions were detected in cycloheximide treated cells than in other cells, even though there were no differences in the isolation rates (105,106). However, there has been a report that C. trachomatis strains isolated in cycloheximide-treated McCoy cells are difficult to culture on subpassage (107).

e. DEAE-dextran (Diethylaminoethyl-dextran)

DEAE-dextran is a polycations compound which transforms the physicochemical properties of the surface of Hela229 cells, will enhance attachment and phagocytosis of TRIC agents of C. trachomatis (108).

When using McCoy cells, treatment with DEAE also increases the chlamydial inclusion count, however the count was lower than when were treated with cycloheximide (87). It has been found that

cycloheximide treated McCoy cell is the most sensitive technique for the isolation of C. trachomatis (105).

1.8 Centrifugation

Centrifugation of cell culture inoculated with chlamydia is the most essential technique to obtain optimal culture results (94). At centrifugation forces of 3000 to 6000 g , the organisms are pelleted onto the cell monolayer so as to increase the chlamydiae-cell contact (93). Use of higher centrifugation forces involves several problems, such as, lack of test tube that can stand such forces. In addition, centrifugation force up to 15,000 g result in only an approximately 5 percent increase in the recovery rate compared with 3000 to 6000 g (109).

The temperature during centrifugation and incubation is important. In practice, specimens are centrifuged onto the cell culture at 35-37°C to obtain maximum interaction between parasite and host cell (100). A suitable time limit for such centrifugation is one hour. Lastly, they also found the greastest number of inclusions after incubating the inoculated monolayers for 48 hr at 35°C (100).

1.9 Staining

Three general staining techniques have been used for detecting chlamydial inclusions in cell monolayers.

a. Giemsa Staining(86)

Giemsa's stain is useful for detecting C. trachomatis inclusion, when combined with dark field microscopy. Bright field microscopy can also be used but it is more difficult to detect the inclusion than the former is used. C. psittaci inclusion do not

"autofluoresce" when examined by dark-field microscopy.

b. Iodine Staining(86)

The various stain use to detect glycogen matrix found in C. trachomatis inclusion is iodine staining, which stains the matrix brown. Iodine staining is probably the simplest of all the staining techniques used for detecting chlamydial, the advantage is requiring a simple-bright-field microscopy for detection of inclusions. In general, C. psittaci do not contain a glycogen matrix, thus iodine staining is not suitable for detecting C. psittaci inclusions.

Either Giemsa or Iodine staining is used after the inoculated had been incubated at 35-37°C for 2 to 3 days.

c. Immunofluorescence Staining

Immunofluorescence staining is used to detect both C. trachomatis and C. psittaci inclusions(86). A comparison of both immunofluorescence and Giemsa staining for the detection of C. trachomatis inclusions was shown that both stains were of similar sensitivity(110). Although the inclusions were seen after incubated the organism 19 hr at 35-37°C , the former would have more inclusions than the latter method (110).

2. Cytologic Study

Cytological procedure can be used to diagnose chlamydial infections by demonstrating inclusions in the patient's cells but are not recommended because of poor sensitivity(5). Comparative study have shown that inclusions can be demonstrated in only 15 percent (by Giemsa staining) and 60 percent (by immunofluorescence staining) of urethral specimens from men who are shown to be infected by isolation procedures

(111). Cervical or adult conjunctival specimens are more likely to yield inclusions,but the sensitivity of these procedures from cervical specimen are 41 percent and 66 percent of culture by Giemsa technique and immunofluorescence technique, respectively (112). In neonatal conjunctivitis these procedures are more sensitive that the percent of positive results is reached to 95% of culture either by Giemsa or immunofluorescence method (112). As a result from this study immunofluorescence procedure is more sensitive than Giemsa staining for detection of inclusion.

In neonatal conjunctivitis, the Giemsa stain represents a valuable diagnostic tool. Not only will it provide accurate and rapid diagnosis in most cases of severe chlamydial conjunctivitis in neonates but since sometimes there is overlap of gonococcal infections. Thus, it seems reasonable to recommend Giemsa stain because both gonococci within polymorphonuclear leukocytes and typical chlamydial inclusions within epithelial cells can be demonstrated (1,5). However, a good result in inclusion conjunctivitis of the newborn requires special attention to obtaining an adequate sample of epithelial cells(1,86).

To summarize the advantage and disadvantage of the stain, iodine staining is faster and simpler than Giemsa or immunoflurescent staining, it is less sensitive (112,113). It cannot be used for staining genital specimens as these often contain other materials that stain with iodine (86).

Giemsa staining is cheap and easy to perform, but examination is time consuming and care must be taken to exclude artifacts. In addition, the types of inflammatory cells present can easily be seen and these have been used as a guide to the nature of

the disease, and hence the likelihood of finding inclusions (114,115).

Although immunofluorescent techniques have been shown to be more sensitive than the Giemsa method for demonstrating inclusions in epithelial scrapings from the eye and the genital tract (111,112), it requires the use of expensive reagents and a microscope equipped with ultraviolet or blue-light illumination (86).

3. Serology

There are many methods used to detect chlamydiae such as, indirect hemagglutination, hemolysin-in-gel, and so on. At the moment, however , the principal generally accepted methods remain the complement fixation (CF) and microimmunofluorescence test. Micro-IF or MIF is also called (22,116).

a. Complement Fixation Test (CF Test)

CF test was first used to detect antibodies to chlamydia in cases of psittacosis (117) and later lymphogranuloma venereum (LGV).

It is sufficiently sensitive to detect antibodies in the systemic infections of psittacosis and LGV, but its sensitivity is limited in localized chlamydial infections (118). The test makes use of detecting only antibodies produced against a 2-keto-3-deoxyoctanoic acid (119), which is common to all members of the chlamydia genus. Thus, this technique does not differentiate between antibodies produced against C. psittaci or C. trachomatis, it is a genus-specific test. In general, a titer of 1 : 64 or higher is suggestive of recent chlamydial infection, but a fourfold rise in titer (without a rapid fall) is still more convincing (120).

b. Micro-Immunofluorescent test (MIF Test)

MIF test was developed to serotype strains of C. trachomatis (17) but was soon adapted to detect antichlamydial antibody (18) which is the type specific antibody (17). It can be applied to patients with LGV, trachoma and oculogenital infections which is the advantage of this test over the CF test (22,116,120). In addition,it can also use for detection of various immunoglobulin classes of the reactive antibodies such as IgM, IgG or IgA (22,116,120)

The type specific antigens involved are trypsin-labile protein located on the surface of chlamydial elementary bodies and are part of the major outer membrane protein (121). This antigen originally the whole range of C. trachomatis serotypes(designated A, B, Ba, C... K, L_1, L_2, L_3) plus some C. psittaci agents were applied separately as individual antigens in the test (17), the test has been simplified by pooling antigens, either on the basis of similarities of serological cross reaction (173), or into epidemiologically similar groups (20).

Antibody may be sought in serum, whole blood, local secretion or discharge (eg. tear fluids, cervical secretions), and the use of pre-cut cellulose sponges to absorb these specimens facilitates their collection and handling (21).

In general, serological test alone are of little value in the definitive diagnosis of certain chlamydial infections because chlamydial genital infections such as urethritis or cervicitis tend to be chronic and the IgM antibody response may last for approximately one month following infection (18). So the changing titer of IgG antibody or the present of IgM antibody are rare.

It is recommended that the significant level in the MIF test has been chosen as 1 : 16 (122), which represent that the patients once had chlamydial infection. As a single serum, the antibody titers suggesting active chlamydial infection were taken as IgG $\geqslant$ 1:32 or IgM $\geqslant$ 1:8 for men and IgG $\geqslant$ 1:64 or IgM $\geqslant$ 1:8 for women (21). In cervical sercetion of female, IgG or IgA antibody at a level of $\geqslant$ 1:8 was closely associated with the isolation of C. trachomatis and non-specific genital infection (21).

In acute salpingitis and perihepatitis high levels of chlamydial IgG antibody are generally elicited, but for definitive serodiagnosis it is necessary to demonstrate a fourfold or greater change in antibody titer (123).

Infant with proven C, trachomatis pneumonia invariably have high IgM antibody levels. A great majority of $\geqslant$ 1:256, and only the atpical young infant appears to have lower, but still appreciable, level $\geqslant$ 1:64. On the other hand, infant with uncomplicated inclusion conjunctivitis have much lower titers (124). It was noted that maternal IgG antibodies to C. trachomatis can be demonstrated in infant, in this reason, IgM antibody must be detected (124).