CHAPTER II

REVIEW OF THE LITERATURES

History

Chlamydia trachomatis is responsible for a wide range of human infections. Trachoma,a disease caused by *C. trachomatis,* is a desease known since the ancient time. There were outbreaks in Europe following the home - bound Crusaders and during Napoleon's campaign in Egypt (32). The organism was first demonstrated in 1907 by Halberstadter and von Prowazek, who found the characteristic intra-cytoplasmic inclusions in eye scraping from orangutans infected with material from the eyes of patients with trachoma. Later in 1909, they found the similar inclusions in smears of secretion from the eyes of infants with ophthalmia neonatorum and from the urethra of their mothers (33,34). In 1911, Lindner et al (35) found these inclusions in neonatal conjunctivitis. These chlamydial infection were , therefore , referred to as " inclusion blennorrhea " or " paratrachoma ". In addition, they confirmed the connection between the disease in neonates and genital disease of the parents and induced inclusion conjunctivitis in monkeys with material obtained from infants with inclusion blennorrhea (36).

In 1930, *C. psittaci* that cause psittacosis, a human respiratory disease, was first isolated from affected humans and

psittacine birds by Bedson et al (37). Almost at the same time, the causative agent of lymphogranuloma venereum (LGV), a sexually transmitted disease caused by *C. trachomatis* serotype L1, L2 and L3 , were also isolated by Hellerstrom et al (38). These isolations were done by intracerebral inoculation in mice.

The first isolation by inoculating of LGV into the yolk sacs of embryonated hens' eggs was succeeded by Tang et al (39) in 1957. Later on, Jones et al (40) were the first who could isolate *C. trachomatis* from the eye of an infant suffering from conjunctivitis, from the cervix of the mother and subsequently in 1964 from the urethra of the father of an infant with chlamydial ophthalmia neonatorum (41). Accordingly, the organisms were refered to as "TRIC agent" to indicate their origin from trachoma (TR) and paratrachoma or 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 (22). The cell culture technique was developed by Gordon and Quan (42) in 1965. Irradiated McCoy cells were originally used as normal replicating cells for the isolation of *C. trachomatis*. Recently , cycloheximide was introduced as alternative agents for pretreatment of the eukaryotic cells. The advantage of using cycloheximide is that pretreatment of the cells can be done in conjunction with sample

inoculation. Accordingly , the growth of *C. trachomatis* in cycloheximide treated McCoy cells becomes one of the standard cell culture techniques for *C. trachomatis* isolation and the most reliable means of diagnosing infection with this organism (43).

Biology and Taxonomy

1) Characteristics of chlamydiae

Chlamydiae are nonmotile , gram negative , obligate intracellular bacteria (44). 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 for by the infected host cells. Thus, the organisms were called "energy parasites" (45). They replicate by binary fission within the cytoplasm of host cells, forming characteristic intracellular inclusions that can be seen by light microscopy (46).

Although chlamydiae are classified as bacteria,they share properties with viruses and bacteria. For instance , chlamydiae grows only intracellularly like viruses and the cultivation of *C. trachomatis* can only be done by cell culture. However,like bacteria, they contain both DNA and RNA , multiply by binary fission, possess cell walls similar in structure to those of gram negative bacteria, and are susceptible to many broad-spectrum antibiotics (46). They possess a unique developmental cycle that differentiates them from all other microorganisms (44).The

compared properties of chlmydiae and other microorganisms are shown in table 1 (22,47,48,52).

Table 1. Characteristics of chlamydiae in relation to those of mycoplasmas, bacteria, and virus.

Properties	Bacteria	Mycoplasmas	Chlamydiae	Viruses
Cell wall	rigid wall muramic acid	membrane lipid	wall muramic acid	protein
Free living	+	+	-	-
Size	0.2-0.5 um	0.25-0.5 um	350 nm	23-300 nm
Nucleic acid	DNA & RNA	DNA & RNA	DNA & RNA	DNA or RNA
Reproduction	fission	budding	complex cycle fission	eclipse, synthesis &assembly
Cultivation	artificial media	artificial media	cell	cell
Antibiotic sensitivity	+	+	+	-
Ribosomes	+	+	+	-
Energy production	+	+	-	-

2) Developmental cycle

C. trachomatis exists in two distinct forms depending on its environment and the stage in its growth cycle (developmental cycle). The elementary body (EB), an infectious particle of 300 nm in diameter , is adapted for extracellular survival when released from the cell. The recticulate body (RB), the metabolically active particle of up to 1,000 nm , is initial body which replicates by binary fission to form the inclusions in cytoplasm of host cells (13).

The first step in the infectious process involves attachment of the EB to a susceptible host cell and is ingested by a mechanism similar to receptor-mediated endocytosis (49). The EB enters the cell within an endosome often termed a phagosome. Like some other obligate intracellular parasites, the organism inhibits phagolysosomal fusion, and the entire growth cycle is completed within the expanding endosome. Within a few hours after entry, the EB reorganizes into a RB . After approximately 10 to 15 hrs, the RB begins dividing by binary fission. At 20-30 hrs after infection , some of the RBs develop central condensation of cytoplasmic contents , decrease in size , and become typical EBs. When the transition from RBs to EBs begins, many of the RBs continue to multiply until the host cell cytoplasm is almost filled by the colony. At 40 to 60 hrs after infection, the EBs are released to initiate another cycle of infection (13). The developmental cycle of chlamydiae is shown in Fig.1.

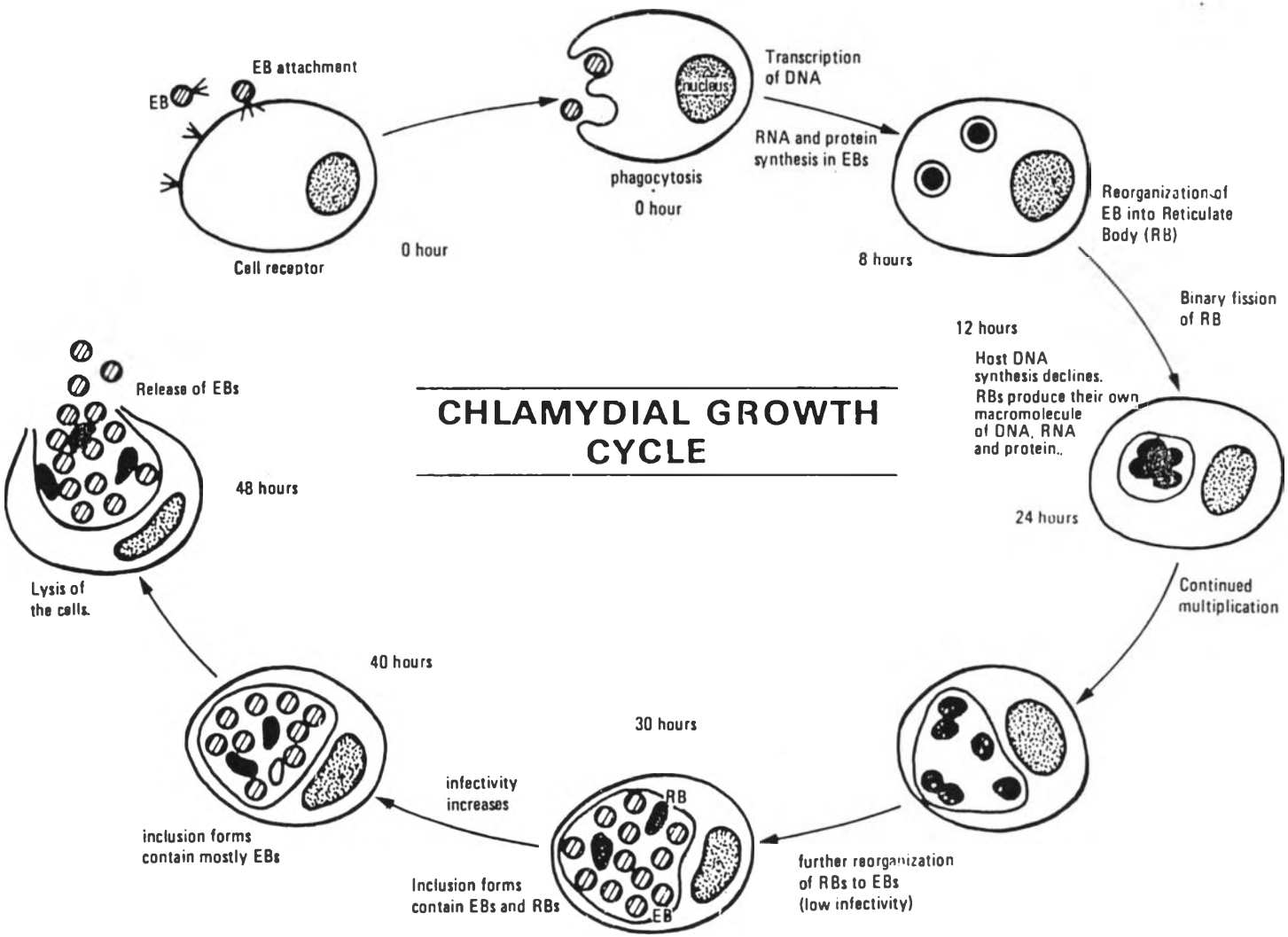

FIGURE 1. Chlamydial growth cycle. EB, elementary body; RB, reticulate body. (52)

3) Cellular morphology and structure

As mentioned above, the chlamydiae exist in two distinct forms depending on the stage of their developmental cycle.:-

The EB is a spherical particle , 0.25 to 0.3 um 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 lack a peptidoglycan layer. The outer membrane (OM) contains lipopolysaccharide , which exhibit characteristic endotoxin - like property, and hemagglutinin. In addition , it has a major outer membrane protein (MOMP) of 39,000 to 45,000 Da, which represents approximately 60 % of the weight of the outer membrane. Inhibition of the synthesis of the cell wall subunit by penicillin (50) suggests the cell wall structure analogous to peptidoglycan. The presence of penicillin - binding proteins suggests the presence of a related cross - linked structure. The structural rigidity of the EB appears to depend on disulfide crosslinking of MOMP molecules with each other and with other cysteine-rich proteins (which appear late in the growth cycle, when EBs are formed). An inner layer of the OM is composed of protein subunits arranged hexagonally.

The RB is much larger than EB and differs in many respects. It is surrounded by a trilaminar envelope that the subunit layer is disrupted and disappeared, fragile and flexible so that pleomorphism results. Ribosomes and other cytoplasmic constituents are distributed homogeneously throughout the

cytoplasm (13,51). A summary of different characteristics of EB and RB is shown in Table 2.

Table 2. Characteristics of Elementary Body (EB) and Reticulate Body (RB).

Characteristics	EB	RB
Morphology	small, dense centered	large, homologous
RNA : DNA	1:1	3:1
Sonication	resistant	sensitive
Effect of trypsin	resistant	sensitive
Infectivity	+	-
Toxicity	+	-
Hemagglutinin	present	absent
Permeability	slight	marked
Envelope subunit	present	absent
Location	extracellular	intracellular
Metabolic activity	inactive	active
Size	0.2-0.3 um	1 um

4) Antigens of chlamydiae

The chlamydiae possesses various antigens that are present on their surface as follows.

4.1) **Genus - or group - specific antigens** ,shared by all members of the genus, are the lipopolysaccharide (LPS), with a ketodeoxyoctanoic acid as the reactive moiety (53).

4.2) **Species - specific antigens** are heat - labile proteins expressed on the outer membrane with molecular weight ranged from 40 KDa to approximately 155 KDa (54).

4.3) **Type - specific antigens** are proteins associated with the MOMP with molecular weight ranged from 30 KDa to 40 KDa. They are heat-stable , trypsin sensitive , non-lipid components (55). With the use of micro-immunofluorescence devised by Wang and Grayston (56) as an immunotyping test, 15 serotypes of *C. trachomatis* have been identified.

5) Taxonomy

Chlamydiae are placed in their own order,the *Chlamydiales*, family *Chlamydiaceae*, with one genus , *Chlamydia*. There are four species , *C. trachomatis* , *C. psittaci* , *C. pneumoniae* , and *C. pecorum* (44,57). (Diagram 1 : Taxonomy)

Diagram 1 Taxonomy of chlamydiae

Order : *Chlamydiales*

Family : *Chlamydiaceae*

Genus : *Chlamydia*

Species : *Chlamydia trachomatis*

C. psittaci

C. pneumoniae

C. pecorum

All of the chlamydial species are similar in their life cycles, necessity for obligate intracellular existence , the presence of both DNA and RNA, and their dependency on host adenosine triphosphate (ATP) production (58). They are distinguished by the symptom of clinical diseases, the staining characteristics (iodine - stained glycogen is formed only by *C. trachomatis*), antibiotic susceptibility patterns , surface epitopes, and limited DNA sequence homology (12,59-63). Within each species of chlamydiae, it can be divided into biovars and serovars on the basis of host range ,disease pattern, and antigen composition.

C. trachomatis specifically parasitizes humans and has no known animal reservoirs. It can be divided into three biovars: the trachoma, lymphogranulcma venereum [LGV], and murine biovars, associated with different diseases. The trachoma biovar appears to infect squamocolumnar cells and results in ocular and genital infections, while the LGV biovar infects endothelial and lymphoid cells and results in lymphogranuloma venereum. The murine biovar is represented by the mouse pneumonitis agent (13,64).

C.trachomatis can be divided into 15 serovars (also called serotypes) on the basis of antigenic variation in their major outer membrane protein (MOMP) (65,66). Each serovar is designated by a letter (A-K,Ba,L1,L2,and L3)(67). Recently, three additional *C.trachomatis* serovars have been identified (Da, Ia, and L2a) from chlamydial strains isolated from patients with chlamydial genital and eye infection (68). All 15 serovars can be grouped into 3 major serogroups on the basis of their infections as follows :

1) The trachoma group includes the serovars A,B,Ba,and C. They have been associated with endemic trachoma,the most common preventable form of blindness.

2) The oculogenital group includes the serovars D - K . They have been found to be the cause of chlamydial genital diseases and neonatal diseases (conjungtivitis and pneumonia).

3) The **lymphogranuloma venereum** (LGV) **group** includes the serovars L1, L2, and L3 that are responsible for lymphogranuloma venereum (69,70,71).

Summary of the association between serovars and clinical spectrum of *C. trachomatis* infections is presented in table 3.

Table 3. Serovars and clinical spectrum of *C. trachomatis* infections.(52)

Serovars	Host	Infection	Complications
L1,L2,and L3	women,men	lymphogranulomar venereum	-vulvar / rectal carcinoma -rectal strictures
A,B,Ba,and C	women,men children	trachoma	blindness

Table 3.(continue)

Serovars	Host	Infection	Complications
D,E,F,G,H,I, J,and K	men	urethritis postgonococcal urethritis *rectal subclinical conjunctivitis	epididymitis prostatitis reiter's syndrome sterility
D,E,F,G,H,I, J,and K	women	cervicitis urethritis subclinical conjunctivitis	salpingitis perihepatitis sterility, dysplasia postpartum endometritis prematurity stillbirth neonatal death
D,E,F,G,H,I, J,and K	infants	conjunctivitis pneumonia asymptomatic pharyngeal carriage asymptomatic gastrointestinal tract carriage otitis media	

6) Genome

Chlamydia trachomatis genome is composed of circular double-stranded DNA with a molecular weight of approximately 6.6 x 10^8 Da (1.1 x 10^6 base pairs) (72). In addition , *C. trachomatis* possesses a plasmid (extrachromosomal DNA) that is approximately 4.4 x 10^6 Da (7.5 x 10^3 base pairs) which has been identified in all 15 serovars (73,74). It has been suggested that these plasmids are involved in DNA binding and replication. However, this vital function is more suspicious on the basis of the most recent finding of an L2 isolate that does not contain a 7.5 Kb plasmid (75,76). The restriction endonuclease cleavage map of the plasmid from *C. trachomatis* serotype L2 (pCHL2) was shown in Fig 2.

The intraspecies DNA homology between different *C. trachomatis* strains is estimated to be 96-97 %. The homology among *C. trachomatis, C. pneumoniae, C. psittaci,* and *C. pecorum* is less than 10 % (57,77). The G+C content (G+C %) of *C. trachomatis* and *C. psittaci* are 44.4 % and 41.2 % respectively (78).

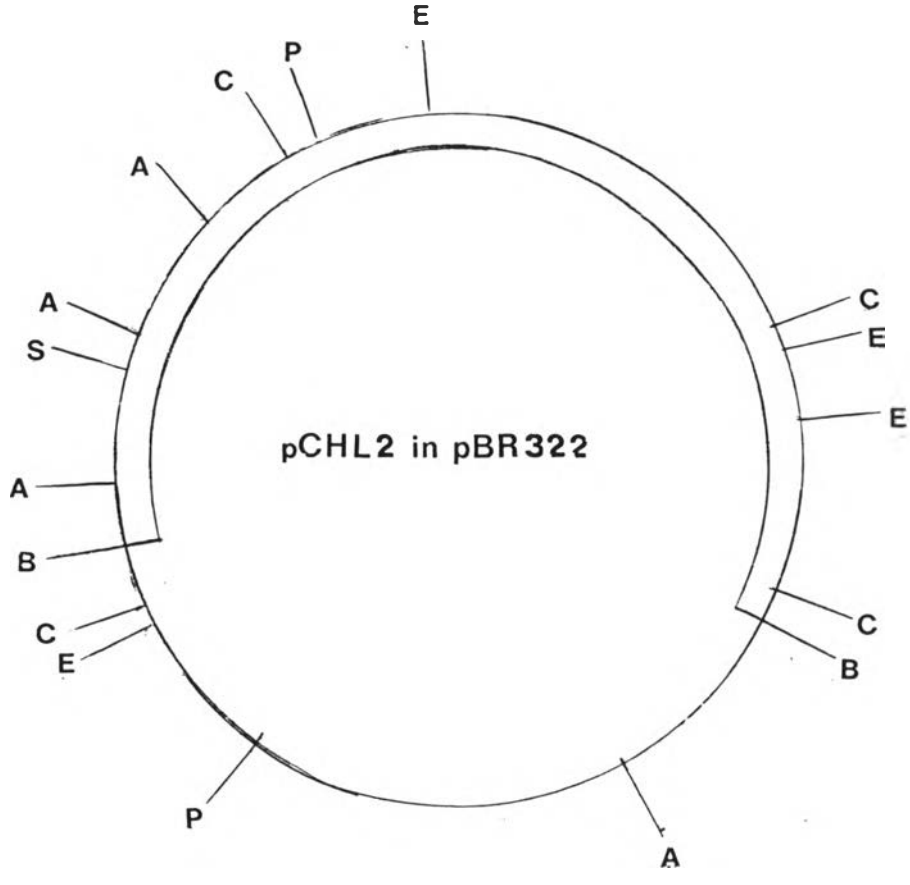

Fig.2 : The restriction endonuclease cleavage map of 7.5 kb pCHL2 in pBR 322. The single lined part of the circle represents pBR 322 sequence , the double line represents the pCHL2 sequence . (B) BamHI,(P) PstI,(E) E.coRI,(C) ClaI,(S) SmaI (A) AvaI.

Clinical feature

C. trachomatis is essentially a pathogen of mucosal surface, infecting and replicating within epithelial cells. Chlamydial infection transmitted to a new host cell is not limited to the lower genital tract but may occur at other anatomic site, such as the eyes or respiratory tract (eg. in trachoma patients and neonates born to mother with cervical infection), oropharynx or rectum (79,80,81).

1) Infections in male

The most common clinical genital syndrome seen in the male is nongonococcal urethritis (NGU) which approximately 50 - 60 % of cases are caused by *C. trachomatis*. The chlamydial urethritis is more likely to be asymptomatic or tend to be milder symptomatic than gonococcal urethritis . Co-infection with *Neisseria gonorheae* occurs about 15-30 % and 5 % in heterosexual and homosexual men, respectively (82,83,84,85). Co-infected men, treated with a penicillin or cephalosporin alone , develop postgonococcal urethritis (PGU) approximately 80 % and approximately 30 - 50 % of PGU cases are attributed to *C. trachomatis* (86).

In male with chlamydial urethritis , *C. trachomatis* may spread from urethra to epididymis, results in epididymitis. This painful serious complication may result in male sterility. In homosextual males, proctitis may occur following receptive

anal intercause. In addition , the L1 to L3 serovars of *C. trachomatis* cause lymphogranuloma venereum which involves regional lymph nodes and produces systemic manifestation.

In Thailand , Pongpun N. et al (87) have studied the incidence of *C. trachomatis* , *Mycoplasma hominis* , and *Ureaplasma urealyticum* infections in males with NGU and found that 27 % of cases of NGU are caused by *C.trachomatis.*

2) Infections in women

In women , most chlamydial infections of lower genital tract are asymptomatic and undetected. Many cases go untreated resulting in ascending of the microorganism to the upper genital tract that leads to pelvic inflammatory disease, tubal damage, and adverse outcomes of pregnancy (13).

Approximately 20-30 % of the cases of pelvic inflammatory disease (PID) in the United States are attributed to *C. trachomatis* (93,94,95). High fever is not a common manifestation of chlamydial PID. Common physical findings include a mucopurulent cervical exudate, uterine and adnexal tenderness , and pain elicited with motion of the cervix. Tenderness over the liver may indicate chlamydial perihepatitis , Fitz Hugh-Curtis syndrome (86).

Approximately 50 % of women with mucopurulent cervicitis appear to possess histological evidence of endometritis

(88,89). However, the culture of the endometrium and fallopain tube for *C. trachomatis* are often negative (86). The symptoms typical of endometrial chlamydial infection include heavy , prolonged , or irregular mense (86).

Salpingitis usually presents with lower abdominal pain often described as atypical or mild (90,91). The onset of symptoms often coincides with the onset of menses , similar to that seen with gonococcal PID (92).

Infertility is a common complication of PID. After a single episode of PID, approximately 15 % of women will be infertile as a result of peritubal adhesions or tubal occlusion. Numerous retrospective studies have demonstrated a consistent correlation between the presence of elevated antibodies to *C. trachomatis* and tubal factor infertility. Up to 75 % of women with tubal factor infertility (diagnosed by laparoscopy or hysterosalpingogram) have significant titers of anti-chlamydial antibody. In contrast, approximately 20 % of fertile "control" patients have significant titers of antibody to *C. trachomatis* (96,97,98).

Ectopic pregnancy continues to be a major cause of maternal morbidity and mortality in the United States occuring as frequently as one to two in 100 pregnancies. PID is associated with a 5-to-7-fold increased risk for ectopic pregnancy, and approximately 50 % of women with an ectopic pregnancy will be infertile. Women with tubal factor infertility or ectopic

pregnancies are more likely to have high titers of antichlamydial antibody detected in their serum (86). A recent case-control study reported a 2-to-3-fold increased risk for ectopic pregnancy in women in an IgG titer equal to or exceed 1:64 (99). Gravett et al.(100) and Sweet et al.(101) confirmed that IgM-seropositive women were at significantly increased risk for preterm labor and premature rupture of membranes. Recently, Cohen et al (102) and Ryan et al (103) suggested that treatment of cervical chlamydial infection during the second trimester may improve pregnancy outcome by reducing the incidence of premature rupture of membranes.

3) Neonatal infections

Approximately one in three of infants born through a chlamydia-infected birth canal develops inclusion conjunctivitis of the newborn (ICN) which has an incubation period of 5 to 21 days and usually resolves in a few months without treatment. In 1986, Sanpavat,S. et al (104) have been studied the prevalence of *C. trachomatis* conjunctivitis in 36 neonates born in Chulalongkorn Hospital and found that the prevalence was 19.4 %.

About one in six exposed infants develops a characteristic pneumonia syndrome. The incubation period is usually between 2 and 12 weeks. The infants often have a prodrome of rhinitis, and many will have conjunctivitis. Affected infants are usually afebrile , markedly tachypneic and occasionally apneic, and have a staccato cough. There are

long-term consiquences of this disease. Most infants with chlamydia pneumonia develop chronic respiratory tract disease.

During the year 1986- 1988, Limudomporn, S. et al (105) have been studied 112 infants younger than 6 months of age with the diagnosis of afebrile pneumonia in Chulalongkorn Hospital and found that 30 cases (26.8 %) were caused by *Chlamydia trachomatis*.

4) Trachoma

In addition to genital tract and newborn infection, *C.trachomatis* is also an infectious agent that causes trachoma. It is one of the oldest known human diseaces , well described in ancient Egyptian and Chinese writings. Once common throughout the world, it is now a major problem only in certain developing countries. Still,trachoma is the world's leading preventable cause of blindness, with more than 400 million people affected and several millions blinded. Trachoma is a disease of poverty, associated with poor environmental sanitation and personal hygiene. In the hyperendermic area, trachoma is a disease of young children, and virtually all will be infected before the age of 2 years.

Although onset can be insidious , the disease begins as a mucopurulent conjunctivitis , developing into a follicular keratoconjunctivitis. Over time, some of the follicles necrose, resulting in scarring of the conjunctivae. The scars may slowly

contract, distorting the eyelid and causing an inturning of the eyelashes so that they abrade the cornea. This complex represents the blinding lesions of trachoma,called trichiasis and entropion. Active inflammatory disease usually disappears by 10 years or earlier. Blindness develops later in life, in up to 25 % of those more than 60 years old. Endemic trachoma is associated with *C. trachomatis* serovars A, B, Ba, and C. (13).

Treatment,Prevention,and control

The treatment of choice for chlamydial infections in men and non-pregnant women is tetracycline (500 mg per 16 hrs for 7 days) or Doxycycline (two 100 - mg doses per day for 7 days) (86). For those allergic to the tetracyclines , safe effective alternatives include erythromycin base (500 mg per 6 hrs for 7 days) and clindamycin (300 mg per 6 hrs for 7 days). Because of fetal and maternal tetracycline toxicity during pregnancy, a nonestolate erythromycin is the most commonly recommended. In addition , amoxicillin and clindamycin have been used safely and effectively to eradicate chlamydia during pregnancy too. In lymphogranuloma venereum may require multiple long courses of therapy (Doxycycline 100 mg per 12 hrs for 21 days or Erythromycin base 500 mg per 6 hrs for 21 days). For the newborns , neonatal infection can be prevented by treatment during the last trimester of pregnancy. Topical prophylaxis alone is ineffective in preventing transmission to the newborn since multiple sites (conjunctiva , oropharynx , and lungs) are frequently infected. Treatment of neonatal infections include

Erythromycin 50 mg / kg / day in 4 doses for 7-14 days (for inclusion conjunctivitis) or 14-21 days (for pneumonia) (86). To preventing blindness , not to eradication of trachoma, trachoma control is currently based on mass treatment of all affected individuals within a village setting with topical tetracycline ointment and on surgical intervention to correct lid deformities.

Laboratory diagnosis

Since *C.trachomatis* is responsible for a wide range of diseases and serious sequelae in human, the rapid identification of chlamydial infections is essential for the proper treatment of infected patients , prevention of transmission to susceptible individuals, and reduction the risk of consequent complication. The organism may be isolated by cell culture or demonstrated by antigen detection of clinical specimens. Serologic study may also be used to demonstrate rising antibody titers to chlamydial antigens.

1. Isolation of *C.trachomatis* by cell culture

Cell culture has long been considered the gold standard to diagnose *C. trachomatis* infections and provided 100 % specificity. There are several significant steps in this method as follow :

1.1) Collection of specimens

Because *C. trachomatis* is an intracellular parasite , found intracellularly in epithelial cells (69,70,22), the specimens should contain many of these cells rather than exudate, which is less often positive on culture (106).In differrent specimens, there are slight differences of collecting proceders. In cervix , *C. trachomatis* 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. Accordingly 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 (106,107). In male, uretral specimens were taken by introducing the swab 2-3 cm up into the urethra , rather than from the uretral meatus (108),then rotated before withdrawn (109). For conjunctival specimens, the samples 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 mediun (106). For nasopharyngeal specimens, the samples recived by introducing the swab through the nostrils to the pharyngeal wall, before withdrawal (109).

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 (109). 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 (110,111).The least toxic type of swab was a cotton-tipped metal stick (E.N.T.,Medical wire and Equipment Co., Ltd., Corsham, England)(110,111). A rayon-tipped plastic swab was also comparatively non toxic (110).

1.3) Transport tubes

It is reccommended that the use of certain plastic instead of certain glass tubes produced more chlamydial inclusions (110).

1.4) Specimen handling and storage

A suitable transport medium for chlamydial samples is 0.2 M sucrose phosphate (2SP) (112). The formular for 2SP is given in Appendix I. All media used for storage and transport of chlamydiae should contain antibiotics, which will inhibit contaminating microorganisms but will not interfere with chlamydial isolation (112). Such antibiotics are streptomycin (50-100 ug/ml) or gentamicin (10-20 ug/ml),together with vancomycin (100 ug/ml) and an antifungal agent such as nystatin (2.5 ug/ml) or amphotericin B (2.5-5 ug/ml) (106,109). The formular of transport medium is given in Appendix I.

When the specimens were obtained, the swab should be inplaced into 2SP transport medium and stored at 4°C. If the laboratory cannot process within 8 hrs , then the specimens should be stored at lower than -60°C. (113).

1.5) Type of cells

Since *C. trachomatis* is an intracellular parasite, growth in cell is need for its isolation. The first isolation and growth of chlamydia in embryonated hen's eggs was succeeded in 1955. Later on, a number of useful cell lines have been developed to cultivate chlamydia (14). The cell that used must have some basic biological characteristic as described below (45).

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 several cell lines generally used for the culture of *C. trachomatis* (106,109,114). One of the cell line that has been utilized is McCoy cell which is believed to have originated from synovial cell , but has apparently been contaminated with cells of a mouse karyotypes similar to L-929 cells (115). They were originally used as normal replicating cells by Gordon et al.,in 1963, for growth of chlamydiae (116).McCoy cells which are used in laboratories all over the world are mouse fibro blast (115). Hela 229 cells is an alternative cell lines used for chlamydial culture. They have originated from a human cervical cancer (117) and have been used since 1966 (118). BHK 21 cells have also been used for chlamydial culture. They were derived from baby hamster kidney (119). Among these cell lines, McCoy cell is the most widely used cell line for isolation of *C. trachomatis* (114).

1.6) Cell culture media

Eagle minimum essential medium (120), medium 199 (101), and RPMI 1640 (107,109) 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 (107,109). The composition of a complete cell culture medium is shown in Appendix I.

1.7) Pre-treatment of the cell line

A number of monolayer cell replication inhibitors have been used to increase the sensitivity of *C. trachomatis* isolation including irradiation, cycloheximide, IUdR , cytochalasin B, and DEAE-dextran .

a. Irradiation

Irradiation cell may be used in cell culture for isolation of chlamydiae (42,115). The cells should be exposed to 4000 to 6000 rads -radiation from cobalt-60 , 6 to 10 days before being used for specimen inoculation (109,116). After irradiation, the cells become non replicating giant cells.

b. 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 DNA and protein synthesis. This renders pretreatment unnecessary. In addition, this substance does not affect prokaryotic cells such as chlamydiae (122).

Comparing various 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 (123,124). However, there

has been a report that *C. trachomatis* strains isolated in cycloheximide-treated McCoy cells are difficult to cultivate on subpassage (125).

c. 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 (126).

d. Cytochalasin B

Cytochalasin B is another cytostatic drug that has been recommended for pretreatment of cells to be used for chlamydial isolation (127). They used cytochalasin B in a similar way to IUdR for treatment of McCoy cells.

e. DEAE-dextran (Diethylaminoethyl-dextran)

DEAE-dextran is a polycations compound which transforms the physicochemical properties of the surface of Hela 229 cells. It enhances attachment and phagocytosis of TRIC agents of *C. trachomatis* (128).

When using McCoy cells, treatment with DEAE also increases the chlamydial inclusion count. However the count was lower than that treated with cycloheximide (109). It has been

found that cycloheximide treated McCoy cell is the most sensitive technique for the isolation of *C. trachomis* (123).

1.8) Centrifugation

Centrifugation of cell culture inoculated with chlamydiae is the most essential technique to obtain optimal culture results (114). At centrifugation forces of 3,000 to 6,000 g, the organisms are pelleted onto the cell monolayer so as to increase the chlamydiae - cell contact . 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 % increase in the recovery rate compared with 3,000 to 6,000 xg (129).

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 (120). A suitable time limit for such centrifugation is one hour. Lastly, they also found the greatest number of inclusions after incubating the inoculated monolayers for 48 hrs at 35°C (120).

1.9) Staining

There are three general staining techniques for detecting chlamydial inclusions in cell monolayers.

a. Giemsa staining

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. *C. psittaci* inclusion does not "autofluoresce" when examined by dark-field microscopy (106).

b. Iodine staining

The stain used 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 chlamydiae. The advantage is the use of a simple-bright-field microscopy for detection of inclusions. In general, *C. psittaci* does not contain a glycogen matrix, thus iodine staining is not suitable for detecting *C. psittaci* inclusions (106). Either Giemsa or Iodine staining is used after the inoculation 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. The inclusions are seen after incubating the organism 19 hrs at 35-37°C (106). Comparison of both immunofluorescence and Giemsa staining for the detection of *C. trachomatis* inclusions shows that both stains are

of similar sensitivity (130).

Although the cell culture method provides high specificity but involves several steps in the collection, transportation, and culture of chlamydiae that may compromise viability because of the fastidious nature of the organisms (131, 132,133). In addition, culture techniques are labor intensive, time consuming , and expensive , and they require strict maintenance of a cold chain if there is a delay of more than 8 hours in processing the samples (113).

Therefore, other methods were developed in recent years which detect chlamydial antigen directly or detect chlamydial DNA in patient samples and thereby circumvent specimen-viability concerns. Current available methods include direct antigen detection in specimens with fluorescein-labeled antibodies (DFA), enzyme immunoassay (EIA), DNA probes, and recently the polymerase chain reaction (PCR) method.

2. Direct fluorescence antibody (DFA) test

The DFA test has been considered an attractive alternative for confirmation of infections. This technique employs a fluorescent-labeled monoclonal antibody that detects the major outer membrane protein (MOMP) of the infectious EBs of chlamydiae which is species - specific and , therefore, will not stain *C. psittaci* or *C. pnuemonia* (46).

Gann,P.H. et al (134) have compared the DFA with a standard cell culture technique for the detection of *C. trachomatis* infection in 268 women in an urban family practice setting (low-prevalence population). The sensitivity was 50 % and the specificity was 98 % . Peterson, E.M. et al (135) have compared the DFA (MicroTrak ; Syva Corp., Palo Alto, Calif), with cell culture for detection of *C. trachomatis* in 196 clinical cervical samples. They found that the sensitivity of the DFA was 75 % and the specificity was 99 % . Another group of investigators, Thomas, B.J. et al (136), have compared the DFA with cell culture for the detection of *C. trachomatis* in clinical specimens from 7 babies with conjunctivitis, 100 men with NGU, 35 female contacted of men with NGU, and 100 men with gonorrhoeae. They found that the DFA had a sensitivity of 100 % and specificity of 100 % in both the babies with conjunctivitis and the female contacted of men with NGU. In the men with NGU, the DFA had a sensitivity of 100 % and specificity of 83 %. In the men with gonorrhoeae, the DFA had a sensitivity of 91 % and specificity of 95 %.

Although the DFA is a rapid method that provides high specificity but the disadvantage of the DFA is the necessity for a fluorescence microscope and clinical experience in differentiating chlamydial staining from specimen artifacts. The use of greater number of EBs as positive criteria to improve specificity decreases sensitivity (136,137).

3. Enzyme immunoassay (EIA)

The enzyme immunoassay is considered to be an alternative diagnostic test for detection of *C. trachomatis* because of the ability to rapidly handle large numbers of specimens. In 1985, Mumtaz,G. et al (139) have used Chlamydiazyme (Abbott laboratories),which measures several antigenic components of *C. trachomatis*, to detect *C. trachomatis* antigen in urethral swabs from 61 men with NGU and 79 cervical swabs from women (contacts cases) for rapid diagnosis of chlamydial genital infection. The EIA test had a sensitivity of 92.5 % and specificity of 97.2 % when compared with cell culture (prevalence of 27.1 %). In 1988, Lefebvre,J. et al (140) have compared the EIA with the DFA and cell culture for detection of *C. trachomatis* in endocervical specimens from asymptomatic women and found that the EIA had a sensitivity of 78.4 % and a specificity of 81.1 % (prevalence of 6.7 %) . In 1989 , Mohony,J. et al (141) have compared two enzyme immunoassays detecting different chlamydial antigens , IDEIA (CellTech Diagnostic) which measures lipopolysaccharide antigen and Chlamydiazyme with cell culture for detection of *C. trachomatis* in urethral swabs from 235 men attending a clinic for sexually transmitted diseases (prevalence of 14.9%) and in 458 endocervical swabs from women attending planned parenthood and obstetrics-gynecology clinics (prevalence of 5.9 and 7.7 % , respectively). The percentage of sensitivities and specificities for IDEIA were 62.5 and 99.5, respectively, for specimens from men and 96.3 and 97.9 , respectively , for specimens from women ; results by

Chlamydiazyme for specimens from men were 81.8 and 99.5 , respectively, and for specimens from women, results were 85.2 and 99.3 , respectively.

Although the EIA systems were particularly attractive because of their ability to rapidly handle large numbers of specimens, the sensitivity of the assays varied widely ranging from 62-92 % and in general is less than that of culture.

4. Nucleic acid probe

Recently, DNA probes have been introduced for the detection of *C. trachomatis*. In 1985, Hyppia,T. et al.(142) have evaluated the sensitivity and specificity of the spot hybridization assay by using whole DNA from *C. trachomatis* serotype L1 elementary bodies as a probe (^{32}P-labelled Probe). The sensitivity of detection was 10-100 pg L1 DNA and 10 infected cells. Later on,in 1986, Quinn,T.C. et al.(143) have used plasmid DNA from *C. trachomatis* serotype L2 as a diagnostic probe for detection of *C. trachomatis* from cervical smear. When in situ hybridization was performed, the sensitivity was 91% and specificity was 80 %. Recently , A nonisotopic DNA probe (Gen-Probe PACE system, Gen-Probe, Inc.,San Diego,CA) which used an acridinium ester-labeled, single stranded DNA that is complementary to *C. trachomatis* ribosomal RNA, has become commercially available. Sensitivity and specificity of this probe assay range from 60% to 90% and 59% to 92% respectively (144,145). Later on, this DNA probe test was modified

(Gen-Probe™ PACE 2 system) to reduce technical time and to decrease turn around time. In 1990, Iwen,P.C. et al.(146) have evaluated the Gen-Probe™ PACE 2 for detection of *C. trachomatis* in endocervical specimens. The sensitivities and specificities of the DNA probe compared with cell culture were 93% and 98%, respectively.

5. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was developed by Mullis, K.B. et al (147) in 1984. It is a specific enzymatic amplification of DNA in vitro via a polymerase-catalyzed chain reaction. The amplification is obtained by repeating a three-step reaction run at different temperature. The reaction is based on the annealing and extension of two oligonucleotide primers that flank the target region in duplex DNA. The initial step is denaturation of target DNA and then each primer hybridizes to one of the two separated strands (primers annealing). The annealed primers are then extended on the template strand with a DNA polymerase from 3'hydroxyl end is directly toward 5'end (primers extension).By repeating the reaction n times, the amount of DNA theoretically rises to 2^n.

Recently, the PCR has been developed for detecting *C. trachomatis* by using DNA primers derived from different DNA target sequences. In 1990 , Ostergaard, L. et al (31) have used the PCR for detection of *C. trachomatis*. The pair of synthetic oligonucleotide primers derived from *C. trachomatis*

plasmid sequence were used and demonstrated the sensitivity for detection of 10^{-15} g of DNA , which corresponds to the detection of 100 copies of the plasmid. In addition, when PCR was used to detect *C. trachomatis* from 228 clinical samples comparing with cell culture and EIA (IDEIA : Medico Nobel, Boots- Celltech Ltd., Berkshire, United Kingdom), PCR showed a sensitivity of 100 %, a specificity of 99 %. Wordsworth, B.P. et al (148) have also reported the use of PCR to detect the presence of a plasmid essential for the growth of *C. trachomatis.* As few as 10 copies of the plasmid in the initial reaction mixture were detected by using this technique.

From the studies of several investigators showed that the PCR technique provided high sensitivity and specificity for detection of *C. trachomatis.* In addition , It is not time consuming and comparatively easy to perform. Accordingly, the PCR could be considered to be a valuable diagnostic tool to detect *C. trachomatis* from the clinical specimens. However, because of the high sensitivity of the PCR technique, false positive from carryovercontamination of the previous amplified products (amplicons) often occurs. The technique that will resolve this problem should be used. One of this technique is performed by digestion of amplicon prior to PCR , using incorporation of dUTP instead of dTTP and uracil DNA glycosylase in the reaction.