CHAPTER III

LITERATURE REVIEW

History

In 1943, Joe Smadel questioned the source of infection of seven patients who had no known contact with birds and these cases thought to be psittacosis on the basis of complement fixation antibody (4). Similar epidemics of chlamydial respiratory infection without avian contact had been documented in Scandinavian countries in 1950s. Later studies of archived serum samples, *C. pneumoniae* is considered responsible for these epidemics. The organism was first isolated in the yolk sac of an embryonated chicken egg from the conjunctiva of a Taiwanese child participating in trachoma vaccine trial in 1965 and it was designated as TW-183 (the type strain of *C. pneumoniae*). The second strain, IOL-207 had been obtained from the same type of specimen of an Iranian child with trachoma. Despite the conjuctival source of these two isolates, serologic studies suggested that the organism was not related to eyes disease (23).

The organism role as a human pathogen has not been defined until 1983, when the first respiratory isolate (AR-39) was obtained in Seattle from a university student with pharyngitis (3). In more detailed studies, the organisms were shown to represent a totally new chlamydial species. In 1989, TWAR was then established as a third species of *Chlamydia* named *Chlamydia pneumoniae* . The strain name TWAR was derived from the laboratory designation of the first conjunctival and respiratory isolates (TW-183 and AR-39) (6).

The MIF test developed by Wang in 1970 has currently been the most sensitive and specific method for diagnosis of *C. pneumoniae* infections. The technique uses fixed whole elementary bodies as antigen of which the target epitopes are on the major outer membrane protein. This test, though technically demanded, is sensitive and species-specific, and can differentiate Ig M from Ig G antibody responses (36).

Classification

At present, the order *Chlamydiales* has one family, the *Chlamydiaceae*, containing one genus and four species. Two of these four species, *Chlamydia trachomatis* and *Chlamydia pneumoniae*, naturally infect humans. *C. trachomtis* is the leading cause of preventable blindness in developing countries, a major cause of sexually transmitted diseases throughout the world and a cause of respiratory tract infections in infants (37,38). *C. pneumoniae* is a major cause of upper and lower respiratory tract infections and has been associated with cardiovascular disease (22,30). *Chlamydia psittaci* mainly infects animals, including birds, and is a cause of zoonotic respiratory tract infections. *Chlamydia pecorum* is not known to infect humans but infect cattle and sheep (39). Recently, two *Chlamydia*-like strains, "Simkania negevensis" or Z-agent and "Parachlamydia aacanthamoeba" or Bn_9, have been described (40). The characteristics of these 4 species were summarized in Table 1 (34).

Table 1. Characteristics of the four chlamydial species (34)

| Characteristics	Chlamydial species			
	C. trachomatis	*C.pneumoniae*	*C. psittaci*	*C. pecorum*
Natural hosts	Humans, mice, pigs	Human, horses	Birds, mammals, occasionally humans	Cattle and sheep
EB morphology	Round	Round or pear shaped	Round	Round
Inclusion	Oval, vacuolar	Oval, dense	Variable, dense	Oval, dense
Iodine staining	Yes	No	No	No
Sulphonamide	Yes	No	No	No
No. of serovars	At least 15	1	Undefined	3
Characteristic infections	Genital and ocular mucosa. Often inapparent. Intermittent shedding. Rarely systemic.	Chronic respiratory tract Infection. Possible association with heart disease.	Frequently systemic: pneumonia, abortion, etc.	CNS, respiratory and gut. Often inapparent. Prolongedcarriage.
DNA: Mol %G+C	39.8	40.3	39.6	39.3
Homology % relative to				
C. trachomatis	92			
C. pneumoniae	1-7	94-96		
C. psittaci	1-33	1-8	14-95	
C. pecorum	1-10	10	1-20	88-100

Morphology

Chlamydia have a unique dimorphic replication cycle that distinguishes them from all other groups of bacteria. This developmental cycle consists of 2 main forms of the microorganism, The infectious cell type for extracellular survival and transmission is termed the elementary body (EB). The intracellular, vegetative cell type is called the reticulate body (RB).

Elementary body(EB)

EB of *C. pneumoniae* consists of pear-shaped or occasionally round shaped particles of 200 - 300 nm in diameter, with an irregular core of DNA. The studies of freeze-etched whole EBs or of ruthenium red-stained sectioned EBs show the presence of flower-like rosettes in the EB envelope. Fine projections of 5 - 6 nm in structure attached on the inside to the cytoplasmic membrane protrude from the surface. These projections are common to all members of the genus *Chlamydia* and are possibly crucial for pathogen-host interaction. The inner aspect of the EB cell wall consists of a regular array of hexagonal structures that may correspond with the outer membrane proteins (6,41).

Reticulate body (RB)

The RB is about 800 - 1000 nm in diameter and in thin section, looks like a typical Gram-negative cocci, with a homogenous interior feature containing numerous 70S ribosomes. The outer envelope of the RB is much less rigid than that of the EB, with a tendency to form pleomorphic outer envelope blebs. Projections and rosettes are present on the RB surface in similar number to the EB (6,41). Electron micrograph of *C. pneumoniae* EB and RB was shown in Figure 1 (34).
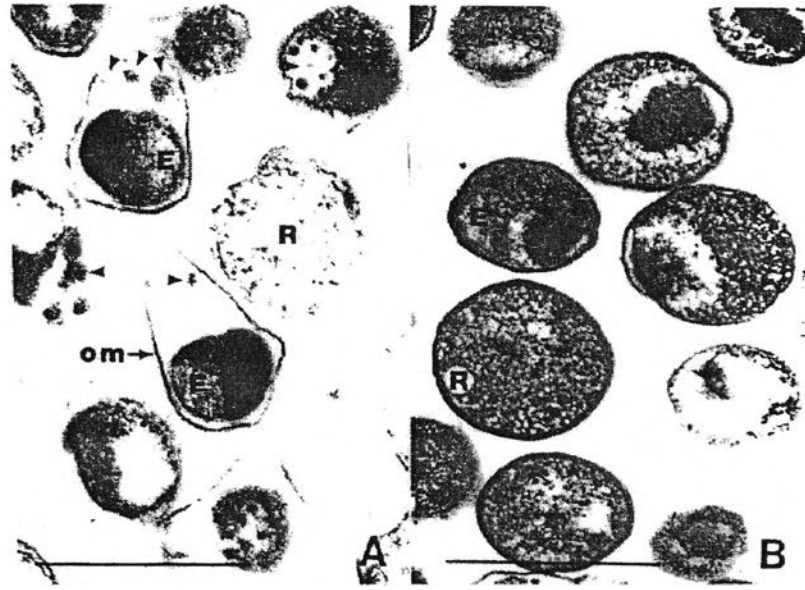
Fig 1. Electron micrograph of (A) *C. pneumoniae* and (B) *C. trachomatis* (bar=0.5 μm). E: elementary body; R: reticulate body; om: outer membrane; arrowhead: small electron-dense bodies of miniature bodies (4).

Developmental cycle of *C. pneumoniae*

Like all chlamydia, *C. pneumoniae* undergoes a developmental cycle in which two functionally and morphologically distinct cell types are the elementary body (EB) and the reticulate body (RB). *C. pneumoniae* EBs appear to interact initially with microvilli prior to endocytosis by the host cell. Recent evidence suggests that it uses heparan sulfate as a bridge between receptors on its surface and the target cell (42). Once internalized, the EBs still retaining their characteristic size and condensed nucleoid structure, remain within individual tightly membrane-bound vesicles. By 8 h postinfection, differentiation into RBs is evidenced by the dissociation of the nucleoid and some increase in size is apparent, although EBs still containing a condensed nucleoid may be observed. At 12 h postinfection, morphologically typical RBs are present, and by 19 h postinfection, multiplication has begun. The initial event includes new synthesis of chlamydial proteins, reduction of disulfide bonds so that membrane protein are no longer cross-linked, and activation of ATPase. During growth and replication, chlamydiae obtain high-energy phosphate compounds from the host cell(43). At 24 h and 36 h postinfection the RBs continue to multiply and accumulate within the inclusion and no EBs are detected yet. The developmental cycle becomes asynchronous by 48 h postinfection as the differentiation of RBs back into EBs is detected. Condensation of RBs into EBs leads to reduction in size with extensive of membrane blebs containing lipopolysaccharide and compaction of the chromatin into an electron dense nucleoid. Although EBs and intermediate developmental forms are detected, some typical RBs apparently in the process of binary fission, are still observed. By 60 and 72 h postinfection, the inclusion consists of RBs and an increasing percentage of EBs that take on the characteristic pear shape

of *C. pneumoniae* EBs. In addition, the cytoplasmic miniature bodies which are typical only of *C. pneumoniae* EBs become apparent. The intracellular growth of *C. pneumoniae* is completed with lysis of the host cell after 84 h postinfection to release progeny EBs for subsequent rounds of infection (44). The developmental cycle of chlamydiae was shown in Fig. 2.
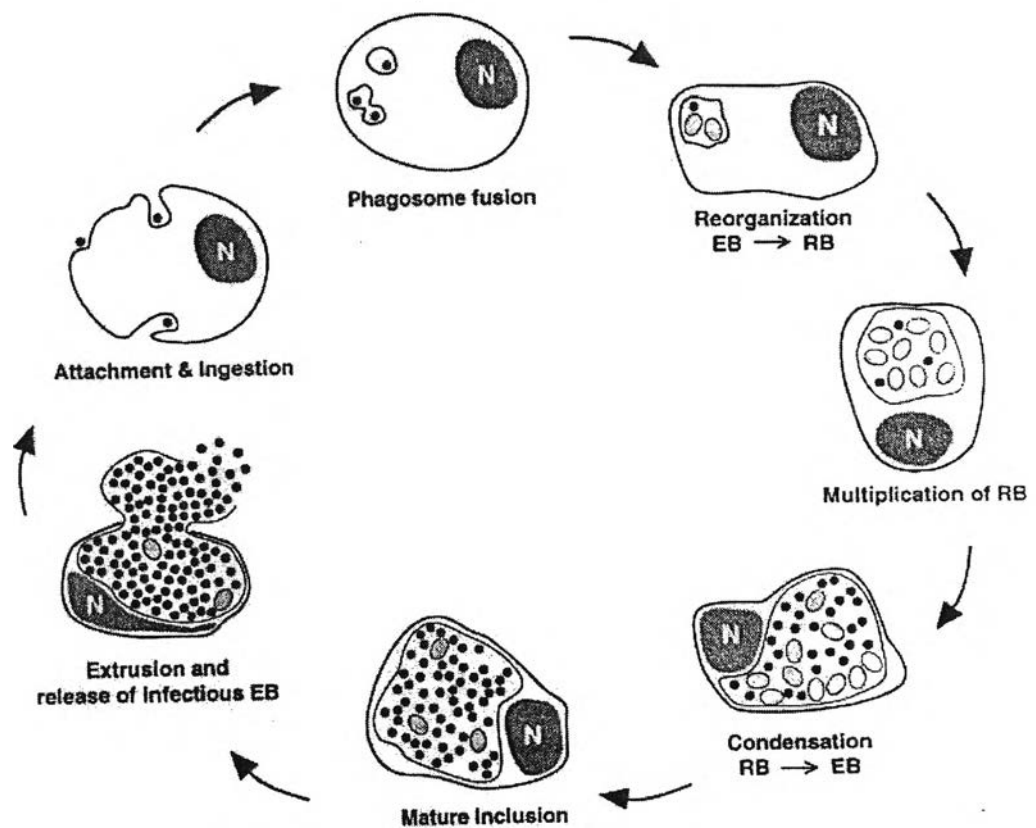
Fig 2. Chlamydial developmental cycle. EB: elementary bodies; RB: reticulate bodies (45).

Antigenic structure

The chlamydiae possess a rough type lipopolysaccharide (LPS) carrying a chlamydial genus-specific epitope consisting of 3-deoxy-D-manno-octulosonic acid (KDO) linked $_\alpha$KDO(2$_{\rightarrow}$8) $_\alpha$(2$_{\rightarrow}$4) $_\alpha$KDO, in which the $_\alpha$(2$_{\rightarrow}$8) linked disaccharide is immunodominant and unique to chlamydia. This epitope is surface exposed and immunoaccesible on RBs and Ebs. It was chemically synthesized and used in commercially serological assay for chlamydial antibody (45).

The immunodominant antigen at surface of the infectious chlamydial EB is the major outer membrane protein (MOMP), encoded by *omp1*. Antigenic variation within MOMP in *C. pneumoniae* appears much more limited. Two geographically distinct isolates of *C. pneumoniae* had an essentially identical *omp1* sequence (46). The serological and genetic uniformity of *C. pneumoniae* strains isolated from various geographical areas for more than 30 years is remarkable (47). In fact, the current thinking about *C. pneumoniae* MOMP is that it is not surface exposed. It is less immunodominant than *C. trachomatis* MOMP (48). Wolf et al. used immunoprecipitation with monoclonal antibody and mass spectrometry analysis to show that MOMP is localized on the surface of the organism and is an immunogenic protein (49). The sequenced genome contains 21 candidates for surface proteins and the actual structure of the surface is not known. *C. trachomatis* is able to avoid the immunological defence by modifications of its MOMP but the mechanisms by which *C. pneumoniae* avoids this immunological pressure of it host are unknown.

C. pneumoniae is more homogeneous than the other species. In all isolates examined, protein profiles were identical with a prominent 39.5 kDa band analogous

to the MOMP of other chlamydiae. In addition to MOMP, cysteine-rich proteins of 15.5, 60, and 98 kDa have been found in the Sarkosyl-insoluble fraction demonstrating their association with the outer membrane complex. The 98 kD cysteine-rich protein appears to be present only in the outer membrane complex of *C. pneumoniae*. It was postulated that the presence of a 98 kDa cysteine-rich protein might provide a more rigid membrane structure to sustain a pear-shaped morphology. (47,50).

Genomic structure

C. pneumoniae has a genome size of 1,230,230 bp, and an estimated 1052 protein coding genes. The *C. pneumoniae* strain AR-39 and the strain CWL029 reported previously have the most similar published genomes to date (51). *C. pneumoniae* does not appear to contain any plasmid whereas some strains of *C. psittaci* and all strains of *C. trachomatis* contain a 7.5 kb cryptic plasmid. Read et al. reported the discovery of a 4,524 nt circular ssDNA bacteriophage genome during sequencing of the *C. pneumoniae* AR39 DNA. This phage is homologous to members of microviridae class of bacteriophages. The phage genome bears 49 % nucleotide sequence identity to the Chp1 phage from *C. psittaci* (52). The whole genome sequence comparison between *C. pneumoniae* strain J138 and CWL029 showed that three DNA segments (G2, G4 and G6), ranging in size from 27 to 84 nt are unique to the J138, while five DNA segments (G1, G3, G5, G7 and G8) ranging in size from 89 to 1649 nt are unique to the CWL029 genome. With the exception of these regions, the nucleotide sequences throughout the genomes are almost identical (identity 99.9 %). Recent analysis of the genomic polymorphism revealed that the genome of *C. pneumoniae* is highly conserved, since the AFLP fingerprints of world-

wide-derived isolates were almost identical, in agreement with previously reported genomic RFLP analysis results, whereas that of *C. trachomatis* is diverse (53,5). The genome sequence of *C. pneumoniae* AR39 which has been found to be identical to the sequence of CWL029 except a truncated ORF (~300 bp shorter in AR39) and bacteriophage, provides additional information on the genomic stability of this organism (51).

Pathogenesis

C. pneumoniae enters the human via the respiratory tract and replicates within bronchial ciliated epithelial of the lung. As a result of respiratory infection, lung macrophages become infected with *C. pneumoniae* (54). After infections foci are initiated by infected macrophages, the organisms may be transmitted to other susceptible cells at the lesion site to establish and expand the disease process. *C. pneumoniae* was detected by immunocytochemistry (ICC) in macrophages, smooth muscle cells, and endothelial cells in human atheromatous lesions. In addition, it was found in the aortic lesions of mouse and rabbit in the animal experiment of *C. pneumoniae* atherosclerosis (55,56). The ability of *C. pneumoniae* to multiply in cell of monocyte-macrophage lineage allows it to disseminate from lung to other sites by hematogenous or lymphatic routes. These findings indicate an important role of macrophages in the pathogenesis of *C. pneumoniae*.

C. pneumoniae are Gram negative bacteria possessing a lipopolysaccharide (LPS). The multiplication of chlamydia is connected with massive production of LPS. The LPS of chlamydia is relatively nontoxic but a potent inducer of cytokines,

apparently due to terminal KDO residues. *C. pneumoniae* infection is associated with the production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-$_\alpha$) and adhesins, such as intercellular adhesive molecule-1 (ICAM-1), macrophage inflammatory protein-1 (MIP-1), which likely contribute to disease pathogenesis (57,58).

Heat shock protein (Hsp) 60 of *C. pneumoniae* also appears to play an important role in immunopathogenesis of chlamydial infection. Hsp 60 is also produced in massive amounts in chlamydial infection. It induces expression of cytokines and adhesins as well as immunological cytotoxicity in vascular endothelium (59,60).

Presence of a large gene family in a microorganism with a small genome will generally be considered to be a potential pathogenicity factor. The outer membrane protein named the proteins Omp 4 – 15 or the GGAI proteins which is found formed a stable protective layer on the surface of the EBs. The *omp 4 – 15* genes have the potential to encode proteins that may vary the surface of the EBs which may be of importance for EBs adherence to different cell types and to escape the host immune system and thereby have a potential role as pathogenicity factors (48).

Immune response

Infection with *C. pneumoniae* induces serum immunoglobulin M (IgM), IgG and IgA response. These species-specific antibodies can be detected by Microimmunofluorescence (MIF) test or Enzyme-linked immunosorbent assay

(ELISA). Antibodies against group-specific LPS also develop and can be demonstrated by complement fixation (CF) or ELISA. Two patterns of antibody response to acute *C. pneumoniae* infection have been identified; in primary infection, prompt CF antibody response is seen, TWAR MIF IgM antibody appear later about 3 weeks after the onset of illness. Antibody in the IgG fraction may not appear until 6 to 8 weeks after onset. In reinfection, CF and IgM antibody titer rises quickly, often in 1 to 2 weeks. Understanding these patterns is important in interpreting serologic studies of *C. pneumoniae* infection. IgA antibodies, which have very short half-life and may demand a continuous antigenic stimulus to be present, seemed to be a better indicator of a chronic *C. pneumoniae* infection (24). Comparison of MIF IgM and IgG TWAR antibody responses are shown in Fig 3.
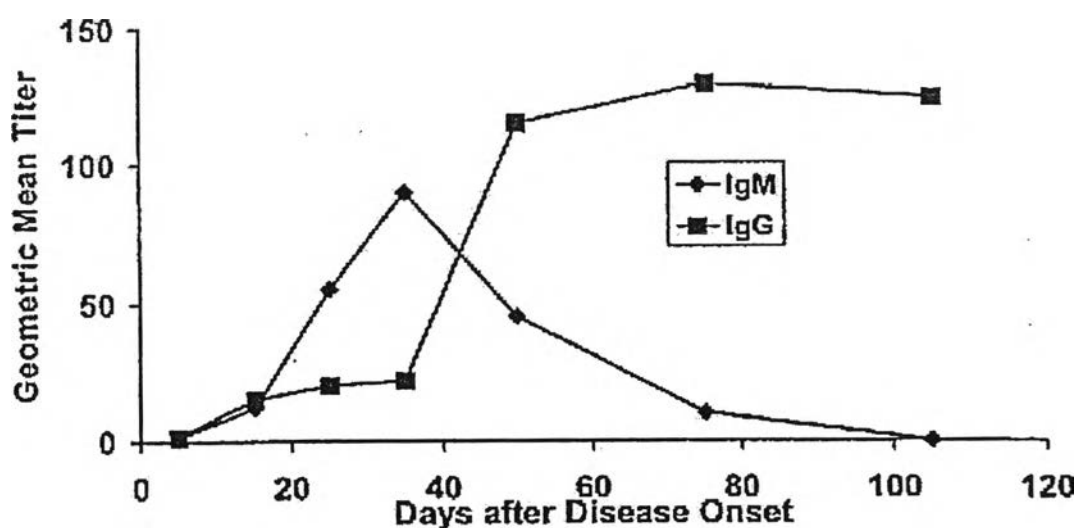

Fig. 3 Comparison of IgM and IgG MIF *C. pneumoniae* antibody response (61).

C. *pneumoniae* can infect a number of cell types, including monocytes and macrophages. Activation of cell-mediated immune (CMI) response is supposedly important for protective immunity. Person infected with C. *pneumoniae* develops a cell-mediated immune response as demonstrated by the lymphocyte transformation assay with peripheral blood or synovial lymphocytes (62,63). Moreover, Halme et al. found that a primary C. *pneumoniae* infection induces the development of lymphocyte proliferation and also the secretion of interferon gamma. C. *pneumoniae* induced lymphocyte activation involves CD8$^+$ T cells in the early phase of infection but CD4$^+$ T-cells in the later stage. However, *in vivo* activation of CD4$^+$ T-cell during the acute disease may relate to their role in recovery from infection (64). Penttila et al. have shown that CD8$^+$ T-cell are necessary for protection from both primary C. *pneumoniae* infection and reinfection (65).

Transmission

C. *pneumoniae* is believed to be transmitted from person to person by respiratory tract secretion but direct evidence is lacking. The incubation period of infection due to C. *pneumoniae* has been estimated for about 1 month, which is longer than that of many other respiratory pathogens (66). Epidemics even in closed population have spreaded slowly. It appears that many infected persons are ineffective transmitters of the organism and that some persons with asymptomatic infections play role in spread of the disease (67).

Epidemiology

Considerable information on the epidemiology of *C. pneumoniae* has been obtained from seroepidemiologic studies of serum banks from past investigations of respiratory infections. It has been shown that infections with this organism were occurring as frequently in 1963 as today. The evidence suggests that the organism is not new but is only newly recognized (68,7). Antibody prevalence to *C. pneumoniae* increases with age. Infection appears to be most common among school-aged children, with children under 5 years affected much less frequently. The prevalence increases dramatically from ages 5 through 14 years, and by age 20 years approximately 50 % of persons throughout the world have detectable levels of antibody to the organism(69,70,71). Due to the decline of antibodies after convalescence, the high prevalence of *C. pneumoniae* antibodies in adults suggests that most people have had more than 1 *C. pneumoniae* infection during their life time. Persistent or chronic *C. pneumoniae* infection seem to be common in the general adult population as judged by the continuous presence of elevated levels of *C. pneumoniae* specific IgG and IgA antibody titers and of immunecomplexes containing chlamydial lipopolysaccharides and proteins (24). Infection with *C. pneumoniae* is endemic, but epidemics tend to occur every 2 - 3 years followed by 3 - 10 years with lower incidence (4).

Clinical Manifestations

The clinical manifestation of an acute *C. pneumoniae* infection can range from asymptomatic to life-threatening. Although pneumonia and bronchitis remain the

most frequent recognized illness, asymptomatic infection or unrecognized, mildly symptomatic illness are the most common result of *C. pneumoniae* infection. The patient's age at the time of infection influences the clinical response (72,73). No set of symptoms or signs is unique to pulmonary infection with *C. pneumoniae*.

1. Pneumonia

The name *C. pneumoniae* was chosen for this organism because pneumonia has been the most commonly recognized disease caused by infection with the organism. Pneumonia due to *C. pneumoniae* is more common among the elderly and less common among persons less than 20 years of age. In a series of studies, about 6 - 20% of cases of pneumonia were caused by *C. pneumoniae* and identified as the third or fourth most common cause (16,74,75). *C. pneumoniae* causes pneumonia in about 6 - 20 % of cases and is rarely severe except in the elderly or those with chronic disease. There are some clinical presentations that are often associated with *C. pneumoniae* pneumonia. Although some cases have an acute onset with immediate signs and symptoms of pneumonia, a more gradual onset is typical. Manifestation of the biphasic illnes, first phase of disease may include pharyngitis, hoarseness, and fever. Patients often suffer from sinusitis that may persist throughout both phases of disease. In the second phase, which begins with cough and malaise, fever is uncommon. Most patients are not very ill unless they are elderly or suffer from underlying pulmonary disease. Reinfection is common, and hospitalization due to pneumonia caused by *C. pneumoniae* is usually required for older patients who have reinfection in which comorbidities undoubtedly play a significant role in the clinical course. *C. pneumoniae* is often found in association with other pathogens, particularly

S. pneumonaie, and the associated pathogen appears to influence the clinical course of pneumonia (4,22,76).

2. Bronchitis

C. pneumoniae bronchitis is often a subacute illness in which symptoms last for many days or weeks. The subacute onset is often preceded or accompanied by pharyngitis. These patients may not come to the medical attention for several weeks. Some patients with bronchitis may have unrecognized pneumonitis early in the course of their illness. In young adults, about 4 % of bronchitis has been shown to be associated with *C. pneumoniae* infection. The symptom responds to appropriate antibiotic therapy (4,69).

3. Sinusitis

Sinusitis, both alone and in association with other syndromes, has been consistently found in the studies of *C. pneumoniae*. About 5 % of primary sinusitis in young adults has been associated with *C. pneumoniae* infection and at least 5 % of patients with lower respiratory tract *C. pneumoniae* infection have had evidence of sinusitis (4,69)

4. Pharyngitis

Primary pharyngitis with or without fever have occurred as separate illness associated with *C. pneumoniae* infection. Pharyngitis is often relatively severe with hoarseness and accompany lower respiratory tract *C. pneumoniae* infection   Up to 80 % of those with *C. pneumoniae* lower respiratory tract infection have sore throat

and less than 1 % of patients with pharyngitis who did not develop lower respiratory tract involvement had evidence of *C. pneumoniae* infection (4,69,77).

5. Coronary artery disease

A series of investigation have found an association between *C. pneumoniae* and atherosclerosis coronary artery disease. The evidence supporting its pathogenic role in atherosclerosis is now substantial including epidemiologic association based on serology, and on the detection of the organism in disease tissue by culture, electron microscopy, immunohistochemical staining, *in situ* hybridization and PCR (24,77,78). Further studies have implied the pathogenic role of this organism by replicating the disease process in animal models and through growth of the organism *in vitro* in coronary artery endothelial cells (79,80). Clinical trials are now underway to determine whether antibiotic treatment can prevent further progression of coronary atherosclerotic disease.

6. Other syndromes

The tendency to cause chronic inflammation is typical of chlamydiae. Chronic *C. pneumoniae* infections of the lung has been associated with asthma, sarcoidosis and reactive arthritis (81,24). It has been found to cause central nervous system infection resulting in meningitis, encephalitis, meningoencephalitis and Guillain Barre syndrome (82,83,84). Recently, *C. pneumoniae* has been suggested as the possible cause of multiple sclerosis; genomic DNA and viable organisms were detected in the CSF of an overwhelming majority of multiple sclerosis patients, and the patient was successfully treated by antibiotics (85).

Treatment

In vivo, *C. pneumoniae* infections have shown a remarkable tendency to recur, often requiring repeated courses of antibiotics. For this reason and for the prevention of chronic infections that may have serious sequelae, prolonged treatment has been recommended. Treatment with tetracycline or macrolide/azalide derivatives should be continued for 2 or preferably 3 weeks (3). For adults, the recommended regimen is doxycycline or erythromycin (2 g/d for 2-3 weeks) or azithromycin (1.5 g over 5 days); for children, it is either erythromycin or clarithromycin administered as a suspension for 10 days to 2 weeks (86).

Vaccine development

Since *C. pneumoniae* has been associated with chronic diseases, including asthma, chronic bronchitis, atherosclerosis and acute myocardial infarction. Vaccination against *C. pneumoniae* would be an effective strategy for preventing or controlling *C. pneumoniae* infections as antimicrobial therapy used in the treatment of acute infections may not be effective in resolving the infection in associated chronic conditions. Immune response induced by intramuscular DNA immunization with *C. pneumoniae omp1*, *omp2* or *Hsp60* genes were studied. Immunization with *omp2* resulted in a strong serum antibody response against Omp2 protein, but it failed to protect the mice. These vaccines did not reduce the severity of histologically assessed pneumonia, but immunization resulted in significantly higher lymphoid reaction in the lung indicating immunological memory (87).

Laboratory diagnosis

Since 1985, *C. pneumoniae* has emerged as a common and important respiratory pathogen worldwide. More attention has recently been directed to diagnosis since the acute infection is treatable with antibiotics. Rapid and reliable diagnostic test will provide an appropriate therapy early in the course of the disease. This strategy may prevent the development of chronic *C. pneumoniae* infection. It is impossible to diagnose *C. pneumoniae* infection on the basis of clinical symptoms and signs although its epidemiological data and clinical course can help in the diagnosis. The final etiologic diagnosis of *C. pneumoniae* infection must always be based on microbiological criteria.

1. Culture

In contrast to *C. trachomatis* which culture has been the diagnostic gold standard, *C. pneumoniae* is difficult to be isolated from clinical samples. Only few laboratories have reported numerous isolates, negative results have been commonly reported. The method currently used by most laboratories for culturing *C. pneumoniae* was first described in 1988 (88). In serologically verified cases of *C. pneumoniae* infection, the sensitivity of culture in cell lines commonly used for isolation of *C. trachomatis* such as McCoy and HeLa 229 has been about 50% (3,4). The most sensitive cell lines for isolation are HL (89,90) and HEp-2 cell lines (91). Tjhie et al. reported that the pretreatment of host cell monolayer with polyethylene glycol (PEG) before inoculation increases recovery of *C. pneumoniae* slightly and that extending culture time to 7 days with additional centrifugations on days 3, 4, and 5 in combination with PEG pretreatment improves recovery by over 300-fold (92).

Kazuyama et al. reported that pretreatment of patient specimens with trypsin before the inoculation increased inclusion formation by 3 to 4 logs depending on the strains used(93). Additional centrifugations and a 7-days culture time resulted in a 500- to 5,000-fold increase in the number of detectable inclusion-forming units (94).

2. Antigen detection

2.1 Enzyme immunoassay (EIA)

Antigen detection by EIA used genus specific antibody to measure common lipopolysaccharide (LPS) group antigen that is present in all chlamydiae. Thus, this test can be used for the detection of *C. pneumoniae* LPS in respiratory tract samples. The EIA procedure is quick, simple, and suitable for processing large numbers of samples. Boman et al. found that in nasopharyngeal samples, the sensitivity and specificity seems to be equal to those of nested PCR. Since EIA is *Chlamydia* genus-specific, positive results should be confirmed by another method if information about *Chlamydia* species is desired. When sputum and throat samples are used, the sensitivity and specificity of EIA were lower than in nested PCR. The EIA sensitivity with throat samples was only 42%. For sputum and throat samples, further evaluation and calculations with other cutoff values might improve the performance of EIA (95,34).

2.2 Direct fluorescent antibody (DFA) test

Direct fluorescent antibody (DFA) staining is based on direct visualization of EBs with the characteristic morphology, using a genus specific or a species specific monoclonal antibody. It has been used successfully in the diagnosis of *C. trachomatis* infection. However, it has been shown to be insensitive for the demonstration of *C.*

pneumoniae in throat swab samples. The sensitivity of this technique in culture and serology controlled studies has ranged from 20 - 60 % and the specificity depends on the skill of the interpreter. The DFA has the advantage of allowing microscopist to assess quality of the specimen. Being more labor intensive, the DFA is less suited to processing large numbers of specimens (24,96).

3. Serological tests

3.1 Complement fixation (CF) test

Complement fixation test has been used to diagnose chlamydial respiratory infections (97). This assay used an enriched lipopolysaccharide (LPS) antigen derived from *C. psittaci* in the detection of *Chlamydia* genus-specific antibodies. It is sensitive only in the diagnosis of primary infections in young adults (3). The result of this test is often negative in case of reinfection (98). In one study, fewer than 33% of patients with other serological evidence for *C. pneumoniae* infection had complement fixation antibodies (16). CF test is widely available and remains a convenient screening technique but it is technically demanded and no information about the immunoglobulin classes involved in the reaction is obtained.

3.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA test is based on a chemically pure structure of a recombinant DNA lipopolysaccharide (rDNA LPS) which contains a genus-specific epitope of *chlamydia* spp. pathogenic for human as an antigen (99). The ELISA test allows the quantitative detection of genus-specific anti-chlamydia IgG, IgA and IgM antibodies. A chemically defined LPS antigen fragment is used that theoretically excludes cross reactivity with other organisms. It is now accepted that LPS antibodies appear very

early in the course of infection. The advantage of ELISA is the rapid development of anti-LPS antibodies (100). Also, ELISA results are observer independent and can easily be standardized for routine diagnosis. One of the disadvantages of using LPS is the inherent serological cross-reactivity among the *Chlamydia* species (38). Recently, purified elementary bodies of *C. pneumoniae* have been used as antigen to detect specific antibody to *C. pneumoniae* infection by using ELISA technique (81).

3.3 Microimmunofluorescence (MIF) test

The MIF test, devised in 1970 for *C. trachomatis*, is the current method of choice for the laboratory diagnosis of acute *C. pneumoniae* infection (36). MIF is an indirect fluorescent antibody technique that enables observation of the binding of antigen antibody that is detected secondarily by fluorescein-conjugated anti-globulin to the corresponding antibody molecules. The antigens used in the test are whole EB chlamydia organisms. In order to differentiate the immunoglobulin class of antibody, fluorescein conjugates of anti-IgM or anti-IgG are used. This test is supposed to be specific for *C. pneumoniae* infection. It can distinguish between antibodies in the IgM and IgG serum fractions, which is helpful in distinguishing recent infection from past infection and reinfection from primary infection. The appearance of MIF antibody is slow therefore, a 3- to 4- week interval is recommended for obtaining the convalescent serum sample. Debate continues over the serological criteria for diagnosing acute infection, but a fourfold or greater rise in IgM or IgG antibody between acute and convalescent serum samples is the considered diagnostic and a single IgM titer of $\geq$ 1:16 or IgG titer $\geq$ 1:512 has been accepted as evidence of acute infection. False positive MIF IgM antibody tests may occur if patients have circulating rheumatoid factor, especially when elderly patients are involved.

Therefore, removal of IgG rheumatoid factor for MIF IgM-positive sera is recommended (102). The value of MIF has been disputed since the test is not well standardized. It is technically demanding, depending on the reader and may yield questionable results (103,18).

4. Nucleic acid amplification techniques

Polymerase chain reaction (PCR) is one of a few *C. pneumoniae* detection strategies that can give a result in a clinical-relevant time frame. In contrast to cell culture and serology, PCR provides a more rapid alternative for identification of *C. pneumoniae* infection. Several diferrent targets for amplification have been used, including a cloned *Pst* I fragment, the gene encoding the major outer membrane protein (*omp*1), and the 16S rRNA gene (103,35,33). The PCR protocol described by Tong and Sillis, in which a nested touchdown PCR with a target within the *omp1* gene was used. The external primers amplified a 333-bp product from both *C. pneumoniae* and *C. psittaci* but the internal primers is specific for *C. pneumoniae*. Therefore, only the first-stage product from *C. pneumoniae* can be amplified in the nested PCR, yielding a 207-bp product. It has been proved to be both sensitive and specific compared with cell culture, DFA and MIF serology (35). Gaydos et al. used 16S rRNA gene as the target and combined PCR and enzyme immunoassay (EIA) method. The 463-bp PCR product is detected in an EIA after hybridization to a biotinylated RNA probe complementary to a part of the amplified 16S rRNA gene. It has proved to be specific and fairly sensitive for detection of *C. pneumoniae* in respiratory specimens, although its sensitivity need to be improved (33). Jantos et al. have developed a simplified PCR-EIA based on the 16S rRNA primers described by Gaydos et al. using biotin-labeled *C. pneumoniae* PCR products hybridized to a

digoxigenin-labeled probe. The complex is captured in streptavidin-coated microtiter plates and then detected with an antidigoxigenin-peroxidsae conjugate and a colorimetric substrate. The PCR-EIA was found to be more sensitive than single step PCR and sensitive as Southern blot hybridization (34).

Successful amplification of *C. pneumoniae* DNA from clinical specimens has been reported by several researchers. However, one major concern to the reliability of PCR method is that it may generate false positive and false negative result. In order to minimize the risk of amplification product carryover, enzyme uracil-N-glycosylase (UNG) was used to degrade contaminating amplification products from previous PCR for detection of *C. pneumoniae* (34). Recently, construction of positive controls DNA to identify inhibitors of the PCR in clinical specimens has also been reported (104,105).