DETECTION OF *CHLAMYDIA PNEUMONIAE* IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE WITH ACUTE RESPIRATORY ILLNESS

SOMYING NGAMURULERT

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SOMYING NGAMURULERT: DETECTION OF *CHLAMYDIA PNEUMONIAE* IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE PATIENTS WITH ACUTE RESPIRATORY ILLNESS. THESIS ADVISORS: SONTANA SIRITANTIKORN, Dr.rer.nat., KHUN NANTA MARANETRA, M.D., PILAIPAN PUTHAVATHANA, Ph.D., URAIWAN KOSITANONT, M.Sc., AMORNRUT LEELAPORN, Ph.D. 98 p.ISBN 974-663-439-9

Chamydia pneumoniae has emerged as a common cause of acute respiratory tract infection. Diagnosis of *C. pneumoniae* infection is difficult. The purpose of this study is to develop polymerase chain reaction (PCR) and use it for detection of *C. pneumoniae* DNA in clinical specimens i.e., throat swab, nasal swab and sputum of chornic obstructive pulmonary disease (COPD) patients with acute respiratory illness (ARI). The PCR and serological methods i.e., ELISA and microimmunofluorescense (MIF) are used for diagnosis of acute *C. pneumoniae* infection and the role of *C. pneumoniae* infection in COPD patients with ARI is evaluated.

Primers derived from 16s rRNA gene were used in nested PCR. The result showed that nested PCR was sensitive and specific for detection of *C. pneumoniae* DNA. PCR could detect 0.005 inclusion body forming unit which was 1000 times more sensitive than isolation in cell culture. The specificity of PCR was determined by using *C. pneumoniae* primers to amplify DNA of *Moraxella catarrhalis. Haemophilus influenzae*, and *Streptococcus pneumoniae.* No amplified products were obtained. The PCR method provided a detection rate of *C. pneumoniae* DNA in throat swabs superior to sputum and nasal swabs. *C. pneumoniae* DNA was found in 40.2% of COPD patients with ARI and 6.7% of the healthy control group. The rates of *C. pneumoniae* DNA detection among the COPD patients with common cold, influenza-like illness and acute exacerbation were not different.

The serological response of COPD patients was investigated by ELISA and MIF. High sensitivities were obtained for IgG- and IgA-ELISA but not for IgM-ELISA when compared to the MIF test. Seroprevalence of IgG, IgM and IgA antibody to *C. pneumoniae* in convalescent sera were found in 85.5%, 4.5%, 88.7% respectively. Both IgG frequency and mean of cut of index (COI) in COPD patients with ARI and without ARI were significantly higher than in the healthy control group. Frequency of *C. pneumoniae* IgA in COPD patients with ARI was higher than in both control groups. This investigation showed *C. pneumoniae* may plays a significant role in acute respiratory tract illness in COPD patients.

PCR and serological methods were used for diagnosis of *C. pneumoniae* infection in COPD with ARI patients. Acute infection of *C. pneumoniae* determined by PCR and/or MIF was 69.6%. Detection rate of *C. pneumoniae* infection diagnosed by PCR (56.5%) is more than MIF (37.0%). Sensitivity for diagnosis of acute respiratory tract infection in COPD patients was 81.3% by PCR and 53.1% by MIF. ELISA-IgG COI $\geq$ 2.5 and IgA COI $\geq$ 3 were suggested for detection of acute *C. pneumoniae* infection. We concluded that *C. pneumoniae* may be a significant etiologic pathogen of acute respiratory tract infection in COPD patients. Combination of PCR and serology methods could be used for diagnosis of acute infection of *C. pneumoniae* in COPD patients; however, the rate of detection by PCR was higher than serology.

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สมหญิง งามอุรุเลิศ : การตรวจหาเชื้อคลามัยเดีย นิวโมนิอิ ในผู้ป่วยโรคปอดอุดกั้นเรื้อรังที่มีอาการทางระบบทางเดินหายใจเฉียบพลัน (DETECTION OF *CHLAMYDIA PNEUMONIAE* IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE PATIENTS WITH ACUTE RESPIRATORY ILLNESS) คณะกรรมการควบคุมวิทยานิพนธ์: สนทนา ศิริตันติกร,Dr.rer.nat.,คุณนันทา มาระเนตร์,MD.,พิไลพันธ์ พุธวัฒนะ, Ph.D., อุไรวรรณ โฆษิตานนท์, M.Sc., อมรรัตน์ ลีลาภรณ์, Ph.D. 98 หน้า ISBN 974-663-439-9

เชื้อคลามัยเดีย นิวโมนิอิ เป็นสาเหตุก่อโรคติดเชื้อทางระบบทางเดินหายใจเฉียบพลัน การวินิจฉัยโรคติดเชื้อคลามัยเดีย นิวโมนิอิ ยังเป็นเป็นเรื่องยากและเป็นปัญหา ในการศึกษาครั้งนี้จึงได้พัฒนาวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส เพื่อตรวจหาดีเอ็นเอของเชื้อคลามัยเดีย นิวโมนิอิ ในสิ่งส่งตรวจ คือ จากลำคอ โพรงจมูก และเสมหะ ในผู้ป่วยโรคปอดอุดกั้นเรื้อรัง (COPD) ที่มีอาการทางระบบทางเดินหายใจเฉียบพลัน และใช้วิธีปฏิกิริยาลูกโซ่โพลีเมอเรส และวิธีการตรวจทางน้ำเหลือง คือ วิธี enzyme-linked immunosorbent (ELISA) และ วิธี microimmunofluorescent (MIF) มาใช้ในการวินิจฉัย การติดเชื้อคลามัยเดีย นิวโมนิอิ แบบเฉียบพลัน และศึกษาบทบาทการติดเชื้อคลามัยเดีย นิวโมนิอิ ในผู้ป่วย COPD ที่มีอาการทางระบบทางเดินหายใจเฉียบพลัน

ปฏิกิริยาลูกโซ่โพลีเมอเรสรอบในใช้ primers ลำดับเบสของ 16s rRNA ยีน ปฏิกิริยาลูกโซ่โพลีเมอเรสที่พัฒนาขึ้นมาจะมีความไว และความจำเพาะต่อการตรวจหาเชื้อ ความไวของปฏิกิริยาลูกโซ่โพลีเมอเรสนี้สามารถตรวจหาเชื้อได้ที่ปริมาณ 0.005 IFU/ml ซึ่งมีความไว 1,000 เท่า เมื่อเปรียบเทียบกับวิธีการเพาะเลี้ยงเชื้อ ส่วนความจำเพาะทำการทดสอบกับดีเอ็นเอของเชื้อ *Moraxella catarrhalis, Haemophilus influenzae* และ *Streptoccous pneumoniae* พบว่า primers ไม่ขยาย DNA ของเชื้อเหล่านี้ นำมาตรวจหาเชื้อคลามัยเดีย นิวโมนิอิ ดีเอ็นเอ ในกลุ่มผู้ป่วย COPD ที่มีอาการทางระบบทางเดินหายใจเฉียบพลัน พบร้อยละ 40.2 และพบเพียงร้อยละ 6.7 ในกลุ่มคนปรกติ พบว่าในสิ่งส่งตรวจมาจากลำคอ สามารถพบเชื้อได้บ่อยมากกว่าในเสมหะ และในโพรงจมูก อัตราการพบเชื้อระหว่างผู้ป่วย COPD ที่มีอาการของ common cold, influenza-like illness และ acute exacerbation ไม่ต่างกัน

วิธีการตรวจทางน้ำเหลืองทั้งสองวิธี ในผู้ป่วย COPD ที่มีอาการทางระบบทางเดินหายใจเฉียบพลัน พบว่าการตรวจโดยใช้วิธี ELISA หาแอนติบอดิต่อ IgG และ IgA มีความไวสูงแต่ แอนติบอดิต่อ IgM มีความไวต่ำ เมื่อเปรียบเทียบกับวิธี MIF ความชุกของ IgG IgM และ IgA แอนติบอดิต่อเชื้อคลามัยเดีย นิวโมนิอิ ในเลือดระยะฟื้นจากโรคพบเป็น 85.5%, 4.5%, 88.7% ตามลำดับ ทั้งความถี่และค่ากลางของ cut of index (COI) ของแอนติบอดิต่อ IgG ของผู้ป่วย COPD ทั้งที่มีและไม่มีอาการทางระบบทางเดินหายใจเฉียบพลันมีค่าสูงกว่ากลุ่มควบคุมในขณะที่ความถี่ของแอนติบอดิต่อ IgA เฉพาะในผู้ป่วย COPD ที่มีอาการจะมีค่าสูงกว่าในกลุ่มควบคุมทั้งสองกลุ่ม แสดงถึงเชื้อคลามัยเดีย นิวโมนิอิ อาจมีบทบาทสำคัญต่อการก่อโรคระบบทางเดินหายใจ ในผู้ป่วย COPD

วิธีปฏิกิริยาลูกโซ่โพลีเมอเรส และวิธีการตรวจทางน้ำเหลือง ควรใช้ควบคู่กันในการตรวจวินิจฉัยโรคติดเชื้อคลามัยเดีย นิวโมนิอิ เมื่อใช้วิธีปฏิกิริยาลูกโซ่โพลีเมอเรส และ/หรือ วิธี MIF พบการติดเชื้อคลามัยเดีย นิวโมนิอิ แบบเฉียบพลัน 69.6% ในผู้ป่วย COPD ที่มีอาการทางระบบทางเดินหายใจเฉียบพลัน อัตราการติดเชื้อคลามัยเดีย นิวโมนิอิ ตรวจด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส พบร้อยละ 56.5 มากกว่าวิธี MIF ซึ่งพบร้อยละ 37.0% ความไวของการตรวจวินิจฉัยโรคด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส เป็นร้อยละ 81.3 และด้วยวิธี MIF เป็นร้อยละ 53.1 ELISA-IgG COI $\geq$2.5 และ IgA COI $\geq$3 เป็นค่าที่นำมาวินิจฉัยเพื่อตรวจหาการติดเชื้อคลามัยเดีย นิวโมนิอิ แบบเฉียบพลัน ในผู้ป่วยCOPD ได้ กล่าวโดยสรุปเชื้อคลามัยเดีย นิวโมนิอิ อาจเป็นสาเหตุก่อโรคระบบทางเดินหายใจเฉียบพลันในผู้ป่วยCOPD โดยการวินิจฉัยการติดเชื้อคลามัยเดีย นิวโมนิอิ ที่เป็นสาเหตุก่อโรคควรจะใช้วิธีปฏิกิริยาลูกโซ่โพลีเมอเรส และวิธีการตรวจทางน้ำเหลืองร่วมกัน อย่างไรก็ดีวิธีปฏิกิริยาลูกโซ่โพลีเมอเรสสามารถตรวจพบเชื้อได้มากกว่าวิธี MIF

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LIST OF ABBREVIATIONS

Bp	Base pair
µl	Microliter
DFA	Direct immunofluorescent assay
DNA	Deoxyribonucleic acid
EB	Elementary body
ELISA	Enzyme linked immunosorbent assay
hr	Hour
LPS	Lipopolysaccharide
M	Molar
MIF	Microimmunofluorescent
min	Minute
ml	Milliliter
mM	Millimolar
MOMP	Major outer membrane protein
°C	Degree celcius
PCR	Polymerase chain reaction
RB	Reticulate body
RLU	Relative light unit
rpm	Revolution per minute
sec	Second
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Genus *Chlamydia* is divided into four species i.e., *Chlamydia trachomatis, C. psittaci, C. pneumoniae,* and *C. pecorum.* The Chlamydiae are non-motile, gram-negative, obligatory intracellular bacteria (1). Their unique developmental cycle differentiates them from all other microorganisms in which there are two morphologically and functionally distinct cell types : the infectious, elementary body (EB) with a diameter of 0.25 to 0.35 μm and reticulate body (RB) with a diameter of 0.5 to 1 μm (2). Chlamydiae replicate within the cytoplasm of host cells. They differ from viruses by processing both RNA and DNA and their cell walls are quite similar in structure to those of gram-negative bacteria. Chlamydiae are susceptible to many broad-spectrum antibiotics, possess a number of enzymes, and have a restricted metabolic capacity. Thus they have been considered energy parasites that use the ATP produced by the host cell for their own requirement (3,4).

Chlamydia pneumoniae, strain TWAR has recently been classified as the third species of the *Chlamydia* genus by means of ultrastructural and DNA homology analysis (5,6). *C. pneumoniae* is a worldwide respiratory pathogen associated with the full range of respiratory tract infections including pharyngitis, otitis media, bronchitis and exacerbation of asthma and chronic obstructive pulmonary disease (COPD). It has been implicated as a cause of 6-20% of community-acquired pneumonia and approximately 5% of bronchitis (7). Several studies have recently stressed the

importance of this agent in the development of respiratory disease, showing a high incidence and prevalence of infections worldwide. The disease spreads via the respiratory route from person to person (8-10). Infection with *C. pneumoniae* has also been associated with extrapulmonary disease such as reactive arthritis, Guillain-Barre' syndrome, meningoencephalitis, and atheromatous disease of the coronary and carotid arteries (11-12). Since acute infection, asymptomatic infection and a chronic carrier state have been described for *C. pneumoniae* infection, the clinical relevance of its infection in some circumstances has been questioned (13).

Laboratory diagnosis of *C. pneumoniae* infection is preferably based on the isolation of the organism from respiratory specimens, polymerase chain reaction (PCR), and/or serology (14). The complexity of isolation and/or demonstrating the presence of the organism is difficult. The presence of inclusions of *C. pneumoniae* in infected cells are small and often lost during passage (15,16). The isolation of *C. pneumoniae* by cell culture takes 3 to 7 days for obtaining the results. Serology is the most commonly used as a diagnostic tool for detection of respiratory chlamydial infections in routine clinical practice. The tests rely on time for antibody production; thus, they are not early-detection methods. Microimmunofluorescent test has been used as a gold standard method for diagnosis of *C. pneumoniae* infection. It provides a great sensitivity & specificity. ELISA test for the detection of chlamydia specific antibodies is introduced mainly into large routine laboratory for diagnosis of *C. pneumoniae* infection and epidemiology study (16). The development of the PCR method has provided an alternative diagnostic method for detecting etiologic agents that are difficult to culture, this method has been used successfully for detection of

C. pneumoniae DNA and offered as a diagnostic tool for screening large numbers of clinical specimens. An important parameter for successful PCR analysis is the quality of the specimens used (17-20).

This research attempts to develop PCR method and use it for determine the detection rate of *C. pneumoniae* in clinical specimens of throat swab, nasal swabs and sputum of COPD with ARI. The established PCR method is employed for determining the relationship of acute infection of *C. pneumoniae* in COPD patients with ARI and the presence of *C. pneumoniae* DNA as well as the responses of *C. pneumoniae* antibody detected by MIF and ELISA. The established PCR and the obtained information may provide for diagnosis of *C. pneumoniae* infection and for study the role of *C. pneumoniae* infection in COPD patients.

CHAPTER II

OBJECTIVES

The objectives of this study are:

1. To develop polymerase chain reaction technique for detection of *C. pneumoniae* DNA.
2. To determine the rate of *C. pneumoniae* in clinical specimens of chronic obstructive pulmonary disease patients showing acute respiratory illness by using established polymerase chain reaction (PCR).
3. To determine the relationship between acute respiratory tract illness in chronic obstructive pulmonary disease patients and detection of *C. pneumoniae* by using PCR, enzyme-linked immunosorbent assay (ELISA) and microimmunofluorescent test (MIF).

CHAPTER III

LITERATURE REVIEW

Properties and morphology of *Chlamydia.*

The family *Chlamydiaceae* are among the most common pathogens throughout the animal kingdom. *Chlamydiae* are obligate intracellular parasites that are classified as bacteria because of the composition of their cell wall and their growth by binary division. *Chlamydiae* have a unique biphasic life cycle: a smaller extracellular form, the elementary body (EB) and a larger replication intracellular form (21,22)(Figure 1). They multiply within the cytoplasm of host cells, forming characteristic intracellular inclusions that can be seen by light microscopy after Giemsa staining *Chlamydiae* are small, about 0.25-1 μl in size. They differ from viruses by processing both RNA and DNA and have cell wall quite similar in structure to those of gram negative bacteria (3). They are susceptible to many broad - spectrum antibiotics, possess a number of enzymes, and have a restricted metabolic capacity. None of these metabolic reactions results in the production of energy. Thus, they have been considered energy parasites that use the ATP produced by the host cell for their own energy requirement.

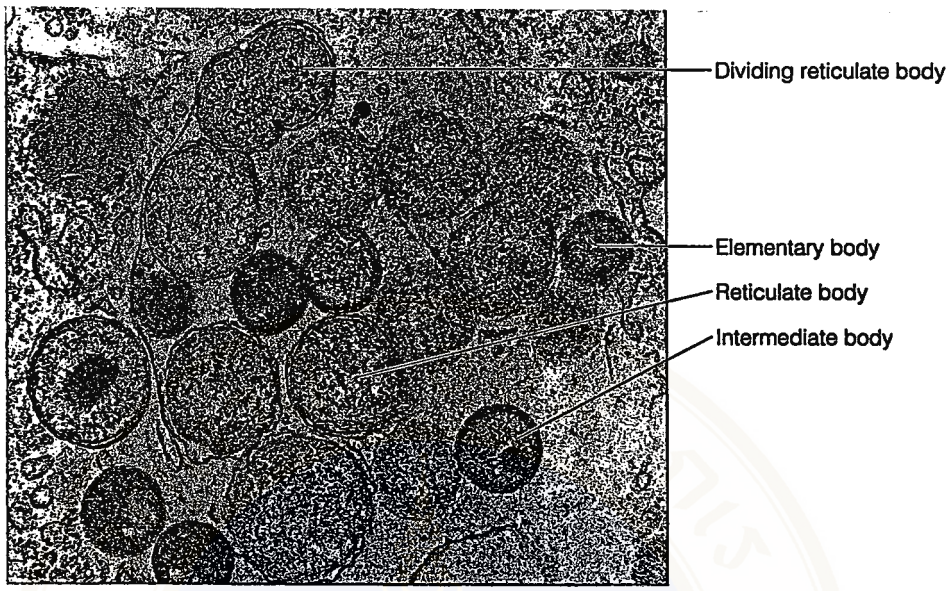

Figure 1. Electron micrograph of *Chlamydiae* in the slice of the cytoplasm of infected cell.

(From Tortora GJ, Funke BR, Case CL, editors. Microbiology an introduction. 5th ed. California : The Benjamin/Comings Publishing ; 1995)

The dense, dark, relatively small elementary bodies have thin walls similar to those of other gram-negative bacteria (A).

The *Chlamydiae* reproduce within the host cell as reticulate bodies (B).

The intermediate bodies appear between the morphology an elementary body and a reticulate body (C).

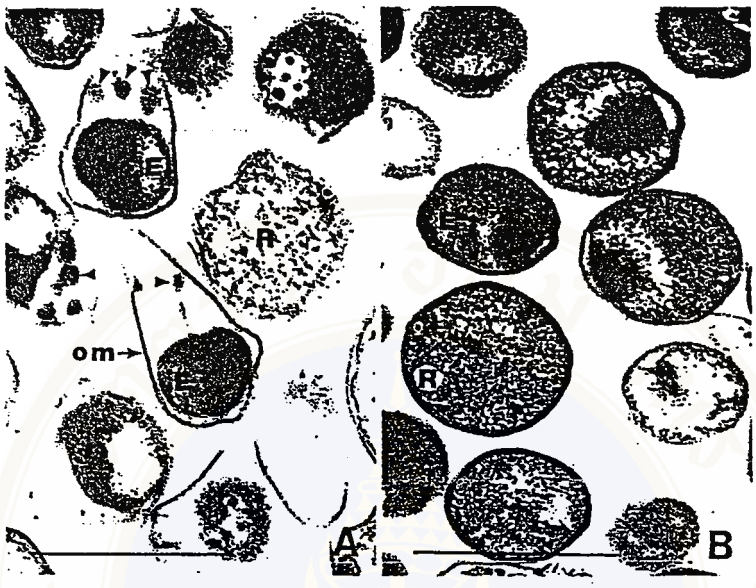

Figure 2. Electron micrographs of *C. pneumoniae* (A) and *C. trachomatis* (B).

(From Grayston JT. A new respiratory tract pathogen: *C. pneumoniae* strain TWAR. J Infect Dis 1990, 161 : 619)

E: elementary body, R: recticulate body, om: outer membrane. Arrowheads indicate small electron-dense bodies (minibodies).

Classification

Chlamydiae are presently placed in Order *Chlamydiales,* family *Chlamydiaceae*, with one genus; *Chlamydia. Chlamydia* species were grouped according to their biological and biochemical properties and 16s ribosomal RNA sequences (23). into four species; *Chlamydia trachomatis, C. psittaci, C. pneumoniae* and a fourth species, *C. pecorum.* The role of *C. pecorum* as a pathogen is not clear, and specialized reagents are required for its identification (24). Only 3 species of *Chlamydia* are human pathogens. *C. trachomatis* includes the organisms causing trachoma, inclusion conjunctivitis , lymphogranuloma venereum (LGV), and genital tract diseases. *C. psittaci* infects many avian species and mammals, producing diseases such as psittacosis, ornithosis, feline pneumonitis , and bovine abortion. *C. pneumoniae* has less than 10% DNA relatedness to the other species and has pear-shape elementary body (EB) rather than round elementary bodies (25). It appears to be exclusively a human pathogens. *C. pneumoniae* has been identified as the cause of a variety of respiratory tract diseases and is distributed worldwide (1,26). Table 1 shows some of the basic characteristics and properties which distinguish *C. pneumoniae* from the other chlamydial species.

Table 1. Characteristics and properties of the *Chlamydiae* species that infect humans.

(From Kuo CC. *Chlamydia pneumoniae* (TWAR). Clin Microbiol Rev 1995,8:452)

Characteristics properties	*C. pneumoniae*	*C. trachomatis*	*C. psittaci*
Major disease	Pneumonia, bronchitis	Trachoma, sexually transmitted diseases, infant pneumonia	Pneumonia, fever of unknown origin
Natural host	Humans	Humans	Birds and lower mammals
No. of servovars	1 (TWAR)	15	Unknown
DNA homology to TWAR (%)	94-100	<5	<10
Morphology of EB on electron microscopy	Pear shaped	Round	Round
MOMP contains species-specific antigens	No	Yes	Yes
Inactivation of specific antigen by methanol	Yes	No	No
Susceptibility to tetracycline and macrolide	Yes	Yes	Yes
Susceptibility to sulfa drugs	No	Yes	No

Developmental cycle of *C. pneumoniae*

The TWAR organism was to named *C. pneumoniae*, a new species of genus *Chlamydiae*, thus far only one strain or serovar has been identified. The strain name TWAR came from the laboratory identifying letters of the first two isolates : TW-183 and AR-39. TW-183 was isolated in 1965 from the eye of a child in a trachoma vaccine study in Taiwan. AR-39 came from a throat swab of a University of Washington student with pharyngitis in 1983 (11). *Chlamydiae* are ingested by susceptible host cells by a mechanism that is not yet completely defined but is likely to be similar to receptor-mediated endocytosis. The uptake process is directly influenced by the *chlamydiae*, and ingestion of *chlamydiae* is specifically enhanced. After attachment, the EB enters the cell in an endosome, within which the entire growth cycle is completely. The *chlamydiae* prevent phagolysosomal fusion. Once the EB has entered the cell, it reorganizes into a reticulate body (RB) that is larger than the EB and richer in RNA. After approximately 8 hr, the RB begins diving by binary fission. Approximately 18 to 24 hr after infection, the RB becomes EB by a poorly understood reorganization or condensation process. The EB is then released to initiate another cycle of infection. The EB is specifically adapted for extracellular survival and is the infectious form of *chlamydiae*. The metabolically active and replicating form, the RB, does not survive well outside the host cell but seems to adapt for an intracellular milieu (27) (Figure 3).

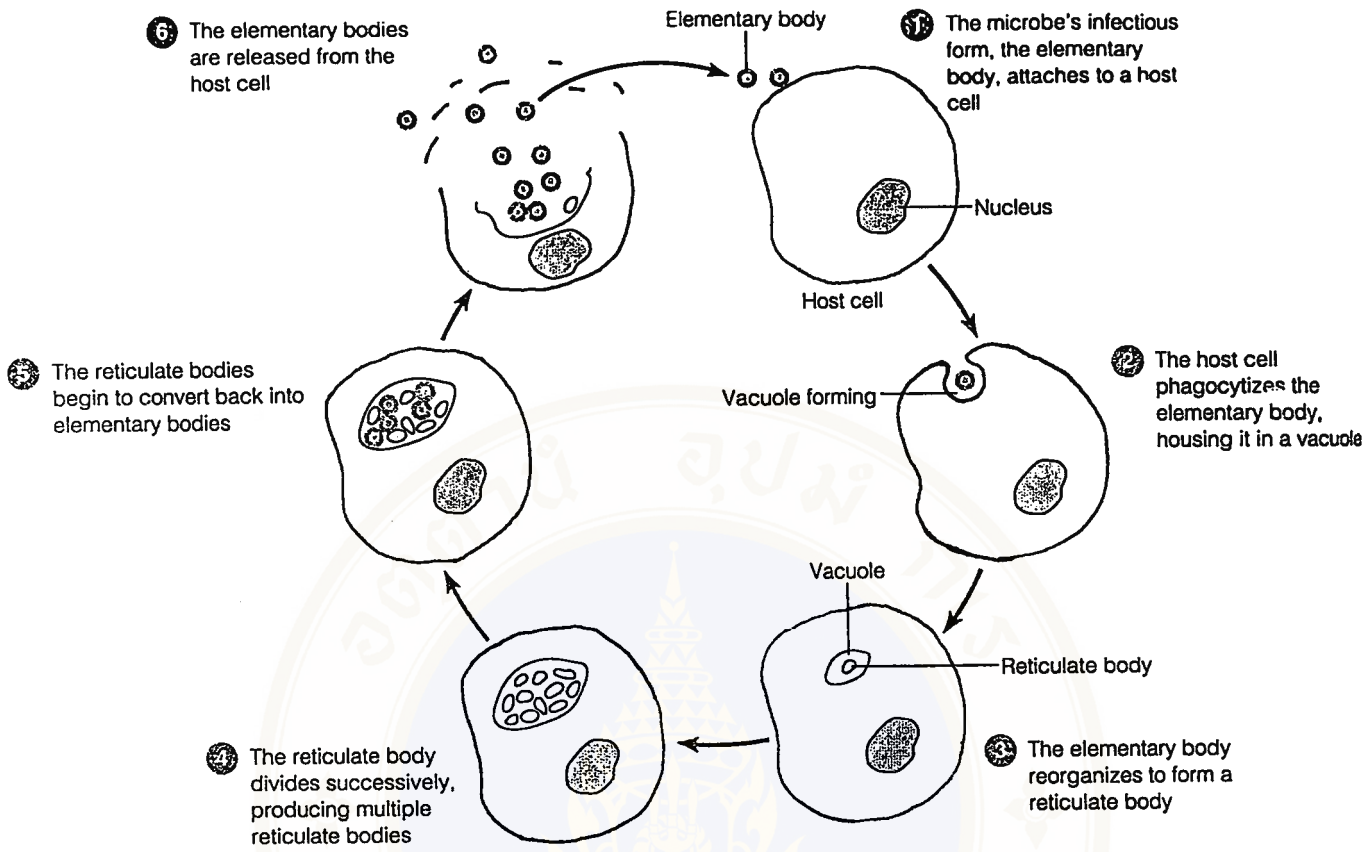

Figure 3. Developmental cycle of *C. pneumoniae*

(From Tortora GJ, Funke BR, Case CL, editors. Microbiology an introduction. 5th ed. California: The Benjamin/Comings Publishing; 1995)

The *Chlamydiae*'s developmental cycle begins as infectious form, the EB, attachment to a host cell. The host cell phagocytes the EB housing it in a vacuole. EB reorganizes to form a reticulate body and reverts to EB form prior to release from the host cell.

Pathogenic mechanisms

1. Host cell tropism

The interaction between *Chlamydiae* and their host cells includes several stages: attachment, entry to the cells, chlamydial developmental cycle inside the cells, energy parasitism, cell destruction, liberation of new infective particles and the persistence of chlamydial infection in the cells. In the case of *C. pneumoniae*, the host is generally the mucosal epithelial cell, and is able to infect several different cell types including macrophages (28). Table 2 shows the cell types chlamydial species tend to infect.

Table 2. Host cell tropism of *Chlamydia* species.

(From Leinonen M. Pathogenetic and epimiology of *C. pneumoniae*. Eur Heart J 1993; 14(suppl k) : 58)

Species	Host cell
C. trachomatis	Epithelial cells
C. psittaci	Monocytes, macrophages
C. pneumoniae	Mucosal epithelial cells, macrophages, endothelial cells, epithelial cells and smooth muscle cells

2. Structural components as virulence factors

Chlamydia is gram-negative bacteria with a typical cell-wall component, lipopolysaccharide (LPS). The chemical composition of LPS from *C. trachomatis and C. psittaci* . has been described, and they contain typical components of gram-negative LPS. However, the longer fatty acid chains have been observed in chlamydial than

enterobacterial LPS. Chlamydial LPS is of deep-rough type having only an acidic Kdo (Ketodeoxyoctonate) trisaccharide unit linked to the lipid A part of the molecule. Acidic hydrophilic Kdo-molecules are on the surface of chlamydial cells and can contribute to the acidity of the bacterial cell surface.

The endotoxin activity of chlamydial LPS seems to be much lower than that of LPS from gram-negative enterobacteria (29). The decreased toxicity might be an advantage to *chlamydial* LPS. LPS secreted into the circulation can effect the coagulation system- it is active in clotting test –and induce production of cytokines in vascular endothelium. Demonstrating the presence of chlamydial LPS containing immune complexes in diseases associated with possible chronic chlamydial infections, such as acute myocardial infarction and chronic coronary heart disease (30,31).

Chlamydia's outer membrane proteins have been studied intensively in recent years. Most important proteins include the major outer membrane proteins (MOMPs) that comprise about 60% of whole mass. MOMPs from different chalmydial species exhibit high sequence homology, but still contain species-, biovar- and serovar-specific epitopes. The isoelectric point of MOMP is about 5.5 and it can also relate to the acidity of the cell surface.

Chlamydiae are adapted to gain access to their host cells. The 40 kDa major outer membrane of *Chlamydiae* may have a role and secondary ligands such as heparin sulphate are involved. A number of invasine, ligands or adhesin are implicate in the specific process of invasion of epithelial cells by *C. trachomatis*, but this remains a controversial areas of research. (32,33).

Chlamydia's outer membrane also contains cysteine-rich proteins that are responsible for the rigidity of the cell wall, and several highly cross-reaction heat

shock protein that may have an important role in the pathogenesis of chlamydial infections (28). Heat shock (or stress) proteins (hsp) have important functions in cellular metabolism and they aid cells in dealing with adverse environmental stimuli. A group of heat shock proteins, may be of great importance in terms of damaging delay-type hypersensitivity (DTH) response. These proteins are conserved and prevalent in most organisms. However, regions of the *chlamydial* heat shock proteins are also unique to the bacterium. Recent studies have shown that *chlamydiae* possess at least two heat shock proteins, the 57 kDa protein belonging to 60 hsp family and the 70 kDa protein belonging to the 70 hsp family (33-35). The chlamydial heat shock proteins response related to pathogenesis has two directions. One is autoimmunity, where cross-reactive epitopes are shown with chlamydial and host heat shock proteins. Additional heat shock proteins, including hsp 10, the GroEL homologue which is functionally associated with hsp 60, have also been implicated in DTH responses, whether the *C. pneumoniae* GroEL gene product might play a similar role in imunopathology of chronic *C. pneumoniae* infections is unknown (36).

The chlamydial developmental cycle has been well characterized in cell culture, and it is presumed that a similar process occurs during natural infection. In cell culture, 72 hr after EB endocytosis, lysis of the infected host cell can release hundreds of infectious EBs, each of which can infect a new host cell. If similar events occur in natural *chlamydial* infections, the disease would progress rapidly with overwhelming, acute pathogen-mediated immunological damage. However, *Chlamydia* is known for its insidious ability to mediate a prolonged, generally subclinical disease. In primary infections, infectious *Chlamydiae* are detected but in chronic diseases, scarring progress without the isolation of the organisms. Chronic inflammation indicates that

the immune response is being stimulated by a quiescent form of *Chlamydiae* or by persisting chlamydial antigens (37). The direct interaction between *Chlamydia* and its host cell is complex. In the setting of a disease process that includes a system of immune-regulated factors and other variables, the interaction is even more confounding. Components of the immune system, nutrients, hormones or other infectious organisms, alone or in concert, may alter the *chlamydiae*-host-cell relationship resulting in persistent (latent) chlamydial development.(36).

Immune response in *C. pneumoniae*

Infection with *C. pneumoniae* induces humoral immunity response, serum immunoglobin IgG, IgA, and IgM. The primary immune response to *Chlamydiae* in IgM class antibody appears early in the infection within 2 to 4 weeks, and a delayed IgG and IgA responses follow the initially IgM response closely within 6-8 weeks. After acute *C. pneumoniae* infection, IgM antibodies are usually lost within 2 to 6 months. IgG antibody titers are usually decrease slowly, whereas IgA antibodies tend to disappear rapidly. In primary chlamydial infection is suspected, the detection of IgM is used for diagnosis. In chronic infection, the prevalence of IgM is low and therefore the absence of IgM does not exclude on persistence infection (11,38). In reinfection, IgM level may be rarely detected while IgG and IgA levels rise quickly, often in one to two weeks. IgA antibodies have shown to be reliable immunological marker of chronic and recurrent infections. (13). The persistence of elevated IgA antibody titers is generally considered as a sign of chronic infection (39). However, high levels of IgG antibodies may indicate an on going chronic or systemic infection.

The otherwise, the role of host response in the resolution of a chlamydial infection is unclear. There is increasing evidence from experimental *C. trachomatis* infections to suggest that cell-mediated immunity (CMI) plays an important role in host defenses against *Chlamydiae* (40,41). In addition, interferon-γ (IFN-γ) seems to be the single most important factor for protective immunity. IFN-γ is mainly produced by T helper type 1 (Th 1) immune cells but is also produced by natural killer cells which may participate in the regulation of immune response and activative of macrophages in the early phase of infection (42). The immunological data concerning *C. pneumoniae* infections are mainly based on serological analyses and CMI, which appear after *C. pneumoniae* infection in human (40).

Epidemiology

Much of the current information on the epidemiology of *C. pneumoniae* infection is derived from serologic studies with the *C. pneumoniae*-specific MIF test. These studies indicate that *C. pneumoniae* is a common cause of infection throughout the world, with a seroprevalence of over 50% among adults in the United States and many other countries (43,44). In addition, although *C. pneumoniae* was first recognized as a respiratory pathogen in 1983, testing of banked serum specimens had revealed that it was not a new pathogen but had been a frequent cause of infection since at least 1963 (12,45).

1. Age distribution

Infection appears to be most common among school-aged children, with children under age 5 years affected much less frequently. The seroprevalence of *C.*

pneumoniae antibody by age was studied in the Seattle population (46). A very small percentage of children under 5 years of age has serologic evidence of past infection with *C. pneumoniae*. The prevalence then increases dramatically from ages 5 through 14 years, and by age 20 years approximately 50% of persons have detecTable levels of antibody to the organism. The seroprevalence continues to increase among older age groups, but at a slower rate, and reaches approximately 75 % in the elderly. These prevalence rates exist despite the fact that fist infection induces a time-limited antibody response (3 to 5 years), suggesting that most people are infected and reinfected throughout life (2).

A similar trend in age distribution of acute infection was shown when a series of serum samples from individuals in a long-term study of Seattle families was tested (45). Children 5 through 9 years of ages had the highest incidence of acute infection, as evidenced by a fourfold or greater rise in antibody titer. The incidence among children 10 to 14 years of ages was slightly lower. Study of Swedish children showed a similar age distribution of acute infection, with annual seroconversion rates of 8.0% in children ages 8 through 12 years and 5.9% in children ages 12 through 16 (2).

2. Sex distribution

Seroprevalence is approximately equal in both sexes under 15 years of age; however, seroprevalence among adult men is considerably higher than that among adult women . This sex difference among adults has been demonstrated in all countries from which sera have been tested. To date, no explanation for the increased frequency among males has been found.

3. Global distribution

C. pneumoniae appears to have a worldwide distribution, although the prevalence of infection due to this organism may vary by regions. Studies of adult sera from 10 areas of the world have shown a higher population prevalence in tropical, less developed countries than in more northerly, developed countries, with Canada, Denmark, and Norway having the lowest rates. There is some evidence that infection among children less than 5 years of ages, uncommon in the United States, may be both more common and more severe in tropical countries (47).

Transmission

C. pneumoniae is believed to be transmitted from human to human. All evidence suggests that *C. pneumoniae* is a primary human pathogen that is transmitted from human to human by respiratory tract secretion (48,49). Humans are the only known reservoir of *C. pneumoniae*, and transmission is believed to be relatively inefficient. When contacts of patients with *C. pneumoniae* infection are investigated, few cases of secondary transmission are detected, as evidenced by follow-up of contacts of University of Washington students with documented *C. pneumoniae* infection (50) and by longitudinal seroepidemiologic study of Seattle families (14). Laboratory studies have indicated that *C. pneumoniae* can survive in aerosol at room temperature in conditions of high relative humidity. Although there is a rapid decline of infectivity with time, a decrease by half in the first 30 seconds, survival in these conditions supports the possibility of direct person-to-person transmission in a crowded humid environment. The organism can remain viable on Formica countertops for 30 hrs and on tissue paper for 12 hrs, suggesting that transfer from fomites may occur (51).

The incubation period of infection due to *C. pneumoniae* is several weeks, which is longer than that for many other respiratory pathogens. This has been demonstrated by serologic testing of family and household contacts of patients with *C. pneumoniae* infection, showing an interval of 3 weeks between seroconversion for pairs with the closest contact (spousal pairs) (51).

Diseases caused by *C. pneumoniae* infection

1. Chronic obstructive pulmonary diseases (COPD)

COPD is a disorder characterized by reduced maximum expiratory flow and slow forced emptying of the lungs ; features which do not change markly over several months. Three conditions may contribute to airflow limitation to varying degree in each patient that are chronic bronchitis, adult chronic bronchiolitis and emphysema (52,53).

COPD is a major cause of morbidity and mortality. In Europe COPD and asthma, together with pneumonia, are the third most common cause of death. In North America, the mortality rates and prevalence of COPD are increasing (54) and lead to be the fourth cause of death. In the study by Blasi,(55) the COPD in patients were categorized to severe or mild to moderate on the basis of lung function measurements. Four different markers of *C. pneumoniae* i.e., serum antibodies (the elevated IgG and IgA criterion), sputum antibodies (secretory IgA), sputum PCR, and circulating immunocomplexes were evaluated in those patients. All markers were significant difference between COPD patients and pneumonia. The proportion of all markers in positive subjects was highest among the patients with severe COPD, second highest

among patients with milder COPD and lowest among patients with community-acquired pneumonia. The overall prevalence of chronic *C. pneumoniae* infection in patients with severe COPD was 71% and with milder disease was 46%. The PCR test providing direct evidence of the presence of the organism, was positive in nearly 60% of the patients with severe disease. The stable IgA levels, the almost complete absence of seroconversions between the paired samples, and the frequent presence of circulating immunocomplexes strongly suggest that the infection in COPD patients is chronic. (9,55-57).

2. Pneumonia.

In a series of studies 10% of case of pneumonia, on average, were caused by TWAR. Pneumonia due to TWAR is more common among the elderly and less common among persons less than 20 years of age. In most studies TWAR was the fourth most common cause of pneumonia. (12). Most cases of pneumonia are relatively mild and do not require hospitalization. Even in mild cases, the complete recovery is slow, cough and malaise may persist for many weeks after the acute illness. Older adults appear to have, on average, a more severe clinical course than young adults.

The available evidence also suggests that underlying illnesses and current infection with other bacteria, such as the pneumococci, are associated with more severe diseases caused by *C. pneumoniae* infection. Studies of patients hospitalized with *C. pneumoniae* pneumonia have found that the majority had one or more underlying illnesses including the complication such as pneumococcal bacteremia.

Epidemics have occurred in communities and in closed population groups. Although pneumonia has been the most common syndrome associated with TWAR

infection, serologic studies during epidemics in military trainee companies at the beginning and end of their training period have suggested that only 1 of 9 or 10 of *C. pneumoniae* infections resulted in pneumonia. This suggests that many TWAR infections are mild or asymptomatic and are not recognized (11,58). The evidences of *C. pneumoniae* infection was shown in asymptomatic persons and in pneumonitis patients (59).

3. Bronchitis

TWAR bronchitis is often a subacute illness in which symptoms last for many days or weeks. Some patients with bronchitis may have unrecognized pneumonitis early in the cause of their illness. (12). TWAR bronchitis often has an insidious onset. The subacute onset is often preceded or accompanied by pharyngitis. In young adults 4% of bronchitis have been shown to be associated with TWAR infection (50,60).

4. Pharyngitis

primary pharyngitis with or without fever and sinusitis or otitis have occurred as separate illness associated with TWAR infection (12). Pharyngitis, often relatively severe with hoarseness, is frequently associated with *C. pneumoniae* infection. Up to 80% of those with TWAR lower respiratory tract infection have sore throat, less than 1% of patients with pharyngitis who did not develop lower respiratory tract involvement in TWAR infection. Pharyngitis showed higher percentage with *C. pneumoniae* infection in persons with development of lower respiratory tract disease or with pneumonia or bronchitis with untreated antibiotics (11).

5. Sinusitis

C. pneumoniae infection has been found in sinusitis, both alone and in association with other syndromes. Otitis is less commonly associated with TWAR

infection. About 5% of primary sinusitis in young adults has been associated with TWAR infection and 5% of patients with lower respiratory tract TWAR infection have had evidence of sinusitis.

6. Myocarditis and endocarditis

Myocarditis and endocarditis have been caused by the other *Chlamydia* species (45). Many reports associated of these syndromes with *C. pneumoniae* infection, both alone and with pneumonia. The initial observation came from Finland, where TWAR antibody was associated with chronic coronary heart disease and acute myocardial infraction. A seroepidermiology showed an association of *C. pneumoniae* with coronary artery disease and atherosclerosis (61,62). In addition, *C. pneumoniae* organisms showing pear-shaped structure by EM could be demonstrated in atheromatous plaques of coronary arteries and aorta (63). There is also evidence of association of TWAR infection with sarcoidosis (12).

7. Other syndromes

Some of the spectrum of milder TWAR syndromes that usually are unrecognized. Fever of undetermined origin and influenza-like illness have been associated with TWAR infection found in many studies (48). Other associations have been reported between serological evidence of *C. pneumoniae* infection and meningo-encephalitis, the Guillain-Barre' syndrome, arthritis, erythema nodusum and acute chest syndrome of sickle cell disease.

The seroprevalence of *C. pneumoniae* in HIV-1 infected intravenous drug users (IDUs) was significantly higher than in both HIV-1 negative IDUs and immunocompetent subjects (64). Recent reports suggest a possible role of *C. pneumoniae* as an etiologic agent of pneumonia in HIV-1 positive subjects (65). The

epidemic occurred in a small group of 26 subjects living and working in the same place. Their rate of HIV-1 positivity was remarkable high (50%). A higher *C. pneumoniae* infection rate was observed more than 76% of HIV-1 positive patients compared to only 38% of HIV negative subjects.

Laboratory diagnosis of *C. pneumoniae* infection

It is impossible to diagnose *C. pneumoniae* infection on the basis of clinical symptoms and signs. Although its epidemiological features and clinical course can sometimes be diagnostically helpful, the final etiologic diagnosis is based on microbiological laboratory methods. Four main diagnostic techniques used are isolation, antigen detection, serological antibody tests and identification of specific DNA sequences by the polymerase chain reaction (PCR). Diagnosis of *C. pneumoniae* infection remains difficult, due in part to the limited availability of facilities capable of performing culture, serology and the lack of a commercially available rapid non-culture test. There is even controversy on which method would be the gold standard for diagnosing infection. Since infections with *C. pneumoniae* are treaTable with antimicrobial agents, the rapid sensitive and specific methods for diagnosis are needed.

1. Isolation

The cultivation of *Chlamydia* strains was done by methods used for many years, both in the yolk sac of embryonated chicken egg and in cell culture (66). The yolk sac is employed for preparing antigens for the microimmunofluorescent test and generally used only for production of highly concentrated antigen for serologic tests, isolation

and propagation of the fastidious *C. pneumoniae*. Isolation by using cell culture is used for evaluation of clinical specimen (67). Centrifugation of the inoculated specimen onto cell monolayers and incorporation of cycloheximide into the culture medium to inhibit host cell metabolism enhance the sensitivity of isolation. Identification of the *C. pneumoniae* inclusions in cell culture was stained by a TWAR-specific monoclonal antibody conjugated with fluorescein (68).

A number of cell lines have been used to support the growth of *Chlamydia*. It does not appear that any single cell line is markedly superior to others. The optimal cell line for the isolation of *C. pneumoniae* have not been found yet (14,69).McCoy and HeLa cells are most commonly used for *C. pneumoniae*. HL cells and HEp-2 cells may be more sensitive for the recovery of *C. pneumoniae*. In contrast of *C. trachomatis* for which culture has been the diagnostic gold standard, *C. pneumoniae* is difficult to be isolated from clinical samples. *C. pneumoniae* grows poorly in culture, and the inclusions formed are smaller. Specimens for isolation are usually obtained from swabs of the oropharynx, as well as nasopharyngeal swab samples. Negative results have been much more common. The sensitivity of isolation by culture technique may be 50% (66,67,70).

The level of *C. pneumoniae* elementary bodies and antigens seems to be low in samples of the pharyngeal epithelium. The isolation from sputum is not so sensitive because of the inhibitors in the samples (18). *C. pneumoniae* can multiply at other sites and it is difficult to obtain a proper samples for the detection of organisms that have invaded deeper layers (71).

2. Antigen detection

2.1 Lipopolysacchalide enzyme immunoassay (LPS EIA) A number of commercial kit of EIA procedures are available for detecting chlamydial antigen in clinical specimens. Most of these products will detect chlamydial LPS, which is more soluble than MOMP. The EIA kits used for the diagnosis of *C. trachomatis* infection will detect common LPS group antigen that is present in all *Chlamydia* . Most of them are less sensitive and less specificity compared to culture. To increase the specificity, the positive samples are repeated in the presence of a monoclonal antibody directed against the chlamydia-specific epitope on the LPS (73).

2.2 Fluorescent antibody staining

In the 1980s, rapid diagnosis of *C. pneumoniae* infection is to use fluorescein-conjugated monoclonal antibodies staining EBs in smears (43,66). The early commercial direct immunofluorescent (DFA) reagents were plagued with problems of cross-reaction with the other bacteria and had been improved. Current experience indicates that the DFA test has approximately 90% sensitivity and 98 to 99% specificity compared with culture.

Monoclonal antibodies to chlamydial MOMP showed species and immunotype specificity while monoclonal antibodies to *C. pneumoniae*-MOMP are currently avalaiable for detecting *C. pneumoniae*. As several DFA configurations are commercially available and have a variety of monoclonal antibodies directed against MOMP or against LPS. Monoclonal antibody to LPS will stain alll *Chlamydia spp.*, but the quality of the fluorescence is somewhat mitigated by uneven distribution of LPS on the chlamydial particle.

3. Serology

3.1 Complement fixation test (CF-test) Complement-fixation test is genus specific, it can measure antibodies to all *Chlamydia spp.* and had been used for presumptive diagnosis of psittacosis. Since TWAR are separated from *C. psittaci* as a new species in 1983 and *C. pneumoniae* infections are much more common than *C. psittaci*, it is probable that many cases of TWAR infections may misdiagnosed as psittacosis on the basis of the CF test in the past time(66). The CF test may be performed in either the tube system or the microtiter system. The microtiter systems are most useful in screening large number of sera , the positive sera will be retested in the tube system furture on. the CF test is more useful in young persons with primary *C. pneumoniae* infection and less useful in older persons, who are often experiencing reinfection. (12).

3.2 Enzyme Immunoassays (EIAs) based on elementary-body antigens also measure antibodies to LPS that are common to all *Chlamydia.* Antibodies to LPS are rapid development as little as 5 days and tend to be IgM class (74). The specificity of the tests has been improved by the removal or destruction of LPS (16). Chemically pure antigens would be ideal for the measurement of antibodies. So far, the recombinant LPS containing a second immunogenic determination of chlamydial LPS has recently developed from Re-type LPS of mutant enterobacteria and chlamydial LPS. Commercially pure antigens has been isolated for the detection of antibodies against chlamydial LPS and this has been developed into a commercially available recombinant DNA LPS ELISA (75) which contains a genus-specific epitope of *Chlamydia spp.* Both of these tests are sensitive but lack of species specificity (76) are widely used Enzyme-Linked immunosorbent assays (ELISA). There are ELISA

genus-specific and have not been specifically evaluated for the diagnosis of *C. pneumoniae* infection. Recently, purified elementary bodies of *C. pneumoniae* are used as antigens to detect the antibody response to *C. pneumoniae* infection (8).

3.3 MIF test A micro-immuofluorescence introduced in the 1970s utilizes purified elementary bodies (EB) as the substrate anntigen. EBs from all chlamydial species and serotypes can be purified and pooled, or used as individual substrate antigen(77,78). The test was originally developed for *C. trachomatis* and was used to differentiate the 15 serovars of *C. trachomatis*. The MIF test is be employed currently for *C. pneumoniae* serological diagnosis as well as a gold standard for *C. pneumoniae* infection. MIF tested performed with properly collected paired sera and interpreted by an expert, is more sensitive than culture or even PCR of throat specimens (50). Specificity and sensitivity of MIF are approximately 95% and 99%, respectively.

The appearance of MIF antibody is slow; therefore, a 3-4 week interval is recommended for obtaining the second, convalescent, serum sample. Failure to detect MIF antibodies in patients with *C. pneumoniae* isolated may occur (79) which usually due to the slow antibody response. It is possible that young children, after their first *C. pneumoniae* infection, develop the antibodies that are detected by the MIF test slowly, and at low titer, or with strict strain specificity. Antibiotic therapy can also prevent the formation of antibody (80). False positive MIF IgM antibody tests may occur if patients have circulating rheumatoid factors increasing with age. Therefore, removal rheumatoid factor positive sera is recommended for IgM MIF test (81).

4. Nucleic acid detection

The most promising non-culture methodology for *C. pneumoniae* infection appears to be DNA amplification, i.e., PCR. PCR can detect 10^{-16} g of DNA,

approximately equivalent to one chlamydial elementary body (77). PCR method uses repeated cycles of oligonucleotides directed DNA synthesis to perform *in vitro* replication of target nucleic sequences. The oligonucleotides which sequences are determined by the target nucleic acid are synthesized to be complementary to their annealing sites within the two different strands of a target DNA sequence.

Each cycle of PCR consists of three steps ; 1) a denaturing step which the target DNA is incubated at high temperature so that double strands of DNA will be seperated, and thus made accessible for hybridization by specific oligonucleotide primers ; 2) an annealing step, which the reaction mixture is cooled down to allow the primers to anneal to their complementary target sequences and ; 3) an extension step ; the reaction is usually done at intermediate temperature, in which the primers are extended on the DNA template by a DNA polymerase.

Several *C. pneumoniae*-specific primers have been used in PCR detection of organisms. The targets amplified include a *C. pneumoniae*-specific sequence of unknown coding function, rRNA gene sequences, outer membrane protein genes or the 60 kDa cystein rich protein (23,82). To increase the sensitivity and specificity of PCR, nested PCR, touchdown PCR and PCR enzyme immunoassay have been applied (19). Primers from the 16s rRNA coding gene and the amplification products being detected by EIA had been developed by Gaydos and her colleagues (16,54) and were used for screening large numbers of clinical specimens.

Treatment and development of vaccines

C. pneumoniae is usually more sensitive to antibiotics *in vitro* than the other chlamydial species.(83). Currently, the tetracyclines and erythromycin are drugs in the treatment for genital *C. trachomatis* infections. However, the limited data are available on the use of these antibiotics for treatment of *C. pneumoniae* (11).

The azalide antibiotic, azithromycin, and the macrolide, clarithromycin, are similar to erythromycin and doxycycline in their clinical activity against chlamydiae. Azithromycin in particular has been recognized as an effective treatment for *C. trachomatis* infection. It is not directly toxic to chlamydial elementary bodies, but rapidly inhibits chlamydial protein synthesis of reticulate bodies. This action is probably exerted at peptidyl donor site on the chlamydial 50S ribosomal subunit. Both azithromycin and clarithromycin have excellent tissue and intracellular penetration, especially into bronchial epithelium and alveolar macrophages. Azithromycin has a serum half-life of 30-68 hrs and a tissue half-life of up to 5 days. These antibiotics are active *in vitro* against *C. pneumoniae* (3).

The searchs for an effective chlamydial vaccine were investigated only in *C. trachomatis* but a vaccine against *C. pneumoniae* has not yet been developed (3). Protective immunity against chlamydial ocular infection caused by *C. trachomatis* in primates which correlates with the presence of persistent, serovar-specific secretory IgA antibodies in tears was found. Epitopes on the major outer membrane protein of *C. trachomatis*, particularly within variable domains 1, 2 and 4 have been identified as important targets for the development of vaccines. It was reported that a recombinant poliovirus encoding part of the serovar A variable domain 1 was highly

immunogenic in rabbits, and passively neutralized chlamydial infectivity in cynomologous monkey conjunctiva *in vitro* (70).

CHAPTER IV

MATERIALS AND METHODS

Materials

1. Study population

1.1 Chronic obstructive pulmonary diseases (COPD) patients who attended the Chest Clinic at Siriraj Hospital during the period of June, 1997 to September, 1998 (n=120) were enrolled in this study. The patients were divided into two groups;

1.1.1 COPD patients with acute respiratory illness (n=72). They were 70 males and 2 females and had mean age of 66.4±7.91 years.

1.1.2 COPD patients without acute respiratory illness (n=48). They were 44 males and 4 females and had mean age of 62.4±3.2 years.

1.2 Healthy controls. One hundred and four subjects with mean age of 65.4±4.6 years composed of 31 males and 73 females were participated in this study.

2. Clinical specimens

In COPD patients with acute respiratory illness, throat swabs and nasal swabs were collected from each patient at the same time Throat swabs were collected in healthy controls and COPD patients without acute respiratory illness. Sera of subjects were obtained for serology detection.

2.1 Throat swabs

The posterior wall of throat was rubbed up and down three times with a cotton-tipped swab. The specimens were collected in transport media and kept at –70°C before performing the test.

2.2 Nasal swabs

A rayon tipped swab (Syva MicroTrak,USA) was inserted through the nasal rubbed and stood for 15s. The swab was dipped and squeezed in vial.

2.3 Sputum

Sputum samples were collected at the initial visit before breakfast in a sterile tube after "deep cough".

2.4 Serum

A serum specimen was collected at the time of acute respiratory illness (acute serum). The convalescent serum specimens, collected about 30 days after the first specimen collection. Blood samples were collected by venipucture. All sera were separated from the clotted blood within 6 hours after collection and stored at –20 °C until tested.

3. Cell lines

Cell lines : Two types of cell lines were used: HEp-2 cell line derived from carcinoma cells of human larynx and McCoy cell lines established from mouse synovial cells.

Both cell lines were obtained from the Division of Virology, Department of Microbiology , Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

4. Bacteria

4.1 *C. pneumoniae* (strain TWAR) was provided by Prof. Dr. Hakiwara, Japan.

4.2 Bacterial strains; *Moraxella catarrhalis, Haemophilus influenzae, Streptococcus pneumoniae* were obtained from clinical specimens and identified by colonial morphology, Gram-stain characteristics and biochemical properties. These organisms were cultured on chocolate agar and incubated in CO_2 incubator at 35±2 ^{o}C. They were provided by the Division of Bacteriology, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University Bangkok, Thailand.

5. Oligonucleotides

C. pneumoniae PCR primer sequences as described by Messmer TO, *et al* were employed (84). The target sequences was 16S rRNA gene. The first-step PCR was genus-specific, and the second-step PCR was species-specific for *C. pneumoniae.* Beta-globin primers (85), specific for sequence of β-globin gene, was used as internal control. Primers of PS3 and PS4 (86), overlapping sequences of bacterial ribosomal RNA genes, were used to amplify and give products of 479 bp fragment. Nucleotide sequences of these four primer pairs are shown in Table 3.

Table 3. Oligonucleotide sequences used as primers to amplify each target sequence.

Primers	Target	Nucleotide sequences 5' to 3'	Length of amplified product in base pair
C07	Sense strand of *Chlamydia* spp.	ACG GAA TAA TGA CTT CGG	436
C08	Antisense strand of *Chlamydia* spp.	TAC CTG GTA CGC TCA ATT	
C11	Sense strand of *C. pneumoniae*	ATA ATG ACT TCG GTT GTT ATT	221
D01	Antisense strand of *C.pneumoniae*	CGT CAT CGC CTT GGT GGG CTT	
GH20	Upstream β-globin	GAA GAG CCA AGG ACA GGT AC	268
PC04	Downstream β-globin	CAA CTT CAT CCA CGT TCA CC	
PS3	Sense strand of bacterial gene	GGA ATT CAA A(T/G)G AAT TGA CGG GGG C	479
PS4	Antisense strand of bacterial gene	CGG GAT CCC AGG CCC GGG AAC GTA TTC AC	

6. Commercial kit reagents for serology tests (MIF & ELISA) (See appendix).

Methods

1. Culture

1.1 Cultivation for HEp-2 cell and McCoy cell lines

HEp-2 cell and McCoy cell were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 0.3 mg/ml L-glutamine, 0.02M HEPES and adjusted to pH 7.4 with 2 mg/ml $NaHCO_3$. Antibiotics; 10 (g/ml gentamicin, 2 μg/ml of fungizone and 50 μg/ml of streptomycin (see appendix) were added in media to protect the growth of other bacteria and fungus.

Cell lines were grown in 150 ml tissue culture flask. The confluent monolayers were trypsinized and propagated in other tissue culture flask. The monolayers usually develop within 3-4 days. Preparation of cell monolayers were as the following :

1.1.1 Decant the medium from the confluent cell monolayers of a culture flask.

1.1.2 Wash the monolayer with 5 ml of PBS (Mg++ and Ca++ free)

1.1.3 Add 5 ml of trypsin to cover the cell monolayers.

1.1.4 Remove trypsin and incubate the culture until the cell detach from the surface.

1.1.5 Add 5 ml of growth media into the culture flask and suspend the cells by pipeting back and forth.

1.1.6 Count the number of cells present in the suspension by using a hemocytometer and read by using 10X microscope objective.

1.1.7 Calculate for number of cells as the following formula :

$$\text{Number of cells/ml} = \frac{\text{No. of cell counted}}{\text{No. of squares counting}} \times \text{Volume of counting chamber} \times \text{dilution factor}$$

1.1.8 Adjust with growth medium to cell concentrations of $1x10^5$ cells/ml

1.1.9 Pipette 10 ml of this cell suspension to a new tissue culture flask.

1.1.10 Incubate the cell culture at 37° C in CO_2 incubator.

1.2 Preparation of cell monolayers for isolation

The fresh cell lines suspension was prepared under the same procedure as described above, and adjusted to the concentration of $2x10^5$ cells/ml. One ml of the cell suspension was distributed into each of sterized vial. The cells were incubated at 35±2°C for 24 hrs or until a monolayer of the cells were formed.

1.3 Inoculation and propagation of *C. pneumoniae* in cell culture

Isolation of *C. pneumoniae* was performed by centrifugation of specimens onto the cell monolayers in presence of cycloheximide. The step of inoculation were as follows :

1.3.1 Remove the growth medium from cell culture.

1.3.2 Inoculate each 0.2 ml of the positive control, the negative control and the clinical specimens into each of the corresponding labeled vials.

1.3.3 Centrifuge the culture at 3,000 rpm for 1 hr at 35° ± 2°C.

1.3.4 Remove the inoculum.

1.3.5 Add 1 ml of culture medium containing 0.25 mg/ml of cycloheximide to each vial.

1.3.6 Incubate the cultures at 35°C for 72 hrs.

1.4 Indirect immunofluorescent staining was performed after incubation period as the followings.

1.4.1 Remove the media from the inoculated cultures.

1.4.2 Gently rinse the cell monolayers with PBS (1 ml).

1.4.3 Add 1 ml of cold acetone to each vial.

1.4.4 Fixed cells for 10 mins at 4°C.

1.4.5 Remove the acetone and allow the cover slips to air-dry. Fix dried cover slips on slide.

1.4.6 Add 15 µl of monoclonal antibody against *C. pneumoniae* (DAKO, Gostrup, Denmark).

1.4.7 Incubate 30 mins at 37° C in moisturechamber.

1.4.8 Wash the preparation in 2 changes of PBS. Agitate for 10 mins each time and then remove PBS.

1.4.9 Add 15 µl of fluorescine isothiocyanate-labelled anti-mouse immunoglobulin–G (DAKO, Gostrup, Denmark). Incubate slide at 37°C for 30 mins in moisture chamber.

1.4.10 Wash slides with PBS on agitator for 10 mins and counter stain in 1% of Evan's blue for 10 mins.

1.4.11 Rinses the slides in distilled water.

1.4.12 Examine the stained slide with a fluorescence microscope (Optiphot,Nikon,Japan). The inclusion bodies were stained and showed apple green in cytoplasm of the infected cells.

2. Polymerase chain reaction (PCR)

2.1 Preparation of specimens

The throat swabs or nasal swabs collected in transport media (2SP media) (see appendix) were centrifuged at 12,000 rpm 30 mins at 4°C. The pellet was obtained and processed by washing with double distilled water and spinning at 12,000 rpm 30 mins at 4°C for 2 times. The pellet was resuspended in 50 µl of lysis buffer containing 10 mg/ml of proteinase K (see appendix) and incubated at 56°C for 1 hr. The lysate was heated at 100 °C for 10 mins and then kept at -20°C until tested by PCR.

Sputum treated with N-acelyl-L-cysteine-sodium hydroxide (NALC-NaOH) (Mucomyst ; Draco, Sweden) (see appendix) and equal volume of specimen, then, centrifuged at 12,000 rpm 30 min at 4°C. The supernatants were discarded and the cell pellets were washed with double distilled water by spinning at 12,000 rpm 30 mins at 4°C. The processed pellets were treated with proteinase K as mentioned above.

2.2 Preparation of bacterial lysates

Bacteria were harvested from culture plates. One isolated colony was picked up with a loop and transferred into each tube containing 50 µl of lysis buffer. The lysate was heated at 100°C for 10 mins and then kept at -20°C until tested by PCR.

2.3 Method of PCR

Reaction volume of 50 µl, contains 10 mM Tris HCl (pH 8.3) 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 0.01% bovine serum albumin (Sigma,St. Louis,USA) 1.25 U of Taq polymerase (Promega,WI ,USA) 0.2 µM each outer primers (GIBCO BRL,NL,USA), and 5 µl of samples. PCR was performed by using the automate Gen Amp PCR system 9700 and 2400 DNA Themalcycle Cycler (Perkin Elmer Cetus,CT,USA). The reaction was amplifed at 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 30s and 72°C for 1 min.

The second round PCR programmed the same as the first round PCR cycles, the 50 µl reaction contained 1 µl of the amplified product and 0.2 µM of each inner primer. Amplified product sized was 221 bp.

First round of PCR was performed in every specimen by β-globin gene the same condition as described for *C. pneumoniae*. The PCR primers PS3 and PS4, were used to give a 479 bp in size. Five microliters of DNA samples was added. In one cycle of amplification composed of : denaturation at 94 °C for 30s, annealing at 55 °C for 15s, and DNA extension at 72°C for 7 min. The reaction for 30 cycles.

2.4 Detection of amplified product by gel electrophoresis

The amplified PCR products were detected by running gel electrophoresis using 1.5% agarose gel (GIBCO BRL, NY, USA).

2.4.1 Preparation of 1.5% agarose gel.

2.4.1.1 Melt 1.5 grams of agarose powder (agarose type I-A) (Sigma, St. Louis, USA.) in 100 ml of 0.5x TBE buffer (see appendix) by boiling until it was completely dissolved.

2.4.1.2 Pour cooling at 60-80°C dissolved gel in a gel-casting platform with a well performer.

2.4.1.3 After that leaving the gel at room temperature for 30 min and take off the well former.

2.4.1.4 Transfer gel with casting platform to an electrophoresis tank and add TBE buffer to cover the gel to a depth of about 1 mm.

2.4.2 Running gel electrophoresis.

2.4.2.1 Mix five µl of PCR product or 0.6 µg of 100 base pair DNA marker (GIBCO BRL, USA.or Promega, WI, USA.) with one µl of gel loading buffer (see appendix) and load into each well of the gel.

2.4.2.2 The electrophoresis was carried out at constant voltage about 100 volts. The running gel was stopped when the dye at three fourth of the gel.

2.4.2.3 Stain the gel with 5 µg/ml of ethidium bromide solution (see appendix) for 10-15 mins, follow by destaining it in distilled water for 5 mins.

The amplified DNA in the gel was observed under an UV transluminator (Spectronic, New York,USA)and was photographed with a Polaroid camera (Polaroid, Cambridge, MA, USA.) fitted with a red orange filter.

3. Serology

3.1 Enzyme-Linked-Immunosorbent-Assay (ELISA)

For the detection of *C. pneumoniae* IgG, IgA and IgM antibodies, each well of microtiter plate was coated with purified *C. pneumoniae* elementary body antigens A sera were diluted as 1:21 for IgM, 1:105 for IgG and IgA before testing. Test procedure were as following

3.1.1 Wash 3 times with wash buffer

3.1.2 Add 50 µl of 1:300 diluted horseradish peroxidase (HRP) Conjugate

3.1.3 Cover plate and incubate 1h at 37 °C in 100% humidity

3.1.4 Wash 3 times with wash buffer

3.1.5 Add 100 µl of 3,3′,5,5′ - tetramethylbenzidine (TMB) substrate

3.1.6 Cover plate and incubate 15 mins at room tempertatureadd 100 µl of stop solution

3.1.7 Read absorbance at 450 nm

3.1.8 Calculate and interpret results by using cut of index value as follow:

$$COI = \frac{\text{Absorbance of the serum sample at 450 nm}}{COV}$$

The cut-off value is calculated according to the following formula:

$$COV = NCx2$$

Cut-Off Value (COV) and of Cut-Off Index (COI)

NC= The average absorbance at 450 nm of the negative control.

Table 4. Correlation between the absorbance at 450 nm and the presence of *C. pneumoniae* antibodies. (Savyon Diagnostic Ltd., Ashdod, Israel)

Absorbance at 450 nm	COI	Results
O.D.≤COV	<1.1	Negative
COV≤O.D.≤O.D.x 1.1	1-1.1	Borderline
O.D.≥COV x 1.1	>1.1	Positive

3.2 Microimmunofluorecent test (MIF)

Test procedure was followed by MRL Diagnosis test kit. One slide had twelve wells. Each well contained four spots: one yolk sac control spot and three individual antigen spots of *C. pneumoniae, C. trachomatis* and *C. psittasi* consisting of EBs suspended in yolk sac matrix.

Test procedure are following :

3.2.1 Remove slide from cold storage and allow it to reach room temperature before opening slide packets.

3.2.2 Apply 25 µl of positive control and negative control to each the well.

3.2.3 Dilute sera sample with buffer for IgG and IgA while pretreated sample of IgM with pretreatment diluent. Add approximately 25 µl of the diluted sample (see appendix) to an appropriate slide well. For titration the titer; IgG were diluted to 1:256 and 1:512, IgA were diluted to 1:16 and 1:32 and IgM were diluted 1:10.

3.2.4 Incubate slide in a humid chamber for 90±2 mins in IgM ,while in IgG and IgA were incubated for 30± 2 mins at 35 to 37°C.

3.2.5 Remove slide from the humid chamber and gently rinse slide with PBS . Wash slide by submersing the rinsed slides into Coplin or slides staining jars containing PBS for 10 mins.

3.2.6 Dip the washed slides briefly in distilled water, and allow the slides to air dry.

3.2.7 Add approximately 25 µl specific Ig conjugate to each slide well.

3.2.8 Incubate slides in a humid chamber for 30±2 mins at 35-37°C.

3.2.9 Repeat wash steps 3.2.6

3.2.10 Place a few drops of mounting medium on the slide and cover with a 24x25 mm coverslip.

3.2.11 View wells at a final magnification of 400x on a properly equipped fluuorescence microscope.

For optimum fluorescence, read slides the same day the assay is performed. If this is not possible, store in the dark at 2 to 8 °C. up to 24 hours.

4. Statistical analysis

Statistical calculations were done by using EpiInfo. Version 6. (CDC, Atlanta and WHO, Geneva). The x^2 test with Yates's correction (or Fisher's exact test if any expected cell frequency was less than five) was used for comparisons of COPD patients and control groups. The difference of means$\pm$SD antibody level among groups of COPD and controls were assessed by the two-tailed unpaired *t* test. The Kappa statistic was used to calculate agreement between two tests. All analyses were undertaken by using the Statistical Product and Service Solution (SPSS Version 9.) packages (SPSS: Chicago, IL, USA).

CHAPTER V

RESULTS

1. Comparison on the sensitivity of two types of cell line in the cultivation of *C. pneumoniae.*

C. pneumoniae was cultured in HEp-2 and McCoy cell lines. The duration of propagation was 3 days for the first passage and 2 days for the second and third passages. One hundred µl of two dilutions (1:10 and 1:100) of *C. pneumoniae* were inoculated into monolayer cells. One hundred µl of the number of inclusion body present in infected cultures of each passage could be counted in triplicate shell vials at dilution of 1:100. In each passage the number of the inclusions were not different, but the inclusions of the second and the third passages were significantly larger in size.The data are shown in Table 5.

Table 5. Comparison on the number of inclusion body (IFU/100 µl) in two cell lines used for cultivation of *C. pneumoniae.*

Passage	Mean of IFU/µl detected	
	HEp-2	McCoy
1	3.25×10^4	3.5×10^4
2	2.9×10^4	3.7×10^4
3	3.4×10^4	4.0×10^4
Mean	3.18×10^4	3.73×10^4

2. Sensitivity and specificity of PCR

2.1 Sensitivity of nested PCR

The sensitivity of the nested PCR was determined by the quantitative assay of the number of inclusion body of *C. pneumoniae* reference strain (Table 6) in McCoy cell culture. The quantity of the *C. pneumoniae* in McCoy cell culture was determined as a number of inclusion forming unit (IFU)/ml. The 2,000 IFU of *C. pneumoniae* inclusions were obtained by the average value of the numbers of inclusions cultured in each triplicate shell vials. The cells were lysed in 200 µl of lysing solution. The undiluted sample containing 50 IFU/5µl was serially diluted in a ten-fold dilution. Five µl of each diluted lysate were used in the PCR reaction. Therefore, the amount of *C. pneumoniae* inclusions were equal to 50, 5, 0.5, 0.05, 0.005, 0.0005 and 0.00005 IFU per reaction tube for each dilution. Ten µl of amplfied product of each dilution of *C. pneumoniae* were electrophoresed in gel. The products stained with ethidium bromide were visualized by UV transilluminator (Figure 4). The 436 bp fragment of the first PCR product used the outer primer pair of *C. pneumoniae* (C07 and C08) could be visualized at undiluted and dilution of 1:10 of the culture. The inner primer pair (C11 and D01) was used to amplify the first PCR product yielding the 221 bp fragment. The minimum detectable level of nested PCR was at the dilution of 10^{-4}. The result showed that the nested PCR was 1,000 times more sensitive than isolation method in cell culture.

Table 6. Sensitivity of the nested PCR for detection number of IFU of *C. pneumoniae* obtained from McCoy cell culture at serial ten fold dilution.

Dilution of *C. pneumoniae*	Inclusion forming unit (IFU) in 5 µl	Detection of *C. pneumoniae* DNA
Undiluted	50	Positive
10^{-1}	5	Positive
10^{-2}	0.5	Positive
10^{-3}	0.05	Positive
10^{-4}	0.005	Positive
10^{-5}	0.0005	Negative
10^{-6}	0.00005	Negative

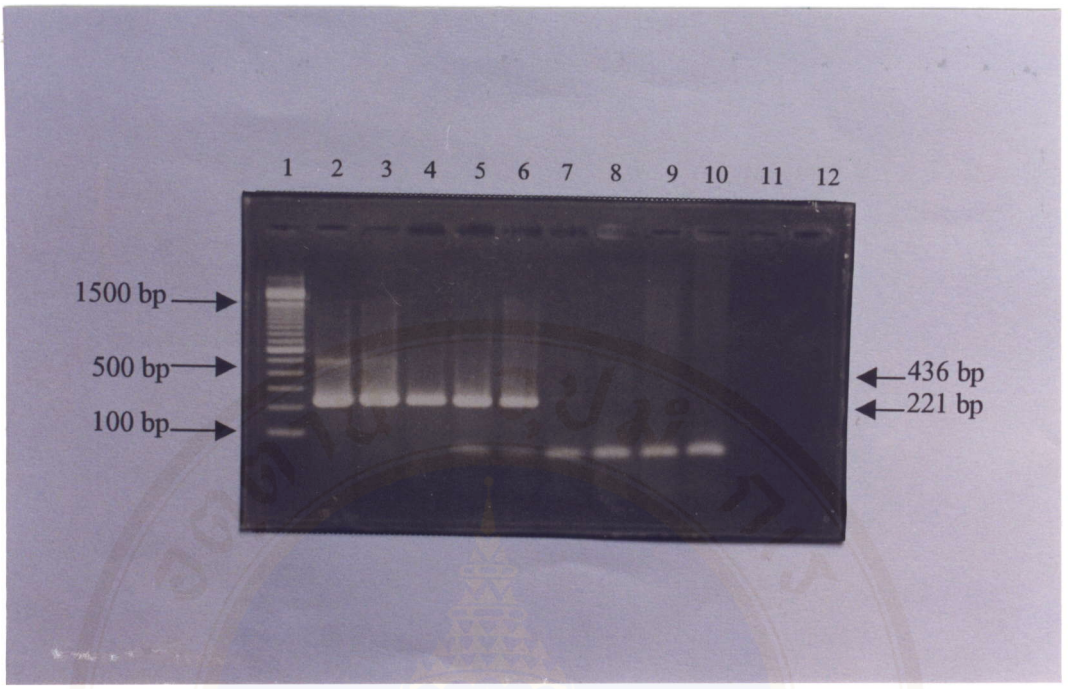

Figure 4. Agarose gel electrophoresis showing the PCR products in serial ten-fold dilution of *C. pneumoniae* (undiluted concentration = 50 IFU/5 µl). Product size of primary PCR and nested PCR were 436 bp and 221 bp, respectively.

Lane 1 : DNA size markers (100 bp ladder)

Lane 2 : Undiluted *C. pneumoniae* DNA

Lane 3-10 : Serial ten-folded dilutions of *C. pneumoniae* DNA, i.e., 10^{1}-10^{-8}

2.2 Specificity of the nested PCR

Two sets of outer (genus-specific) and the inner (species-specific) primers used for *C. pneumoniae* detection were tested with other bacterial DNA. Those bacteria i.e., *Moraxella catarrhalis, Haemophilus influenzae, Streptococcus pneumoniae* are known to cause respiratory tract infections and are predominate in causing acute exacerbation of COPD. PS3 and PS4 primers which are specific for bacterial ribosomal RNA genes were used to amplify DNA from mentioned bacteria in order to show the existence of bacterial DNA in the samples. PCR products were 479 bp fragments and shown in Figure 5. After knowing that all of these bacterial DNA existed in the samples, the baterial samples were tested in PCR for specificity of *C. pneumoniae* primers. The PCR were performed using *C. pneumoniae* DNA as a positive control. The results are shown in Table 7. None of the bacteria tested except *C. pneumoniae* showed band of 221 bp in size (Figure 6).

Table 7. *C. pneumoniae* primers used in nested PCR to amplify bacterial DNA products.

Microorganisms tested*	Detection of *C. pneumoniae* DNA band at 221 bp.
Moraxella catarrhalis	Negative
Haemophilus influenzae	Negative
Streptococcus pneumoniae	Negative
Chlamydia trachomatis	Negative
Chlamydia pneumoniae	Positive

* *C. pneumoniae* primer sequences published by Messmer TO, *et al* (84) were tested for cross-reactivity in the PCR assay with the following microorganisms: *Acinetobacter* species, *Alcaligenes faecalis*, *Bordetella pertussis*, *Corynebacterium maruchotis* strains B1 G5048 and BC F124, *Corynebacterium straitium* strains D9110 (A) and E4684, *Corynebacterium xerosis* strains G676 and G3375, *Ehrlichia chaffeensis*, *Flavobacterium meningosepticum*, *Haemophilus influenzae* strains KC818A, KC1050B, KC1051C, KC819D, KC528E and KC529F, *Kingella kingae*, *Legionella pneumophila* serogroup 1, *Mycobacterium tuberculosis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.

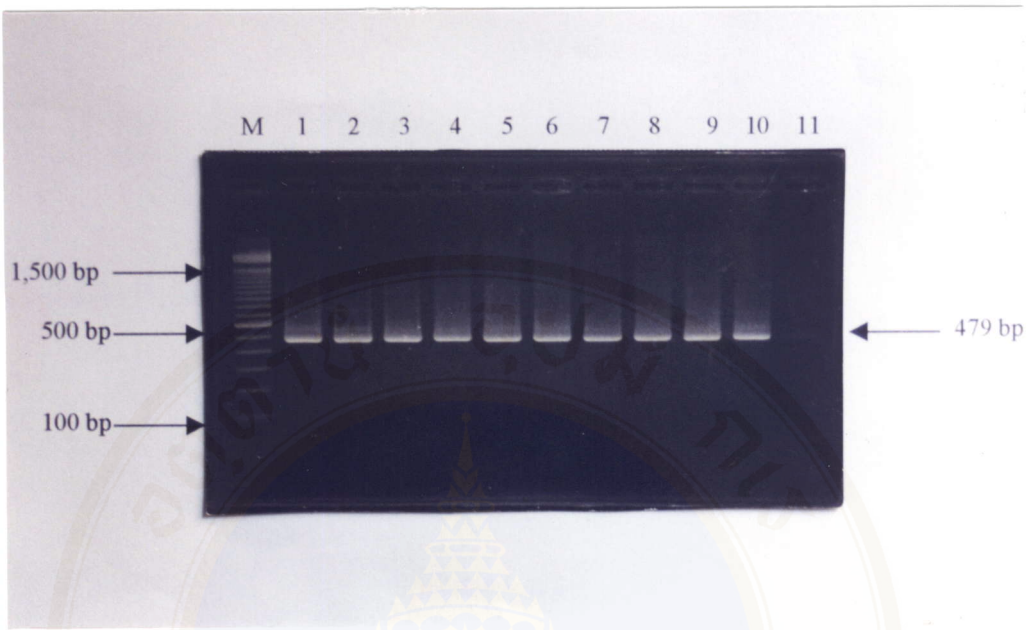

Figure 5. Results of the bacterial DNA amplification using PS3 and PS4 primers. The PCR products of 479 bp in size were demonstrated.

Lane M. DNA size markers (100 bp ladder)

Lane 1,2 *Moraxella catarrhalis*

Lane 3,4 *Haemophilus influenzae*

Lane 5,6 *Streptococcus pneumoniae*

Lane 7,8 *Chlamydia pneumoniae* (HEp-2 cell)

Lane 9,10 *Chlamydia pneumoniae* (McCoy cell)

Lane 11 Reagent blank

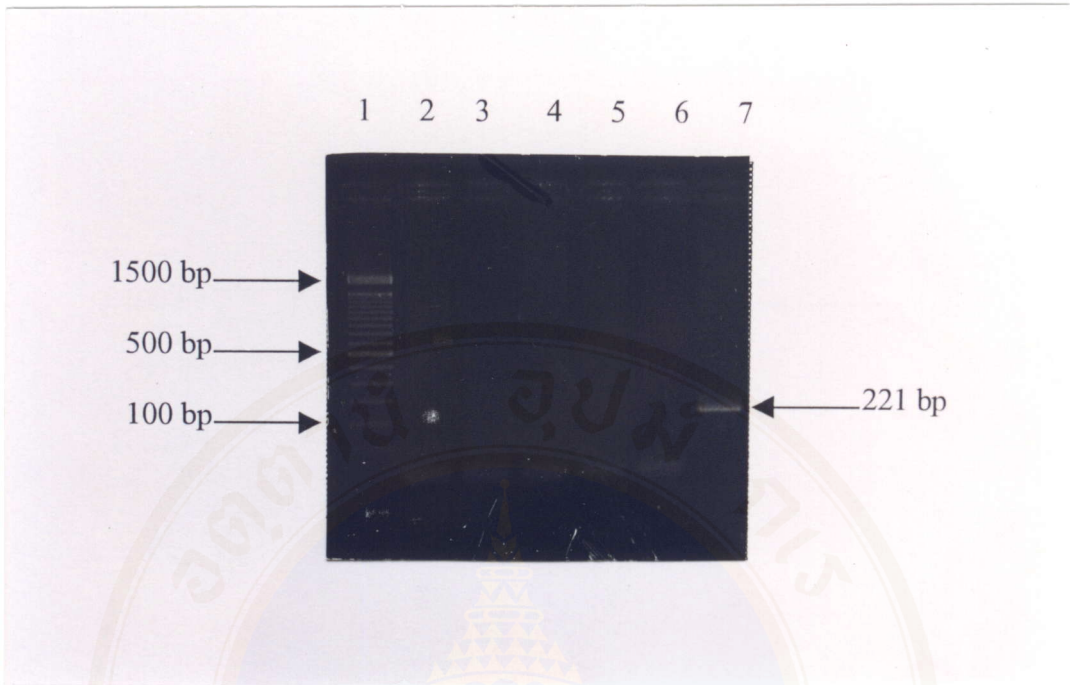

Figure 6. Agarose gel electrophoresis of the nested PCR products of bacteria amplified by specific *C. pneumoniae* primers.

Lane M DNA size markers (100 bp ladder)

Lane 1 *Moraxella catarrhalis*

Lane 2 *Haemophilus influenzae*

Lane 3 *Streptococcus pneumoniae*

Lane 4 Reagent blank

Lane 5 Negative control

Lane 6 *Chlamydia pneumoniae*

3. Detection of *C. pneumoniae* DNA by PCR in COPD patients with ARI

Only the processed specimens which can be amplified by β-globin primers were used for testing *C. pneumoniae*-PCR. The β-globin positive samples were obtained for detection of *C. pneumoniae* DNA from 72 COPD patients with ARI. They consisted of 24 patients with common cold, 35 with acute exacerbation of COPD, 9 with influenza-like illness and 4 with unknown symptoms. *C. pneumoniae* were found in 29 (40.2%) of COPD patients with ARI which included 11 (45.8%) COPD patients with common cold, 12 (34.3%) with acute exacerbation, 4(44.4%) with influenza-like illness, and 2 (50%) with unknown diagnosis of ARI. Rate of *C. pneumoniae* infection detected by presenting of *C. pneumoniae* DNA in COPD patients with ARI was highly significant difference from that in COPD without ARI symptom (0%) and from healthy control (6.7%) ($p<0.001$) (Table 8.).

Table 8. Detection rate of *C. pneumoniae* DNA by PCR from COPD patients with acute respiratory illness and controls.

Subjects	Diagnosis of acute respiratory illness	No. of subjects tested	No. of PCR positive subjects (%)
COPD patients	Acute exacerbation of COPD	35	12 (34.3)
	Common cold	24	11 (45.8)
	Influenza-like illness	9	4 (44.4)
	Unknown	4	2 (50)
	Total	72	29 (40.2)*
Controls	COPD without respiratory illness	8	0 (0)
	Healthy control	30	2 (6.7)

*P< 0.001 using chi-squared test to compare COPD patients with ARI and control.

The majority of the *C. pneumoniae* DNA positive samples of 29 COPD patients with ARI revealed that throat swab specimen was the most common site to discover *C. pneumoniae* DNA. *C. pneumoniae* DNA was detected in 43.6% of throat swab specimen, only 19.2% and 8.6% were found in specimens of nasal swab and sputum, respectively (Table 9).

Table 9. Detection rate of *C. pneumoniae* DNA by PCR in various specimens collected from COPD patients with ARI (n=72).

Specimen type	No. of specimens tested	No. of PCR positive specimen (%)
Sputum	58	5 (8.6)
Throat swab	55	24 (43.6)
Nasal swab	52	10 (19.2)

Table 10 shows distribution of *C. pneumoniae* present in various specimen types of 38 COPD patients with ARI in whom all three types of specimens could be collected at the same times. *C. pneumoniae* DNA could be detected in 3 types of specimens from COPD patients with acute exacerbation. Detection rate of *C. pneumoniae* DNA in throat swab (39.5%) was highly significant different from nasal swab (13.2%) and sputum (7.9%) ($p < 0.01$).

Table 10. Distribution of *C. pneumoniae* DNA samples in various specimens types collected from COPD patients with ARI (n=38).

Diagnosis of acute respiratory illness in COPD patients	No. tested of *C. pneumoniae* positive sample						
	SP	TS	NS	TS+NS	TS+SP	SP+NS	SP+TS+NS
Acute exacerbation of COPD (n=20)	1	3	1	0	0	0	2
Common cold (n=12)	0	5	0	1	0	0	0
Influenza-like illness (n=6)	0	3	0	1	0	0	0

SP: Sputum, TS: Throat swab, NS: Nasal swab

4. Serological diagnosis of *C. pneumoniae* infection in COPD patients with acute respiratory illness

4.1 Comparison of ELISA and MIF methods

MIF is a gold standard method for detecting antibody to *C. pneumoniae*. The serological method of ELISA was compared to MIF. Measurement of agreement was used Kappa (κ) statistic. *C. pneumoniae* IgG ELISA compared to MIF, κ value was 0.656 and $p < 0.0001$ (Table 11). Table 12 shows κ value of *C. pneumoniae* IgA ELISA compared to MIF was –0.055, p-value was 0.674. Table 13 shows κ value of *C. pneumoniae* IgM ELISA compared to MIF was 0.646, p-value was 0.001.

Table 11. Sensitivity and specificity of *C. pneumoniae* IgG ELISA compared to *C. pneumoniae* IgG MIF.

Antibody to *C. pneumoniae* IgG ELISA	MIF-IgG		Total
	Positive	Negative	
Positive	43	0	43
Negative	1	1	2
Total	44	1	45

$\kappa = 0.656$, $(p<0.0001)$

Table 12.. Sensitivity and specificity of *C. pneumoniae* IgA ELISA compared to *C. pneumoniae* IgA MIF.

Antibody to *C. pneumoniae* IgA ELISA	MIF-IgA		Total
	Positive	Negative	
Positive	45	4	49
Negative	2	0	2
Total	47	4	52

κ = -0.055, (p= 0.674)

Table 13. Sensitivity and specificity of *C. pneumoniae* IgM ELISA compared to *C. pneumoniae* IgM MIF.

Antibody to *C. pneumoniae* IgM ELISA	MIF-IgM		Total
	Positive	Negative	
Positive	1	0	1
Negative	1	21	22
Total	2	21	23

κ = 0.646, (p= 0.001)

4.2 Antibody to *C. pneumoniae* determined by ELISA.

Sera of seventy-two COPD patients with ARI were tested for antibody to *C. pneumoniae* by ELISA. The results are shown in Tables 14 and 15. *C. pneumoniae* IgG, IgA, and IgM were found in 13/14 (92.8%) , 46/52 (88.5%) and 1/67 (1.5%) of acute sera of COPD patients with ARI, respectively. In convalescent sera (BC) of COPD patients with ARI, 53/62 (85.5%), 55/66 (88.7%) and 3/66(4.5%) were positive for IgG, IgA, and IgM antibodies, respectively. The frequency of IgG, IgA and IgM antibodies in acute sera did not show significantly different from convalescent sera. Frequency of IgG antibody to *C. pneumoniae* were found in 41/48 (85.4%) and IgA in 9/17 (53.0%) of COPD without respiratory illness patients. Among healthy controls, frequencies of IgG, IgA and IgM were 65/100 (65.0%), 55/99 (55.6%) and 0/41 (0%), respectively. The frequencies and means of cut of index (COI) of *C. pneumoniae* IgG in sera of COPD patients with or without ARI showed values higher than healthy control ($p<0.05$). Frequency of *C. pneumoniae* IgA in COPD patients with ARI was higher than in both control groups. Only mean of COI of COPD with ARI patients showed significantly higher than the healthy control. The 4.5% of IgM positive sera were detected in COPD with ARI patients and none was found in healthy control (0/41). COI IgA borderline were found in 2 acute and 4 convalescent sera of COPD with ARI patients, as well as 5 sera of healthy controls. IgG borderline were found in 4 acute sera of each study groups.

Table 14. Antibody responses to *C.pneumoniae* in COPD patients and controls determined by ELISA tests.

Subjects	Diagnosis	serum	No. of subjects with positive *C. pneumoniae* antibody (%)					
			n	IgG	n	IgM	n	IgA
COPD								
	Common cold	Acute	4	4 (100)	22	0 (0)	15	13 (86.7)
		Convalescent	22	21(95.5)	22	0 (0)	22	19 (86.4)
	Acute exacerbation	Acute	7	6 (85.7)	34	1(2.9)	29	14 (82.8)
		Convalescent	30	25(83.3)	33	3(9.1)	30	26 (93.3)
	Influenza-like illness	Acute	3	3 (100)	9	0 (0)	7	7 (100)
		Convalescent	8	7 (87.5)	9	0 (0)	8	8 (100)
	unknown	Acute	0	ND	2	0 (0)	2	2 (100)
		Convalescent	2	0 (0)	2	0 (0)	2	2 (100)
	Total	Acute	14	13 (92.8)*	67	1(1.5)	52	46(88.5)*,***
		Convalescent	62	53 (85.5)*	66	3(4.5)	62	55(88.7)*,***
Control								
	COPD without respiratory illness	Single	48	41(85.4)**	0	ND	17	9(53.0)
	Healthy control	Single	100	65(65.0)	41	0 (0)	99	55 (55.6)

*P< 0.05, compare COPD patients with ARI with healthy control in corresponding antibody tested

** P< 0.05, compare COPD patients without ARI with healthy control in corresponding antibody tested

***P< 0.05, compare COPD patients with ARI and COPD patients without ARI in corresponding antibody tested

Table 15. Mean of cut of index (COI) of antibody to *C. pneumoniae* in COPD patients and controls determined by ELISA tests.

Subjects	Diagnosis	serum	Mean of COI of *C. pneumoniae* ± 1.96SE					
			n	IgG	n	IgM	n	IgA
COPD								
	Common cold	Acute	4	1.86±(0.08)	22	0.42±(0.08)	16	1.98±(0.61)
		Convalescent	23	1.87±(0.27)	23	0.45±(0.1)	23	1.9±(0.37)
	Acute exacerbation	Acute	7	2.1±(0.59)	34	0.51±(0.06)	29	2.15±(0.51)
		Convalescent	33	1.88±(0.27)	33	0.55±(0.1)	30	1.98±(0.41)
	Influenza-like illness	Acute	3	1.68±(0.57)	9	0.5±(0.14)	7	2.53±(0.65)
		Convalescent	8	1.69±(0.57)	8	0.58±(0.14)	8	2.19±(0.53)
	unknown	Acute	0	ND	2	0.35±(0.06)	2	1.83± (0.2)
		Convalescent	2	0.93±(0.04)	2	0.33±(0.01)	2	0.92±(0.55)
	Total	Acute	14	1.94±(0.31)*	67	0.51±(0.04)	54	2.14±(0.33)*
		Convalescent	66	1.81±(0.18)*	66	0.53±(0.06)	66	1.97±(0.24)*
Control								
	COPD without respiratory illness		52	1.59±1.96 (0.16)**	0	ND	17	1.47±(0.49)
	Healthy control		104	1.37±1.96 (0.12)	41	0.46±(0.06)	104	1.36±(0.18)

* P< 0.05, compare COPD patients with ARI with healthy control in corresponding antibody tested

**P< 0.05, compare COPD patients without ARI with healthy control in corresponding antibody tested

4.3 Antibody response to *C. pneumoniae* determined by MIF.

The convalescent blood sera of COPD patients using commercial MIF with ARI was investigated for *C. pneumoniae* IgG, IgA and IgM by using commercial MIF technique. The data are shown in Table 16. The existing of *C. pneumoniae* IgG, IgA and IgM antibody was considered at titer of ≥ 16, ≥ 16 and ≥ 10. High levels of *C. pneumoniae* IgG titer of ≥ 256 and ≥ 512 of COPD patients with ARI were found in 37 of 65 (56.9%) and 11 of 66 (16.7%), respectively. *C. pneumoniae* IgG antibody titer of ≥ 256 detected in COPD patients diagnosed as common cold, acute exacerbation, influenza-like illness and unknown were 13/23 (56.5%), 19/31 (61.3%), 4/8(50.0%) and 1/3 (33.3%), respectively. The presence of *C.pneumoniae* IgG antibody titer of ≥ 512 in patients with those symptoms were 4/23 (17.4%), 6/33 (18.2%), 0/7(0%), and 1/3 (33.3%). IgA antibody titer of ≥16 were demonstrated in 11 of 19 (57.9%) in COPD patients with common cold, 20/25 (80.0%) with acute exacerbation, 5/6 (83.3%) with influenza-like illness and 3/3 (100%) with unknown symptom. The MIF IgA titer of ≥ 32 were detected in 3/17 (17.5%) of COPD patients with common cold, 2/17 (11.8%) with acute exacerbation, 1/5 (20%) with influenza-like illness but not found in patients with unknown symptom. IgM titer of ≥10 were determined in 1/10 (10%) of patients with common cold, 1/9 (11.1%) of acute exacerbation, and 1/3 (33.3%) in influenza-like illness but was not found in unknown symptom. Therefore current *C. pneumoniae* infection in COPD with ARI determined by IgG titer of ≥ 512, IgA titer of ≥ 32 and IgM titer of ≥ 10 were 11/66 (16.7%), 6/41 (14.6%) and 3/41 (12.5%), respectively.

Table 16. Current infection of *C. pneumoniae* in COPD patients with ARI diagnosed by MIF IgG titer of ≥ 512, MIF-IgM titer of ≥ 10 and IgA titers of ≥ 32.

Diagnosis of COPD with ARI	No. of patients with positive MIF *C. pneumoniae* antibody in COPD patients with ARI / Total no. of COPD patients with ARI		
	IgG titer of ≥512 (%)	IgM titer of ≥10 (%)	IgA titer of≥ 32 (%)
Common cold	4/23 (17.4)	1/10 (10)	3/17 (17.5)
Acute exacerbation	6/33 (18.2)	1/9 (11.1)	2/18 (11.1)
Influenza-like illness	0/7 (0)	1/3 (33.3)	1/5 (20)
Unknown	1/3 (33.3)	0/2 (0)	0/3 (0)
Total	11/66 (16.7)	3/24 (12.5)	6/41 (14.6)

5. Diagnosis of acute infection of *C. pneumoniae* in COPD patients with ARI by PCR method and serology methods.

Means of COI of ELISA antibody (IgG, IgA) used for determining acute infection of *C. pneumoniae* were calculated according to MIF IgG titer of ≥ 512 and IgA titer of ≥32 (Figure 7 and 8). Means of COI±SD of IgG, IgA antibody were 2.1±0.98 and 2.36±1.85. If means of COI±0.5SD (2.5 for IgG and 3 for IgA) were selected to be COI values for determining acute infection of *C. pneumoniae*, the acute infection in COPD patients with ARI found by IgG-ELISA (COI≥2.5) and IgA-ELISA (COI≥3) were 16.1% and 14.5%, respectively.

Diagnosis of acute infection of *C. pneumoniae* by PCR was compared to MIF. *C. pneumoniae* DNA were detected in 5/26 (19.2%), 6/26 (23.1%) and 3/24 (12.5%) of the COPD patients with ARI existing IgG titer of ≥ 512, IgA titer of ≥ 32 and IgM titer of ≥ 10, respectively (Table 17). Diagnosis of acute infection of *C. pneumoniae* by PCR together with MIF antibody tests was evaluated in fourty-six COPD patients with ARI. Acute infection of *C. pneumoniae* was determined in 32 (69.6%) patients, 26 (56.5%) patients diagnosed by PCR and 17 (37%) patients diagnosed by MIF. The sensitivity of PCR and MIF were 81.3% and 53.1%, respectively. The data were tested the correlation between PCR with MIF and ELISA. The result of correlation was found only between PCR and MIF-IgA.

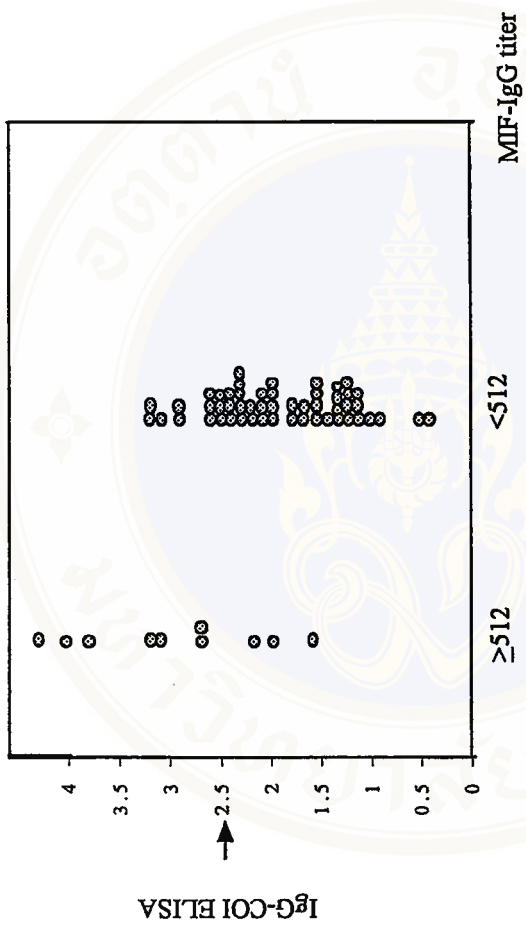

Figure 7. Correlation between MIF IgG titer and COI (ELISA) IgG to *C. pneumoniae*
● : Case number
▲ : Mean of COI ± 0.5SD

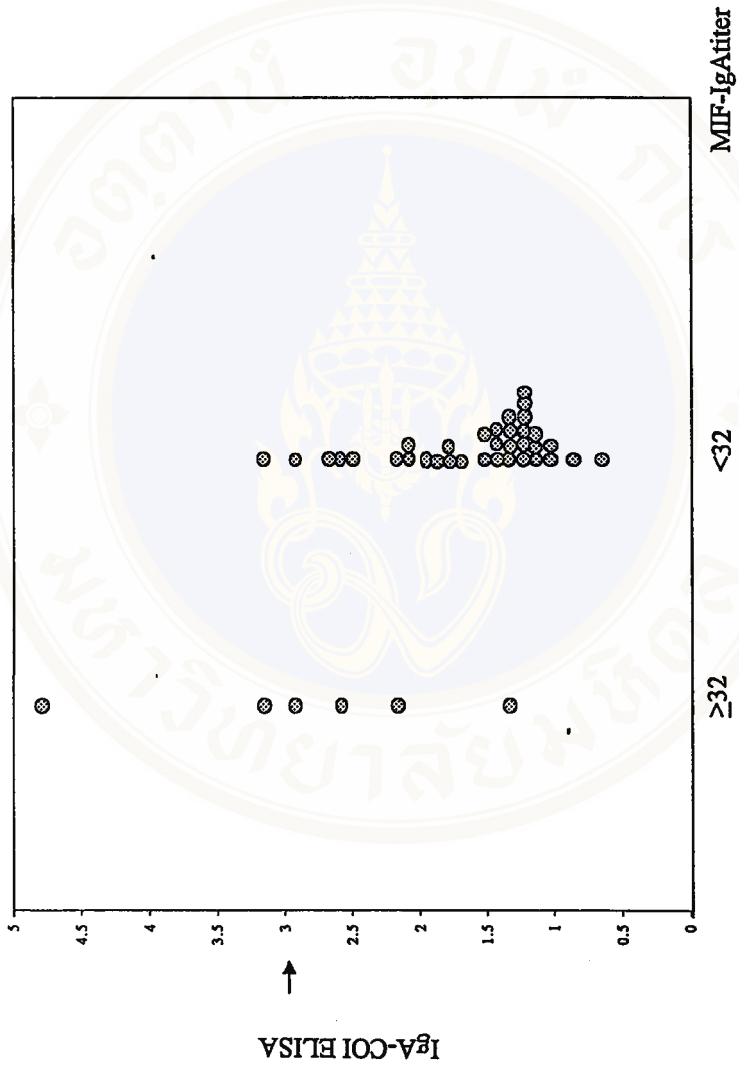

Figure 8. Correlation between MIF-IgA titer and COI (ELISA) IgA to *C. pneumoniae*
⊗ : Case number
↑ : Mean of COI ±0.5SD

Table 17. Detection of *C. pneumoniae* DNA (PCR) compared to antibody to *C. pneumoniae* by MIF and ELISA tests.

C. pneumoniae DNA (PCR)	Antibody to *C. pneumoniae*				
	MIF titer			ELISA COI (%)	
	IgG	IgM	IgA	IgG COI≥2.5	IgA COI≥3
	≥512	≥10	≥32		
Positive	5/26 (19.2)	3/24(12.5)	6/26 (23.1)	3/24(12.5)	2/24 (8.3)
Negative	6/40 (15.0)	ND	0/15 (0)	7/38 (29.2)	7/38 (29.2)
Total	11/66(16.7)	3/24(12.5)	6/41(14.6)*	10/62 (16.1)	9/62 (14.5)

*P<0.05, compare the correlation between PCR and MIF IgA by κ statistic

CHAPTER VI

DISCUSSION

Chlamydia pneumoniae is a common respiratory pathogen causing acute respiratory tract infection. It has been implicated as a cause of 10% of community-acquired pneumonia. *C. pneumoniae* infection have several characteristics of the clinical presentation. It has been associated with brochitis, pharyngitis, sinusitis and otitis in children and adults. Laboratory diagnosis of *C. pneumoniae* infection is needed since *C. pneumoniae* infection is treatable with antimicrobial agents. However the laboratory method for diagnosis remains difficult due in part to the limited available of facilities capable of performing culture or serology and the lack of a commercially available rapid non-culture test. At the present time, antibody detected by MIF is considered as gold standard method for diagnosis of *C. pneumoniae* infection. Detection of DNA by PCR method is more convenient, rapid, sensitivity and can be applied for the diagnosis of acute infection. The primers derived from 16s rRNA genes were described by Pollard *et al.* The primers can distinguish *C. pneumoniae* from other *Chlamydia* spp (87-89). The primer sets of *C. pneumoniae* in this study were derived from 16s rRNA gene and the sequence were synthesized followed the publishing of Messmer *et al* and others (64,65,84). Since 16s rRNA sequence are highly conserved among species, PCR primers designed from these sequences are likely to recognize the diversity of *C. pneumoniae* strains (23). Antigenic difference has recently been described for *C. pneumoniae*, but the variation

in nucleic acid sequences among *C. pneumoniae* strains is largely unknown (10). The primers selected here should detect diverse strains of *C. pneumoniae* in clinical specimens.

The sensitivity of nested PCR was evaluated by number of inclusion forming unit of *C. pneumoniae* (IFU) in the cell culture. We used nested PCR to amplify chlamydial culture containing 5 IFU/5ul (undiluted sample) and found that PCR product could be detected from undiluted to 10^{-3}diluted (0.005 IFU/5µl) samples. The PCR sensitivity was 1000 times more than the isolation in cell culture. Previous study reported that the limit of detection was less than 5 IFU. The same primer sets, and the amplification cycles were employed. We used *C. pneumoniae* elementary body (EB) and reticulate bodies instead of only elementary bodies (EB) as a target DNA. It represents of natural life cycle of *C. pneumoniae*.

To demonstrate specificity of primers used for detection of chlamydia DNA in throat swabs, nasal swabs and sputum, the target DNA derived from the most three common bacterial pathogens usually causing acute exacerbation in COPD patients were subjected to be amplified (84). Those bacteria are *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pneumoniae*. We obtained those bacterial culture from sputum of the patients which were cultured at Microbiology laboratory, Siriraj Hospital. The result showed that *C. pneumoniae* primers used for detecting *C. pneumoniae* DNA in the PCR is specific. They could not amplify DNA of *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*. Tests of specificity of these primer sets with other bacterial DNA could be demonstrated by others (84).

Important factors involving in detection of *C. pneumoniae* infection by PCR are the quality and types of the specimens. We investigated 3 types of specimens,

which were throat swab, sputum and nasal swab. Sputum often contains PCR inhibitors. Performing two steps or nested PCR and the DNA purification of the sample can eliminate inhibitors in the samples. Nested PCR can reduce the effect of inhibitor and increase sensitivity and specificity of the tests. The sample purification was not used in our test since it is inconvenient for the routine work and it may increase risks of the contamination. In this study, centrifugation and washing cell pellet were carried out to reduce the inhibitor in the sputum and other soluble inhibitors in the sample. In addition, we controlled false negative reaction and specimen quantity by performing β-globin gene amplification of the samples used for *C. pneumoniae* DNA detection (Table 9). All specimens were tested with β-globin primers. Therefore the presence of amplified product implies that there is enough DNA content and no inhibitory effect in PCR. The result in our study showed that throat swab yielded the highest detection rate of *C. pneumoniae* DNA by PCR among three types of specimens. Detection of *C. pneumoniae* DNA from throat swabs superior to nasopharygeal swab from patients with acute respiratory infections was reported by Gnarpe J. *et al* (17,90,91). The report of Verkooyen RP. *et al* (20) showed that chlamydial DNA could be detected in nasopharyngeal specimen more than sputum. In their study chlamydial DNA could not be detected from the sputum samples. In contrast, Boman *et al.* (19) reported that chlamydial infection was more frequently obtained from sputum samples than from nasophaygeal and throat swab samples in the patients who were diagnosed with a possible indication of acute *C. pneumoniae* infection. In our study, positive *C. pneumoniae* DNA sputum were detected only from the COPD patients with acute exacerbation. This finding suggested that *C. pneumoniae* may frequently colonize and could be detected in the throat of

patients with upper respiratory tract infection. During the invasive infection such as in acute exacerbation, *C. pneumoniae* can invade deeper and could be detected both in throat swab and in sputum. In patients who can not produce representative sputum, throat swab samples are appropriate specimens collected for DNA detection. The advantage of throat swab specimen is that it is easy to obtain, no technical problems and practical difficulty in collecting the samples. In the study *C. pneumoniae* DNA was detected in 43.6% of throat swab specimens of the COPD patients with ARI. Chlamydial DNA could be detected from the sputum of patients with the invasive infection i.e., acute exacerbation, but not in the sputum of COPD patients with common cold and with influenza-like illness.

Detection rate of *C. pneumoniae* DNA in COPD with ARI patients (40.2%) was higher than both in healthy control (6.7%) and in COPD without respiratory illness. The infection rates was not difference among each group of COPD with acute exacerbation, common cold and influenza-like illness. We concluded that *C. pneumoniae* plays an important role in causing acute infection in both upper and lower respiratory tract of COPD patients. Previous study of Von Hertzen *et al* (51) showed that *C. pneumoniae* DNA was detected nearly 60% of the COPD patients with severe symptom. The previous studies suggested that underlying illnesses and concurrent infection with other bacteria, such as the pneumococci, was associated with more severe diseases in COPD patients (21,25,35). Marrie *et al* (92) showed that the majority of the patients hospitalized with *C. pneumoniae* pneumonia had one or more underlying illnesses. In this study we did not investigated for other bacteria infection (12,26,48).

In serological study, the commercially serological tests both microimmunofluorecent (MIF) test, and the enzyme-linked immunosorbent assay (ELISA) are available. Purified *C. pneumoniae* elementary bodies (EB) were used as an antigen to evaluate the *C. pneumoniae* antibody ELISA assay. The reticulate body which comprise in the inclusion bodies and express genus specific epitopes are eliminated and the purified EB should be removed the genus-reactive LPS antigen before using as an antigen substrate in ELISA test. There are some reports showing the low sensitivity and specificity of the commercial ELISA kits. So it is important to evaluated the ELISA kits before use of detecting the antibody. Correlation of ELISA and MIF was investigated. MIF assay, the gold standard method for diagnosis of *C. pneumoniae* infection, has been developed since early 1970s and revealed to be suitable for routine diagnosis of chlamydial infection (73). The correlation of both methods was acceptable only for detecting IgG- and IgM-antibody, but not for IgA antibody. More sera may be needed for testing IgA by both methods.

Serological response to *C. pneumoniae* infection was investigated by ELISA test. In our study higher prevalence of *C. pneumoniae* infection in COPD patients with ARI (85.5%) and without ARI (85.4%) compared to the healthy control (65%) were demonstrated. The prevalence of antibodies against *C. pneumoniae* in a general population is varied from 25%-75% (21,92). The seroplevalence found in children under 5 years is less and then increase dramatically from ages 5 through 14 years, and by ages 20 years approximately 50%. The seroprevalence continues to increase among older age groups, but at slower rate, and reaches approximately 75% in the elderly (1,48). In general, primary infection is demonstrated by the presence of IgM antibody. The IgG response after primary infection may be slowly increase. Study of

Ekan *et al* (20) demonstrated the four-fold rising of IgG antibody in convalescent sera shown after 4-6 weeks of infection. In recurrent infection or reinfection the IgG antibody response may increase in 1 week after the acute illness. We tested antibody in convalescence sera which were collected in 1 month after acute illness to cover the period of antibody response of the primary infection. However, reinfection was commonly found in COPD patients with ARI since we could detect the DNA and high titers of IgG and IgA in the pre-existing IgG patients (93,94,95). ELISA-IgM was found in 4.5% of the COPD patients with ARI. We detected IgM in sera of 2 patients with continuously high IgA COI and 1 case with low levels of IgG and IgA. IgM response occurs not only in primary infection but also in active chronic infection or reinfection of the COPD patients (9,44,57). In the present study more frequency and higher level of COI of IgA were found in COPD with ARI than in healthy control. It indicated that *C. pneumoniae* causes acute respiratory illness in COPD patients. No difference of *C. pneumoniae* IgA COI in COPD patients with ARI and COPD patients without ARI may reveal a chronic *C. pneumoniae* infection present in COPD group. Elevated IgA titres or level in the absence of IgM antibody indicate acute or chronic infection (9,57). Persistent infection with chronic *C. pneumoniae* associated with chronic obstructive pulmonary disease has been reported by Beaty CD (9). To support this explanation more cases of COPD patients without ARI should be investigated for IgA-COI level.

Mean of COI values of IgG and IgA were determined according to the COI values found in the patients with IgG titer $\geq$ 512 and IgA titer of $\geq$ 32. Means of COI$\pm$0.5SD of the IgG and IgA were 2.5 and 3, respectively. Acute infection of *C. pneumoniae* determined by high IgG COI ($\geq$ 2.5) and IgA COI ($\geq$ 3) were found in

16.1% and 14.5% of COPD patient with ARI, respectively. To confirm that those existing IgA represented a chronic infection, detection of IgA should be followed up until 3-6 months. Chronic infection of *C. pneumoniae* exceeding 63% was reported in COPD patients with acute exacerbation compared to 46% in controls (55).

In MIF test, purified elementary bodies are used to detect specific *Chlamydia* IgM, IgG and IgA antibodies in sera. Antibodies detected by MIF- IgG titer ≥ 512 or -IgA titer ≥ 32 and –IgM ≥ 10 were applied to diagnosis of acute infection of *C. pneumoniae* in COPD patients with ARI. Data showed that acute infection determined by IgG, IgM and IgA were 16.7%, 12.5% and 14.6% in COPD patients with ARI, respectively. Acute *C. pneumoniae* infection occurred in 5% of COPD patient with excerbations was conducted by Beaty et al (9).

Combined methods of PCR and high titer of MIF can be used as a tool for diagnosis of acute respiratory infection of *C. pneumoniae* in COPD patients with ARI. The disparity between PCR and the MIF serological tests occurred in this study. Detection of acute *C. pneumoniae* infection by PCR was not correlated with serology. Diagnosis of acute infection could be detected by PCR (56.6%) more than MIF (37.0%). We detected *C. pneumoniae* DNA only in 19.2% of those COPD patients with acute infection of *C. pneumoniae* diagnosed by IgG titer of ≥ 512. Many reports demonstrated that result of PCR was not correlated with the serology (55,71,93). We suggested that both PCR and serological test should be used for the diagnosis of *C. pnueumoniae* infection, however detection rate by PCR is better than MIF. Negative PCR result may occur in patients harbouring low number (less than 0.005 IFU/5ul) of the organism. Chronic infection or asymptomatic infection of *C. pneumoniae* may cause elevated IgA without *C. pneumoniae* DNA detected in respiratory tract. ARI in

COPD patients may cause by other organisms. *C. pneumoniae* DNA could be detected in the patients without serological evidence of acute infection. *C. pneumoniae* may colonize superficial in respiratory tract. They could not stimulate the immune response. Invasion of the organism can stimulate better immune response.

CHAPTER VII

CONCLUSION

Detection of *C. pneumoniae* DNA by PCR methods was developed for diagnosis of *C. pneumoniae* infection. The primers were derived from DNA encoding *C. pneumoniae* 16s rRNA which are conserved region in order to detect diverse *C. pneumoniae* strains. The sensitivity of nested PCR was evaluated by comparing to the number of inclusion forming unit in the cell culture. The sensitivity of PCR is 1000 times more than isolation method. The specificity of primers was tested in PCR by amplifying the target DNA of bacteria predominated in respiratory tract infection of exacerbation of COPD patients which are *Haemophilus influenza, Moraxella catarrhalis* and *Streptococcus pneumoniae.* The result showed that *C. pneumoniae* primers could not amplify those bactrial DNA.

The established PCR method was used to detect *C. pneumoniae* DNA in the specimens of throat swab, nasal swab and sputum of the COPD patients with ARI. The specimens were centrifuged and washed in order to reduce the inhibitor in the sputum fluid and other soluble inhibitors. The β-globin gene amplification was used for internal control. The result showed that chlamydial DNA was detected mostly in throat swab. Thus, throat swab is specimen suggested for diagnosis of *C. pneumoniae* infection in the COPD patients with both upper and/or lower respiratory tract infections. In addition of throat swab, sputum should be collected in patients with acute exacerbation.

Detection rate of *C. pneumoniae* DNA in COPD with ARI patients (40.2%) was higher than in healthy control (6.7%) and in COPD without ARI (0%). No difference of detection rates were found among each group of COPD with acute exacerbation, with common cold and with influenza-like illness. We concluded that *C. pneumoniae* may play an important role in causing both upper and lower ARI in COPD patients.

Serological study of *C. pneumoniae* infection in COPD patients with ARI by microimmunofluorecent (MIF) test, and by enzyme-linked immunosorbent assay (ELISA) was performed. The correlation of ELISA and MIF was investigated. The correlation was found only in IgG and IgM, but not in IgA. The correlation of IgA-ELISA and IgA-MIF may be found if more number of sera are tested. Since the sensitivity and specificity of commercial ELISA kit are varied, it is important to evaluate the ELISA kits compared to MIF before using. In our study the IgG prevalence of *C. pneumoniae* infection in COPD patients with ARI (85.5%) and in COPD patients without ARI (85.4%) were higher than healthy control (65%). The presence of high frequency of IgG in both COPD with and without ARI indicated the association of COPD patients with *C. pneumoniae* infection. More frequency and higher level of COI of IgA could be detected in COPD with ARI than in healthy control. ELISA IgA antibody could be used for supporting acute infection of *C. pneumoniae*. However the level of IgA COI in those acute infection should be further investigated because no difference of IgA COI in COPD patients with ARI and without ARI was observed.

MIF antibody titer of IgG $\geq$ 512, IgM $\geq$10 and IgA $\geq$ 32 determined for acute infection of *C. pneumoniae* were found in 16.7%, 12.5% and 14.6% of COPD patients

with ARI, respectively. The means of ELISA IgG COI of ≥ 2.5 and IgA COI of ≥ 3 were used for diagnosis of acute infection. Acute infection of *C. pneumoniae* evaluated by ELISA COI of IgG (≥ 2.5) and IgA (≥ 3) were found in 16.1% and in 14.5% of COPD with ARI, respectively.

Acute infection in COPD patients with ARI caused by *C. pneumoniae* was the reinfection since the DNA as well as high titer of IgG and IgA were obtained in the pre-existing IgG patients. IgM response is normally found in patients with primary infection, however we could detect IgM antibody in COPD patients with reinfection and with acute chronic infection.

PCR and MIF can be used as a tool for diagnosis of acute infection of *C. pneumoniae* in COPD patients with ARI. Detection of *C. pneumoniae* by PCR should be used for diagnosis of *C. pneumoniae* infection in COPD patients with ARI which is better than serology. Acute infection of *C. pneumoniae* in COPD patients with ARI diagnosed by PCR and/or MIF was 69.6%. Detection rate by PCR was 56.5% and by MIF only 37.0% . PCR method showed higher sensitivity (81.3%) than MIF (53.1%). PCR result was not correlated with serology (Table 17). We detected *C. pneumoniae* DNA in the specimens of COPD patients with ARI who had no evidence of acute *C. pneumoniae* infection by serology. *C. pneumoniae* may colonize at the respiratory tract without stimulating the immune response. *C. pneumoniae* DNA negative patients but serological diagnosis of acute *C. pneumoniae* infection could be found in our study. The reason may be less number of *C. pneumoniae* DNA present in the samples. PCR combined with serology methods are suggested for diagnosis and study of *C. pneumoniae* infection, however the detection rate by PCR is better than serology.

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APPENDIX

1. Transport media; 2SP (0.2M sucrose - 0.02M phosphate)

Sucrose (Sigma, USA.)	68.46	g
K_2HPO_4 (Merck, Germany)	2.01	g
KH_2PO_4 (Merck, Germany)	1.01	g
0.2% Phenol red (Merck, Germany)	5.00	ml

Dissolve in double distilled water to 1,000 ml. Autoclave at 121°C for 15 minutes.

Fetal bovine serum (Gibco, NY)	100	ml
Gentamicin (40mg/ml)	0.25	ml
Fungizone (5mg/ml)	0.4	ml
Streptomycin (200mg/ml)	0.5	ml

Dispense 2 ml of 2 SP into steriled screw cap tubes (13 x 100 mm). Store at 4°C for up to 1 month.

2. Media and reagents for cell cultures

2.1 1 M HEPES buffer (10X concentration)

HEPES (Ameresco,USA)	4.77	ml
(N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid)		
Double deionized distilled water	100	ml

Dissolve HEPES and incubate in 56°C overnight. Adjust the pH to 7.5 with 1N NaOH. Sterilized by membrane (0.2μm) filtration. Store at 4°C

2.2 Growth medium (pH 7.5)

Eagle's Minimum Essential Medium (10X) (Gibco, USA)	100	ml
1M HEPES buffer	20	ml
L-Glutamine (Fluka, Switzerland)	300	mg
Glucose (Difco, USA)	5	g
$NaHCO_3$ (May & Baker Englang)	2	g
Double deionized distilled water to	1,000	ml

Adjust the pH to 7.5 with 1N NaOH or 1N HCl Sterilize by membrane (0.2 μm) filtration.

Add supplement

Fetal calf serum (Gibco, NY)	100	ml
Gentamicin (40mg/ml)	0.25	ml
Fungizone (5mg/ml)	0.4	ml
Streptomycin (200mg/ml)	0.5	ml

Mix together and dispense 100 ml into steriled bottle. Store at -20°C.

2.3 Phosphate buffered saline (PBS), Mg^{++} and Ca^{++} free

NaCl (Merck, Germany)	8	g
KCl (Merck, Germany)	200	g
Na_2HPO_4 (Merck, Germany)	1.15	mg
KH2PO4 (Merck, Germany)	200	mg
0.2% phenol red	5	ml
Double distilled water to	1,000	ml

Autoclave at 121°C with pressure 15 lb/square inch for 15 minutes, then store at 4°C.

2.4 Trypsin 0.25%

Trypsin powder 1:300 (Gibco,USA)	250	mg
0.2% Phenol	0.5	ml
PBS (Mg^{++} and Ca^{++} free)	100	ml.

Sterilize by membrane (0.2 μm) filtration. Dispense 10 ml into steriled bottle. Store at –20°C.

3. Media for *C. pneumoniae* culture

3.1 Stock cycloheximide (200ug/ml)

Cycloheximide (Fluka, Switzerland)	20	mg
Double deionized distilled water to	100	ml

Sterilize by membrane (0.2 μm) Filtration. Dispense 10 ml into steriled brown bottle. Store at 2°-8°C and protect from light.

3.2 Chlamydia culture media

Cycloheximide (200ug/ml)	0.12	ml
Growth media for cell culture	100	ml

Freshly prepare before use.

4. Reagents for PCR technique

4.1 Digestant-decontaminant solution for sputum sample [N-Acyetyl-L-Cystein-Sodium Hydroxide (NALC-NaOH)]

NALC	0.25	g

4% NaOH	25	ml
2.9% Na citrate $2H_2O$	25	ml

Stored in sterile screwcap flasks for later use. After adding NALC the digestant must be used within 24 hrs.

4.2 PCR lysis solution

4.2.1 Lysis buffer

1M KCl	5	ml
1M Tris HCl (pH 8.3)	1	ml
1M $MgCl_2$		
Tween-20	0.5	ml
Double deionized distilled water	100	ml

Sterilize by autoclaving at 121°C with pressure 15 lb/ square inch, for 15 minutes and store at 4°C.

4.2.2 Proteinase K (10mg/ml)

Proteinase K	10	mg
10 mM Tris HCl (pH 7.5)	1	ml

4.3 5X PCR buffer

Tris-HCl, pH 8.4	0.5	Mm
KCl	2.5	mM
$MgCl_2$	0.125	mM
0.1% BSA	5	ml
Double deionized distilled water	1.875	ml

4.4 Deoxynucleotide triphosphate(dNTPs) : Each dATP, dGTP, dCTP, and dTTP is supplied in a vial of concentration 100 mM.

4.4.1 Preparation of 2.5 mM concentration of working dNTPs.

4.4.2 Preparation of 10 mM stock solution of each dNTP.

dNTP	100	µl
Double deionized distilled water	900	µl

Use one µl of each dNTP in PCR.

4.5 Taq DNA polymerase (500 units) (GIBCO, USA. and Promega, USA.)

4.6 Loading buffer (6X)

Bromophenol blue	100	mg
Sucrose	20	g
TBE buffer (0.5X)	50	ml

4.7 TBE buffer (10X)

Tris base	108	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml

4.8 Ethidium bromide (10 mg/ml)

Ethidium bromide	1	g
Distilled water	100	ml

Store in the dark at room temperature.

4.9 Agarose gel for electrophoresis

Agarose type I-A (Sigma, USA.)	0.48	g
TBE buffer (0.5X)	32	ml

Melt and warm before pouring in a gel cast with 17 teeth.

5. Commercial kits for chlamydial antibody detection

5.1 SeroCP^{TM} for IgG IgA and IgM antibody (Savyon Diagnostics Ltd., Ashdod, Israel)

5.2 MRL for MIF –IgG, IgA and IgM (MRL Diagnostic Ltd.,California,USA)

5.3 Interpretation of the results of MIF-IgG, IgA and IgM tests

Read the slides from fluorescent intensity of elementary bodies, and grade the fluoresence as follow:

2 to 4+ : Moderate to intense apple-green fluorescence.

1+ : Definite, but dim fluorescence

Negative : No fluorescence or fluorescence equal to that observed in the corresponsing yolk sac control spot or in the negative control well.

In IgM endpoint titer of $\geq$10, IgG and IgA titers of $\geq$ 16 are presumptive evidence of infection.

6. Definition of acute respiratory illness in chronic obstructive pulmonary diseases (COPD) patients.

Common Cold : Infection of the upper respiratory tract with predominating rhinitis and pharyngitis

Influenza-like illness : Generalized aches, fever, and headache with or without upper respiratory tract symptoms

Acute exacerbation of COPD was diagnosed when at least two of the three

: symptoms (Increased dyspnea, increased sputum volume, and increased acute exacerbation of

COPD could be diagnosed by showing one of those mentioned symptoms in addition to at least one of the following findings:

1. Upper respiratory tract infection (sore throat, nasal discharge) within the past 5 days
2. Fever without other cause
3. Increase wheezing
4. Increase cough
5. Increase in respiratory rate or heart rate by 20% as compared with baseline

BIOGRAPHY

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