

**King Saud University**  
**Faculty of Science**  
**Botany and Microbiology Department**

**Surveillance and in-vitro cell response to *chlamydia*  
*pneumoniae* infection.**

“Thesis submitted for partial fulfillment of the requirement  
of the Degree of Doctor of Philosophy (Ph.D) in  
Microbiology (Bacteriology) at the department of Botany  
and Microbiology , King Saud University”

**Prepared By:**

**Mohammed Ali M. Marie**

**Supervised By:**

**Dr. Rasheed M. Al-Ssum**

**1425-2004**

## **ABSTRACT**

*Chlamydia pneumoniae* is a major cause of acute respiratory tract diseases in human and has been responsible for both endemic and epidemic pneumonia. In addition, this organism is thought to play some roles in other clinical manifestations, like coronary artery diseases, asthma and sarcoidosis

This is the first Saudi Arabian study that investigates the prevalence of this organism in Saudi Arabia, its pathogenesis and immunological response. The study employed a wide range of advanced techniques and methods, including culturing ,detection by immunoflourescence, polymerase chain reaction (PCR), electron microscopy, antichlamydial activity assays and the most recent advanced technique of microarray technology.

Five hundred seventy two respiratory tract specimens were obtained over a period of 12 months from patients diagnosed with different respiratory diseases. *Chlamydia pneumoniae* was detected in respiratory specimens by culture and PCR and the prevalence was 11.9% by both techniques.

In this study, the surveillance of *C. pneumoniae* infection among all age groups was 50% in small children less than 4 years, decrease rapidly at the age of 4-10 years to (8%) and in adults less than 40 years old (6%). A dramatic increase was noticed in group aged 40 year and over (36%). The infection occurred all year-round and the highest rate of *C. pneumoniae* was during winter (November (38%)), while there is noticeable drop in its detection in other months. More than one half of the patients (63%) presented with pneumonia, and about one third (33%) presented with upper respiratory tract infections. In some cases, *C.*

*pneumoniae* co-existed with other bacteria, such as *S. pneumoniae* and *M. pneumoniae*, and viruses according to the patient record. These mixed infections might be responsible for the high rate of pneumonia and may mislead the treatment.

In the present study, a detailed description based on transmission and scanning electron microscopy for temporal events throughout *chlamydia pneumoniae* developmental cycle. Morphologically, *C. pneumoniae* have 2 stages : dormant stage (Elementary Bodies-EBs) are typically pear shaped dense in color, while the vegetative stage (Reticulate Bodies-RBs) appear round shape and lighter.

The developmental process of *chlamydia pneumoniae*, showed that EBs interact initially with microvilli , then EBs inject type three secretion (TTS) protein into the cytoplasm to modify the environment and make it suitable for multiplication. This protein play an important role in the success of the infection. Elementary Bodies enters the host cell by an invagination process, this process is completed when the host cell membrane completely encircles the EBs and form inclusion bodies in which EBs transformed into RBs, which multiply by binary fission, and finally maturation to EB leaving the cell in volcanic eruption phenomenon. This study proved the invasion of the host nucleus leading to its destruction by *chlamydia pneumoniae*.

Cytokine have been well described as potentiators of the cellular immune response and consequently inhibit the replication of several species of chlamydia.

The data presented here support the possibility of a similar role for some cytokine for inhibiting the intracellular multiplication of *C. pneumoniae* in Hep-2

cells. The addition of IFN- $\gamma$  at 10 IU/ml 24 hours prior to infection was capable of completely inhibiting the multiplication of *C. pneumoniae* in the Hep-2 cell monolayers. This study demonstrates that IFN- $\gamma$  can alter the intracellular fate of this organism and thus may play an important role in the pathogenesis of human infection.

The expression of cytokine genes by *C. pneumoniae* infection may play an important role in the pathogenesis of *C. pneumoniae*, specially in chronic respiratory infections. In this study, it has been found that treated infected Hep-2 with *C. pneumoniae* had elevated gene expression of the proinflammatory cytokine TNF- $\alpha$ , IL-15 and other cellular genes

To my knowledge it is the first time to show the involvement of IL-15 gene in *C. pneumoniae* infection. Other cellular genes seem to play antichlamydiale role.

# TABLE OF CONTENTS

	<b>PAGE NO</b>
Abstract	
Table of content	
List of tables and Figure	
<b>CHAPTER ONE</b>	
<b>INTRODUCTION</b>	
1.1. Taxonomy	1
1.2. History of <i>Chlamydiae Pneumoniae</i>	4
1.3. Developmental cycle	4
1.4. Structure	7
1.5. The role of cytokines in chlamydial infection	9
1.6. Pathogenesis	11
1.7. Surveillance	12
1.8. Clinical manifestations	14
1.9. Diagnosis	18
1.10. Treatment	25
1.11. Objective of this study	26
<b>CHAPTER TWO</b>	
<b>MATERIALS AND METHODS</b>	
2.1. Fundamentals	27
2.1.1. Cell line	27
2.1.2. Interferon Preparation	27
2.1.3 Bacterial preparation and bacterial stock	28
2.2 Chlamydia pneumoniae culture and PCR	30
2.2.1. Patients	30

2.2.2. Clinical specimens	30
2.2.3. Laboratory assays for <i>C. pneumoniae</i>	30
2.2.4. PCR (Polymerase chain Reaction)	32
2.3. Electron microscopy	34
2.3.1. Plan for study the life cycle of <i>C. pneumoniae</i>	34
2.3.2. Scanning Electron Microscopy (SEM) procedure	34
2.3.3. Transmission Electron Microscopy (TEM) procedure	36
2.4 Antichlamydial assay	38
2.4.1. Crystal violet stain Assay for cytopathic effect	39
2.5. Microarray-Genisphere Method	40
2.5.1 Cell culture, treatment and sample preparation for Microarray	40
2.5.2 Agarose Gel Electrophoresis for RNA	42
2.5.3. Genisphere 3DNA indirect labeling of cDNA targets	42
<b>CHAPTER THREE</b>	
<b>RESULTS</b>	
3.1. Age and sex distribution	46
3.1.1. <i>Chlamydia pneumoniae</i> Culture	46
3.1.2. Mixed <i>C. pneumoniae</i> With Other Microorganisms	46
3.1.3. Polymerase Chain Reaction (PCR)	54
3.2. Ultrastructural study by Electron microscopy	57
3.2.1 Morphology of Hep2 cells	57

3.2.2 Morphology of <i>C. pneumoniae</i> elementary bodies and reticulate bodies	58
3.2.3 The process of infection as illustrated by Electron microscopy	58
3.3. Antichlamydial activities	70
3.3.1 Cell Response to IFNs treatment	70
3.4 Expression Profiling of cytokine and other cellular genes Using Microarray	73
<b>CHAPTER FOUR</b>	
<b>DISCUSSION</b>	
4.1 Surveillance of <i>chlamydia pneumoniae</i> infection	81
4.2 Ultrastructural study of <i>C. Pneumoniae</i>	86
4.2.1 Morphology of Chlamydia pneumoniae	87
4.2.2 The process of <i>C. pneumoniae</i> infection	88
4.2.3 The intracellular development	90
4.3 Antichlamydial activity	95
4.4 Expression Profiling of cytokine and other cellular genes Using Microarray	97
References	102

## LIST OF TABLES

	PAGE NO.
Table 1. The family Chlamydiaceae	3
Table 2. Acute and chronic diseases associated with <i>C. pneumoniae</i>	17
Table 3. Laboratory methods for diagnosing <i>C. pneumoniae</i> infection	24
Table 4. <i>Chlamydia pneumoniae</i> finding and co-pathogen over 12-months study period	Appendix
Table 5. Epidemiological characteristics of 68 patients with <i>C. pneumoniae</i> infection	48
Table 6: Seasonal variation of <i>C. pneumoniae</i> isolation in 68 patients with <i>C. pneumoniae</i>	50
Table 7: Clinical manifestation of culture proven <i>C. pneumoniae</i> infection in 68 patients.	52
Table 8. Frequency Distribution of Additional microorganisms in 68 Patients Infected With <i>C. pneumoniae</i>	53
Table 9: Up- and down-regulated genes between Set-1 (infected Hep-2 cells) and Set-2 (treated infected Hep2-cells)	78

## LIST OF FIGURES

	PAGE NO.
Figure 1 : Developmental cycle of <i>Chlamydia</i>	6
Figure 2 :The action of Dextran on Cytoplasmic membrane of Hep-2 cells	31
Fig 3: Sex and age distribution of <i>chlamydia pneumoniae</i> .	49
Fig 4: The prevalence of <i>chlamydia pneumoniae</i> in both sex, Male 65% and female 35%.	49
Fig 5: The distribution of positive and negative results in all months.	50
Fig 6: DNA sequence of <i>C. pneumoniae</i> -specific 437 bp PstI restriction fragment	55
Fig 7 : Amplification of <i>C. pneumoniae</i> DNA from clinical specimens	56
Fig 8 Mophology of Hep-2 cells	61
Fig 9 Morphology of elementary bodies and reticulate bodies	62
Fig 10 The start of infection (Ruffling)	63
Fig 11 The stage of nesting	64
Fig 12 The invasion of the cell	65
Fig 13 Formation of projecting structure	66
Fig 14 Maturation of RBs	67
Fig 15 Releasing of EBs	68
Fig 16 Non-Successful infection	69
Fig 17 The effect of IFNs on Hep-2 cell line	72

*Chapter One*  
*INTRODUCTION*

# ***1. INTRODUCTION***

## **1.1. Taxonomy of *Chlamydiae***

*Chlamydiae* were first considered as protozoa and later as viruses, they are gram-negative obligate intracellular bacteria. Originally, they were taxonomically categorised into their own order *Chlamydiales*, with one family, *Chlamydiaceae*, and a single genus, *Chlamydia* (Moulder *et al.* 1984). The genus included four species: *C. trachomatis*, *C. psittaci* (Moulder *et al.* 1984), *C. pneumoniae* (Grayston *et al.* 1989) and *C. pecorum* (Fukushi & Hirai 1992).

In 1999, it was recommended by Everett *et al.* (1999) that the genus *Chlamydia* should be divided in two genera, *Chlamydia* and *Chlamydophila*, containing altogether nine species (Table 1). However, the proposal to change the taxonomic nomenclature for the *Chlamydiaceae* family has not been generally accepted in the field (Schachter *et al.* 2001).

Two species, *C. trachomatis* and *C. pneumoniae*, are common human pathogens, whereas the other species occur mainly in animals. *C. trachomatis* has been isolated only from humans and comprises two human biovars (trachoma and lymphogranuloma venereum, LGV), including a total of 18 serovars, whereas *C. pneumoniae* has one human biovar (TWAR) and two animal biovars, one infecting horses (biovar

equine) and the other infecting frogs and koalas (biovar koala) (Everett et al. 1999).

**Table 1. The family Chlamydiaceae as proposed by Everett et al.  
(1999)**

<b>Species</b>	<b>Host</b>	<b>Route of entry</b>
<b><i>Chlamydia</i></b>		
<i>C. muridarum</i>	Mouse, hamster	Pharyngeal, genital
<i>C. suis</i>	Swine	Pharyngeal
<i>C. trachomatis</i>	Human	Pharyngeal, ocular, genital, rectal
<b><i>Chlamydophila</i></b>		
<i>C. abortus</i>	Mammals	Oral, genital
<i>C. caviae</i>	Guinea pig	Pharyngeal, ocular, genital, urethral
<b><i>C. felis</i></b>	Cat	Pharyngeal, ocular, genital
<i>C. pecorum</i>	Mammals	Oral
<i>C. pneumoniae</i>	Human, frog, koala, horse	Pharyngeal, ocular
<i>C. psittaci</i>	Birds	Pharyngeal, ocular, genital

## **1.2. History of *Chlamydia Pneumoniae***

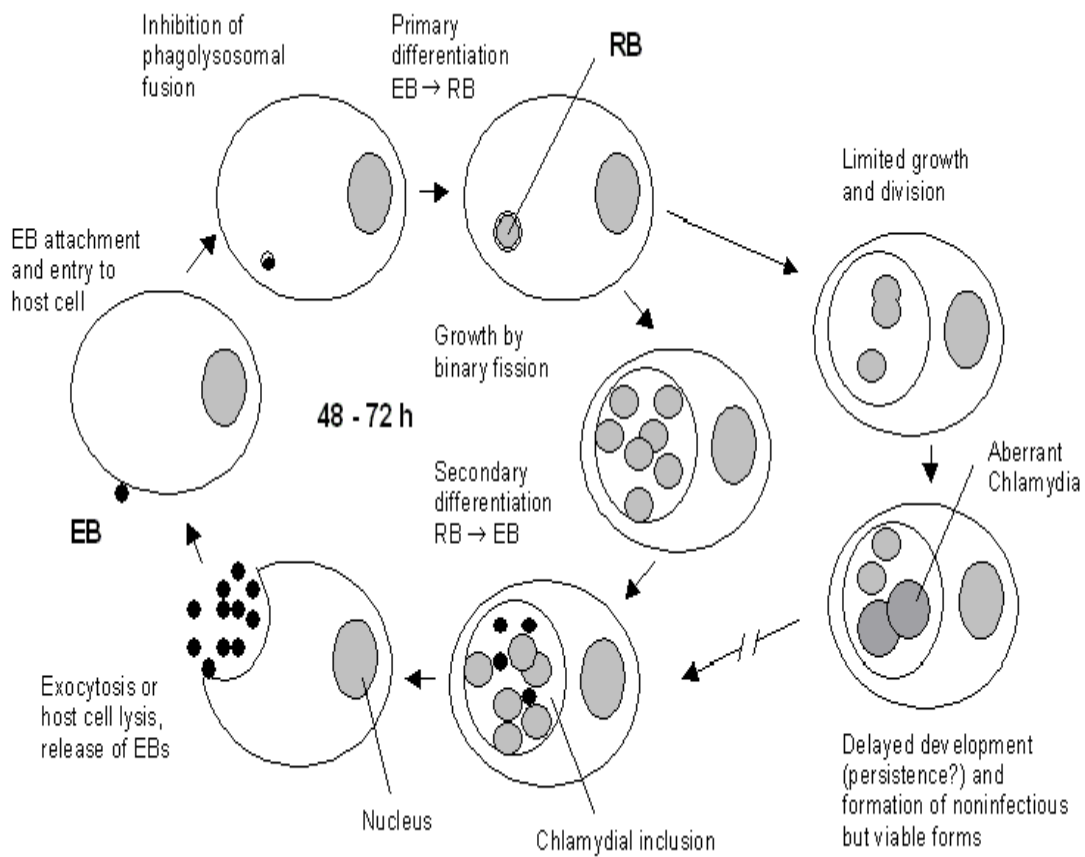
*C. pneumoniae* was described quite recently. In the mid-1980s, an atypical strain of *C. psittaci* was found to be responsible for the epidemic of mild pneumonia that had occurred in Finland in 1978 (Saikku *et al.* 1985). The strain was called TWAR, which was an acronym of the first two Seattle isolates: TW-183, isolated in 1965 from the eye of a child during a trachoma vaccine trial in Taiwan, and AR-39, isolated in 1983 from a throat swab of a university student with pharyngitis in Seattle (Grayston *et al.* 1986). In 1989, the strain was identified as a separate species within the genus *Chlamydia* and named *Chlamydia pneumoniae* (Grayston *et al.* 1989). Since then, it has been proposed that the species should be rather placed under the new genus called *Chlamydophila*, as distinct from the genus *Chlamydia*, and be renamed as *Chlamydophila pneumoniae* (Everett *et al.* 1999). But in this thesis we are going to use *Chlamydia pneumoniae* because it is widely used and accepted in publications.

### **1.3. Developmental cycle**

*Chlamydiae* are intracellular bacteria that have a unique biphasic developmental cycle with two distinct morphological forms. The extracellular, infectious form (0.3  $\mu\text{m}$ ) is called elementary body (EB), and the intracellular, replicating form (1.0  $\mu\text{m}$ ) is called reticulate body (RB). Infectious EBs start the cycle by attaching to a susceptible host cell membrane. They gain access into the host cell via either parasite-specified

phagocytosis or receptor-mediated endocytosis. When inside the cell, the *chlamydiae* remain within an enlarging intracellular vacuole, a characteristic inclusion, avoiding lysosomal fusion and hence destruction. During the first few hours, EBs differentiate into metabolically active RBs. By using the host cell's energy and nutrient resources, RBs begin to multiply by binary fission. After multiple rounds of division, RBs start to transform back to EBs. Finally, by exocytosis or host cell lysis, the infectious EBs are released out of the host cell to initiate new cycles in the other new host cells. (Hatch 1999)

In cell culture conditions, the duration of the developmental cycle is between 2 and 3 days. In natural infections, the situation is more complicated, and the normal development of *Chlamydia* is easily disturbed due to nutrient deficiency, interferon-gamma and antibiotics may result in morphological alterations of RBs and the emergence of enlarged, atypical chlamydial forms (Beatty *et al.* 1993). These altered forms may persist inside the host cell in a viable but culture-negative state for a long time. The cycle of both normal and altered development of *Chlamydia* is presented in Fig. 1.



**Figure 1. Developmental cycle of *Chlamydia* (Beatty *et al.* 1994)**

## 1.4. Structure

At all stages of development, chlamydial cells appear to be surrounded by a double membrane, a characteristic feature of gram-negative bacteria. However, unlike other gram-negative bacteria, *chlamydiae* do not have a peptidoglycan layer in the space between the two membranes (Fox *et al.* 1990). On the other hand, they contain penicillin-binding proteins, and the presence of peptide crosslinks analogous to those between peptidoglycan backbones has been suggested (Barbour *et al.* 1982). The genomic sequence of *C. trachomatis* revealed the presence of genes for peptidoglycan synthesis (Stephens *et al.* 1998). Peptidoglycan has been suggested to have greater role in cell division in *chlamydiae* (Brown & Rockey 2000).

Lipopolysaccharide (LPS), which is a general endotoxin in gram-negative bacteria, is localised on the surface of *Chlamydia*, EBs and RBs (Birkelund *et al.* 1989). Chlamydial LPS is structurally similar to the rough form of LPS found in enterobacteria, having both a cross-reactive epitope and a genus-specific epitope (Brade *et al.* 1987). In addition to the rough-type LPS, a smooth form of chlamydial LPS has also been found (Lukacova *et al.* 1994). The structure of LPS is not identical in all chlamydial species, and compared to the LPS of enterobacteria, chlamydial LPS has much lower endotoxin activity (Ingalls *et al.* 1995).

The outer membrane contains proteins named outer membrane proteins (Omp). The most abundant of them is the major outer membrane protein (MOMP) of 38 to 42 kDa, comprising about 60% of total Omps (Caldwell *et al.* 1981). MOMP contains serovar-, subspecies- and species-specific epitopes that can be identified by monoclonal antibodies (Perez Melgosa *et al.* 1991). MOMP is surface-localised on all *chlamydiae* species (Wolf *et al.* 2001), but the MOMP of *C. pneumoniae* appears to be less immunogenic and antigenically complex than that of the other *chlamydiae* (Campbell *et al.* 1990, Perez Melgosa *et al.* 1991).

A small cysteine-rich protein, Omp3, is synthesized late in the developmental cycle, and it is not exposed at the surface of *Chlamydia* (Collett *et al.* 1989). Omp2 is a 60-kDa cysteine-rich protein, and it has been suggested to be surface-exposed (Stephens *et al.* 2001). On the other hand, it has been suggested that Omp2 is the structural element for the hexagonally arrayed structures which has been seen inside host cells (Mygind *et al.* 1998).

*Chlamydiae* also contain heat shock proteins (Hsp). The genes encoding Hsp10, Hsp60 and Hsp70 have been cloned and sequenced (LaVerda & Byrne 1997). These genes are continuously expressed throughout the developmental cycle. The Hsps are highly conserved within chlamydial species, including *C. pneumoniae* (Kornak *et al.* 1991). All

three Hsps can be found in the outer membrane complexes of both EBs and RBs (Brunham & Peeling 1994). Chlamydial Hsp60 and Hsp70 are highly immunogenic during natural infection (Brunham & Peeling 1994).

In the inclusion membrane, there is a group of proteins called inclusion membrane proteins (Inc). The first of them was demonstrated in *C. psittaci* by Rockey *et al.* (1995) and named inclusion membrane protein A (IncA). Since then, six other Incs, from IncB to IncG, have been characterised (Scidmore-Carlson *et al.* 1999). However, a genome search of *C. trachomatis* revealed 46 candidates as potential members of Incs (Bannantine *et al.* 2000). Six of the genes were selected for antibody production, and five of these were shown to be located in the inclusion membrane. The genome of *C. pneumoniae* contains an even higher number of hypothetical Inc proteins. The potential to export such a high number of Incs to the inclusion membrane suggests that the inclusion membrane may have several functions in vesicle trafficking, inclusion development, avoidance of lysosomal fusion, nutrient acquisition and signalling associated with EB-RB-EB reorganization (Rockey *et al.* 2000).

### **1.5. The role of cytokines in chlamydial infection.**

Cytokines are chemical substances produced by cells, which affect the function of other cells. Cytokines are important mediators in host defenses against bacterial and viral infections. Cytokines such as tumor necrosis

factor alpha (TNF- $\alpha$ ), gamma interferon (IFN- $\gamma$ ), interleukin 1 (IL-1), and lymphotoxin have been shown to inhibit chlamydial infection, ( Geng et al. 2000).

*C. pneumoniae* has also been shown to induce cytokine such as IL-1B, IL-6, IL-8, IL-12, TNF- $\alpha$ , IFN- $\gamma$ , and intracellular adhesion molecule-1 in various systems such as human peripheral blood mononuclear cells, alveolar macrophages, and various mouse cell models ( Geng et al. 2000). *C. pneumoniae* infection can also stimulate the production of anti-inflammatory cytokines such as IL-10, which can down regulate the expression of major histocompatibility complex class I molecules.

Bronchial epithelial cells are a first line of defense during *C. pneumoniae* airway infection. It is known that epithelial cells at the mucosal surface are capable of secreting chemoattractants and proinflammatory cytokines, which are important mediators in both lung defense and inflammation, in response to bacterial infection. This suggests that epithelial cells can act as early warning system for local immune and inflammatory cells (Levine. 1995)

## 1.6. Pathogenesis

Different chlamydial species as well as different biovars infect different cell types. The human biovar of *C. pneumoniae* has been shown to be able to infect and multiply in endothelial cells, smooth muscle cells, monocytes/macrophages and lymphocytes *in vitro* (Haranaga *et al.* 2001). The dissemination of *C. pneumoniae* has been studied in mouse models. After intranasal inoculation, *C. pneumoniae* spreads systemically in mice, and it can be isolated from lungs, spleen and peritoneal macrophages (Yang *et al.* 1995). Intravenous and subcutaneous inoculations also result in disseminated infections. It has further been shown, also in mice, that *C. pneumoniae* has an ability to disseminate systematically via infected macrophages along hematogenous and lymphatic routes (Moazed *et al.* 1998).

The host defence mechanisms seem to be unable to eradicate *Chlamydia* or to provide protection from re-infections. Therefore, repeated infections with *Chlamydia* are common (Ward 1995). Repeated and persistent chlamydial infections are associated with adverse outcomes, in which Hsps seem to have a role (Beatty *et al.* 1994). Chlamydial Hsp60 has been shown to elicit an ocular delayed hypersensitivity response (Morrison *et al.* 1989), and its expression has been shown to be increased in persistent infection with *C. trachomatis* (Beatty *et al.* 1994). An enhanced immune

reaction against chlamydial Hsp60 is more typically associated with chronic upper genital tract conditions than with acute infections of the lower genital tract (Peeling & Mabey 1999). Serum antibodies to chlamydial Hsp60, as well as Hsp60-specific T cell responses, have been shown to be associated with blinding trachoma, salpingitis, pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility following ocular and genital *C. trachomatis* infections (Kinnunen *et al.* 2001). In diseases associated with *C. pneumoniae* infection, antibody responses to chlamydial Hsp60 in asthma, arteriosclerosis and acute anterior uveitis have been reported. Since Hsp60 is highly conserved, an autoimmune response to human Hsp60 may have a role in chlamydial pathogenesis (Huhtinen *et al.* 2001).

## **1.7. Epidemiology**

*C. pneumoniae* is a common worldwide respiratory pathogen. Most likely, it is primarily transmitted from human to human by the respiratory tract without any animal reservoir (Kleemola *et al.* 1988).

*C. pneumoniae* infections appear to be most common among school-aged children (Kuo *et al.* 1995). In some areas, however, infections are already common in children aged 1 to 4 years (Normann *et al.* 1998). The prevalence increases dramatically after the age of 5, and by the age of 20, half of the population are estimated to have detectable antibody levels. Thereafter, seroprevalence continues to increase in adult, but at a slower

rate, and reaching a level of approximately 75% in the elderly (Kuo *et al.* 1995). Seroprevalence rates continue to be high despite the fact that some individuals lose their antibodies over a period of several years, suggesting that the majority of people are infected during their lifetime, and that reinfections are common (Grayston *et al.* 1990).

Seroprevalence is almost equal in both sexes up till adolescence, but higher among adult men than adult women (Kuo *et al.* 1995). In addition to male sex, smoking has been shown to be associated with *C. pneumoniae* infection (Mayr *et al.* 2000).

*C. pneumoniae* infections occur throughout the year, but cyclic variations have been demonstrated. Periods of 2 to 3 years with high incidence rates are followed by 4 to 5 years of lower incidence (Schachter & Grayston 1998). The first observations of *C. pneumoniae* epidemics came from Finland, where *C. pneumoniae* caused two outbreaks in civilian communities in 1978 and four in military garrisons in 1977–1978 and 1985–1987 (Ekman *et al.* 1993). It has been shown that *C. pneumoniae* was endemic in Finland as early as 1958 (Karvonen *et al.* 1992). During these epidemics, 43% of pneumonia cases were caused by *C. pneumoniae* (Kauppinen *et al.* 1995). At other times, *C. pneumoniae* causes approximately 10% of all community-acquired pneumonias (Saikku 1992).

## 1.8. Clinical manifestations

*C. pneumoniae* is a respiratory pathogen that causes both upper and lower respiratory tract diseases. The majority of *C. pneumoniae* infections are asymptomatic or mild upper respiratory tract infections (Saikku 1992). Involvement of *C. pneumoniae* infection has been described in case of common cold, persistent cough, pharyngitis, sinusitis and otitis media (Blasi 2000). Pneumonia and acute bronchitis are the most frequently recognised lower respiratory tract diseases associated with *C. pneumoniae* infection (Kuo *et al.* 1995). In addition to acute respiratory infections, several chronic respiratory tract inflammatory diseases have also been associated with *C. pneumoniae* infection. These include chronic bronchitis and chronic obstructive pulmonary disease (COPD) as well as sarcoidosis (Blasi 2000). A number of studies also speak for an association between *C. pneumoniae* and asthma (Hahn 1999).

In addition to respiratory tract infections, *C. pneumoniae* has been associated with cardiovascular diseases. Subacute inflammatory conditions, such as endocarditis, myocarditis and vasculitis, have been reported to follow *C. pneumoniae* infections (Saikku 2002). The association of *C. pneumoniae* infection with coronary heart disease (CHD) and acute myocardial infarction (AMI) was discovered in 1988 by Saikku *et al.* (1988a). Although no causal association between *C. pneumoniae* infection

and atherosclerosis has been demonstrated, up to 500 papers have been published to support the theory that *C. pneumoniae* infection is involved in the clinical diseases associated with atherosclerosis and its complications, such as AMI, stroke, transient ischaemic attack (TIA) and abdominal aortic aneurysm (AAA) (Ngeh *et al.* 2002).

The role for *C. pneumoniae* in cerebrovascular diseases, such as stroke and TIA, has been suggested in a number of studies, and the organism has been reported to be present in cerebral vessels. There are some reports on an association of *C. pneumoniae* with acute infections of the central nervous system, such as meningoencephalitis and Guillain-Barre syndrome. Furthermore, *C. pneumoniae* may play a role in two major neurological disorders: Multiple Sclerosis (MS) and Alzheimer's disease. (Saikku 2002)

*C. pneumoniae* infection has also been associated with cancer: small and squamous cell lung cancer, non-Hodgkin's lymphoma and the rare Szezary's syndrome. Other diseases associated with *C. pneumoniae* infection include erythema nodosum, Reactive Arthritis (ReA), Sweet's syndrome and eye diseases (conjunctivitis, iritis and uveitis). (Saikku 2002).

Table 2.

**Table 2. Acute and chronic diseases associated with *C. pneumoniae***

<b><i>Respiratory tract diseases</i></b>	<b><i>Cardiovascular diseases</i></b>	<b><i>Neurological disorders</i></b>	<b><i>Others</i></b>
Common cold	Carditis	Headache	Lung cancer
Persistent cough	Vasculitis	Encephalitis	Non-Hodgkin lymphoma
Pharyngitis	Cardiomyopathy	Guillain-Barre syndrome	Szeczary's syndrome
Sinusitis	Hypertonia	Multiple sclerosis (MS)	Erythema nodosum
Otitis media	CHD	Alzheimer's disease	Reactive arthritis (ReA)
Pneumonia	AMI		Sweet's syndrome
Bronchitis	Stroke		Conjunctivitis
COPD	TIA		Iritis
Sarcoidosis	AAA		Uveitis
Asthma			
COPD, chronic obstructive pulmonary disease; CHD, coronary heart disease; AMI, acute myocardial infarction, TIA, transient ischaemic attack; AAA, abdominal aortic aneurysm (Saikku 2002)			

## **1.9. Diagnosis**

### **1.9.1. Culture**

Although culturing of the organism is the gold standard in chlamydial diagnosis, and *C. trachomatis* is relatively easy to culture in acute infections, the task of isolating and growing *C. pneumoniae* is more difficult. Isolation is best performed by cell culture, the most sensitive cell lines being HL and Hep-2 (Roblin *et al.* 1992). The sensitivity of cell culture in the diagnosis of acute *C. pneumoniae* respiratory infection is approximately 60% compared to serology, while specificity is close to 100%. However, isolation from the chronic stage is much more difficult. The probable reason for the difficulties of isolation in chronic stages is that deeper tissues are involved, such as lung interstitial macrophages, arterial wall macrophages and smooth muscle cells. These sites are not readily accessible by routine sample collection methods. Additionally, chlamydial titres are low due to poor growth in these cells. The lesions in chronic infections are also extensively affected by activated defence mechanisms. (Saikku 1999). Table 3.

### **1.9.2. Serology**

So far, serology has been the most frequently used method for diagnosing *C. pneumoniae* infections. The best serological evidence of acute infection is a four-fold rise in IgG or IgA antibody titre between paired sera taken several weeks apart. A positive IgM antibody titre is also

considered a marker of a current or recent infection. In primary infection, IgM antibodies are produced about 3 weeks after the onset of the illness, whereas IgG and IgA antibodies may not appear until 6–8 weeks after onset. In reinfection, on the other hand, IgM antibodies appear only at low titres, if at all. IgG and IgA titres rise quickly, within 1 or 2 weeks, and may reach very high levels. IgM titre usually begins to fall within 2 months and disappears within 4–6 months. IgA antibodies also have a short half-life, whereas IgG antibodies persist in the body and may be detectable for more than 3 years. Especially older patients, who have probably had multiple *C. pneumoniae* infections, may have persistently high IgG titres. (Kuo *et al.* 1995)

Serology is an inadequate indicator of chronic infection (Saikku 1999). It does not indicate the locality of the possible chronic process, and the high frequency of *C. pneumoniae* antibodies in people makes it difficult to prove an association with a specific disease. In spite of these problems, continuously elevated antibody titres have been considered a reliable marker of chronic infection (Saikku 1999). Persistent production of IgA antibodies, compared to long-lasting IgG antibodies, seems to be a better marker in chronic infections (Laurila *et al.* 1997).

The diagnosis is generally made with the microimmunofluorescence (MIF) test, which was developed in the early 1970s (Wang & Grayston

1970). When properly performed and read, this test is the most sensitive and specific method for diagnosing acute *C. pneumoniae* infections. The test measures antibodies against *C. pneumoniae* using EBs as antigen. It is able to measure separately antibodies in the IgA, IgM and IgG classes and is therefore suitable for distinguishing recent from past infections as well as primary from reinfections (Kuo *et al.* 1995). The antibodies may be measured not only from serum samples, but also from circulating immune complexes (IC) after precipitation and from sputum samples (Linnanmäki *et al.* 1993). ICs are complexes of microbial antigens and antibodies produced in defence against pathogens. Their consistent presence in the circulation is a sign of continuous production of microbial antigens and, thus, a potential marker of persistent infection. This is typical in many chronic viral and bacterial diseases (Saikku 1999).

Enzyme immunoassay (EIA) is also able to differentiate between the three antibody classes. EIA kits with LPS-extracted EBs or synthetic peptides unique to *C. pneumoniae* as antigen are commercially available. However, problems with sensitivity and specificity have been observed. If the absorbance threshold is raised to increase the specificity of the assay, sensitivity decreases, and *vice versa*. (Peeling and Mabey 1999)

The complement fixation (CF) test detects antibodies against chlamydial LPS. It is therefore unable to differentiate between the species.

Although lacking in specificity, the CF test is technically much less demanding than MIF and has objective endpoints. Another thing in favour of the CF test is that LPS antibodies are produced very early in primary infection. The sensitivity of the CF test in primary infection is about 60%. In reinfections, on the contrary, the CF test is not a suitable method: complement-fixing LPS antibodies are rarely detectable by the CF test, whose sensitivity is only 10%. ( Peeling and Mabey1999). Table 3.

### **1.9.3. Antigen detection**

Monoclonal antibodies specific for *C. pneumoniae* enable the detection of *C. pneumoniae* EBs in various samples. Their performance in direct fluorescent antibody (DFA) tests appears to be fairly comparable (Montalban *et al.* 1994). The sensitivity of DFA is 20 to 60% compared to culture or serology. It is somewhat higher for specimens from deep sites (Peeling and Mabey1999). EIA kits designed for *C. trachomatis* can be used for the detection of *C. pneumoniae* , because the capture antibody used in these kits is the genus-specific LPS (Peeling 1999). LPS antigens have also been detected by EIA from circulating ICs. The method is not easy and does not seem equally sensitive as antibody detection. In cases of chronic *C. trachomatis* infection, antigen detection has proved suitable compared to isolation, since antigen detection does not require the presence of viable organisms (Saikku 1999). Table 3.

#### **1.9.4. Polymerase chain reaction (PCR)**

The ability of the PCR technique to amplify small amounts of specific nucleic acid has made it an important and convenient diagnostic tool with a potential to detect *C. pneumoniae* rapidly and reliably. Several different targets (16S rDNA, MOMP, pmp4), primers and reaction protocols have been described for the detection of *C. pneumoniae* DNA. PCR detects as few as 10–100 EBs. Nested PCR with amplification in two steps utilising two different primer pairs may greatly enhance both sensitivity and specificity (Boman *et al.* 1997). Recently, a quantitative real-time PCR technique has also been developed for the detection of *C. pneumoniae* (Mygind *et al.* 2001). Suitable specimens include nasopharyngeal and throat swabs, bronchoalveolar lavage (BAL), sputum, gargled water, blood and tissue from biopsy or autopsy. As PCR can detect the presence of *C. pneumoniae* DNA from non-infectious RBs and non-viable EBs, PCR tests are expected to be more sensitive than culture methods. It has been estimated that PCR, in general, is at least 25% more sensitive than culture. Detection of cDNA by reverse transcriptase-PCR of mRNA may be a useful complement to cell culture in assessing whether the infection is active or productive (Khan *et al.* 1996). Guidelines have been developed to minimise the risk of false-positive as well as false-negative results. One important issue is the standardisation of protocols, in which the increased use of

automation and the introduction of commercial diagnostic kits are playing an important role. (Boman & Gaydos 1999). Table 3.

**Table 3. Laboratory methods for diagnosing *C. pneumoniae* infection**

	<b><i>Culture</i></b>	<b><i>Serology</i></b>	<b><i>Antigen detection</i></b>	<b><i>PCR</i></b>
<b>Detection</b>	Organism	Antibodies	Antigens	DNA
<b>Specimen</b>	NP/throat swab, BAL, sputum	Blood	NP/throat swab, BAL	NP/throat swab, BAL, sputum, gargled water, blood, tissue from biopsy/autopsy
<b>Sensitivity</b>	50–75%	60–80%	20–60%	10–100 organisms
<b>Specificity</b>	100%	90–100%	70–95%	95–100%
<b>Time frame</b>	3–12 days	1–2 days*	1 hour	1–2 days
<b>Specimen transport</b>	4°C/frozen	RT/4°C	RT	RT
<b>Interpretation of results</b>	Subjective	Subjective	Subjective	Objective
<b>NP, nasopharyngeal; BAL, bronchoalveolar lavage; RT, room temperature. *paired sera nearly always needed Peeling 1999)</b>				

## 1.10. Treatment

A number of different antibiotics have been tested in search for an appropriate treatment for *C. pneumoniae* infection. Azithromycin and clarithromycin are two macrolides which have shown high activity against the organism *in vitro* (Welsh *et al.* 1996). Some of the new fluoroquinolones and ketolides, a new class of macrolides, have also turned out effective (Miyashita *et al.* 2002). The organism is not susceptible *in vitro* to sulpha drugs, and penicillin and ampicillin prevent the growth of the organism, but do not destroy it (Kuo *et al.* 1995). Clinical experience has shown that the symptoms of *C. pneumoniae* infection frequently recur after short or conventional courses of appropriate antibiotics, and intensive long-term therapy is therefore highly recommended (Kuo *et al.* 1995). Inappropriate antibiotic treatment may lead to chronicity of the disease. The insidious nature of *C. pneumoniae* infection makes prevention very difficult, and the development of anti-chlamydial vaccines remains an important goal for researchers.

## **1.11. Objective of this study**

There was no report or article published on the study of chlamydia pneumoniae in developing countries. However, most of the articles came from Europe, USA and Japan. Therefore, these reports could not be considered representative of the prevalence of chlamydia pneumoniae in countries with different environmental changes, social and economical background such as Saudi Arabia. The present study will investigate the following:

1. The surveillance of chlamydia pneumoniae among patient with respiratory tract infections attending hospital.
2. Significance of serious complications related to chlamydia pneumoniae infection.
3. The response of cell to in-vitro chlamydia infection at the level of morphological changes and cytokine release.

*Chapter Two*

*MATERIALS  
AND  
METHODS*

## **2. MATERIALS AND METHODS**

### **2.1. FUNDAMENTALS**

#### **2.1. 1 Cell line**

Hep-2 cells was obtained from Virology laboratory , King Abdulaziz University hospital, Jeddah, Saudi Arabia. This cell line has been originally purchased from the American Type Culture Collection (ATCC CCL23), and maintained in Minimum Essential Medium (MEM) (GIBCO, BRL, Gaithersburg, MD) with 1% penicillin and streptomycin and 10% fetal bovine serum (FBS,GIBCO). Cell line were maintained in 75 cm<sup>3</sup> plastic flasks in standard conditions of 37° C and 5% CO<sub>2</sub> humidified atmosphere. The cells were always passed upon confluence with split of 1:4 weekly. Cells were trypsinized using Trypsin-EDTA (1X) [0.25% porcine tpsin (1:250)] (Biosciences, KS) solution, and were washed in culture medium.

#### **2.1.2 Interferon preparations:-**

Human recombinant IFN- $\alpha$ 2a (HurIFN- $\alpha$ 2a), or Roferon, is highly purified protein containing 165 amino acids. It has an approximate molecular weight of 19,000 daltons. It is produced by recombinant technology using genetically engineered *E. Coli* strain containing DNA that codes for this human protein. HurIFN- $\alpha$ 2a obtained from Hoffman-LaRoche, Switzzland, had a specific activity of  $2 \times 10^8$  IU/mg, as reported

by the manufacturer. A starting solution was made and calibrated with NIHGxa 01-901-535 IFN $\alpha$  reference preparation; the titer was  $10^9$  IU/ml.

Human rIFN- $\beta$ ; purified protein containing 165 amino acids and molecular weight 20,000 daltons. It is produced by recombinant technology using genetically engineered *E. Coli* strain containing DNA that codes for this human protein. Human rIFN- $\beta$  obtained from GIBCO, BRL, Gaithersburg, MD, had a specific activity of  $2 \times 10^8$  IU/mg, as reported by the manufacturer. A starting solution was made and calibrated with NIHGg 23-901-530 reference preparation; the titer was  $10^7$  IU/ml.

Human rIFN- $\gamma$ ; purified protein containing 143 amino acids and molecular weight 17,000 daltons. It is produced by recombinant technology using genetically engineered *E. Coli* strain containing DNA that codes for this human protein. Human rIFN- $\gamma$  obtained from GIBCO, BRL, Gaithersburg, MD, had a specific activity of  $1 \times 10^7$  IU/mg, as reported by the manufacturer. A starting solution was made and calibrated. The titer was  $10^6$  IU/ml.

### **2.1.3 Bacterial preparation and bacterial stock:**

*C. pneumonia* TW-183 was obtained from Virology laboratory, King Abudlaziz University hospital, Jeddah, Saudi Arabia. This strain was originally purchased from American type tissue culture collection (Rockville, Md.) and propagated for 3 days in Hep-2 cells.

***C. pneumoniae* propagation.** *C. pneumoniae* was inoculated onto confluent monolayers of HEp-2 cells, centrifuged at 3500 rpm for 60 min at 25°C, and then incubated at 37°C for 1 h. The inoculum was removed and replaced with growth medium consisting of minimal essential medium containing cycloheximide (1 µg/ml) and incubated for 72 h at 37°C and 5% CO<sub>2</sub>. *C. pneumoniae* was harvested by disruption of HEp-2 cells with glass beads followed by repeated freezing and thawing. Then centrifugation at 2500 rpm to remove cellular debris. Supernatants containing *C. pneumoniae* were centrifuged at 10,000 rpm for 30 min at 4°C to pellet *C. pneumoniae* elementary bodies (EBs). EB pellets were suspended in sucrose-phosphate-glutamate buffer, aliquoted, and stored at -70°C. *C. pneumoniae* titrations were performed on frozen stocks using immunofluorescent staining with a genus-specific fluorescein isothiocyanate-labeled monoclonal antibody (Kallestad, Chaska, Minn.). *C. pneumoniae* titers were expressed as inclusion-forming units per milliliter.

## **2.2 Chlamydia pneumoniae culture and PCR**

### **2.2.1. Patients:**

The studied population included patients attending King Abdulaziz University hospital outpatient pulmonary clinic with clinical feature of bronchitis, pharyngitis and pneumoniae. And those admitted to the same hospital with clinical evidence of pneumoniae. The age groups were ranged from 1 day upto 85 years old.

### **2.2.2. Clinical specimens:**

Nasopharyngeal aspirate and sputum were suspended into 1.5 ml of chlamydia transport media (CTM) (GIBCO, BRL, Gaithersburg, MD) and placed at  $-70^{\circ}\text{C}$  for 24 hours. A small amount of patient specimen was withdrawn for direct immunofluorescence stain as discussed below.

### **2.2.3. Laboratory assays for *C. pneumoniae***

Hep-2 cells should be cultured with MEM in a shell vial containing glass coverslips one day prior to specimens inoculation. The cells were incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  humidified atmosphere. After 24 hours, the medium were removed and the Hep-2 cells were treated for 30 min at  $37^{\circ}\text{C}$  with 1 ml of Dextran (DEAE-D) solution (Sigma chemical Co. St. Louis, Mo.), the action of Dextran is shown in Figure 2. At the same time, patient specimens were thawed, vortexed gently and briefly. 0.5 ml of specimen

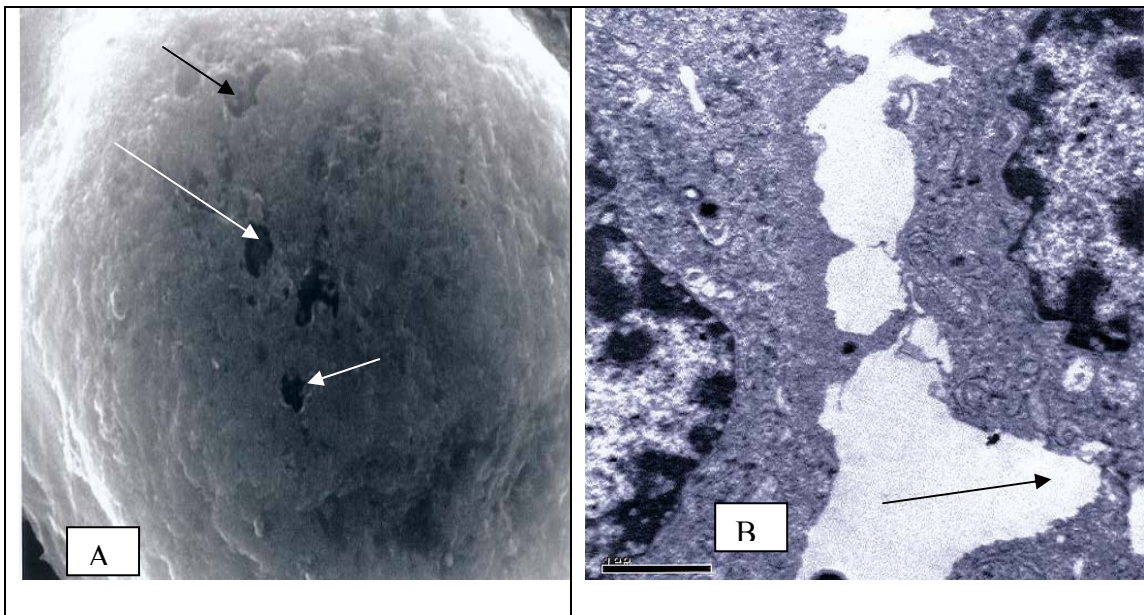


Figure 2 : The action of Dextran on cytoplasmic membrane of Hep-2 cells. Artificial pores created by addition of dextran to facilitate the entrance of chlamydia pneumoniae elementary bodies. A : Scanning Electron Microscope (SEM), B : Transmission Electron Microscope (TEM).

was mixed with 5 ml of CTM, filtered through 0.8 um millipore . Filtered specimen was added to the shell vial (after discarding the dextran) and incubated at 37 C in 5% CO<sub>2</sub> atmosphere for 2-3 days.

By the end of incubation period shell vilas were centrifuged for one hour at 3500 rpm followed by incubation for three days in CTM with daily change. At the end of the third day CTM were discarded and the cell fixed in 95% ethanol (Merck, Darmstadt, Germany) for 30 min at room temperature. The fixed cells were rinsed once with phosphate bufferd saline then the coverslip was removed from the shell vial and stained with a fluorescein conjugated species-specific antibody (DAKO, Hamburg, Germany) which is specific for *C. pneumoniae*. The staining took 30 min then the coverslip washed in phophate buffered salaine and ethanol and examined by flouresence microscope.

#### **2.2.4. PCR (Polymerase chain Reaction)**

DNA isolation from patient samples (sputum or NPA) was performed by using Qiagen method (QIAmp DNA mini kit, QIAGEN inc. USA). According to the manufacturer, in 1.5 ml microcentrifuge tube, 20 µl of Qiagen protease, 200 ul of patient sample were mixed in 200 µl PBS (phosphate buffer saline). 200 µl buffer AL were added, vortexed for 15 min and incubated at 56 C for 10 min. To the mixture, 200 ul of 96% ethanol were added, vortexed then centrifuged. Carfully, the mixture applied to the QIAamp spin column, cap of column was closed and the column centrifuged

at 8000 rpm for 1 min. QIAamp spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate (sediment) was discarded. Carefully the QIAamp spin column was opened and 500 ul of buffer AW1 was added, cap of the column closed and centrifuge at 8000 rpm for 1 min. Again, QIAamp spin column was placed in a clean 2 ml collection tube and the tube containing the filtrate (sediment) was discarded. Repeat the last step but by using AW2 buffer and centrifugation at 14000 rpm for 3 min. Finally, the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. The QIAamp spin column was opened carefully and 200 ul of eluting buffer AE was added, incubate at room temperature for 1 min and centrifuge at 8000 rpm for 1 min. By this step we can have our DNA in the elution buffer.

The nucleotide sequences of the one set primer (from the 5' to 3' end) HL-1 (5'-GTTGTTTCATGAAGGCCTACT-3') and reverse primer HR-1 (5'-TGCATAACCTACGGTGTGTT-3') was used. DNA were amplified for 40 cycles. Each cycle consisted of the following: denaturation at 94 C for 1 min, annealing at 55 C for 1 min, and primer extension at 72 C for 1 min. The buffers and reagents used were those supplied in the GeneAmp Kit (Perkin-Elmer Cetus, Norwalk, Conn). Amplification products were analyzed by electrophoresis through a 1.5% agarose gel.

## **2.3. Electron microscopy:**

### **2.3.1. Plan for study the life cycle of *C. pneumoniae***

By using electron microscopy we aimed to have an idea about the infectivity and life cycle as the main process of pathogenicity. Hep-2 cells were infected by *C. pneumoniae* without using Dextran, centrifuged and were processed at time intervals of 6 (A), 12 (B), 18 (C), 24 (D), 36 (E), 48 (F), 60 (G), 72 (H), and 84 (I) h postinfection for both scanning and transmission electron microscopy.

### **2.3.2. Scanning Electron Microscopy (SEM) procedure:**

Infected cells were washed with sodium phosphate buffer PH.7.4, prefixed in 3% glutaraldehyde (Sigma) for 3 hrs. and washed twice by sodium phosphate buffer.

After prefixation, the coverglass was carefully taken out from the shell vial making sure the upside of coverglass contain the infected cells sample. Coverglass were then transferred to glass petridish containing the sodium phosphate buffer for 10 min, transferred to another petridish which contain 1% of osmic acid (Sigma), kept covered for 1 hr, and processed as follows:

Wash with phosphate buffer for 10 min and transferred to increasing concentration of alcohol (Fisherscient, Fisherchemical, CO.):

1. 50% of alcohol for 10 min
2. 60% of alcohol for 10 min

3. 70% of alcohol for 10 min
4. 80% of alcohol for 10 min
5. 90% of alcohol for 10 min
6. 100% of alcohol for 10 min and carefully put into petri dish containing HMDs solution for 1 hrs.

Stub preparation: very carefully the coverglasss were taken from the petridish with fine forceps and kept for few minutes on filter paper, making sure the side of coverglass with cells is facing upward to dry.

Copper stub with 8 mm diameter used for SEM, with the help of sticky (tabs) adhesive (two sided) thumb sticker were placed first on the copper stub and then immediately the coverglass with cells facing toward upside kept on copper stub so it stick to copper stub properly.

This stub was placed into the vaccum jar to dry, next day stub with infected cells ( coverglass) subjected to carbon coating for 30 second using (TB-500 emscope , carbon coater), coated by gold-cadmium for 3 minutes (SC500 emscope , gold coater) and kept carefully under vaccium. This stub is studied by the JOEL SEM (JOEL JSM-551, JAPAN).

### **2.3.3. Transmission Electron Microscopy (TEM) procedure:**

Infected cells were collected in 3% of glutaradehyde and washed in sodium phosphate buffer PH 7.4 in special small conical tube. Prefixation was done in 3% glutaraldehyde prepared freshly in sodium phosphate buffer for 3 hrs. The tube was then centrifuged at 2000 rpm for 3 min and wash in

sodium phosphate buffer twice, fixed in 1% osmium tetroxide (osmotic acid) (Sigma) for 1 hour, and washed with sodium phosphate buffer twice. Dehydration was done by series of increasing alcohol concentration 50%, 60%, 70%, 80%, 90%, 100%, and propylene oxide (Sigma) for 10 min while shaking to prevent the sedimentation of infected cells and transferred to special conical bean capsules, made for TEM processing. The bean capsules containing the cells and propylene oxide were covered with their lid, fixed in the foam box and centrifuged at 1000 rpm for 10 min. Propylene oxide was discarded carefully without disturbing cells.

Thereafter, bean capsules were filled with mixture of 1:1 (propyleneoxide : araldite (Sigma) mixture), shaken well, covered, left for an hour at room temperature and centrifuged at 1000 rpm for 10 min. The process was repeated with 1:2 and 1:3 mixture of propyleneoxide : araldite while the incubation time was extended to 2 hours after which propyleneoxide was evaporated.

Finally bean capsules were centrifuged at 3000 rpm, liquid was discarded and pure resin was added. Bean capsules were incubated overnight at 35 C, followed by two days incubation at 78 C and araldite blocks were removed from bean capsules.

Blocks were trimmed with one sided blade superficially to take out upper resin, to make the face of block cutting neatly. With diamond knife (diamond, Switzerland) sections of 0.4  $\mu\text{m}$  in thickness were obtained,

stained with toluidine blue (Sigma) and studied by light microscopy to confirm the presence of cells. Thickness was reduced to 80 nm and white ribbon of sections were collected on 300 mesh of copper grids, stained by heavy metal uranyl acetate (Sigma) for 15 min, washed with double distilled deionized water then treated for 10 min in lead citrate, washed in double distilled deionized water and examined using transmission electron microscopy equipped with digital camera and printer, TEM 1010 JEOL (Tokyo, Japan)

#### **2.4 Antichlamydial assay:-**

This assay was used to determine which is the most potent interferon (IFN) against *C. pneumoniae*. Therefore, Hep-2 cells were seeded at  $3 \times 10^4$  cells/0.1 ml into each well of 96 well-microtiter plate (Costar, USA) containing MEM culture medium with 10% FBS and incubated for 24 hours to reach confluence, supernatants were removed and a series of dilution (1 IU/ml – 0.3 IU/ml) of IFN- $\alpha$ 2a, IFN- $\beta$  and IFN- $\gamma$  were added (0.1 ml) to each well in triplicates. Chlamydia and Hep-2 cell were included as controls, positive control consist of Hep-2 cells with chlamydiae while negative control consists of Hep-2 cell with medium (each was in 4 wells replicate). After 16-18 hours, supernatants were removed.

*C. pneumoniae* TW-183 was passed in Hep-2 cells monolayers, titrated, and stored at  $-80^\circ\text{C}$  as stock and used as inoculum. The stock chlamydial suspension was diluted in phosphate buffered saline and a 100- $\mu$ l

aliquot ( $1.7 \times 10^5$  cells per ml) was added to each well containing Hep-2 monolayer and IFN, plates were centrifuged at 1000 rpm for 1 hr at 37 C, incubation for 30 min at 37 C in 5% CO<sub>2</sub>. Liquid was aspirated and replaced with fresh medium and plates were incubated for 72 h at 37 C in 5%CO<sub>2</sub> to induce chlamydia cytopathic effect.

#### ***2.4.1. Crystal violet stain Assay for cytopathic effect:***

The plates were fixed with 5% formalin and 0.9% saline solution, stained with 0.5% crystal violet (Fisher, Inc., Springfield, NJ) solution, washed repeatedly with water, and air dried. The dye was eluted with ethylene glycol monomethyl ether (Fisher, Inc.), and the optical density (OD) was read at 550 nm in an enzyme-linked immunosorbent assay plate reader.

The OD was correlated with the degree of protection from chlamydia -induced cytopathic effect as observed microscopically. Percent of cell protection was calculated as follows:

$$1 - ([\text{dilution OD} - \text{chlamydia control OD}] / [\text{cell control OD} - \text{chlamydia control OD}]) \times 100$$

where OD is the average of optical density of triplicate wells at the concentration specified. Percent cell protection were plotted against serial concentrations of the IFN preparation ( Khabar et al 1996).

## **2.5. Microarray-Genisphere Method**

DNA microarrays allow for rapid measurement and visualisation of differential expression between genes at the whole genome scale (read more about microarray technology at appendix) . The experiment was performed according to the manufacturer procedure (Microarray-Genisphere Method)

### **2.5.1 Cell culture, treatment and sample preparation for Microarray:**

**Cell culture:** HEp-2 cells were grown in 75-cm<sup>3</sup> culture flasks with minimal essential medium (Gibco BRL, Gaithersburg, Md.) containing Earle's salts supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) and 2 mM L-glutamine at 37 C in 5% CO<sub>2</sub> for three days, subcultured into 25-cm<sup>3</sup> flasks (for cDNA array experiments) and allowed to adhere for 24 h at 37 C. The experiment consist of four sets control, treated with IFN, infected with chlamydia, treated+infected. All sets were done under the same condition.

**IFN Treatment:** MEM plus 10 IU/ml of IFN- $\gamma$  were added to monolayer Hep-2 cells, and incubated for 18 hr at 37 C in 5% CO<sub>2</sub>.

**Hep-2 infection protocol.** Hep-2 cells were infected with *C. pneumoniae*. With aid of centrifugation at 4000 rpm and incubation at 37°C in 5% CO<sub>2</sub> for 1 h, attached chlamydia were wiped off and cells were wash twice with phosphate buffer saline and MEM medium containing 0.1% FBS

(but lacking growth supplements) in the presence of cycloheximide. Host cell RNA was isolated after 2 hours of incubation at 37 C in 5% CO<sub>2</sub>.

**Total RNA extraction ( guanidine isothiocyanate method ) :**

Total cellular RNA was extracted from the freshly prepared cells by using Tri Reagent (Trizol) (Molecular Research Center, Cincinnati, OH). A volume of 2 ml Trizol was used of 25 cm<sup>3</sup> flask of confluent growth. Cells were detached by Trizol and homogenized by repeated pipetting. Homogenates were transferred to 1.5 ml sterile Eppendorff tubes, 0.2 ml of chloroform (AnalaR, BDH laboratory supplies, England) was added and the suspension was mixed vigorously. The homogenates were centrifuged at 10800 rpm for 15 minutes at 4° C, and the upper aqueous phase was removed. An equal volume of isopropanol (Fisherscient, Fisherchemical, CO.) was added to precipitate the RNA overnight. The RNA was collected by centrifugation at 10800 rpm for 10 minutes at 4° C and washed with 1 ml of 75% ethanol, centrifuged at 10, 000 rpm for 10 minutes at 4° C, RNA pellets were dried, suspended in sterile RNase-free water, incubated at 55-60° C for 10 minutes, and the RNA was quantified by UV absorbance at 260 and 280 and stored at - 20° C until use.

### **2.5.2 Agarose Gel Electrophoresis for RNA:**

Ten  $\mu$ l of total RNA were suspended in 24  $\mu$ l loading buffer (Sigma) plus RNA free water to a final volume of 30  $\mu$ l. The RNA solution was incubated at 65° C for 10 minutes, cooled on ice and setteled by spinning at 14000 rpm for one minute. The RNA solution was dispensed onto each lane of 1.2% (w/v) agarose gel that contained 6% (2.2 M) formaldehyde and 10 X MOPS. Electrophoresis was performed in 1 X MOPS buffer and was run at approximately 60-75 mV for four hours. The gel was rinsed with sterile distilled water and was photographed under UV light to visualize the banding pattern of the 18 S and 28 S ribosomal RNA and its relative migration as indicators for the purity of the RNA preparation.

### **2.5.3. Genisphere 3DNA indirect labeling of cDNA targets**

In our experiment we divided our culture sets as follows:

Groups (RNA)	Cy3	Cy5
1	Hep-2	Hep-2 + <i>C. pneumoniae</i>
2	Hep-2 + <i>C. pneumoniae</i>	Infected Hep-2 + IFN- $\gamma$

### **cDNA synthesis:**

1. Twenty microliters of total RNA , 5 ul of either Cy3 or Cy5 RT primer and nuclease free water were mixed to a final volume of 10 ul (called RNA-RT primer mix), incubated at 80 °C for 10 min , chilled on ice and 1 ul Rnase inhibitor was added.
2. In a separate iced microtube , a reaction mix of 5X superscript II first strand buffer, dNTP mix (10mM each for dATP, dCTP, dGTP, dTTP) and RT enzyme in a total of volume 10 ul was added to RNA-RT primer mix to final volume of 20 ul, incubate at 42 C for 2 hrs. Reaction was terminated by adding 3.5 ul of 0.5M NaOH in 50 mM EDTA and the mix were incubated at 65 C for 10 min, to denature the DNA/RNA hybrids. Neutralize the reaction with 5 ul of 1 M Tris-HCl, pH 7.5 and combine each group (cy3 and cy5) in one tube.

**Ethanol precipitation of the cDNA:** Three microliters of acrylamide were added to the cDNA mix. 6 ul of 5M NaCl , 540 ul of 100% ethanol, incubated at – 20 C for 30 min, centrifuged at 10800 rpm for 15 min at 4 C and supernatant was discarded. To the precipitant 300 ul of 70% ethanol was added, gently mix, centrifuged at 10800 rpm for 5 min, supernatent was discarded and left at 65 C for 10-30 mins for dryness.

**cDNA Hybridization** : cDNA, were suspended in nuclease free water to a total volume of 10 ul, heated for 10-15 min at 65 C. To a hybridization mixture (17.5 ul of 2 x hybridization buffer, 2 ul of Array 50 dT blocker, 4.5 ul Nuclease free water and 1ul of COT-1 DNA) 10 ul of concentrated cDNA were added. The mixture was vortexed, centrifuged, incubated at 75 C for 10 min, left at 45 C for 15 min and loaded to prewarmed microarray slides (which contain all the genes for cytokine, see table at the end of this section), covered with cleaned coverslip and left overnight in dark humidified chamber at 45 C for hybridization.

**Post cDNA Hybridization Wash:** after hybridization the slides are washed briefly several times to remove unbound cDNA, washed for 10 min sequentially in 2X SSC in buffer in 0.2% SDS pH 7.5 at 55 C , 2X SSC buffer pH 7.5 and 0.2X SSC buffer pH 7.5, 2 minute at room temperature in 95% ethanol, to fix cDNA molecule to the probes. Slides immediately transfer to a dry 50 ml centrifuge tube and centrifuge for 2 min at 1000 rpm.

**3DNA Hybridization:** Another hybridization mixture were made as follows:

<b>3 DNA Hybridization mixture</b>	<b>Quantity (ul)</b>
2X Hybridization Buffer + Anti-Fade	17.5
3DNA capture Reagent (Cy3)	2.5
3DNA capture Reagent (Cy5)	2.5
Nuclease free water	12.5

Hybridization Mixture were incubated first at 75 C for 10 min and left at 45C for 15 min. Microarray slides were pre-warmed to 55 C for 10 min, hybridization mix were added to the prewarmed microarray slides, slides were covered with clean coverslip and incubated 2 hrs in a dark, humidified chamber at 45 C. Finally, post 3DNA hybridization wash was performed as follows: The slides washed for 10 min sequentially in 2X SSC buffer in 0.2% SDS buffer pH 7.5 (65 C) , 2X SSC buffer pH 7.5 and 0.2X SSC buffer pH 7.5. Immediately the arrays were transferred to a dry 50 ml centrifuge tube, centrifuged for 10 min at 1000 rpm to dry, then immediately transferred to a light-protected slide box and scanned on a GenPix 4000A scanner.

*Chapter Three*  
*RESULTS*

## ***3. Results***

### **3.1. Age and Sex distribution**

#### ***3.1.1. Chlamydia pneumoniae Culture***

Five hundred seventy two respiratory specimens were obtained over a period of 12 months (Table 4) from patients diagnosed with different respiratory diseases. Specimens were immediately processed for isolation and identification of *C. pneumoniae*.

Sixty-eight respiratory specimens from sixty-eight patients were found to be positive for *C. pneumoniae*. The demographic and epidemiological characteristics of the patients are summarized in (Table 5). It was noticed that the distribution of infection among all groups (in decreasing order) for elderly above 40 years (36%) , infants (26%) , preschool children (24%) , in young aged 4.1-10 years (8%) and adults aged 20 to 40 years (6%). There was no isolation of *C. pneumoniae* in age 10.1-20 (0%) , (Figure 3). The infection was more common in male (65%) than in female (35%) ,(Fig 4).

The infection occurring all year-round, but was most common in November (38%), less in January (15%) and August (12%) and least common in other months, (Table 6 & Figure 5).

More than half of infected patients (63%) presented with pneumonia, and the rest (37%) with upper respiratory tract infection, Table 7.

### ***3.1.2. Mixed C. pneumoniae With Other Microorganisms:***

Among all of our cases, *C pneumoniae* was identified as the etiologic agent in 68 cases. Of these, *C pneumoniae* was the only pathogen identified in 46 cases (67.6%), and a co-pathogen was diagnosed in 22 cases (32.4%). Table 8 shows the distribution of microorganisms among the 22 cases of mixed *C pneumoniae*. The most common additional pathogens were *S pneumoniae* (9 patients), followed by *M pneumoniae* (4 patients), *Haemophilus influenzae* (4 patients) and influenza A virus (2 patients).

Table 5. Age and sex distribution of 68 patients with *C. pneumoniae* infection:

---

Variable	Frequency (%)
Age Groups:	
0.01-1 year	18 (26%)
1.1-4 year	16 (24%)
4.1-10 year	6 (8%)
10.1-20 year	0 (0%)
20.1-40 year	4 (6%)
40.1-60 year	16 (24%)
60.1-85 year	8 (12%)
Gender:	
Male	44 (65%)
Female	24 (35%)

---

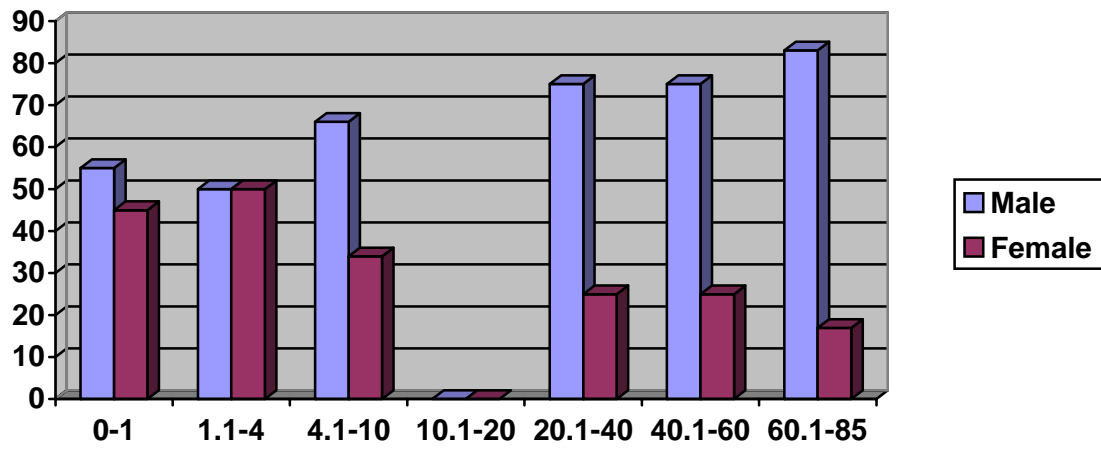


Fig 3: Sex and age distribution of patient with *chlamydia pneumoniae*.

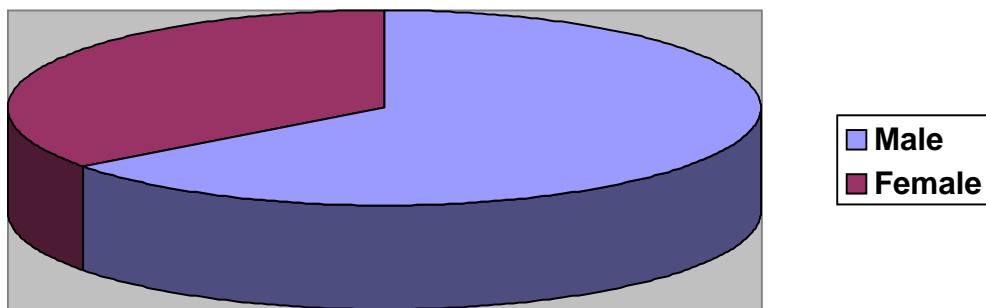


Fig 4: The prevalence of *chlamydia pneumoniae* in both sexes, Male 65% and female 35%.

**Table 6: Monthly variation of *C. pneumoniae* isolation in 68 patients with *C. pneumoniae***

---

Month	Frequency (%)
January	10 (15%)
February	2 (3%)
March	2 (3%)
April	2 (3%)
May	4 (6%)
June	0 (0%)
July	2 (3%)
August	8 (12%)
September	4 (6%)
October	2 (3%)
November	26 (38%)
December	6 (8%)

---

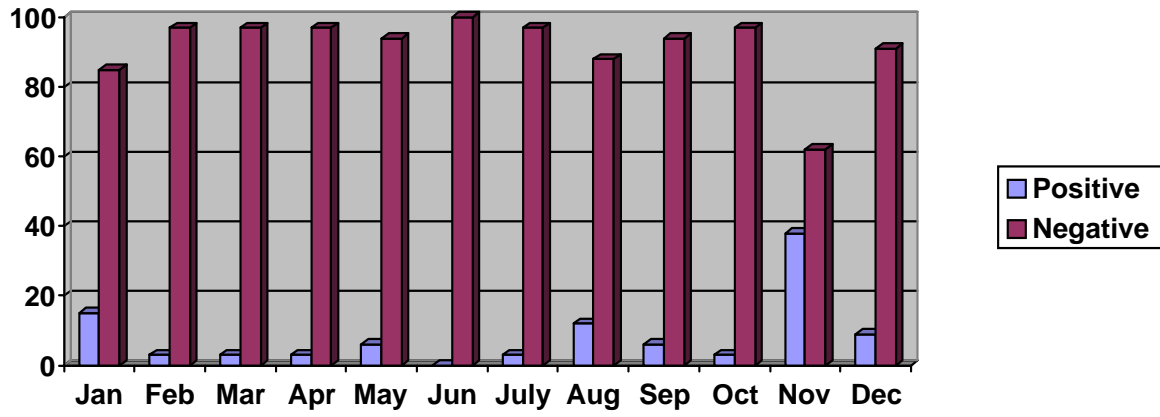


Fig 5: The distribution of positive and negative results of *C. pneumoniae* in different months.

Table 7: Clinical manifestation of culture proven *C. pneumoniae* infection in 68 patients (as recorded by Doctor).

---

Type of infection	Frequency
Pneumoniae	43(63%)
Upper respiratory tract infection	23 (33%)
Bronchitis	2 (4%)

---

Table 8. Frequency Distribution of Additional microorganisms in 68 Patients Infected With *C. pneumoniae*

---

Pathogen	No. (%)
<i>S. pneumoniae</i>	9 (13.2)
<i>M. pneumoniae</i>	6 (8.8)
<i>H. influenzae</i>	4 (5.8)
Influenza A virus	2 (2.9)
<i>Staphylococcus aureus</i>	1 (1.5)
Single agent*	46 (67.6)

---

\**C pneumoniae* was the only pathogen identified.

### ***3.1.3. Polymerase Chain Reaction (PCR)***

The PCR was carried out according to (Campbell *et al*1992) using *C. pneumoniae* species-specific primer set, this primer set amplifies a 437-bp fragment (Fig 6) and consists of the following primer: forward primer HL-1 (5'-GTTGTTCATGAAGGCCTACT-3') and reverse primer HR-1 (5'-TGCATAACCTACGGTGTGTT-3'). The thermal cycling conditions used were described by Campbell *et al* 1992. The PCR products were visualized in 1.5% agarose gel with added ethidium-bromide on an Ultraviolet transilluminator after electrophoresis.

Nasopharyngeal aspirate and other clinical specimens were tested from patient that were culture positive for *C. pneumoniae*. Positive control and negative control were run in parallel with patient samples in each set. Sixty-eight positive samples by culture yielded sixty-eight positive samples by PCR. There was no difference between culture and PCR. The run were repeated twice for confirmation , Fig 7.

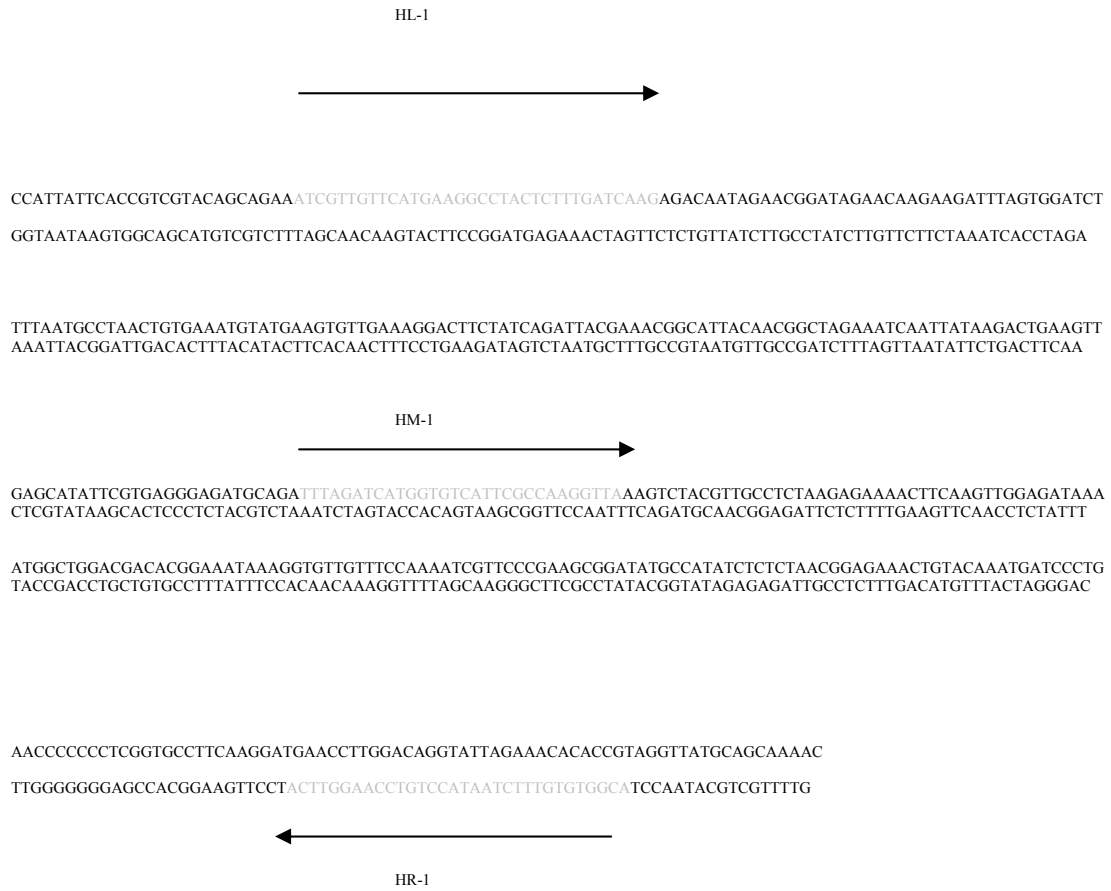


Fig 6: DNA sequence of *C. pneumoniae*-specific 437 bp PstI restriction fragment. Primers are illustrated by dashed lines, with arrows indicating direction of polymerization.

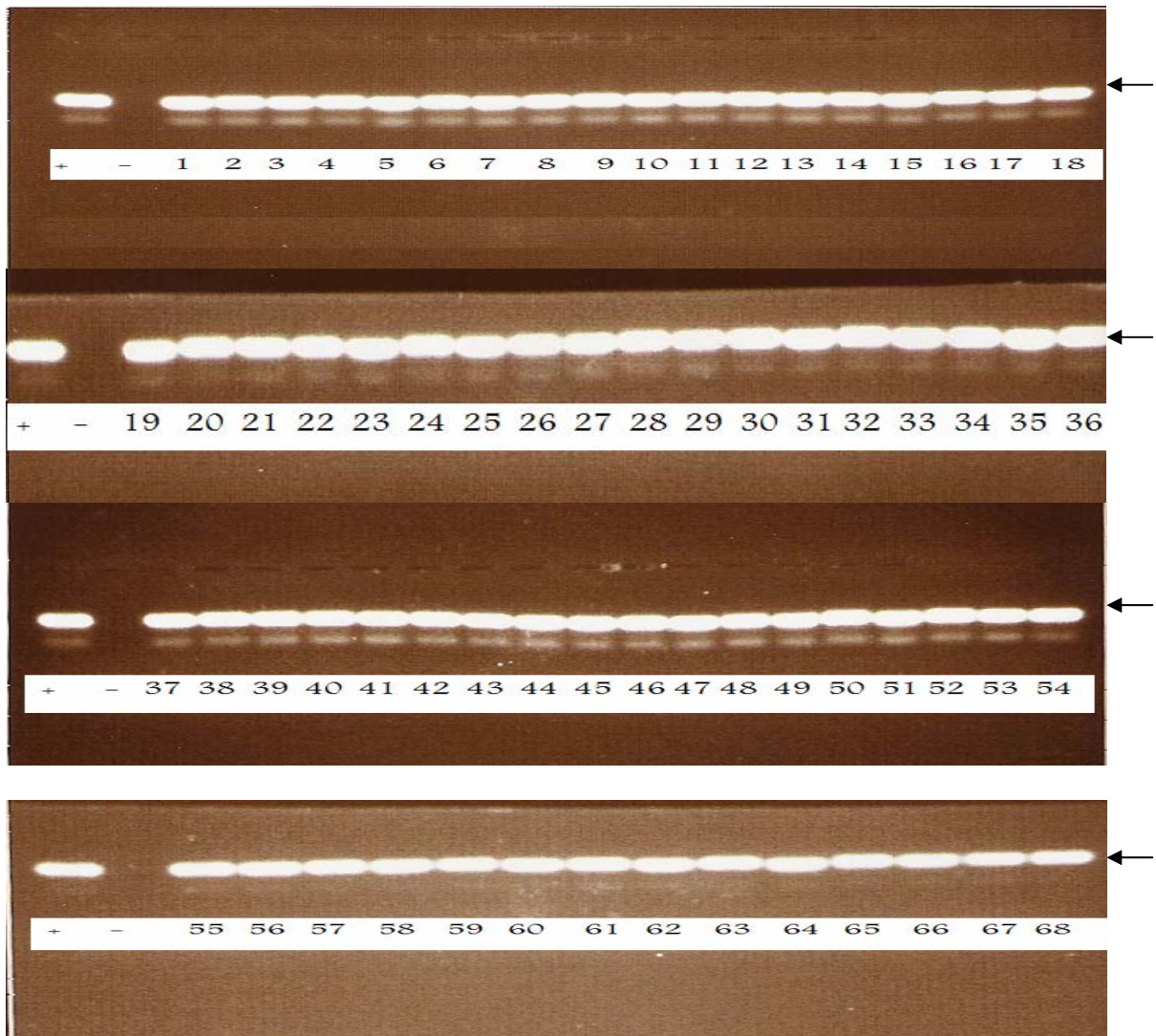


Fig 7: Amplification of *C. pneumoniae* DNA from clinical specimens. Samples were concentrated by centrifugation and amplified by using HL-1-HR-1 primer set. The product amplify and presented by gel electrophoresis . Positive and negative control were run together with clinical samples that were positive by culture (1-68). Arrows indicate 437-bp.

## **3.2.Ultrastructural study by Electron microscopy:**

Confluent monolayer of Hep-2 cells on glass coverslip in a shell vial were infected by *chlamydia pneumoniae*, washed, fixed with 3% glutaraldehyde, and processed for both scanning (SEM) and transmission (TEM) electron microscopy at time intervals of 6 (A), 12 (B), 18 (C), 24 (D), 36 (E), 48 (F), 60 (G), 72 (H), and 84 (I) hours postinfection. Normal non-infected cell were processed in the same way and was used as a reference.

To understand the process of infection its important to describe both the normal shape and morphology of Hep-2 cell and the infective agents, elementary bodies of *C. pneumoniae* as seen by both TEM and SEM electron microscope.

### **3.2.1 Morphology of Hep2 cells:**

SEM microscope showed round shaped cells with a smooth surface. Some foldings were seen on the edge which are believed to be due to the fluidity of the cytoplasmic membrane. The TEM micrograph revealed smooth membrane and typical eukaryotic undisturbed organelles (Fig 8).

### **3.2.2 Morphology of *C. pneumoniae* elementary bodies and reticulate bodies:**

The elementary bodies revealed by TEM microscope strongly support the pear shaped morphology, simply because of different length of axis of the sections obtained or at least it is not round shaped. The size of EB

measured 0.38  $\mu\text{m}$  in diameter with long axis attending 0.44  $\mu\text{m}$  and short axis 0.31  $\mu\text{m}$  and the long-to-short axis ratio was 1.42 (Fig 9 A).

Pleomorphism of EBs may be a result of an intermediate shape that is characterized by clear preplasmic space and less dense colour (Fig 9 A)

The morphology of *C. pneumoniae* reticulate bodies (RBs) was round. The diameter of RBs in the inclusion was  $0.51 \pm 0.15 \mu\text{m}$  in diameter the long axis was 0.56  $\mu\text{m}$ , the short axis was 0.47  $\mu\text{m}$ ; and the ratio of the two axes was 1.19  $\mu\text{m}$ . unlike EBs, the outline of the cytoplasm was inconformity with the outer membrane. There was a narrow but conspicuous periplasmic space (Fig 9 B).

### **3.2.3 The process of *C. pneumoniae* infection as illustrated by Electron microscopy:**

The infection of confluent monolayer of Hep-2 with *C. pneumoniae* is characterized by several stages that led to destruction of Hep-2 cells.

The response of the host cells to the infective agents started by ruffling of the host cytoplasmic membrane, forming distinguished microvilli which in turn provide an attachment or receptors for EBs. The process occur within the first 6 h, (Fig 10).

In further development , EBs rolled over to nest within the microvilli and to come into contact with the cell surface, this stage of nesting should be followed by an engulfment process providing netch for the EBs to develop into RBs. However, the conversion of EBs to RBs seems to be a

consequence of environmental modification of the host cytoplasm, initiated by secretion of Type III secretion protein (tts) into the cytoplasm in favour of the engulfed EBs to emigrate into the cytoplasm, (Fig 11).

In the cytoplasm of the host cell the RBs continue to mature inside the inclusion bodies and some emigrate toward the nucleus membrane which invaginate easing the penetration of inclusion bodies that contain RBs into the nucleus and leaving a damaged nucleus membrane at the site of inclusion bodies entry, (Fig 12). At this stage numerous EBs freed into the cytoplasm and some mature inclusion bodies emigrate toward the cytoplasmic membrane, (Fig 13), forcing it to form a projecting like structure that releases the mature EBs toward the neighboring cells (Fig 14). The releasing of EBs occurs in volcanic manner to reach a distance from the site of infections (Fig 15)

The above mentioned process has been considered a successful and destructive type of infection, as the result has always been the disintegration of infected cell.

It is believed that there are other forms of infection which are characterized by becoming praises or commender, and did not lead to the destruction of the cell, at least during our experimental investigation.

The sequence of the above mentioned infection starts with seeking a point of attachment nesting in between the microvilli, but the engulfment of the EBs happens in the absence of any sign to the type III secretion in the

cytoplasm of the host cell which we think is a very critical step in the modification of the host cell for successful invasion (Fig 16).

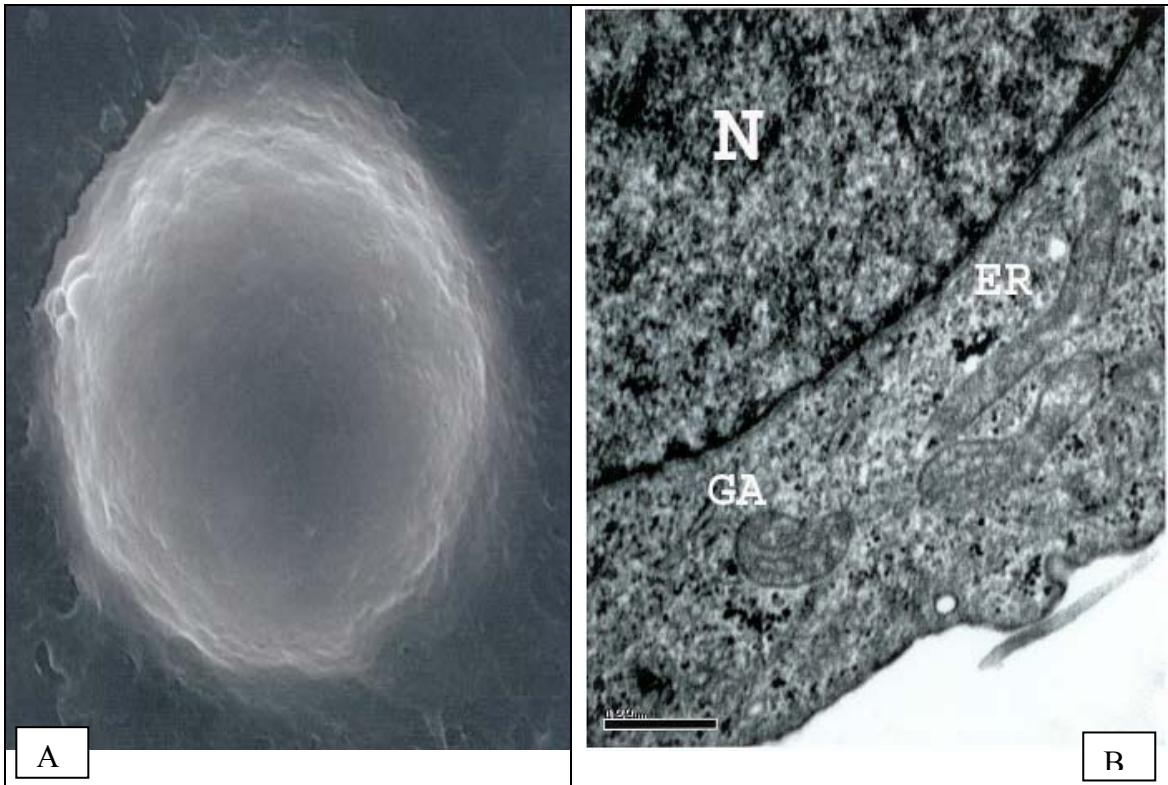
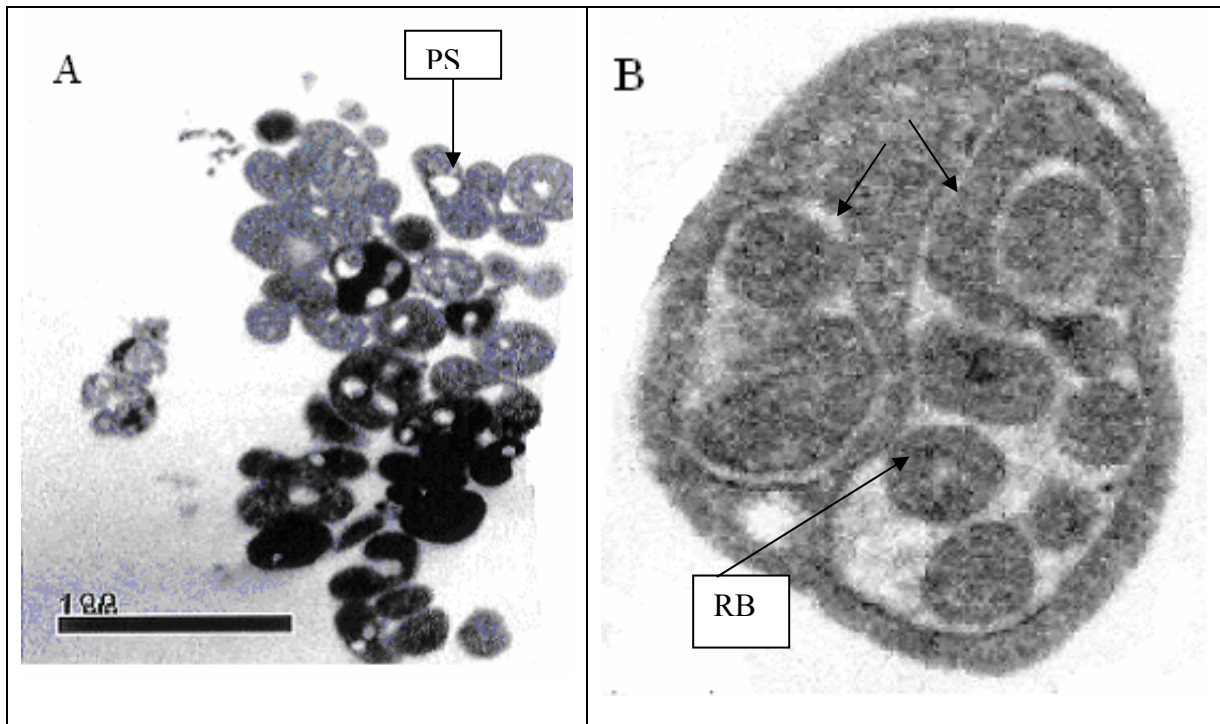


Fig 8: **Morphology of Hep-2 Cell.** Normal cell was seen by SEM (A) and TEM (B). SEM shows smooth surface with folded edge as consequence of fluidity of cytoplasmic membrane. TEM shows a contact cytoplasmic membrane with intact cell organelles and smooth nuclear membrane. (N= nucleus, GA= golgi apparatus, ER= endoplasmic reticulum.).



**Fig 9: Morphology of Elementary bodies and Reticulate bodies.** TEM shows the Pear shape morphology of Elementary bodies (EBs) which appear in dense color with a clear periplasmic space (PS) other form of EBs as a result of an intermediate shape that appears as a clear periplasmic space and less dense color (A), while picture B, showing mature inclusion bodies in which the reticulate bodies (RBs) are inside which is characterized by round shape and narrowing periplasmic space, also it shows bigger size than EBs.

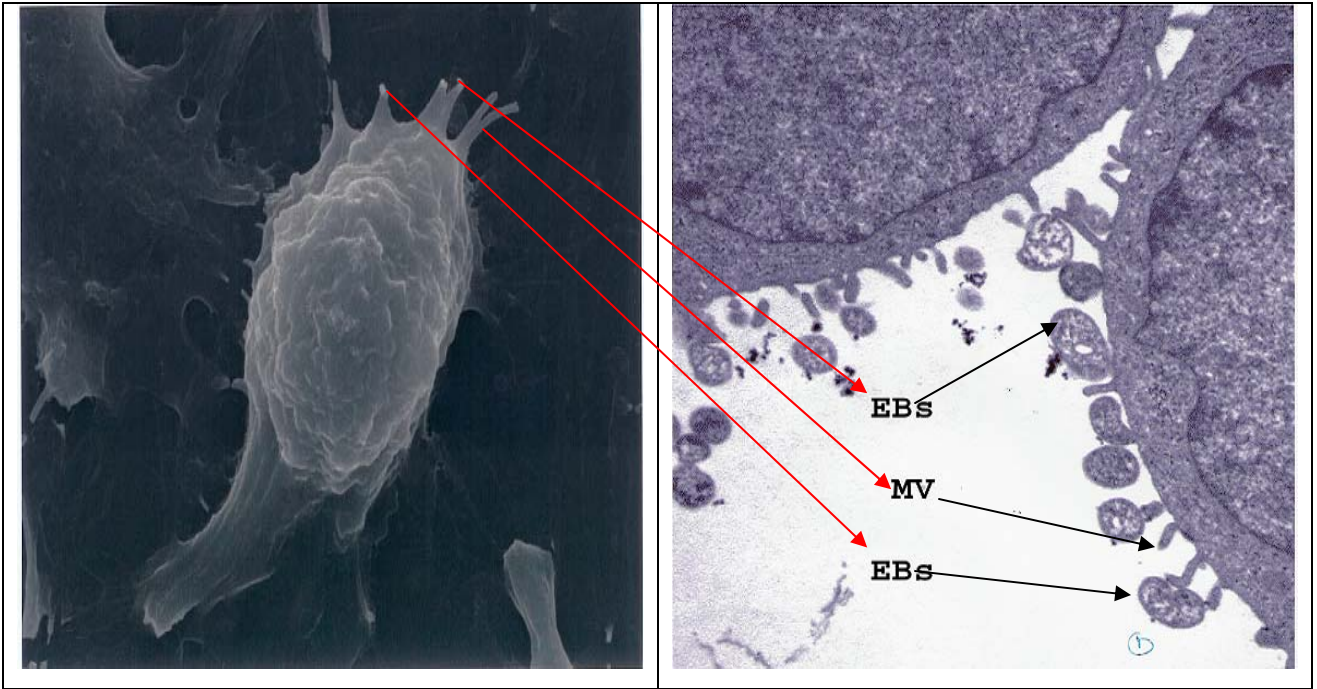


Fig 10: **The start of infection (Ruffling).** Hep2 cells showing ruffling of cytoplasmic membrane and the formation of microvilli. Chlamydiae pneumoniae elementary bodies (EB) seeking entrance of the microvilli . TEM (right) showing the process of seeking attachment. MV =microvilli.

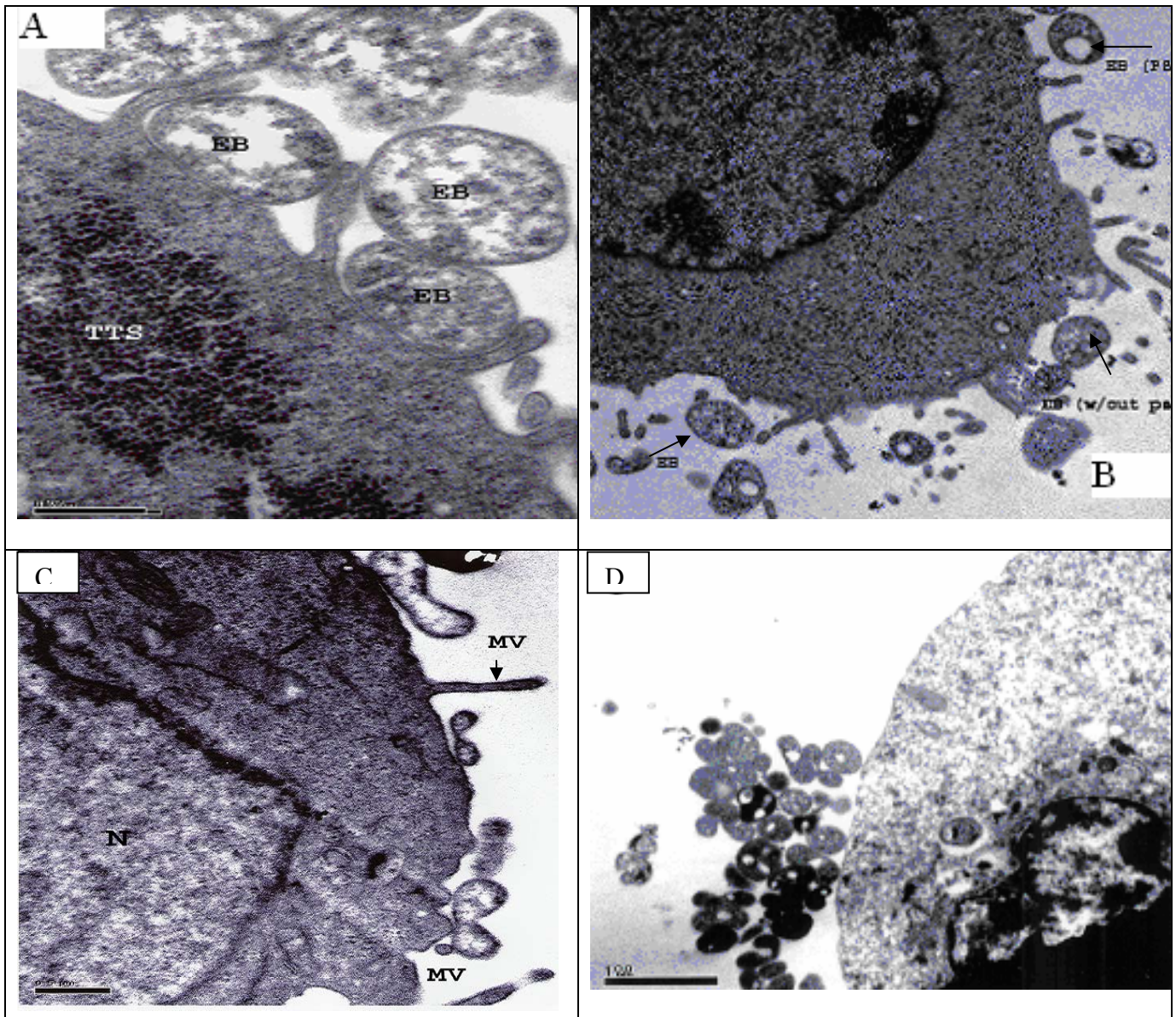


Fig 11: **The stage of Nesting.** The EB s loose the thick wall and the dark dense appearance and converted to an acceptable form by the microvilli for provision of nesting place. During this phase Type three secretion (TTS) appear in the cytoplasm (A). Picture (B) shows that the transformation of elementary bodies before attachment is necessary for the acceptance by microvilli. EB should be converted to light color, (PS = periplasmic space). Picture (C), TEM shows an elaborated seen of two microvilli nursing several EBs during their immigration toward the host cell surface. In picture (D), we can observe that all EBs not converted to acceptable form, cell does not respond at all to infection.

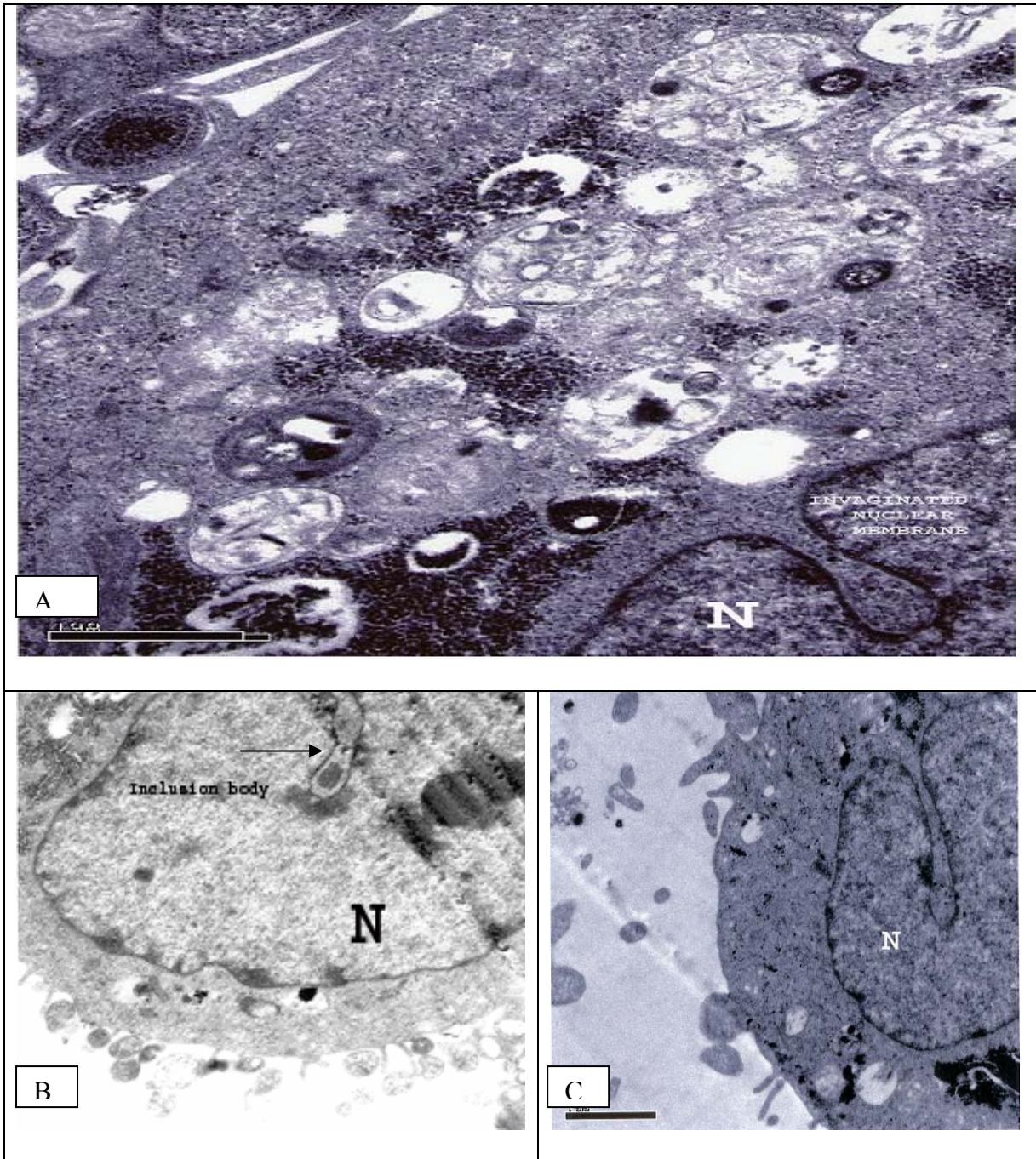


Fig 12: **The invasion of the cell.** TTS protein dominates the cytoplasm and seems to wipe off all the antichlamydial function in the cytoplasm easing the invasion and rapid multiplication characterized by several inclusion bodies. The conversion of EBs to RBs seems to be a consequence of environmental modification of the host cytoplasm initiated by the TTS present in the cytoplasm. In the cytoplasm RBs continue to mature inside the inclusion bodies and some emigrate toward the nuclear membrane. The inclusion bodies seem to seek entrance into the nucleus which shows nuclear membrane invagination to provide a groove like passage for inclusion bodies (A). Pictures B & C show the inclusion bodies are within the groove declaring the invasion of the nucleus.

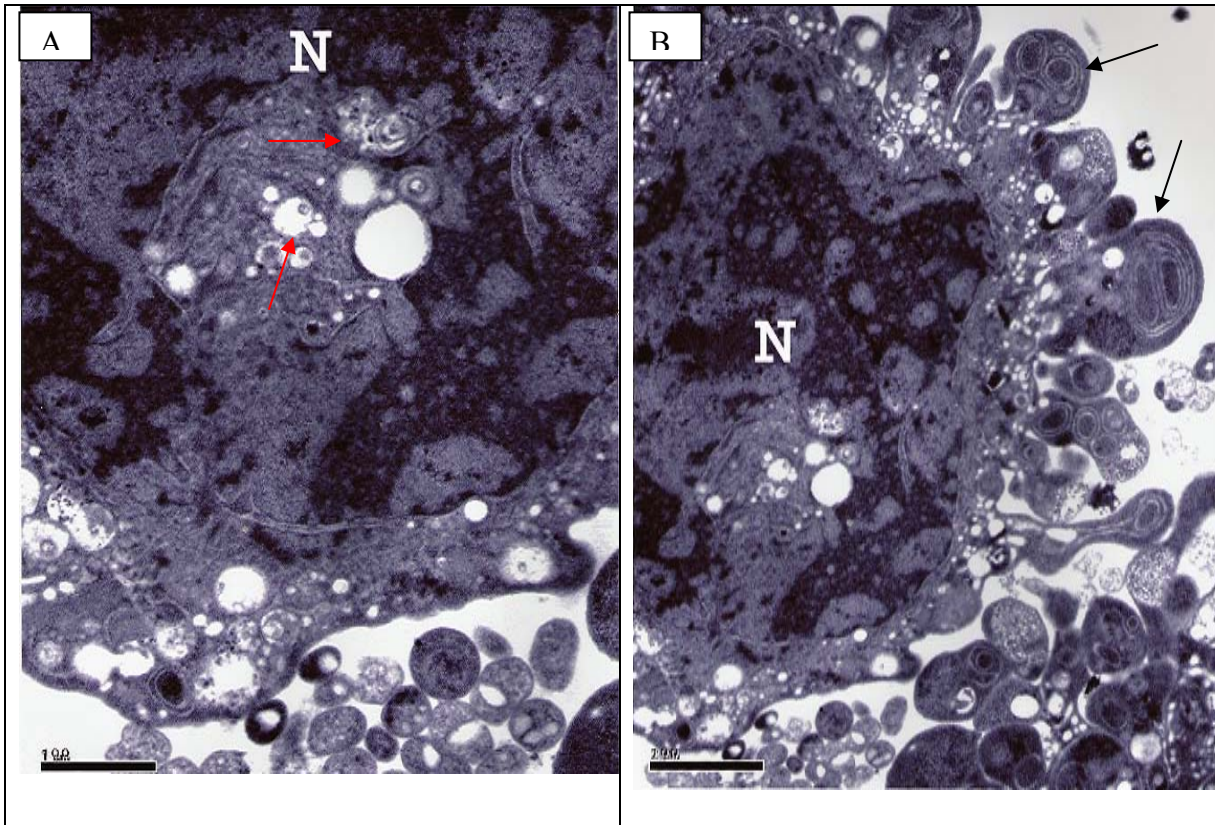


Fig 13: **Formation of projecting structure.** TEM shows the inclusion bodies inside the nucleus and causing a disturbance of the genetic material, declaring the end of the cell as a host (A). The maturation and trafficking of inclusion bodies toward the cytoplasmic membrane causing a projection like structure (B).

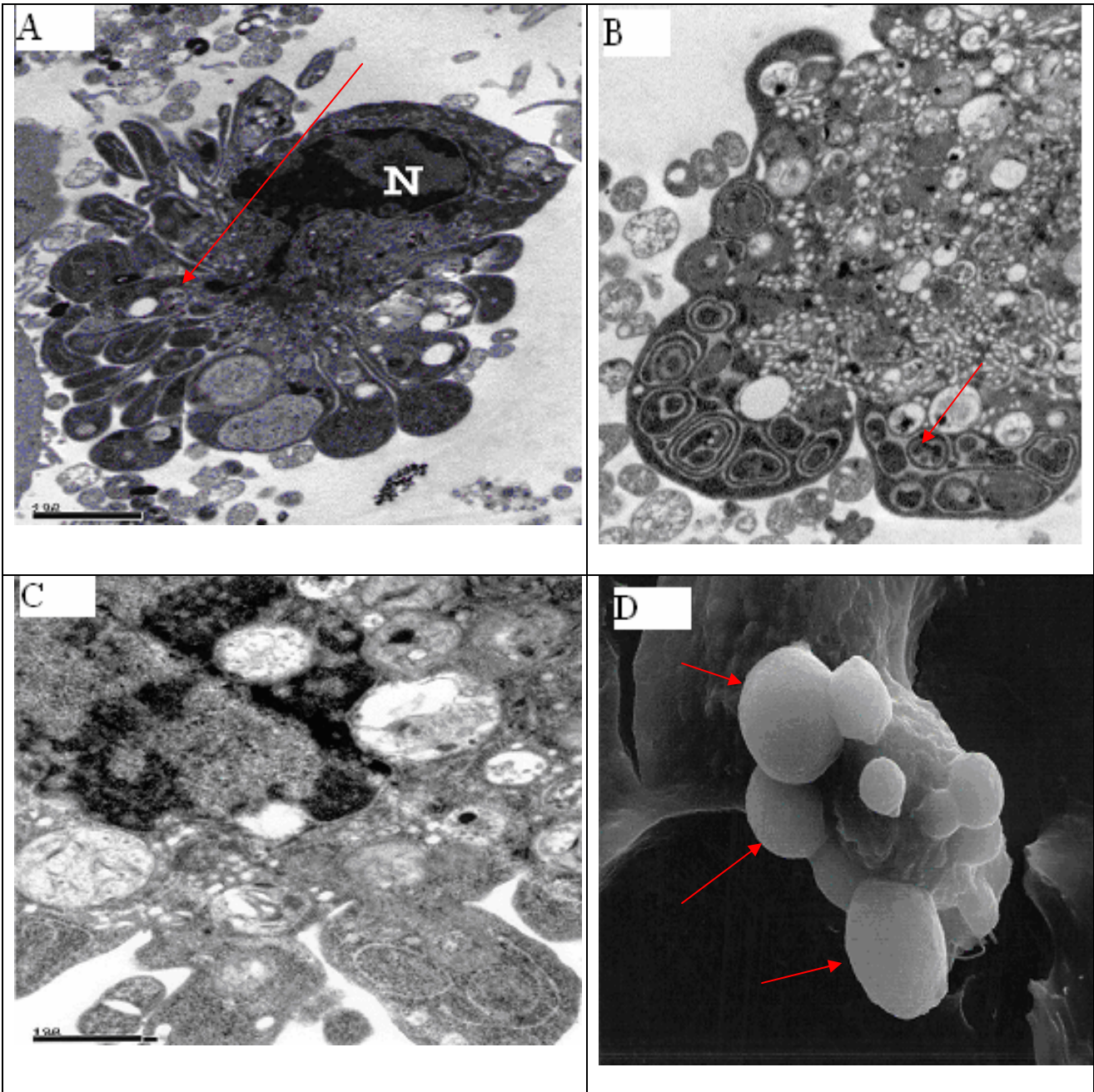


Fig 14: **Maturation of RBs.** TEM shows typical pear shaped EBs are arranged within the projecting structures and some RBs are in process for binary fission (A&B). The process continues, including the maturation of the inclusion bodies and the transformation of RB into EB (B & C). In D, SEM shows enlarged and congested projecting structures ready for releasing progeny EB.

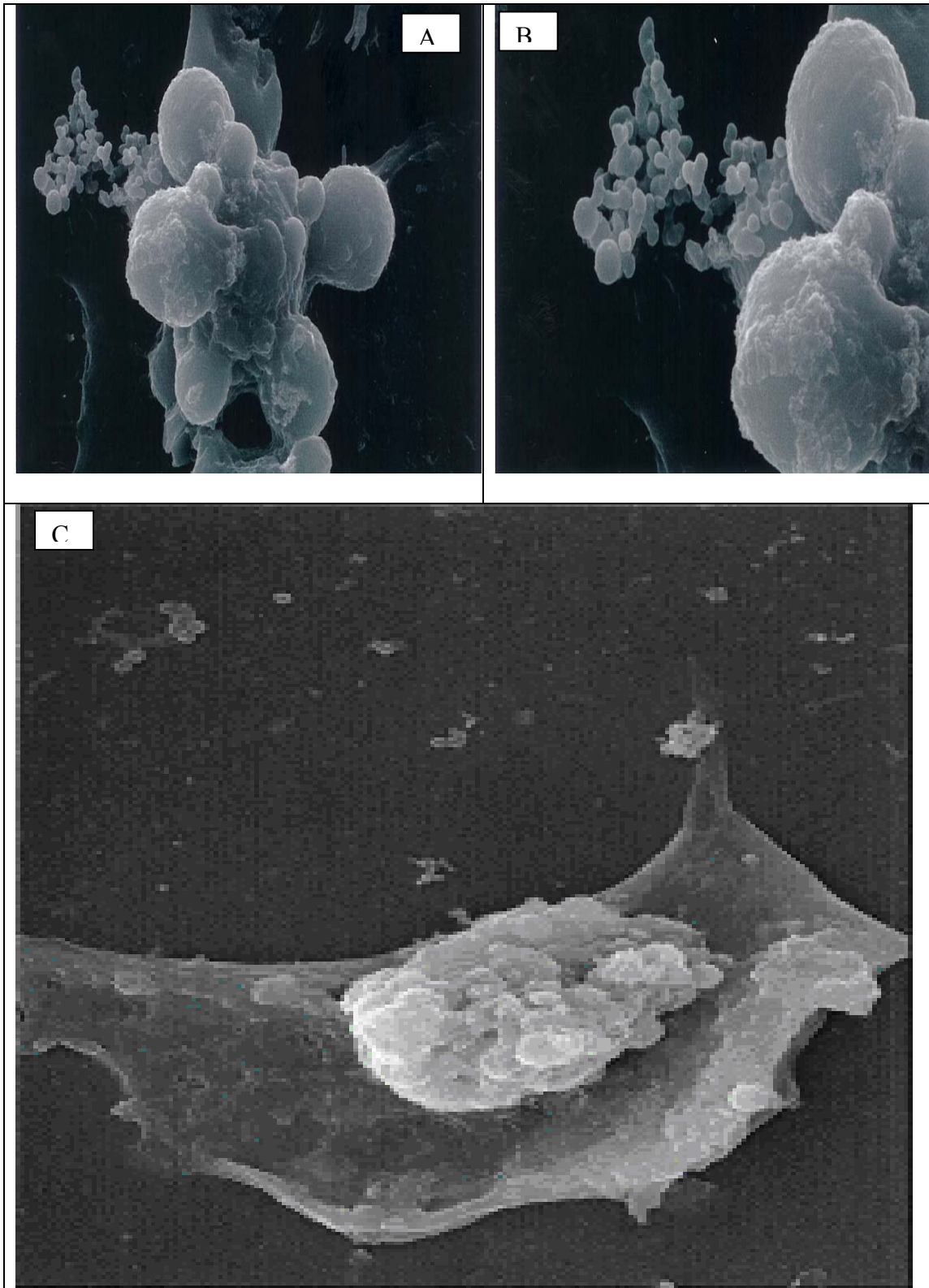


Fig 15: **Releasing of EBs.** The cycle ends with rupture of the projecting structure and volcanic eruption that lead to the release of new EBs (A& B). Picture C shows that host cell was torn and the nucleus filled with EBs after disappearance of the nuclear membrane to exposing a mass of EBs.

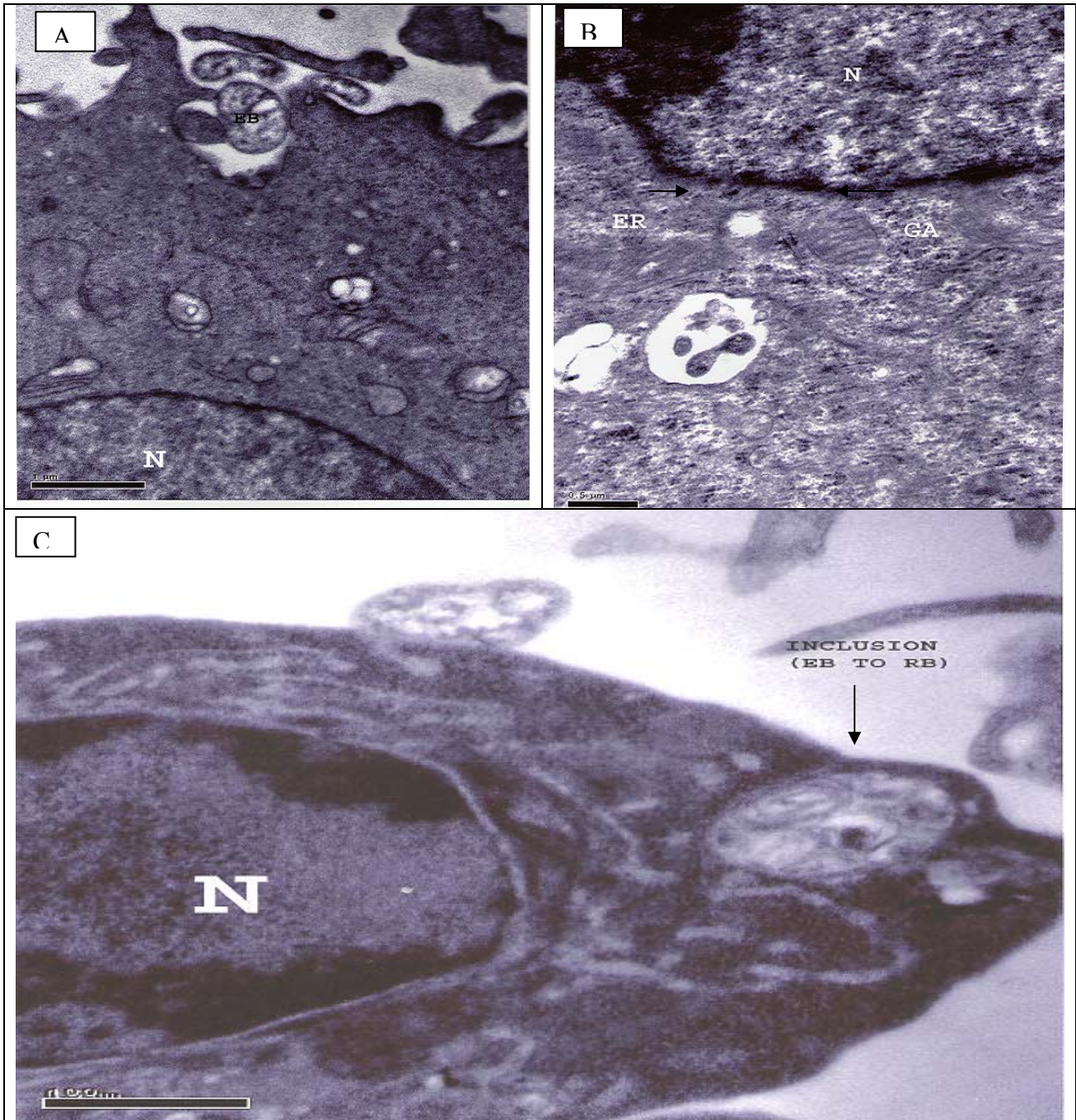


Fig 16: **Non-successful infection.** TEM shows the start of engulfment but it may be led to dormancy because of the cell response is at minimum (A). In (B), it shows undisturbed infected cell characterized by the absence of notable TTS (type III secretion) and the recognition of cell organelles. It is a feature of dormant inclusion body. In (C), TEM shows the localization of EB within the cytoplasm near the cytoplasmic membrane and the start of differentiation to RB. The cell is not responding, it seem the RB entered dormant stage.

### **3.3. Antichlamydial activities:**

The antichlamydial activities of different type of interferons (IFN- $\alpha$ 2a, IFN- $\beta$  and IFN- $\gamma$ ) over a range of concentrations were compared using Hep-2 cell line. The cells were pretreated with increasing amounts of IFNs and then challenged with *chlamydia pneumoniae*. Different IFNs showed a dose-dependent protection against chlamydia-induced cytopathic effect (CPE) and IFNs were able to protect cell in variable degrees.

#### **3.3.1 Cell Response to IFN treatment:**

The antichlamydial effect of IFN- $\alpha$ 2a on Hep2 cell line exhibited dose-dependant effect. IFN- $\alpha$ 2a protected Hep2 cells from CPE induced by *C. pneumoniae*. When Hep2 cells were pretreated with IFN- $\alpha$ 2a, as low as 1.2 IU/ml significant protection (50 %) was achieved, and maximum protection was recorded at 1000 IU/ml (86%) (Fig 17).

Like IFN- $\alpha$ 2a, the effect of IFN- $\beta$  on Hep2 cell line exhibited dose-dependent effect and protected Hep2 from CPE induced by *chlamydia pneumoniae* but in a more potent state. When Hep2 cells were pretreated with IFN- $\beta$ , as low as 0.35 IU/ml protection attained 50% and maximum of 88% at 1000 IU/ml (Fig 17).

IFN- $\gamma$  protect Hep2 cell from CPE induced by *C. pneumoniae* and was the most potent in a dose-dependent manner. When Hep-2 cells were pretreated with IFN- $\gamma$ , as low as less than 0.3 IU/ml significant protection (more than 50%) was achieved, and maximum protection was observed at 10 IU/ml (100%) (Fig 17).

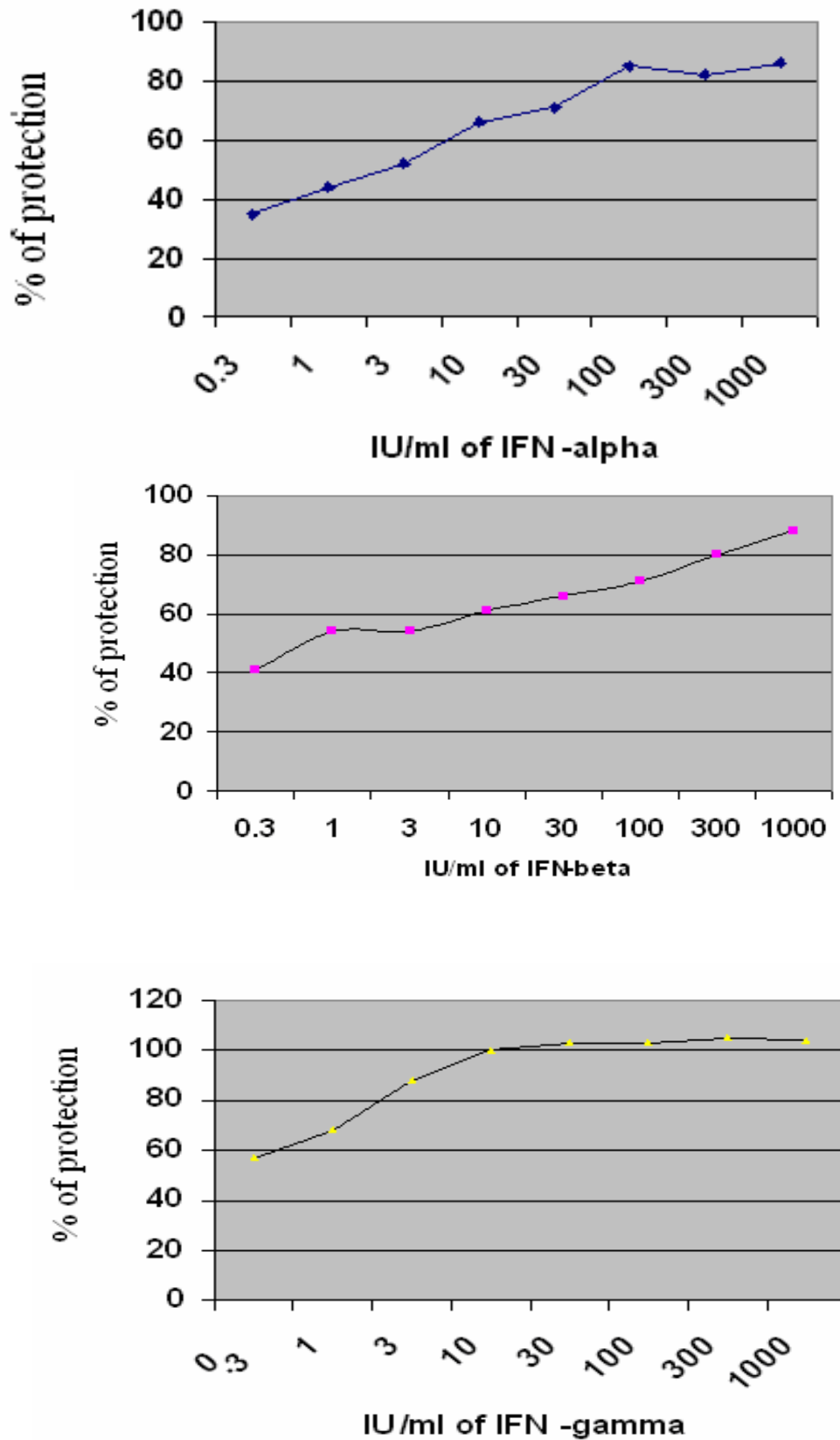


Fig 17: The effect of different IFNs on Hep-2 cell line. Confluent Hep-2 cells were pretreated with indicated concentration of IFNs; cells were then used for antichlamydial assay as described in materials and methods.(cell control = 100% protection & chlamydia control = 47 % protection).

### **3.4 Expression Profiling of cytokine and other cellular genes Using Microarray**

The cDNA expression array encompassing 1113 genes (each gene spotted in duplicate) were hybridized with Cy3-3DNA or Cy5-3DNA-labeled cDNA from infected Hep-2 cells with *C. pneumoniae* (Set 1) and treated-infected Hep-2 Cells (Set 2) and the products visualized by using Microarray GenPix 4000A Scanner.

The results shows significant induction of several cytokine genes expression as detected by microarray. Table 9 shows summary of microarray results for the 11 chosen hits that displayed high levels of gene expression. Expression value of the infected (set 1) compared with infected-treated (set 2) is indicated by +, ++ , +++ ... etc. which represent the fold increase of expression of the treated-infected Hep-2 cells in comparison to the infected Hep-2 cells. These gene expressions are expressed as up regulated genes and down regulated genes according to the value.

The cytokine providing the cell protection e.g. TNF- $\alpha$  (alpha induced protein 3) and IL-15 genes were highly expressed while other cytokines such as IL-13 , IL-6, IL-1 $\beta$  , IL-10, IL-12, IL-11, IL-5 and IL-23 remained unchanged. Other genes related to the cell function that were expressed significantly include cyclin D1 , MHC (Major Histocompatibility) class I polypeptide-related sequence B, methylene tetrahydrofolate dehydrogenase

(NAD<sup>+</sup> dependent), methenyltetrahydrofolate cyclohydrolase, , E2F (Elongation 2 factor) transcription factor 1, colony stimulating factor 1 (macrophage), serine/threonine kinase 17a (apoptosis-inducing), rho/rac guanine nucleotide exchange factor (GEF)2, and death-associated protein kinase1. While the down regulated gene expressed in infected more than treated-infected was that specify protocadherin gamma subfamilyC,3.

Each of the above mentioned genes has a role at the level of the cell function through which we can understand their function in both infected (Set-1) and infected-treated (Set-2) and know the differences between their action in both sets, therefore:-

### ***1. MHC class I polypeptide-related sequence B***

It involved in the presentation of foreign antigens to the immune system.

### ***2. Cyclin D***

Cyclin D1 is a critical component of the core cell cycle machinery. Cyclin D1 is the product of the *CCND1* gene and was first implicated in tumourigenesis following localisation to chromosome 11q13, a region of the genome that is commonly amplified in a range of human carcinomas including breast cancer. Cyclin D1 plays a pivotal role in the regulation of progression from the G<sub>1</sub> to the S phase of the cell cycle through the

formation of active enzyme complexes with cyclin-dependent kinases Cdk4 and Cdk6.

### ***3. Colony stimulating factor 1 (macrophage)***

Colony stimulating factor 1 is a homodimeric glycoprotein which is produced in a variety of adult tissues and which has been shown to modulate proliferation, differentiation and survival of numerous cell types.

### ***4. E2F transcription factor 1***

E2F1 would appear to play dual roles in the control of cell proliferation and cell fate. Overexpression of E2F1 can drive quiescent cells into S phase, and studies of cells deficient for multiple E2F proteins provide evidence of a role for E2F1 in the ability of cells to proliferate. But, in addition to this role, E2F1 appears to have the unique ability to induce apoptosis when expressed in the absence of proliferative signals (Timothy *et al.* 2003).

## ***5. Death-associated protein kinase 1 and Serine/threonine kinase 17a (apoptosis-inducing).***

Apoptosis is a genetically controlled cell death process which is important at various developmental stages as well as for cell maintenance and tissue homeostasis. During the last few years, many of the key players in this process, including receptors, adapter proteins, proteases, and other positive and negative regulators, have been identified. One of the positive mediators of apoptosis, which was cloned, is death-associated protein (DAP) kinase. This protein was discovered by a functional approach to gene cloning, based on transfections of mammalian cells with antisense cDNA libraries and subsequent isolation of death-protective cDNA fragments. The antisense cDNA of DAP kinase protected HeLa cells from gamma interferon-induced cell death. DAP kinase is a calcium/calmodulin (CaM)-regulated serine/threonine protein kinase (160 kDa), associated with actin microfilaments. Overexpression of DAP kinase in various cell lines results in programmed cell death. Several independent lines of evidence proved that DAP kinase is involved in apoptosis triggered by different external signals including gamma interferon and tumor necrosis factor alpha (TNF- $\alpha$ ). Recent studies have implicated several serine/threonine kinases in the regulation of programmed cell death, either as death-promoting or as death-protecting proteins (Boaz *et al.* 2000)

#### **6. *Methylene tetrahydrofolate dehydrogenase (NAD<sup>+</sup> dependent):***

This folate-dependent enzymes are found in the mitochondria as well as the cytoplasm of eukaryotic cells. Isozymes of certain folate-dependent enzymes are present in both compartments, and a number of observations demonstrated that the folate-dependent pathways in mitochondria contribute to total cellular folate metabolism. Both cytoplasmic and mitochondrial isoenzymes occur as trifunctional NADP-dependent dehydrogenase-cyclohydrolase-synthetase (DCS) proteins (Harshila *et al.* 2003).

#### **7. *Protocadherin gamma subfamily C,3 (down regulated)***

Cadherins are calcium-dependent cell-cell adhesion molecules that mediate neural cell-cell interactions. Protocadherins constitute a subfamily of nonclassic cadherins. PCDHGC3 is a member of subfamily C of the gamma cluster of protocadherin genes on 5q31 (Sago *et al.* 1995).

#### **8. *Rho/Rac guanine nucleotide exchange factor (GEF)-2:***

Rho/Rac GEF-2 associates with microtubules and stimulates GTP binding on Rac and Rho. Rho GTPases play a fundamental role in numerous cellular processes that are initiated by extracellular stimuli that work through G protein coupled receptors. The encoded protein may form complex with G proteins and stimulate Rho-dependent signals (Olson *et al.* 1995).

**Table 9: Up- and down-regulated genes between Set-1 (infected Hep-2 cells) and Set-2 (treated infected Hep2-cells)**

Series	Gene	Gene descriptive name	Cluster ID #	Set 1	Set 2
1	TNFAI P3	tumor necrosis factor, alpha-induced protein 3	Hs.211600	+	+++
2	IL 15	interleukin 15	Hs.168132	+	++
3	MICB	MHC class I polypeptide-related sequence B	Hs. 211580	+	++++
4	CCND1	cyclin D1	Hs.82932	+	+++
5	CSF1	colony stimulating factor 1 (macrophage)	Hs.173894	+	+++++
6	E2F1	E2F transcription factor 1	Hs.96055	+	++
7	STK17 A	serine/threonine kinase 17a (apoptosis-inducing)	Hs.9075	+	++
8	DAPK1	death-associated proteinkinase1	Hs.153924	+	+++
9	MTHF D2	methylene tetrahydrofolate dehydrogenase (NAD+ dependent)	Hs.154672	+	++++++
10		Protocadherin gamma subfamily C,3	Hs.173310	+++	+
11	EGR1	rho/rac guanine nucleotide exchangefactor (GEF)-2	Hs.155120	+	++++++

- (+) Down-regulated
- (> ++ ) Up-regulated

*Chapter Four*  
*DISCUSSION*

## ***4. DISCUSSION***

*Chlamydia pneumoniae* is a major cause of acute respiratory tract disease in human and has been responsible for both endemic and epidemic pneumonia (Grayston *et al.* 1990). In addition, this organism has been associated with other clinical manifestation, including coronary artery diseases, asthma and sarcoidosis (Capmpell *et al.* 1992). The finding that 50 to 60% of adults in the diverse geographical places have serological evidence of *chlamydia pneumoniae* infection suggests that it is the most prevalent infectious agent (Grayston *et al.* 1990). So the initiation of unperfounded study as the case in this investigation necessitate the employment of a wide range of validated and recommended techniques and gaining the skill required for performace.

This is the first Saudi Arabian study that give a glance at this organism, its surveillancce, pathogenesis and immunological response. The study employs a wide range of advanced techniques and methods, culturing of chlamydia ,detection by immunoflourescence, polymerase chain reaction (PCR), electron microscopy, antichlamydial assays and the most recent advanced technique of microarray technology.

This study was performed to determine the surveillancce of *chlamydia pneumoniae* in a region of Saudi Arabia and the significance of serious complications related to *chlamydia pneumoniae* infection. To study the

surveillance of *C. pneumoniae* we employed immunofluorescence stain for all cultures and confirmed the positive results by PCR. In the field of *C. pneumoniae* research, there is a problem with the lack of standardized laboratory methods (Dowell *et al.* 2001). The 'gold-standard' for detection of *C. pneumoniae* infection is culturing, but unfortunately *C. pneumoniae* has proved to be slow and difficult to culture and the sensitivity is highly dependent on proper conditions which include specimen collection, transport and processing as well as correct culture procedure. The PCR test is even more associated with laboratory variation, either in the technique procedure or the choice of the primer. The method used in the present study is one of the most validated and has been used in several studies (Campbell *et al* 1992), though the preparation of the specimen is further optimized (Dowell *et al.* 2001).

#### **4.1 Surveillance of *Chlamydia pneumoniae* infection**

*Chlamydia pneumoniae* was detected in respiratory specimens by culture and PCR. The surveillance was 11.9% by both techniques. However, being lower than many published data and higher than others is a consequence of applying different techniques. As mentioned earlier there is a lack of laboratory standard method for such organism. Normann *et al* 1998 used two techniques for studying the surveillance of *C. pneumoniae* in which it showed that serology revealed more infection than MIF (microimmunofluorescence) in children younger than five years, (17% vs.

7%). Other studies employing seroepidemiological methods showed that more than 60% of adults have had exposure to this organism during their lifetimes (Schachter and Grayston, 1998). Chrigwin *et al* (1991) showed that surveillance of *C. pneumoniae* in hospitalized children with respiratory disease was 18.7%, when they combined culturing with serology techniques. No studies were done for the surveillance of *C. pneumoniae* in the respiratory tract infection patients by using both cultures and PCR technology together. All the studies were limited to one of the respiratory diseases such as community acquired pneumoniae, asthma, ..etc. Some studies only concentrated on a certain age group (Grayston 1992), unlike our study which included a spectrum of all the age groups.

In our study, the distribution of the infection with *C. pneumoniae* affected all ages groups that was 50% in small children less than 4 years, decrease rapidly at the age of 4-10 years to (8%) and in adults less than 40 years old (6%) increasing again at the age above 40 years old to reach (36%). These data conflicted with other study done by Aldous *et al.* 1992 in Seattle, in which children between 4-9 years of age had the highest incidence of acute infection with *C. pneumoniae* (40%). The possible explanation of such difference is that the results did not in fact bear any consideration with ours, if we take into account the geographical variation and the narrow spectrum of age. However, if we take in concentration the children at the age

of 4-9 years, it is even worse to find that chlamydia infection claim large proportion of this age group.

The infection occurred all year-round and the highest rate of *C. pneumoniae* was during winter (November (38%)), while there is a dramatic drop in its detection observed in other months. Recently, Lim *et al* (2001) reported that they found *C pneumoniae* to be more common in the winter than in the summer. This high rate during winter may explain the question of the pathogenicity of this bacteria. Hence, the pathogenicity of this organism seems to be implemented by rhinitis, pharyngitis, and some cold associated infections which is common in winter.

The surveillance of the infection was most common in male (65%) than female (35%), and it does agree with data from Grayston 1992 , in which the serosurveillance of *C. pneumoniae* among adult men is considerably higher than that among adult women. This sex difference among adult 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.

More than one half of patients (63%) presented with pneumonia, and about one third (33%) presented with upper respiratory tract infection. This high rate of pneumoniae might be due to participation of other microorganisms such as *S. pneumoniae* and *M. pneumoniae*. To support this explanation a study performed by Marston *et al* (1997), in which we studied

a population of 2776 (in Noninstitutionalized Adult Ohio Residents Hospitalized) with Community Acquired pneumonia (CAP) revealed that the main etiological agents of CAP were *M. pneumoniae* (32.5%), *S. pneumoniae* (12.5%) and *C. pneumoniae* (8.9%). So, our high rate of pneumoniae could be explained in the light of the participation of other organisms.

In some of the cases in this study, *C. pneumoniae* co-existed with bacteria and viruses according to the patient record. These mixed infections might be responsible for misleading the treatment. *C. pneumoniae* was frequently found in mixed infections, in about 13.2% of the cases with *S. pneumoniae* in parallel to data reported in other countries by Lieberman *et al* 1996, Kauppinen *et al* 1995 and Marrie *et al* 1987. Pathogens such as *C. pneumoniae* may play a role in promoting other bacterial infection through their effect on ciliated epithelial cells (Shemer-Avni *et al*, 1995). Second common co-pathogen was *M. pneumoniae* which is distributed in about 8.8% of the patients with *C. pneumoniae*. *M. pneumoniae* is the most common cause of atypical pneumoniae accounting for 5-23% of community-acquired pneumoniae. The presence of *C. pneumoniae* with *M. pneumoniae* could help in the persistence of pneumonia and could be more lethal mixed infection. Other uncommon microorganisms were *H. influenza* (5.8%), Influenza A virus (2.9%) and *staphylococcus aureus* (1.5%). Mixed infections seem to be common and should be taken into account when

planning antimicrobial treatment for pneumonia or other respiratory diseases  
due to *C. pneumoniae*.

## 4.2 ULTRASTRUCTURAL STUDY OF *C. PNEUMONIAE*

*Chlamydia pneumoniae* is an obligate intracellular parasite with developmental cycle believed to be common to all members of the genus Chlamydia. Like all chlamydiae, *C. pneumoniae* undergoes a developmental cycle in which two functionally and morphologically distinct cell types are recognized. The infectious cell type, which is specialized for extracellular survival and transmission, is termed the elementary body (EB). The intracellular, vegetative cell type is called the reticulate body (RB). The developmental cycle is initiated by endocytosis of an EB by a eukaryotic host cell. Chlamydiae remain within an intracellular vacuole, termed as inclusion, for their entire developmental cycle. Shortly after internalization, EBs begin to reorganize and differentiate into RBs, which then begin to multiply by binary fission. Late in the cycle, logarithmic growth ceases as RBs commence to reorganize into EBs, which are released upon lysis of the host cell (Stephens 2002).

We present a detailed description based on transmission and scanning electron microscopy for temporal events throughout *chlamydia pneumoniae* developmental cycle.

First of all the use of Hep 2 cells as a cell line for detecting and illustrating the developmental cycle for *C. pneumoniae* was dictated by the sensitivity of these cells to the chlamydial infection and its continuous use in several international laboratories (Roblin *et al*, 1992)

#### 4.2.1 Morphology of *Chlamydia pneumoniae*

The electron microscopic study has shown that the morphology of *C. pneumoniae* RB is similar to that of other chlamydiae and the sizes of the *C. pneumoniae* RB and EB are within the range of the chlamydia species. However, the electron microscopic observations have demonstrated that the structure of the *C. pneumoniae* EB is distinct from those of other chlamydiae. *C. pneumoniae* EBs are typically pear-shaped and have large periplasmic space. These features contradict to many reports that generally consider the chlamydial EBs as round with a narrow or barely discernible periplasmic space either in host tissues, tissue cultures, or purified preparations. However, such conclusion was the result of studies conducted on avian *C. psittaci* strain in parakeets, the murine biovars of *C. trachomatis* (mouse pneumonitis); and the human biovars of *C. trachomatis* type E (Stirling *et al* 1987).

Explanations for the pear-shaped structure and large periplasmic space are speculative. It may be caused by disturbance in the regulation of outer membrane synthesis, so that excess outer membrane continue to be made or is not cleaved off when the cytoplasmic mass shrinks during the RB-to-EB transformation. Another possibility is that the outer membrane is more rigid in *C. pneumoniae* than other chlamydiae species. The rigidity may prevent the membrane from collapsing on the shrinking cytoplasmic mass, which may suggest a difference in the chemical structure of the outer membrane

between *C. pneumoniae* and other chlamydiae (Chi *et al* 1987). The pear like shape of *C. pneumoniae* has been solely attributed to the rapid binary fission causing lip like structure and seems to be the most acceptable explanation.

#### **4.2.2 The process of *Chlamydia pneumoniae* infection**

The developmental process of *chlamydia pneumoniae* phagosome in general appears to be similar to that of other chlamydiae, i.e., transformation of EB into RB after entering the host cell, multiplication by binary fission, and maturation of RB to EB.

*Chlamydia pneumoniae* EBs appear to interact initially with microvilli prior to endocytosis by the host cell. The point of attachment could suggest the following interpretation of how *C. pneumoniae* attaches and enters host cells. According to Kuo and chi in 1987 and Su, et al 1990, EBs of *chlamydiae pneumoniae* may possess polarity, within the pointed end serving as the primary site of attachment and the chlamydial major outer membrane protein (MOMP) may function as an adhesin mediating both electrostatic and hydrophobic interaction with the host cell surface.

The organism may actively seek targets by directing the tip end toward the host cell. EBs may first attach to the microvilli or the smooth surface of host cells, but most often they attach to the microvilli because of their accessibility.

After this initial contact, the EBs rearrange their outer membranes to form several protruding structures which serve as anchoring sites, unlike those of the other chlamydiae, in which the outer membrane is tightly bound to the cytoplasmic mass, the *C. pneumoniae* EB has a loose outer membrane which appears to facilitate its attachment and anchoring process. The reshaping of the *C. pneumoniae* outer membrane during attachment suggests that the membrane fluidity of *C. pneumoniae* is more dynamic than that of the other chlamydiae. (Kuo and Chi, 1987).

If the *C. pneumoniae* attaches to the microvilli, it is transported toward the base, where it enters the host cell by an invagination process. This process is completed when the host cell membrane completely encircles the EB.

The unique shape of the *C. pneumoniae* EB and its attachment process may explain the differences in morphology of the *C. pneumoniae* endocytic vesicles from that of the other chlamydiae. The endocytosis of the *C. pneumoniae* EB would result in a vacuolar endocytic vesicle, while the engulfment of the circular *C. trachomatis* or *C. psittaci* EB would result in a compact endocytic vesicle (Hodinka *et al*, 1986).

The differences in attachment suggest that the unique shape of the *chlamydia pneumoniae* has a biological function and is not an artifact.

### 4.2.3 The intracellular development:

#### Type three secretion in chlamydiae.

The identification of genes in chlamydiae encoding a type three secretion (tts) system provides a newly understood mechanism by which chlamydiae might interact with the host cell. The tts system functions as a kind of 'molecular syringe', enabling gram-negative bacteria to inject virulence-related proteins into the cytoplasm of host cells, Fig 11. The tts system differs from all other bacterial secretion mechanisms in that it requires and is triggered by the intimate contact of the bacterium with host cell membrane. In the case of *C. pneumoniae*, tts activity may be triggered extracellularly when the EB comes into contact with the host cell membrane as seen in this study, and intracellularly when it comes into contact with the inclusion membrane into which effector proteins are secreted and may become either embedded or alternatively translocated into the host cell cytoplasm (Hsia *et al.*, 1997), possibly through the **surface rosettes and fibres** (Rockey & Matsumoto, 1999). Currently there is little supporting evidence for this hypothesis other than preliminary studies which suggest that some components of the tts system are involved either at the middle of or late in the developmental cycle (Kubo & Stephens, 1998).

Tts play an important role for triggering of binary fission of reticulate bodies which might lead to detachment of RBs from the inclusion membrane while

inactivation of *tts* a possible trigger for late differentiation (Rockey & Matsumoto, 1999).

Bavoil and Hsia, 1998, proposed that the surface of EBs and RBs represent chlamydial type III secretion apparatus which suggest that EBs are poised to secrete virulence protein. Hence, it seems that *tts* disrupt eukaryotic signal transduction pathways to subvert such process and aggravate the state of infection.

### **Ultrastructural observation of intracellular development**

Contact with the host cell and entry of the EB are the first steps in a complicated interaction between the infecting chlamydiae and the invaded host cell. The cell has mechanisms in place to traffic endocytosed material into lysosomal pathway, leading to the death and digestion of the invader. The chlamydiae must modify their environment, and quickly, to avoid this fate. Phagosomes containing chlamydiae quickly leave the lysosomal fusion pathway and reside in the nonacidified exocytic vesicular pathway which called early inclusion. This inclusion moves toward perinuclear area of the host cell (Priscilla 2000), such observations have been mentioned earlier.

A major step in initiation of the chlamydial developmental cycle is the 'conditioning' of the phagosome to become an inclusion. While EB-to-RB transition and the initial phases of a productive developmental cycle are dependent on bacterial RNA and protein synthesis, very little is known about the initial molecular steps in the intracellular life of the EB or the signals

involved in the EB-to-RB transition. The early condition in the intracellular-intravacuolar environment trigger the reduction of major outer membrane protein (MOMP). This may augment the porosity of the EB, which could be a first or very early step in the signaling process allowing early transcription and translation and the initiation of the EB-to-RB transition (Priscilla 2000).

Chlamydial growth occurs inside a membrane-bound inclusion. The nascent inclusion intercepts epithelial exocytic vesicles carrying sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylcholine (PC) and cholesterol (CL) for inclusion membrane expansion. Metabolically active RB produce specific proteins (Inc), which become incorporated in inclusion membrane and regulate its environment while chlamydial projections may assist the inclusion membrane-adherent RB to acquire iron and nutrients from the host cell cytoplasm (Priscilla 2000).

Bavoil and Hsia (1998) stated that the chlamydial projections could serve an alternative function to the type III secretion machinery using transport protein and a cytoplasmic memberane energizing ATPase to deliver secreted molecules into the host cell cytoplasm to modulate the host-parasite interaction. This was an exciting conceptual advancement.

The interesting point was that the host cell nucleus were also infected by *chlamydia pneumoniae* ,which is approved for the first time in this study. This observation was noticed at 48 hrs postinfection in which the RBs were seen inside the nucleus, while at 72 hours postinfection that all nuclear

materials were shown to be eaten by *C. pneumoniae*. This observation clearly supports the invasive nature of this organism in which it can dismantle and stop all the genetic apparatus and consequently paralyzing the signal transduction inside the cell.

Infectious EB that have completed developmental cycle and the maturation process from RB aggregate in the center of the inclusion bodies.

### **Exit**

The signals that determine the end of the developmental cycle and trigger maturation of RB back to infectious EB are unknown. The process involves a combination of events, including a decrease in host cell nutrients, which may signal detachment of RB from the inclusion membrane, and activation of histone proteins, which regulate stage-specific gene expression and DNA condensation (Tao *et al*, 1991). There are reports of gradual disintegration of the inclusion membrane with release of naked EB into host cytosol for lateral entry into uninfected host cells, and an impressive cinematographic capture of rapid inclusion rotation, first counterclockwise and then clockwise, before a volcanic like rupture to release chlamydial progeny within minutes. Finally, there is evidence that the inclusion travels the exocytic pathway to the epithelial surface for fusion with the plasma membrane. This latter fusion event occurs at apical membrane for release of disseminating chlamydiae (Priscilla 2000).

However, our observation to the process may include the dryness of the EBs as the main mechanism of the volcanic rupture of inclusion membrane and cytoplasmic membrane of the host cell followed by the shrinking of the cell and integration.

### 4.3 ANTICHLAMYDIAL ACTIVITY:

Cytokines are chemical substances produced by cells, which affect the function of other cells, and are important in generating inflammation and in sustaining the body's immune response to infection. They may also be involved in the process of scarring and fibrosis that leads to the characteristic tissue damage associated with long term or severe chlamydial infection.

Chlamydial infection generates a cytokine response, both by direct infection of the epithelial cells lining the moist surfaces of the body (the mucosae), and by interaction with cells of the immune system (Fitzpatrick *et al.*, 1991).

One of the cytokines are interferons which are a family of related antiviral proteins, produced by mammalian cells in response to infection (including chlamydial infection) and other stimuli. The interferons also act as intercellular messengers, altering the function of many different kinds of cells. Human interferons have recently been re-classified as IFN- $\alpha$  , IFN- $\beta$  (previously type I, leucocyte and fibroblast respectively) and IFN- $\gamma$ , (previously type II, or immune)

Cytokines have been well described as potentiators of the cellular immune response and the subsequent inhibition of replication of several species of chlamydia. IFN- $\gamma$  has been shown to inhibit the intracellular growth of *C. psittaci* 6BC in L cells and monocyte-derived macrophages

(Rothermel *et al.* 1986). McCafferty *et al.* in 1994 reported an important role for IFN- $\gamma$  in defense against early infection with *C. psittaci* of experimentally infected mice. Beatty *et al.* in 1994, reported that the treatment of HeLa 229 cell monolayer with 0.2 IU/ml of IFN- $\gamma$  completely inhibited the replication of *C. trachomatis* serovar A/Har-13. IFN- $\gamma$  have been identified as human cytokines which can restrict the intracellular replication of *C. trachomatis* (Rothermel *et al.* 1986).

The data presented here support the possibility of a similar role for same cytokine in inhibiting the intracellular multiplication of *C. pneumoniae* in Hep-2 cells. The addition of IFN- $\gamma$  at 10 IU/ml for 24 hours before infection was capable of completely inhibiting the multiplication of *C. pneumoniae* in the Hep-2 cell monolayers. IFN- $\gamma$  appears to be more potent than other IFNs, which exert their actions at higher dose than IFN- $\gamma$  and they cannot inhibit the growth completely.

Overall, in light of the involvement of the cellular immune response to *C. pneumoniae* infection, this study demonstrates that IFN- $\gamma$  can alter the intracellular fate of this organism and thus may play an important role in the pathogenesis of human infection.

#### **4.4 Expression Profiling of cytokine and other cellular genes Using Microarray**

The expression of cytokines by *C. pneumoniae* infection may play an important role in the pathogenesis of *C. pneumoniae* infection specially in chronic respiratory infections. In this study, we found that treated infected Hep-2 with *C. pneumoniae* induced gene expression of the proinflammatory cytokine TNF- $\alpha$  and IL-15. TNF- $\alpha$  is one of the T-cell derived cytokines and its role is well studied as in antichlamydial action with the help of IFN- $\gamma$ . It has four roles includes:

**i)** The activation of phagocytes to rapidly take up and degrade chlamydiae or infected cells (Zhong & de La Maza, 1988); **(ii)** The induction of indoleamine 2, 3-dioxygenase, an enzyme that catalyzes the decyclization of L-tryptophan into N-formylkynurenine (Byrne *et al.*, 1986), thereby limiting the availability of the essential amino acid, and consequently inhibiting chlamydial growth; **(iii)** The activation of the inducible nitric oxide synthase (iNOS), which catalyzes the production of various antimicrobial reactive nitrogen intermediates, most notably nitric oxide (NO) from L-arginine (Chen *et al.*, 1996); and **(iv)** The induction of intracellular iron deficiency, involving the down-regulation of transferrin receptors (Byrd & Horwitz, 1993). Iron deprivation influences the growth of different species of *Chlamydia* (Freidank *et al.*, 2001).

Interleukin-15 (IL-15) is a recently discovered cytokine produced by a wide range of different cell types including fibroblasts, keratinocytes, endothelial cells, and macrophages in response to lipopolysaccharide or microbial infection. To our knowledge it is the first time to show the inhibition of IL-15 due to *C. pneumoniae* infection. IL-15 stimulates the growth of activated T, B, and NK cells and tumor-infiltrating lymphocytes (Armitage *et al.* 1995), acts as a chemoattractant for T lymphocytes, induces lymphokine-activated killer activity in NK cells, and induces the generation of cytolytic effector cells (Bamford *et al.* 1994). There is increasing evidence that IL-15 can also affect phagocytic cells. Recently Badolato *et al.* 1997 showed that IL-15 acts as a proinflammatory cytokine that induces monocytes to secrete IL-8 and monocyte chemoattractant protein 1, other investigators have shown that it induces morphological changes and delays apoptosis in polymorphonuclear leukocytes (PMN) (Girard *et al.* 1996). In this study we add one more function of IL-15 as antichlamydial properties.

As we observed from microarray experiment there were some cellular genes that were up-regulated and others down-regulated, all of these genes play an important role in cell function. One of these genes is MHC class I polypeptide-related sequence B, which is over-expressed in infected-treated cells meaning that the infected cell lost the immunity reflected by this gene. So *C. pneumoniae* decrease the immunity by inhibiting MHC class I polypeptide-related sequence B. Other genes that are related to the cell

proliferation, Cyclin D, Colony stimulating factor and E2F transcription factor 1, were more expressed in infected-treated cells. Such observation supports the idea that *chlamydia pneumoniae* inhibit the proliferative signals of the cell by deactivating the expression of proliferative genes in the cell.

Apoptosis is a programmed individual cell death that occurs normally during development and aging. In our microarray experiment we found that *chlamydia pneumoniae* inhibit the process of apoptosis by down-regulation of Serine/threonine kinase 17a and death-associated protein kinase 1 which are inducer of apoptosis. So, the infected cell cannot perform apoptosis which is characterized by maintenance of cell membrane during the suicide process, allowing adjacent cells to engulf the dying cell, preventing release of its contents and triggering a local inflammatory reaction (Green , 1998).

An over-expressed gene, Methylene tetrahydrofolate dehydrogenase (NAD<sup>+</sup> dependent), which plays an important role in cellular folate metabolism was observed. *Chlamydia pneumoniae* need folate to perform its destructive function, so it got its folate from the host (Moulder, 1991). In our experiment we found that *chlamydia pneumoniae* caused the down regulation of Methylene tetrahydrofolate dehydrogenase (NAD<sup>+</sup> dependent) while in infected-treated cells it was found to be up-regulated. Consequently, the infected cells will be deprived from folate while the normal level of folate would be maintained in infected-treated cells. Another important gene

that was significantly up-regulated in infected cells was Protocadherin gamma subfamily C,3 which is needed in cell-to-cell adhesion that mediate cell-to-cell interactions, alerting the neighboring cell from possible invasion. It is appreciated to observe up-regulation for this gene in infected cell because these cells have been alerted and it should communicate with other cells to take precaution, while it is down regulated in infected-treated cells because the cell does not recognize a dangerous situation.

The gene that was significantly expressed in treated cells is the one called Rho/Rac guanine nucleotide exchange factor (GEF)-2 which is responsible for the formation of microtubules of the cells. This gene was masked in infected cell due to *chlamydia pneumoniae*, so it was down-regulated in infected while it is up-regulated in infected-treated cells. It is speculated that the reason for such phenomenon is that the cell used the gene product for forming microvilli (microtubule), therefore the expression should be down regulated in infected cell and no more microvilli will be formed. By contrast, the expression of the gene in infected-treated cells will stay alerted, so it will be up regulated.

# *REFERENCES*

Aldous MB, Grayston JT and wang SP (1992) Seroepidemiology of *Chlamydia pneumoniae* TWAR in Seattle families, 1966-1978. *J Infect Dis* 166: 646-649.

Armitage, R. J., B. M. Macduff, J. Eisenman, R. Paxton, and K. H. Grabstein ( 1995). IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *J. Immunol.* 154:483-490.

Campell L A, Perez M, Jahmilton DJ, Kuo C and Grayston JT (1992) Detection of *Chlamydia pneumoniae* by Polymerase chain reaction. *J Clinical Microbiology*, 434-439.

Badolato, R., A. Negro Ponzi, M. Millesimo, L. D. Notarangelo, and T. Musso. 1997. Interleukin-15 (IL-15) induces IL-8 and monocyte chemoattractant protein 1 production in human monocytes. *Blood* 90:2804-2809.

Bamford, R. N., A. J. Grant, J. D. Burton, C. Peters, G. Kurys, C. K. Goldman, J. Brennan, E. Roessler, and T. A. Waldmann. 1994. The interleukin (IL) 2 receptor beta chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and the induction of lymphokine-activated killer cells. *Proc. Natl. Acad. Sci. USA* 91:4940-4944.

Bannantine JP, Griffiths RS, Viratyosin W, Brown WJ & Rockey DD (2000) A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell Microbiol* 2: 35–47.

Bannantine JP, Rockey DD & Hackstadt T (1998) Tandem genes of *Chlamydia psittaci* that encode proteins localized to the inclusion membrane. *Mol Microbiol* 28: 1017–1026.

Barbour AG, Amano K, Hackstadt T, Perry L & Caldwell HD (1982) *Chlamydia trachomatis* has penicillin-binding proteins but not detectable muramic acid. *J Bacteriol* 151: 420–428.

Bavoil, P. M. & Hsia, R-C. (1998). Type III secretion in *Chlamydia*. *Molecular Microbiology* 28, 860 - 862.

Beatty WL, Byrne GI & Morrison RP (1993) Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection in vitro. *Proc Natl Acad Sci U S A* 90: 3998–4002.  
Beatty WL, Morrison RP & Byrne GI (1994b) Persistent chlamydiae:

from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol Rev* 58; 686–699.

Beatty WL, Morrison RP & Byrne GI (1994b) Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol Rev* 58; 686–699.

Beatty WL, Belanger AA and Byrne GI (1994) Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect Immun* 62: 3705-3711.

Birkelund S, Lundemose AG & Christiansen G (1989) Immunoelectron microscopy of lipopolysaccharide in *Chlamydia trachomatis*. *Infect Immun* 57: 3250–3253.

Blasi F (2000) *Chlamydia pneumoniae* in respiratory infections. In: Saikku P (ed) *Proceedings of the Fourth Meeting of the European Society for Chlamydia Research*. Helsinki, Finland, August 2000, p 231–234.

Blasi F, Cosentini R, Denti F & Allegra L (1994) Two family outbreaks of *Chlamydia pneumoniae* infection. *Eur Respir J* 7: 102–104.

Boaz I, Gidi S, Ofer C, Joseph L., and Adi K (2000) Death-Associated Protein Kinase-Related Protein 1, a Novel Serine/Threonine Kinase Involved in Apoptosis. *Molec Cell Biol*, 20 (3) 1044-1054

Boman J & Gaydos CA (1999) *Chlamydia pneumoniae*: molecular biology methods. In: Allegra L & Blasi F (eds) *Chlamydia pneumoniae, The Lung and the Heart*. Springer-Verlag, Milan, p 24–32.

Boman J, Allard A, Persson K, Lundborg M, Juto P & Wadell G (1997) Rapid diagnosis of respiratory *Chlamydia pneumoniae* infection by nested touchdown polymerase chain reaction compared with culture and antigen detection by EIA. *J Infect Dis* 175: 1523–1526.

Brade H, Brade L & Nano FE (1987) Chemical and serological investigations on the genus-specific lipopolysaccharide epitope of *Chlamydia*. *Proc Natl Acad Sci U S A* 84: 2508–2512.

Brown WJ & Rockey DD (2000) Identification of an antigen localized to an apparent septum within dividing chlamydiae. *Infect Immun* 68: 708–715.

Brunham RC & Peeling RW (1994) Chlamydia trachomatis antigens: role in immunity and pathogenesis. *Infect Agents Dis* 3: 218–233.

Byrd, T. F. and Horwitz, M. A. (1993). Regulation of transferrin receptor expression and ferritin content in human mononuclear phagocytes. Coordinate upregulation by iron transferrin and downregulation by interferon gamma. *J.Clin.Invest.*, 91, 969-976.

Byrne, G. I., Lehmann, L. K. and Landry, G. J. (1986). Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular Chlamydia psittaci replication in T24 cells. *Infect.Immun.*, 53, 347-351.

Caldwell HD, Kromhout J & Schachter J (1981) Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. *Infect Immun* 31: 1161–1176.

Campbell LA, Kuo CC & Grayston JT (1990) Structural and antigenic analysis of Chlamydia pneumoniae. *Infect Immun* 58: 93–97.

Chen, B., Stout, R. and Campbell, W. F. (1996). Nitric oxide production: a mechanism of Chlamydia trachomatis inhibition in interferon-gamma-treated RAW264.7 cells. *FEMS Immunol. Med. Microbiol.*, 14, 109-120.

Chi E, Kuo CC and Grayston T. (1987) Unique ultrastructure in the elementary body of chlamydia sp. Strain TWAR. *J. Bacter* 3757-3763.

Chirgwin K, Robin PM, Gelling M, and Hammerschlag MR (1991) Infection with Chlamydia pneumoniae in Brooklyn. *J Infect Dis* 163: 757-761.

Christiansen G, Pedersen AS, Hjerno K, Vandahl B & Birkelund S (2000) Potential relevance of Chlamydia pneumoniae surface proteins to an effective vaccine. *J Infect Dis* 181 Suppl 3: S528–537.

Collett BA, Newhall WJ, Jersild RA, Jr. & Jones RB (1989) Detection of surface-exposed epitopes on Chlamydia trachomatis by immune electron microscopy. *J Gen Microbiol* 135: 85–94.

Danilition SL, Maclean IW, Peeling R, Winston S & Brunham RC (1990) The 75-kilodalton protein of Chlamydia trachomatis: a member of the heat shock protein 70 family? *Infect Immun* 58: 189–196.

Domeika M, Domeika K, Paavonen J, Mardh PA & Witkin SS (1998) Humoral immune response to conserved epitopes of *Chlamydia trachomatis* and human 60-kDa heat-shock protein in women with pelvic inflammatory disease. *J Infect Dis* 177: 714–719.

Dowell SF, Peeling RW and Boomen J (2001) Standardizing *Chlamydia pneumoniae* assays. *Clin Infect Dis* 33:492-503.

Ekman MR, Grayston JT, Visakorpi R, Kleemola M, Kuo CC & Saikku P (1993a) An epidemic of infections due to *Chlamydia pneumoniae* in military conscripts. *Clin Infect Dis* 17: 420–425.

Everett KD, Bush RM & Andersen AA (1999) Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* 49 Pt 2: 415–440.

Fitzpatrick, D. R., Wie, J., Webb, D., Bonfiglioli, R., Gardner, I. D., Mathews, J. D., and Bielefeldt-Hoemann, H. (1991). Preferential binding of *Chlamydia trachomatis* to subsets of human-lymphocytes and induction of interleukin-6 and interferon- gamma. *Immunology and Cell Biology* 69, 337 - 348.

Fox A, Rogers JC, Gilbert J, Morgan S, Davis CH, Knight S & Wyrick PB (1990) Muramic acid is not detectable in *Chlamydia psittaci* or *Chlamydia trachomatis* by gas chromatography-mass spectrometry. *Infect Immun* 58: 835–837.

Freidank, H. M., Billing, H. and Wiedmann-Al-Ahmad, M. (2001). Influence of iron restriction on *Chlamydia pneumoniae* and *C. trachomatis*. *J Med Microbiol*, 50, 223-227.

Fukushi H & Hirai K (1992) Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains derived from ruminants. *Int J Syst Bacteriol* 42: 306–308.

Geng, Y., K. Beerencsi, Z. Gyulai, T. Valyi-Nagy (2000) Role of interleukin 12 and gamma interferon in murine chlamydia pneumoniae infection. *Infect. Immun.* 68:2245-2253.

Girard, D., M. Paquet, R. Paquin, and A. D. Beaulieu. 1996. Differential effects of interleukin-15 (IL-15) and IL-2 on human neutrophils: modulation of phagocytosis, cytoskeleton rearrangement, gene expression, and apoptosis by IL-15. *Blood* 88:3176-3184.

Grayston JT, Campbell LA, Kuo CC, Mordhorst CH, Saikku P, Thom DH & Wang SP (1990) A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. *J Infect Dis* 161: 618–625.

Grayston JT, Kuo CC, Wang SP & Altman J (1986) A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. *N Engl J Med* 315: 161–168.

Grayston JT, Mordhorst C, Bruu AL, Vene S & Wang SP (1989) Countrywide epidemics of *Chlamydia pneumoniae*, strain TWAR, in Scandinavia, 1981-1983. *J Infect Dis* 159: 1111–1114.

Hahn DL (1999) *Chlamydia pneumoniae*, asthma, and COPD: what is the evidence? *Ann Allergy Asthma Immunol* 83: 271–288, 291; quiz 291–272.

Haranaga S, Yamaguchi H, Friedman H, Izumi S & Yamamoto Y (2001) *Chlamydia pneumoniae* infects and multiplies in lymphocytes in vitro. *Infect Immun* 69: 7753–7759.

Harshila P, Erminia D & Robert EM (2003) Mammalian Fibroblasts Lacking Mitochondrial NAD<sup>+</sup>-dependent Methylenetetrahydrofolate Dehydrogenase-Cyclohydrolase Are Glycine Auxotrophs. *J. Biol. Chem.*, 278 (21), 19436-19441.

Hatch TP (1999) Developmental biology. In: Stephens RS (ed) *Chlamydia, Intracellular Biology, Pathogenesis, and Immunity*. American Society for Microbiology, Washington, DC, p 29–67.

Hodinka RL and Wyrick PB (1986). Ultrastructural study of mode of entry of *chlamydia psittaci* into L-929 cells. *Infect. Immun.* 54: 855-863.

Hsia, R-C., Pannekoek, Y., Ingerowski, E. & Bavoil, P. M. (1997). Type III secretion genes identify a putative virulence locus of *Chlamydia*. *Molecular Microbiology* 25, 351-359.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial

pathogens of animals and plants. *Microbiology and Molecular Biology Reviews* 62, 379 - 433.

Huhtinen M, Puolakkainen M, Laasila K, Sarvas M, Karma A & Leirisalo-Repo M (2001) Chlamydial antibodies in patients with previous acute anterior uveitis. *Invest Ophthalmol Vis Sci* 42: 1816–1819.

Ingalls RR, Rice PA, Qureshi N, Takayama K, Lin JS & Golenbock DT (1995) The inflammatory cytokine response to *Chlamydia trachomatis* infection is endotoxin mediated. *Infect Immun* 63: 3125–3130.

Kalayoglu MV, Hoerneman B, LaVerda D, Morrison SG, Morrison RP & Byrne GI (1999) Cellular oxidation of low-density lipoprotein by *Chlamydia pneumoniae*. *J Infect Dis* 180: 780–790.

Kalman S, Mitchell W, Marathe R, Lammel C, Fan J, Hyman RW, Olinger L, Grimwood J, Davis RW & Stephens RS (1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* 21: 385–389.

Karvonen M, Tuomilehto J, Naukkarinen A & Saikku P (1992) The prevalence and regional distribution of antibodies against *Chlamydia pneumoniae* (strain TWAR) in Finland in 1958. *Int J Epidemiol* 21: 391–398.

Kauppinen MT, Herva E and Kujala P (1995) The etiology of community-acquired pneumonia among hospitalized patients during a *Chlamydia pneumoniae* epidemic in Finland. *J Infect Dis* 172:1330-1335.

Kauppinen MT, Herva E, Kujala P, Leinonen M, Saikku P & Syrjälä H (1995) The etiology of community-acquired pneumonia among hospitalized patients during a *Chlamydia pneumoniae* epidemic in Finland. *J Infect Dis* 172: 1330–1335.

Khabar KS, al-Zoghaibi F, Dzimir M, Taha M, al-Tuwaijri A, al-Ahdal MN (1996) MTS interferon assay: a simplified cellular dehydrogenase assay for interferon activity using a water-soluble tetrazolium salt. *J Interferon Cytokine Res.* 16:31-3.

Khan MA, Potter CW & Sharrard RM (1996) A reverse transcriptase-PCR based assay for in-vitro antibiotic susceptibility testing of *Chlamydia pneumoniae*. *J Antimicrob Chemother* 37: 677–685.

Kikuta LC, Puolakkainen M, Kuo CC & Campbell LA (1991) Isolation and sequence analysis of the *Chlamydia pneumoniae* GroE operon. *Infect Immun* 59: 4665–4669.

Kinnunen A, Paavonen J & Surcel HM (2001) Heat shock protein 60 specific T-cell response in chlamydial infections. *Scand J Immunol* 54: 76–81.

Kleemola M, Saikku P, Visakorpi R, Wang SP & Grayston JT (1988) Epidemics of pneumonia caused by TWAR, a new *Chlamydia* organism, in military trainees in Finland. *J Infect Dis* 157: 230–236.

Knudsen K, Madsen AS, Mygind P, Christiansen G & Birkelund S (1999) Identification of two novel genes encoding 97- to 99-kilodalton outer membrane proteins of *Chlamydia pneumoniae*. *Infect Immun* 67: 375–383.

Kornak JM, Kuo CC & Campbell LA (1991) Sequence analysis of the gene encoding the *Chlamydia pneumoniae* DnaK protein homolog. *Infect Immun* 59: 721–725.

Kubo, A. & Stephens, R. S. (1998). Temporal differences in transcription of type III secretion genes during the *Chlamydia trachomatis* developmental cycle. Page 539-542. In: *Chlamydial infections. Proceedings of the ninth international symposium on human chlamydial infection.* (Stephens, R. S. et al., eds). International Chlamydia Symposium, San Francisco, ISBN 0-9664383-02.

Kuo CC, and Chi EY (1987) Ultrastructural study of *Chlamydia trachomatis* surface antigens by immunogold staining with monoclonal antibodies. *Infect Immun* 55:1324-1328.

Kuo CC, Jackson LA, Campbell LA & Grayston JT (1995) *Chlamydia pneumoniae* (TWAR). *Clin Microbiol Rev* 8: 451–461.

Laurila A, Anttila T, Läärä E, Bloigu A, Virtamo J, Albanes D, Leinonen M & Saikku P (1997) Serological evidence of an association between *Chlamydia pneumoniae* infection and lung cancer. *Int J Cancer* 74: 31–34.

LaVerda D & Byrne GI (1997) Use of monoclonal antibodies to facilitate identification, cloning, and purification of *Chlamydia trachomatis* hsp10.

J Clin Microbiol 35: 1209–1215.

Levine, S. J. (1995) Bronchial epithelial cell-cytokine interactions in airway inflammation. *J. Investig. Med.* 43:241-249.

Lieberman D, Schlaeffer F, and Boldur I (1996) Multiple pathogens in adult patients admitted with community acquired pneumonia: a one year prospective study of 346 consecutive patients. *Thorax* 51:179-184.

Lim WS, Macfarlane JT, and Boswell TCJ (2001) Study of community acquired pneumonia aetiology (SCAPA) in adults admitted to hospital: implications for management guidelines. *Thorax* 56,296-301.

Linnanmäki E, Leinonen M, Mattila K, Nieminen MS, Valtonen V & Saikku P (1993) Chlamydia pneumoniae-specific circulating immune complexes in patients with chronic coronary heart disease. *Circulation* 87: 1130–1134.

Longbottom D, Russell M, Dunbar SM, Jones GE & Herring AJ (1998) Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the Chlamydia psittaci subtype that causes abortion in sheep. *Infect Immun* 66: 1317–1324.

Lukacova M, Baumann M, Brade L, Mamat U & Brade H (1994) Lipopolysaccharide smooth-rough phase variation in bacteria of the genus Chlamydia. *Infect Immun* 62: 2270–2276.

Marrie TJ, Grayston JT and Wang SP (1987) Pneumonia associated with the TWAR strain of *Chlamydia*. *Ann Intern Med* 106:507-511.

Marston BJ, Plouffe JF and File TM Jr (1997) Incidence of community-acquired pneumonia requiring hospitalizations: results of a population-based active surveillance study in Ohio. Community-based pneumonia incidence study group. *Arch Intern Med* 157:1709-1718.

Mayr M, Kiechl S, Willeit J, Wick G & Xu Q (2000) Infections, immunity, and atherosclerosis: associations of antibodies to Chlamydia pneumoniae, Helicobacter pylori, and cytomegalovirus with immune reactions to heat-shock protein 60 and carotid or femoral atherosclerosis. *Circulation* 102: 833–839.

McCafferty MC, Maley SW and Buxton D (1994) The importance of

interferon gamma in an early infection of chlamydia psittaci in mice. *Immunology* 81: 631-636.

Miyashita N, Fukano H, Yoshida K, Niki Y & Matsushima T (2002) In-vitro activity of moxifloxacin and other fluoroquinolones against *Chlamydia* species. *J Infect Chemother* 8: 115–117.

Moazed TC, Kuo CC, Grayston JT & Campbell LA (1998) Evidence of systemic dissemination of *Chlamydia pneumoniae* via macrophages in the mouse. *J Infect Dis* 177: 1322–1325.

Montalban GS, Roblin PM & Hammerschlag MR (1994) Performance of three commercially available monoclonal reagents for confirmation of *Chlamydia pneumoniae* in cell culture. *J Clin Microbiol* 32: 1406–1407.

Morrison RP, Belland RJ, Lyng K & Caldwell HD (1989a) Chlamydial disease pathogenesis. The 57-kD chlamydial hypersensitivity antigen is a stress response protein. *J Exp Med* 170: 1271–1283.

Morrison RP, Lyng K & Caldwell HD (1989b) Chlamydial disease pathogenesis. Ocular hypersensitivity elicited by a genus-specific 57-kD protein. *J Exp Med* 169: 663–675.

Moulder JW, Hatch JP, Kuo CC, Schachter JT & Storz J (1984) Genus *Chlamydia*. In: Krieg NR & Holt JG (eds) *Bergey's Manual of Systematic Bacteriology*, vol 1. Williams & Wilkins, Baltimore, MD, p 729–739.

Moulder JW, Hatch TP and KUO CC (1984) *Chlamydia* Jones, Rake and Stearns 1945, 55, p. 729-735. in N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology vol.1. the Williams and Wilkins Co., Baltimore.*

Mygind P, Christiansen G & Birkelund S (1998) Topological analysis of *Chlamydia trachomatis* L2 outer membrane protein 2. *J Bacteriol* 180: 5784–5787.

Normann E, Gnarpe J and Wettergren B (1998) *Chlamydia pneumoniae* in children with acute respiratory tract infections. *Acta Paediatrica* 87: 23-27.

Normann E, Gnarpe J, Gnarpe H & Wettergren B (1998) *Chlamydia pneumoniae* in children attending day-care centers in Gavle, Sweden. *Pediatr Infect Dis J* 17: 474–478.

Nurminen M, Leinonen M, Saikku P & Mäkelä PH (1983) The genus-specific antigen of Chlamydia: resemblance to the lipopolysaccharide of enteric bacteria. *Science* 220: 1279–1281.

Olson M, Ashworth A and Hall A(1995) An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* 269 (5228): 1270-1272.

Peeling RW & Mabey DC (1999) Heat shock protein expression and immunity in chlamydial infections. *Infect Dis Obstet Gynecol* 7: 72–79.

Perez Melgosa M, Kuo CC & Campbell LA (1991) Sequence analysis of the major outer membrane protein gene of Chlamydia pneumoniae. *Infect Immun* 59: 2195–2199.

Priscilla BW (2000) Intracellular survival by Chlamydia. *Cellular Microbiology* 4:275-282.

Roblin PM, Dumornay W & Hammerschlag MR (1992) Use of HEp-2 cells for improved isolation and passage of Chlamydia pneumoniae. *J Clin Microbiol* 30: 1968–1971.

Rockey DD, Heinzen RA & Hackstadt T (1995) Cloning and characterization of a Chlamydia psittaci gene coding for a protein localized in the inclusion membrane of infected cells. *Mol Microbiol* 15: 617–626.

Rockey DD, Lenart J & Stephens RS (2000) Genome sequencing and our understanding of chlamydiae. *Infect Immun* 68: 5473–5479.

Rockey, D. D. & Matsumoto, A. (1999). The chlamydial developmental cycle. In: *Prokaryotic Development*, pp. 403 - 425. (Brun, Y. V. & Shimkets, L. J. eds). Washington, D.C.: ASM Press.

Rothermel CD, Rubin BY, Jaffe EA and Murray HW (1986) Oxygen-dependent inhibition of intracellular chlamydia psittaci growth by human monocytes and interferon gamma activated macrophages. *J. Immunol* 137 :689-692.

Sago, H.; Kitagawa, M.; Obata, S.; Mori, N.; Taketani, S.; Rochelle, J. M.; Seldin, M. F.; Davidson, M.; St. John, T. and Suzuki, S. T. (1995) Cloning, expression, and chromosomal localization of a novel cadherin-related protein, protocadherin-3. *Genomics* 29: 631-640.

Saikku P (1999) Chronic Chlamydia pneumoniae infections. In: Allegra L & Blasi F (eds) Chlamydia pneumoniae, The Lung and the Heart. Springer-Verlag, Milan, p 96–113.

Saikku P (2002) Chlamydia pneumoniae – an update on clinical disease. In: Schachter J et al. (eds) Chlamydial Infections. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. Antalya, Turkey, June 2002, p 443–453.

Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Mäkelä PH, Huttunen JK & Valtonen V (1988) Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* 2: 983–986.

Saikku P, Leinonen M, Tenkanen L, Linnanmäki E, Ekman MR, Manninen V, Mänttari M, Frick MH & Huttunen JK (1992) Chronic Chlamydia pneumoniae infection as a risk factor for coronary heart disease in the Helsinki Heart Study. *Ann Intern Med* 116: 273–278.

Saikku P, Wang SP, Kleemola M, Brander E, Rusanen E & Grayston JT (1985) An epidemic of mild pneumonia due to an unusual strain of Chlamydia psittaci. *J Infect Dis* 151: 832–839.

Schachter J & Grayston JT (1998) Epidemiology of human chlamydial infections. In: Stephens RS et al. (eds) Chlamydial Infections. Proceedings of the Ninth International Symposium on Human Chlamydial Infections. San Francisco, CA, June 1998, p 3–10.

Schachter J, Stephens RS, Timms P, Kuo C, Bavoil PM, Birkelund S, Boman J, Caldwell H, Campbell LA, Chernesky M, Christiansen G, Clarke IN, Gaydos C, Grayston JT, Hackstadt T, Hsia R, Kaltenboeck B, Leinonen M, Ocjius D, McClarty G, Orfila J, Peeling R, Puolakkainen M, Quinn TC, Rank RG, Raulston J, Ridgeway GL, Saikku P, Stamm WE, Taylor-Robinson DT, Wang SP & Wyrick PB (2001) Radical changes to chlamydial taxonomy are not necessary just yet. *Int J Syst Evol Microbiol* 51: 249, 251–243.

Scidmore-Carlson MA, Shaw EI, Dooley CA, Fischer ER & Hackstadt T (1999) Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. *Mol Microbiol* 33: 753–765.

Shemer-Avni Y, and Lieberman D (1995) *Chlamydia pneumoniae*-induced ciliostasis in ciliated bronchial epithelial cells. *J Infect Dis* 171: 1274-1278.

Stephens RS (2002) Chlamydial evolution: a billion years and counting. In: Schachter J et al. (eds) *Chlamydial Infections. Proceedings of the Tenth International Symposium on Human Chlamydial Infections*. Antalya, Turkey, June 2002, p 3–12.

Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, Mitchell W, Olinger L, Tatusov RL, Zhao Q, Koonin EV & Davis RW (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* 282: 754–759.

Stephens RS, Koshiyama K, Lewis E & Kubo A (2001) Heparin-binding outer membrane protein of chlamydiae. *Mol Microbiol* 40: 691–699.

Stirling P. and Richmond S (1987) Production of outer membrane blebs during chlamydial replication. *FEMS Microbiol. Lett* 9:103-105.

Su H, Watkins NG, and Caldwell HD (1990) *Chlamydia trachomatis* host cell interaction: role of the chlamydial major outer membrane protein as adhesin. *Infect. Immun* 58:1017-1025.

Tao S, Kaul R and Wenman WM (1991) Identification and nucleotide sequence of a developmentally regulated gene encoding a eukaryotic histone H1-like protein from *chlamydia trachomatis*. *J Bacteriol* 173: 2818-2822.

Timothy C. Hallstrom and Joseph R (2003) Specificity in the activation and control of transcription factor E2F-dependent apoptosis. *PNAS*, 100 (19): 10848-10853

Ting LM, Hsia RC, Haidaris CG & Bavoil PM (1995) Interaction of outer envelope proteins of *Chlamydia psittaci* GPIC with the HeLa cell surface. *Infect Immun* 63: 3600–3608.

Wang SP & Grayston JT (1970) Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. *Am J Ophthalmol* 70: 367–374.

Ward ME (1995) The immunobiology and immunopathology of chlamydial infections. *Apmis* 103: 769–796.

Welsh L, Gaydos C & Quinn TC (1996) In vitro activities of azithromycin, clarithromycin, erythromycin, and tetracycline against 13 strains of *Chlamydia pneumoniae*. *Antimicrob Agents Chemother* 40: 212–214.

Wolf K, Fischer E, Mead D, Zhong G, Peeling R, Whitmire B & Caldwell HD (2001) *Chlamydia pneumoniae* major outer membrane protein is a surface-exposed antigen that elicits antibodies primarily directed against conformation-dependent determinants. *Infect Immun* 69: 3082–3091.

Yang ZP, Kuo CC & Grayston JT (1995) Systemic dissemination of *Chlamydia pneumoniae* following intranasal inoculation in mice. *J Infect Dis* 171: 736–738.

Zhong, G. and de la Maza, L. M. (1988) Activation of mouse peritoneal macrophages in vitro or in vivo by recombinant murine gamma interferon inhibits the growth of *Chlamydia trachomatis* serovar L1. *Infect.Immun.*, 56, 3322-3325.

# *APPENDIX*

Table 4. *Chlamydia pneumoniae* finding and co-pathogen over 12-months study period

Date	No.	Age	Sex	C.Pneumonia	Co-pathogen
Jan 05, 2002	1	1.6	M	Negative	-
Jan 05, 2002	2	49	M	<b>Positive</b>	S. aureus
Jan 05, 2002	3	0.01	M	Negative	-
Jan 06, 2002	4	4	M	<b>Positive</b>	-
Jan 06, 2002	5	6	M	Negative	-
Jan 06, 2002	6	1.6	F	Negative	-
Jan 06, 2002	7	17	F	Negative	-
Jan 07, 2002	8	0.04	M	Negative	-
Jan 07, 2002	9	0.11	M	Negative	-
Jan 08, 2002	10	6	F	Negative	-
Jan 08, 2002	11	50	M	Negative	-
Jan 08, 2002	12	1.6	M	Negative	-
Jan 08, 2002	13	0.4	M	Negative	-
Jan 08, 2002	14	62	M	Negative	-
Jan 08, 2002	15	0.024	M	Negative	-
Jan 09, 2002	16	4	F	Negative	-
Jan 09, 2002	17	4	F	<b>Positive</b>	-
Jan 12, 2002	18	0.2	M	Negative	-
Jan 12, 2002	19	0.015	F	Negative	-
Jan 13, 2002	20	0.5	F	Negative	-
Jan 13, 2002	21	5	M	Negative	-
Jan 13, 2002	22	40	M	Negative	-
Jan 13, 2002	23	4	F	<b>Positive</b>	-
Jan 14, 2002	24	0.5	M	Negative	-
Jan 14, 2002	25	31	F	Negative	-
Jan 14, 2002	26	0.3	M	Negative	-
Jan 14, 2002	27	35	F	Negative	-
Jan 15, 2002	28	8	F	Negative	-
Jan 15, 2002	29	0.8	M	Negative	-
Jan 15, 2002	30	0.7	M	Negative	-
Jan 15, 2002	31	85	M	<b>Positive</b>	-
Jan 15, 2002	32	0.6	M	Negative	-
Jan 16, 2002	33	0.1	M	Negative	-
Jan 16, 2002	34	55	M	<b>Positive</b>	-
Jan 16, 2002	35	1	F	Negative	-
Jan 19, 2002	36	1.2	F	Negative	-
Jan 19, 2002	37	0.1	F	Negative	-
Jan 20, 2002	38	0.1	F	Negative	-
Jan 20, 2002	39	85	M	Negative	-
Jan 21, 2002	40	58	F	Negative	-
Jan 21, 2002	41	2	M	Negative	-
Jan 21, 2002	42	55	M	Negative	-
Jan 21, 2002	43	1	F	Negative	-
Jan 22, 2002	44	2.2	F	<b>Positive</b>	-
Jan 22, 2002	45	12	F	Negative	-
Jan 22, 2002	46	16	M	Negative	-
Jan 22, 2002	47	12	M	Negative	-
Jan 22, 2002	48	1.3	M	<b>Positive</b>	-
Jan 23, 2002	49	11	M	Negative	-
Jan 23, 2002	50	19	F	Negative	-
Jan 23, 2002	51	53	M	Negative	-
Jan 26, 2002	52	54	M	Negative	-
Jan 26, 2002	53	44	M	Negative	-

Date	No.	Age	Sex	C.Pneumonia	Co-pathogen
Jan 26, 2002	54	62	F	Negative	-
Jan 26, 2002	55	35	F	Negative	-
Jan 26, 2002	56	23	M	Negative	-
Jan 26, 2002	57	1.3	M	Negative	-
Jan 26, 2002	58	23	F	Negative	-
Jan 27, 2002	59	65	F	Negative	-
Jan 27, 2002	60	3.4	M	<b>Positive</b>	-
Jan 27, 2002	61	76	M	Negative	-
Jan 27, 2002	62	45	F	Negative	-
Jan 27, 2002	63	3.4	M	Negative	-
Jan 27, 2002	64	34	F	Negative	-
Jan 27, 2002	65	34	F	Negative	-
Jan 27, 2002	66	0.7	F	Negative	-
Jan 27, 2002	67	76	F	Negative	-
Jan 28, 2002	68	11	F	Negative	-
Jan 28, 2002	69	0.2	M	Negative	-
Jan 28, 2002	70	0.6	M	Negative	-
Jan 28, 2002	71	78	M	<b>Positive</b>	-
Jan 28, 2002	72	16	M	Negative	-
Jan 28, 2002	73	0.4	M	Negative	-
Jan 29, 2002	74	34	M	Negative	-
Jan 29, 2002	75	5	F	Negative	-
Jan 30, 2002	76	2	F	Negative	-
Jan 30, 2002	77	1	M	Negative	-
Jan 30, 2002	78	0.4	M	Negative	-
Jan 30, 2002	79	36	M	Negative	-
Jan 30, 2002	80	81	M	Negative	-
Feb 02, 2002	81	12	F	Negative	-
Feb 02, 2002	82	0.025	M-	Negative	-
Feb 03, 2002	83	0.018	M	Negative	-
Feb 03, 2002	84	0.11	M	Negative	-
Feb 03, 2002	85	0.4	M	Negative	-
Feb 03, 2002	86	1	M	Negative	-
Feb 04, 2002	87	0.8	M	Negative	-
Feb 04, 2002	88	0.022	M	Negative	-
Feb 04, 2002	89	2.6	F	Negative	-
Feb 05, 2002	90	3.6	F	Negative	-
Feb 05, 2002	91	2	M	Negative	-
Feb 06, 2002	92	0.5	F	Negative	-
Feb 06, 2002	93	0.5	F	Negative	-
Feb 06, 2002	94	25	F	Negative	-
Feb 06, 2002	95	7.6	F	Negative	-
Feb 06, 2002	96	4	F	Negative	-
Feb 09, 2002	97	3	M	Negative	-
Feb 09, 2002	98	1.6	M	Negative	-
Feb 09, 2002	99	12	F	Negative	-
Feb 10, 2002	100	43	M	Negative	-
Feb 10, 2002	101	3	M	Negative	-
Feb 11, 2002	102	27	F	Negative	-
Feb 11, 2002	103	60	F	Negative	-
Feb 11, 2002	104	8	F	<b>Positive</b>	-
Feb 11, 2002	105	23	M	Negative	-
Feb 11, 2002	106	1	M	Negative	-
Feb 12, 2002	107	0.7	M	Negative	-
Feb 12, 2002	108	0.1	M	Negative	-
Feb 12, 2002	109	5	M	<b>Positive</b>	-

Feb 12, 2002	110	1.9	F	Negative	-
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Feb 13, 2002	111	19	F	Negative	-
Feb 13, 2002	112	12	M	Negative	-
Feb 13, 2002	113	6	F	Negative	-
Feb 13, 2002	114	6	F	Negative	-
Feb 16, 2002	115	3	M	Negative	-
Feb 16, 2002	116	0.5	M	Negative	-
Feb 16, 2002	117	15	M	Negative	-
Feb 16, 2002	118	25	M	Negative	-
Feb 17, 2002	119	6	F	Negative	-
Feb 17, 2002	120	43	F	Negative	-
Feb 18, 2002	121	13	M	Negative	-
Feb 18, 2002	122	6	F	Negative	-
Feb 18, 2002	123	12	M	Negative	-
Feb 18, 2002	124	43	F	Negative	-
Feb 19, 2002	125	23	M	Negative	-
Feb 23, 2002	126	27	M	Negative	-
Feb 25, 2002	127	43	M	Negative	-
Feb 26, 2002	128	5.3	M	Negative	-
Mar 02, 2002	129	40	F	Negative	-
Mar 02, 2002	130	80	F	Negative	-
Mar 03, 2002	131	7	F	Negative	-
Mar 03, 2002	132	0.2	F	Negative	-
Mar 04, 2002	133	0.3	F	Negative	-
Mar 05, 2002	134	0.2	M	Negative	-
Mar 05, 2002	135	0.7	M	Negative	-
Mar 06, 2002	136	0.2	M	Negative	-
Mar 06, 2002	137	71	M	Negative	-
Mar 09, 2002	138	0.021	F	Negative	-
Mar 09, 2002	139	1	M	Negative	-
Mar 10, 2002	140	0.1	F	Negative	-
Mar 11, 2002	141	0.047	M	Negative	-
Mar 11, 2002	142	0.4	F	Negative	-
Mar 11, 2002	143	0.041	F	Negative	-
Mar 11, 2002	144	0.4	F	Negative	-
Mar 12, 2002	145	0.2	M	<b>Positive</b>	-
Mar 12, 2002	146	22	M	Negative	-
Mar 13, 2002	147	28	M	Negative	-
Mar 13, 2002	148	0.8	F	Negative	-
Mar 13, 2002	149	26	F	Negative	-
Mar 16, 2002	150	2.6	F	Negative	-
Mar 16, 2002	151	0.4	F	Negative	-
Mar 17, 2002	152	10	F	Negative	-
Mar 18, 2002	153	64	F	Negative	-
Mar 18, 2002	154	0.2	M	Negative	-
Mar 18, 2002	155	5	M	Negative	-
Mar 18, 2002	156	10	F	Negative	-
Mar 20, 2002	157	23	F	Negative	-
Mar 20, 2002	158	53	M	Negative	-
Mar 20, 2002	159	65	M	Negative	-
Mar 23, 2002	160	0.3	F	<b>Positive</b>	-
Mar 23, 2002	161	0.1	M	Negative	-
Mar 25, 2002	162	0.1	M	Negative	-
Mar 25, 2002	163	0.1	M	Negative	-
Mar 25, 2002	164	64	F	Negative	-
Mar 26, 2002	165	23	F	Negative	-

Mar 26, 2002	166	13	F	Negative	-
Mar 27, 2002	167	0.01	M	Negative	-
Date	No.	Age	Sex	C.Pneumonia	Co-pathogen
Mar 27, 2002	168	7	M	Negative	-
Mar 27, 2002	169	14	F	Negative	-
Mar 27, 2002	170	1	M	Negative	-
Mar 27, 2002	171	14	F	Negative	-
Mar 30, 2002	172	0.2	F	Negative	-
Mar 30, 2002	173	12	M	Negative	-
Mar 30, 2002	174	43	F	Negative	-
Mar 30, 2002	175	1	M	Negative	-
Mar 30, 2002	176	16	M	Negative	-
Mar 31, 2002	177	22	M	Negative	-
Mar 31, 2002	178	41	M	Negative	-
Mar 31, 2002	179	12	M	Negative	-
Mar 31, 2002	180	54	M	Negative	-
Mar 31, 2002	181	0.5	F	Negative	-
Mar 31, 2002	182	3	M	Negative	-
Apr 01, 2002	183	61	F	Negative	-
Apr 02, 2002	184	35	F	Negative	-
Apr 02, 2002	185	1	F	Negative	-
Apr 03, 2002	186	12	F	Negative	-
Apr 03, 2002	187	3.6	F	Negative	-
Apr 06, 2002	188	35	M	Negative	-
Apr 07, 2002	189	29	F	Negative	-
Apr 09, 2002	190	25	M	Negative	-
Apr 10, 2002	191	0.1	M	Negative	-
Apr 10, 2002	192	6	M	Negative	-
Apr 13, 2002	193	51	M	Negative	-
Apr 13, 2002	194	0.3	M	Negative	-
Apr 14, 2002	195	47	M	Negative	-
Apr 14, 2002	196	50	M	Negative	-
Apr 15, 2002	197	2.6	M	Negative	-
Apr 16, 2002	198	6	M	Negative	-
Apr 17, 2002	199	57	M	<b>Positive</b>	-
Apr 17, 2002	200	1.3	F	Negative	-
Apr 20, 2002	201	0.6	F	Negative	-
Apr 20, 2002	202	45	F	<b>Positive</b>	-
Apr 20, 2002	203	0.5	M	Negative	-
Apr 21, 2002	204	28	M	Negative	-
Apr 21, 2002	205	2	M	Negative	-
Apr 21, 2002	206	54	F	Negative	-
Apr 22, 2002	207	32	M	Negative	-
Apr 22, 2002	208	32	M	Negative	-
Apr 22, 2002	209	1	F	Negative	-
Apr 23, 2002	210	1	F	Negative	-
Apr 23, 2002	211	3	F	Negative	-
Apr 24, 2002	212	32	F	Negative	-
Apr 27, 2002	213	30	F	Negative	-
Apr 27, 2002	214	30	M	Negative	-
Apr 27, 2002	215	1	F	Negative	-
Apr 28, 2002	216	4	M	Negative	-
Apr 28, 2002	217	32	M	Negative	-
Apr 28, 2002	218	0.9	M	Negative	-
Apr 28, 2002	219	42	M	Negative	-
Apr 29, 2002	220	10	M	Negative	-
Apr 29, 2002	221	3	F	Negative	-

Apr 29, 2002	222	32	M	Negative	-
Apr 29, 2002	223	50	F	Negative	-
Apr 20, 2002	224	2	F	Negative	-
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Apr 29, 2002	225	16	F	Negative	-
Apr 29, 2002	226	54	M	Negative	-
Apr 29, 2002	227	0.003	M	Negative	-
Apr 30, 2002	228	8	M	Negative	-
Apr 30, 2002	229	5	M	Negative	-
Apr 30, 2002	230	34	F	Negative	-
May 01, 2002	231	60	M	<b>Positive</b>	-
May 04, 2002	232	7	M	Negative	-
May 04, 2002	233	0.9	M	Negative	-
May 06, 2002	234	6	M	Negative	-
May 07, 2002	235	0.1	F	<b>Positive</b>	-
May 08, 2002	236	36	M	Negative	-
May 11, 2002	237	47	F	Negative	-
May 13, 2002	238	3	F	Negative	-
May 13, 2002	239	24	F	Negative	-
May 15, 2002	240	44	F	<b>Positive</b>	-
May 18, 2002	241	0.5	M	<b>Positive</b>	-
May 20, 2002	242	21	M	Negative	-
May 20, 2002	243	63	F	Negative	-
May 22, 2002	244	32	M	Negative	-
May 22, 2002	245	65	M	Negative	-
May 25, 2002	246	4	F	Negative	-
May 26, 2002	247	0.09	M	Negative	-
May 26, 2002	248	38	F	Negative	-
May 27, 2002	249	44	M	Negative	-
May 27, 2002	250	65	M	Negative	-
May 28, 2002	251	43	M	Negative	-
May 28, 2002	252	85	M	Negative	-
May 29, 2002	253	1	F	Negative	-
May 29, 2002	254	1	F	Negative	-
Jun 04, 2002	255	52	M	Negative	-
Jun 08, 2002	256	2	M	Negative	-
Jun 11, 2002	257	0.2	M	Negative	-
Jun 15, 2002	258	4	M	Negative	-
Jun 18, 2002	259	45	M	Negative	-
Jun 24, 2002	260	1	M	Negative	-
Jun 26, 2002	261	13	F	Negative	-
Jun 26, 2002	262	17	F	Negative	-
July 03, 2002	263	5	F	Negative	-
July 06, 2002	264	42	M	Negative	-
July 08, 2002	265	0.2	M	Negative	-
July 09, 2002	266	4	M	<b>Positive</b>	-
July 10, 2002	267	1.6	M	<b>Positive</b>	<b>H. influenza</b>
July 13, 2002	268	73	M	Negative	-
July 14, 2002	269	22	M	Negative	-
July 20, 2002	270	43	M	Negative	-
July 22, 2002	271	1	M	Negative	-
July 24, 2002	272	8	M	Negative	-
July 30, 2002	273	14	M	Negative	-
July 31, 2002	274	21	M	Negative	-

Aug 03, 2002	275	0.7	M	Negative	-
Aug 05, 2002	276	3	F	Negative	-
Aug 06, 2002	277	0.11	F	Negative	-
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Aug 07, 2002	278	0.5	F	Negative	-
Aug 10, 2002	279	0.9	F	Negative	-
Aug 12, 2002	280	6	F	Negative	-
Aug 12, 2002	281	2.6	M	<b>Positive</b>	-
Aug 13, 2002	282	0.021	M	Negative	-
Aug 14, 2002	283	0.3	F	Negative	-
Aug 17, 2002	284	40	F	Negative	-
Aug 17, 2002	285	57	F	<b>Positive</b>	M. pneumoniae
Aug 19, 2002	286	73	M	<b>Positive</b>	-
Aug 20, 2002	287	65	M	<b>Positive</b>	Influenza A virus
Aug 20, 2002	288	0.9	M	Negative	-
Aug 21, 2002	289	48	M	Negative	-
Aug 21, 2002	290	20	M	Negative	-
Aug 24, 2002	291	15	M	Negative	-
Aug 24, 2002	292	2	M	<b>Positive</b>	-
Aug 25, 2002	293	12	F	Negative	-
Aug 25, 2002	294	60	F	<b>Positive</b>	-
Aug 25, 2002	295	35	F	Negative	-
Aug 26, 2002	296	43	F	Negative	-
Aug 26, 2002	297	8	M	Negative	-
Aug 27, 2002	298	8	M	Negative	-
Aug 27, 2002	299	1	M	Negative	-
Aug 27, 2002	300	21	M	Negative	-
Aug 27, 2002	301	80	F	<b>Positive</b>	M. pneumoniae
Aug 28, 2002	302	66	F	Negative	-
Aug 28, 2002	303	66	M	<b>Positive</b>	-
Aug 28, 2002	304	32	F	Negative	-
Aug 31, 2002	305	1	M	Negative	-
Aug 31, 2002	306	1	M	Negative	-
Aug 31, 2002	307	43	M	Negative	-
Aug 31, 2002	308	20	M	Negative	-
Sep 01, 2002	309	45	F	<b>Positive</b>	-
Sep 01, 2002	310	73	F	Negative	-
Sep 01, 2002	311	0.4	F	Negative	-
Sep 01, 2002	312	63	F	Negative	-
Sep 02, 2002	313	0.1	M	Negative	-
Sep 02, 2002	314	0.2	F	Negative	-
Sep 02, 2002	315	9	M	Negative	-
Sep 03, 2002	316	0.9	F	Negative	-
Sep 03, 2002	317	0.11	F	Negative	-
Sep 04, 2002	318	5	M	Negative	-
Sep 04, 2002	319	5.6	M	Negative	-
Sep 04, 2002	320	30	M	Negative	-
Sep 07, 2002	321	6	M	Negative	-
Sep 07, 2002	322	8	M	Negative	-
Sep 08, 2002	323	31	M	Negative	-
Sep 08, 2002	324	2.6	M	Negative	-
Sep 08, 2002	325	0.6	M	Negative	-
Sep 08, 2002	326	4	M	Negative	-
Sep 09, 2002	327	6	M	Negative	-
Sep 09, 2002	328	0.6	M	Negative	-
Sep 09, 2002	329	41	M	Negative	-

Sep 09, 2002	330	6	M	Negative	-
Sep 10, 2002	331	0.1	M	Negative	-
Sep 10, 2002	332	4	M	Negative	-
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Sep 10, 2002	333	8	M	Negative	-
Sep 10, 2002	334	11	M	Negative	-
Sep 10, 2002	335	0.014	F	Negative	-
Sep 11, 2002	336	5	M	Negative	-
Sep 11, 2002	337	0.3	F	Negative	-
Sep 11, 2002	338	61	M	Negative	-
Sep 11, 2002	339	3.6	M	Negative	-
Sep 11, 2002	340	4	M	Negative	-
Sep 11, 2002	341	54	M	<b>Positive</b>	-
Sep 11, 2002	342	0.1	F	Negative	-
Sep 11, 2002	343	0.4	F	Negative	-
Sep 11, 2002	344	2.6	F	Negative	-
Sep 11, 2002	345	5	M	Negative	-
Sep 11, 2002	346	0.3	M	Negative	-
Sep 14, 2002	347	1	M	Negative	-
Sep 14, 2002	348	0.1	M	Negative	-
Sep 14, 2002	349	3	M	Negative	-
Sep 14, 2002	350	44	M	<b>Positive</b>	S. pneumoniae
Sep 14, 2002	351	0.1	F	Negative	-
Sep 14, 2002	352	56	M	Negative	-
Sep 14, 2002	353	7	M	Negative	-
Sep 15, 2002	354	1	M	Negative	-
Sep 15, 2002	355	56	F	Negative	-
Sep 15, 2002	356	0.1	F	Negative	-
Sep 15, 2002	357	0.2	F	Negative	-
Sep 15, 2002	358	41	M	<b>Positive</b>	-
Sep 15, 2002	359	32	M	Negative	-
Sep 15, 2002	360	22	M	Negative	-
Sep 15, 2002	361	51	M	Negative	-
Sep 16, 2002	362	5	M	Negative	-
Sep 16, 2002	363	33	M	Negative	-
Sep 16, 2002	364	2	M	Negative	-
Sep 16, 2002	365	5	M	Negative	-
Sep 16, 2002	366	33	M	Negative	-
Sep 17, 2002	367	3	M	Negative	-
Sep 17, 2002	368	5	M	Negative	-
Sep 18, 2002	369	1	M	Negative	-
Sep 18, 2002	370	43	M	Negative	-
Sep 18, 2002	371	0.2	M	Negative	-
Sep 21, 2002	372	33	M	Negative	-
Sep 21, 2002	373	4	M	Negative	-
Sep 21, 2002	374	0.1	F	Negative	-
Sep 22, 2002	375	65	F	Negative	-
Sep 22, 2002	376	8	F	Negative	-
Sep 22, 2002	377	13	F	Negative	-
Sep 22, 2002	378	0.3	F	Negative	-
Sep 23, 2002	379	4	M	Negative	-
Sep 23, 2002	380	2	M	Negative	-
Sep 23, 2002	381	56	F	Negative	-
Sep 23, 2002	382	3	F	Negative	-
Sep 24, 2002	383	5	M	Negative	-
Sep 24, 2002	384	17	M	Negative	-
Sep 24, 2002	385	65	F	Negative	-
Sep 25, 2002	386	62	F	Negative	-

Sep 25, 2002	387	4	F	Negative	-
Sep 28, 2002	388	23	F	Negative	-
Sep 28, 2002	389	44	F	Negative	-
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Sep 28, 2002	390	13	F	Negative	-
Sep 29, 2002	391	1	F	Negative	-
Sep 30, 2002	392	23	F	Negative	-
Sep 30, 2002	393	5	M	Negative	-
Sep 30, 2002	394	53	M	Negative	-
Oct 01, 2002	395	63	F	Negative	-
Oct 01, 2002	396	0.6	F	Negative	-
Oct 01, 2002	397	41	F	Negative	-
Oct 02, 2002	398	6	M	Negative	-
Oct 02, 2002	399	0.1	F	Negative	-
Oct 02, 2002	400	4	F	Negative	-
Oct 05, 2002	401	8	M	Negative	-
Oct 05, 2002	402	11	M	Negative	-
Oct 05, 2002	403	0.014	M	Negative	-
Oct 07, 2002	404	5	M	Negative	-
Oct 07, 2002	405	0.3	M	Negative	-
Oct 07, 2002	406	61	M	Negative	-
Oct 08, 2002	407	0.3	M	Negative	-
Oct 09, 2002	408	4	M	Negative	-
Oct 09, 2002	409	12	M	Negative	-
Oct 09, 2002	410	0.1	M	Negative	-
Oct 09, 2002	411	0.4	M	Negative	-
Oct 09, 2002	412	2.6	M	Negative	-
Oct 09, 2002	413	5	M	Negative	-
Oct 12, 2002	414	6	F	Negative	-
Oct 12, 2002	415	0.3	F	Negative	-
Oct 12, 2002	416	1	F	Negative	-
Oct 12, 2002	417	0.1	M	Negative	-
Oct 13, 2002	418	3	M	Negative	-
Oct 13, 2002	419	0.2	F	Negative	-
Oct 13, 2002	420	0.1	F	Negative	-
Oct 14, 2002	421	30	F	Negative	-
Oct 14, 2002	422	6	F	Negative	-
Oct 14, 2002	423	54	F	Negative	-
Oct 15, 2002	424	12	M	Negative	-
Oct 16, 2002	425	31	M	Negative	-
Oct 19, 2002	426	2	M	Negative	-
Oct 21, 2002	427	2	F	Negative	-
Oct 21, 2002	428	5	F	Negative	-
Oct 21, 2002	429	17	F	Negative	-
Oct 22, 2002	430	6	M	Negative	-
Oct 22, 2002	431	0.01	M	Negative	-
Oct 23, 2002	432	53	M	Negative	-
Oct 23, 2002	433	54	F	Negative	-
Oct 26, 2002	434	5	F	Negative	-
Oct 26, 2002	435	10	F	Negative	-
Oct 26, 2002	436	22	M	Negative	-
Oct 27, 2002	437	1	M	Negative	-
Oct 27, 2002	438	9	M	Negative	-
Oct 27, 2002	439	11	F	Negative	-
Oct 27, 2002	440	43	F	Negative	-
Oct 28, 2002	441	76	M	Negative	-
Oct 29, 2002	442	34	F	Negative	-

Oct 29, 2002	443	2	M	Negative	-
Oct 30, 2002	444	34	F	Negative	-
Oct 30, 2002	445	44	F	<b>Positive</b>	<i>S. pneumoniae</i>
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Oct 30, 2002	446	52	F	<b>Positive</b>	<i>S. pneumoniae</i>
Nov 02, 2002	447	1	M	<b>Positive</b>	<i>S. pneumoniae</i>
Nov 02, 2002	448	63	M	<b>Positive</b>	-
Nov 03, 2002	449	60	M	<b>Positive</b>	<i>S. pneumoniae</i>
Nov 03, 2002	450	67	M	Negative	-
Nov 03, 2002	451	3	F	<b>Positive</b>	Influenza A virus
Nov 04, 2002	452	0.7	M	<b>Positive</b>	-
Nov 04, 2002	453	4	F	<b>Positive</b>	-
Nov 04, 2002	454	0.2	M	Negative	-
Nov 05, 2002	455	9	M	Negative	-
Nov 05, 2002	456	11	M	Negative	-
Nov 05, 2002	457	0.9	M	Negative	-
Nov 06, 2002	458	0.8	F	Negative	-
Nov 06, 2002	459	6	F	Negative	-
Nov 06, 2002	460	7	F	<b>Positive</b>	<i>M. pneumoniae</i>
Nov 09, 2002	461	41	M	Negative	-
Nov 09, 2002	462	0.9	M	Negative	-
Nov 09, 2002	463	47	M	Negative	-
Nov 10, 2002	464	0.4	M	<b>Positive</b>	<i>H. influenzae</i>
Nov 10, 2002	465	47	M	Negative	-
Nov 11, 2002	466	0.3	M	<b>Positive</b>	-
Nov 12, 2002	467	12	F	Negative	-
Nov 13, 2002	468	0.7	F	Negative	-
Nov 13, 2002	469	61	F	Negative	-
Nov 16, 2002	470	74	F	Negative	-
Nov 16, 2002	471	24	F	Negative	-
Nov 16, 2002	472	0.2	M	Negative	-
Nov 16, 2002	473	27	F	<b>Positive</b>	<i>M. pneumoniae</i>
Nov 16, 2002	474	21	F	<b>Positive</b>	<i>M. pneumoniae</i>
Nov 16, 2002	475	5	F	Negative	-
Nov 16, 2002	476	28	F	Negative	-
Nov 16, 2002	477	1	F	Negative	-
Nov 16, 2002	478	0.7	M	<b>Positive</b>	<i>S. pneumoniae</i>
Nov 16, 2002	479	2	F	Negative	-
Nov 17, 2002	480	0.5	M	<b>Positive</b>	-
Nov 17, 2002	481	0.1	M	Negative	-
Nov 17, 2002	482	52	M	Negative	-
Nov 17, 2002	483	2	M	Negative	-
Nov 17, 2002	484	0.7	F	<b>Positive</b>	-
Nov 17, 2002	485	82	M	<b>Positive</b>	-
Nov 17, 2002	486	45	M	<b>Positive</b>	-
Nov 18, 2002	487	1.3	M	<b>Positive</b>	-
Nov 18, 2002	488	0.1	M	<b>Positive</b>	<i>H. influenzae</i>
Nov 18, 2002	489	2	F	<b>Positive</b>	-
Nov 18, 2002	490	9	M	<b>Positive</b>	<i>S. pneumoniae</i>
Nov 18, 2002	491	1	M	Negative	-
Nov 18, 2002	492	21	M	Negative	-
Nov 18, 2002	493	22	M	Negative	-
Nov 18, 2002	494	1	M	<b>Positive</b>	-
Nov 18, 2002	495	1	M	<b>Positive</b>	-
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Nov 19, 2002	496	23	F	Negative	-
Nov 19, 2002	497	64	F	Negative	-

Nov 19, 2002	498	54	F	Negative	-
Nov 19, 2002	499	4	F	Negative	-
Nov 20, 2002	500	43	F	Negative	-
Nov 20, 2002	501	2	M	Negative	-
Nov 20, 2002	502	11	M	Negative	-
Nov 20, 2002	503	78	F	Negative	-
Nov 23, 2002	504	15	M	Negative	-
Nov 23, 2002	505	1	M	Negative	-
Nov 24, 2002	506	72	F	Negative	-
Nov 24, 2002	507	32	F	Negative	-
Nov 25, 2002	508	13	F	Negative	-
Nov 25, 2002	509	0.2	M	<b>Positive</b>	-
Nov 25, 2002	510	31	M	<b>Positive</b>	-
Nov 26, 2002	511	27	F	<b>Positive</b>	M. pneumoniae
Nov 26, 2002	512	1	M	<b>Positive</b>	-
Nov 27, 2002	513	14	M	Negative	-
Nov 27, 2002	514	0.8	M	Negative	-
Nov 27, 2002	515	43	F	Negative	-
Nov 27, 2002	516	4	M	Negative	-
Nov 30, 2002	517	16	F	Negative	-
Nov 30, 2002	518	0.4	M	Negative	-
Nov 30, 2002	519	56	M	Negative	-
Nov 30, 2002	520	8	M	Negative	-
Dec 01, 2002	521	4.5	M	<b>Positive</b>	S. pneumoniae
Dec 01, 2002	522	3	F	<b>Positive</b>	-
Dec 01, 2002	523	4	F	Negative	-
Dec 02, 2002	524	81	F	Negative	-
Dec 03, 2002	525	15	M	Negative	-
Dec 03, 2002	526	12	M	Negative	-
Dec 04, 2002	527	9	F	Negative	-
Dec 04, 2002	528	75	F	Negative	-
Dec 07, 2002	529	33	M	Negative	-
Dec 07, 2002	530	1	M	Negative	-
Dec 07, 2002	531	5	F	Negative	-
Dec 08, 2002	532	0.7	M	<b>Positive</b>	-
Dec 08, 2002	533	42	F	Negative	-
Dec 08, 2002	534	0.8	F	Negative	-
Dec 09, 2002	535	1.6	M	<b>Positive</b>	S. pneumoniae
Dec 09, 2002	536	23	M	Negative	-
Dec 09, 2002	537	30	M	Negative	-
Dec 09, 2002	538	43	M	Negative	-
Dec 10, 2002	539	20	F	Negative	-
Dec 10, 2002	540	1	F	Negative	-
Dec 11, 2002	541	23	M	Negative	-
Dec 11, 2002	542	31	M	Negative	-
Dec 11, 2002	543	8	M	Negative	-
Dec 11, 2002	544	7	F	<b>Positive</b>	H. influenzae
Dec 14, 2002	545	5	F	Negative	-
Dec 14, 2002	546	14	F	Negative	-
Dec 14, 2002	547	67	F	Negative	-
Dec 15, 2002	548	0.1	F	Negative	-
<b>Date</b>	<b>No.</b>	<b>Age</b>	<b>Sex</b>	<b>C.Pneumonia</b>	<b>Co-pathogen</b>
Dec 15, 2002	549	1	M	Negative	-
Dec 15, 2002	550	70	M	Negative	-
Dec 15, 2002	551	1	M	Negative	-
Dec 15, 2002	552	0.1	M	Negative	-
Dec 16, 2002	553	0.1	F	Negative	-

Dec 18, 2002	554	70	M	Negative	-
Dec 18, 2002	555	47	M	Negative	-
Dec 20, 2002	556	0.2	F	Negative	-
Dec 20, 2002	557	1	M	Negative	-
Dec 24, 2002	558	0.1	F	Negative	-
Dec 24, 2002	559	50	F	Negative	-
Dec 24, 2002	560	0.1	M	Negative	-
Dec 25, 2002	561	1	M	<b>Positive</b>	-
Dec 25, 2002	562	0.1	F	Negative	-
Dec 25, 2002	563	27	M	Negative	-
Dec 28, 2002	564	53	M	Negative	-
Dec 28, 2002	565	20	F	Negative	-
Dec 28, 2002	566	0.6	F	Negative	-
Dec 30, 2002	567	6	M	Negative	-
Dec 30, 2002	568	0.11	M	Negative	-
Dec 30, 2002	569	1	M	Negative	-
Dec 31, 2002	570	60	M	Negative	-
Dec 31, 2002	571	0.1	M	Negative	-
Dec 31, 2002	572	1	F	Negative	-

.

.

.

.

.

.

.% ,

-:

- % %

. %

.%

.% ( )

. %

*M. pneumoniae*

*S. pneumoniae*

.

( )

( EB- )

( RB - )

.

.

( )

.

.

-

/

-

.

IL-15    TNF- $\alpha$

.

المملكة العربية السعودية

جامعة الملك سعود

قسم النبات والاحياء الدقيقة

تتبع حالات الاصابة واستجابة الخلية مخبريا للعدوي بالكلاميديا نيومونيا

"رسالة مقدمة إلى قسم النبات والاحياء الدقيقة بكلية العلوم استكمالاً لمتطلبات

الحصول علي درجة الدكتوراة في الاحياء الدقيقة – تخصص بكتيريا"

رسالة مقدمة من

محمد بن علي بن محمد مرعي

اشراف

الدكتور / رشيد بن موسي الصم

٢٠٠٤ - ١٤٢٥