Chlamydia pneumoniae:

studies on an emerging pathogen

Roel P. Verkooyen

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Cover photo:

Front: Photomicrograph using fluorescence microscopy (x250) of HeLa 229 infected monolayer with *Chlamydia pneumoniae*.

Back: Photomicrograph using fluorescence microscopy (x600) of purified *Chlamydia pneumoniae* elementary bodies (microimmunofluorescence test).

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die de grootste offers hebben moeten brengen

Abbreviations

2SP	sucrose phosphate Chlamydia transport medium
ADV	adenovirus
ATCC	American Type Culture Collection
BAL	broncho-alveolar lavage
BGM	buffalo green monkey
bp	base pairs
bpp	beats per minute
CAP	community-acquired pneumonia
CF	complement fixation
CI_{95}	95% confidence interval
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
Cp	*Chlamydia pneumoniae*
CPE	cytopathologic effect
CRP	C-reactive protein
DEAE	di-ethyl-amino-ethyl
DNA	deoxyribonucleic acid
EB	elementary body
EBs	elementary bodies
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
EMBL	European molecular biology laboratory
EMEM	Eagle minimum essential medium
ESR	erythrocyte sedimentation rate
FCS	fetal calf serum
GuSCN	guanidinium-thiocyanate
IFU	inclusion forming units
Ig	immunoglobulin
IgA	immunoglobulin A antibody
IgG	immunoglobulin G antibody
IgM	immunoglobulin M antibody
LPS	lipopolysaccharide
MIF	microimmunofluorescence
MOMP	major outer membrane protein
MRL	Microbiology Research Laboratories
NA	nucleic acids
NPV	negative predictive value
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHYLIP	phylogeny inference package
PPV	positive predictive value
RA	rheumatoid arthritis
RB	reticulate body
rDNA	recombinant DNA

RF	rheumatoid factor
RLU	relative light units
RNA	ribo nucleic acid
rRNA	ribosomal RNA
RSV	respiratory syncitial virus
SPG	sucrose phosphate glucose chlamydia storage medium
SPSS	statistical package for the social science
STD	sexual transmitted disease
WBC	white blood cells
WIF	whole inclusion fluorescence
WRF	Washington Research Foundation

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CHAPTER 1

EPIDEMIOLOGY AND DIAGNOSTIC ASPECTS OF *CHLAMYDIA PNEUMONIAE* INFECTION

MICROBIOLOGY AND CLASSIFICATION

The Chlamydiae are characterized as bacteria because of the composition of their cell wall and their growth by binary division. However, they are obligate intracellular bacterial parasites of eukaryotic cells that have a unique replication cycle similar to *Rickettsia*. Chlamydial infection of host cells is initiated by the elementary body (ϕ = 300-400 nm) , a stable structure specifically adapted to survive the extracellular phase during transit between cells. According to the knowledge, the infectious elementary body enters the cell via endocytosis (90,118). Intracellular parasites have evolved several ways to avoid being killed by lysosomal enzymes (139). However, the mechanism by which subsequent fagosome/lysosome fusion is avoided, is not completely understood. After endocytosis of the elementary body, it will reorganize into a non-infectious, metabolic active reticulate body (ϕ = 800 - 1000 nm), which is responsible for intracellular replication through binary fission. At both stages of development the chlamydial cell is surrounded by an envelope similar to that of gram-negative bacteria. The envelope consists of two trilaminar membranes, an outer membrane and an inner, cytoplasmic membrane (24). The fissions occur in the original phagosome that enlarges during this process until it resembles a vacuole. This vacuole is referred to as an inclusion body or in short, an inclusion. During replication, the reticulate body obtains adenosine triphosphate, amino acids and sugars from the host cell (7). Each reticulate body will reorganize into one or more elementary bodies (204). After 48-72 hrs, release of infective elementary bodies will take place due to host cell lysis (Figure 1). The order *Chlamydiales* has one family, Chlamydiaceae, and one genus, *Chlamydia*. There are now four recognized species, *Chlamydia trachomatis, Chlamydia psittaci, Chlamydia pneumoniae* and *Chlamydia pecorum*. Of these, the most recently described *C.pecorum* infects sheep and cattle, and has so far not been associated with human disease (58,59). At this moment, 19 different *C.trachomatis* serovars or serotypes are known, i.e. distinguished from each other by polyclonal antibodies that were formed in mice after they have been infected with a single type Chlamydia (201).

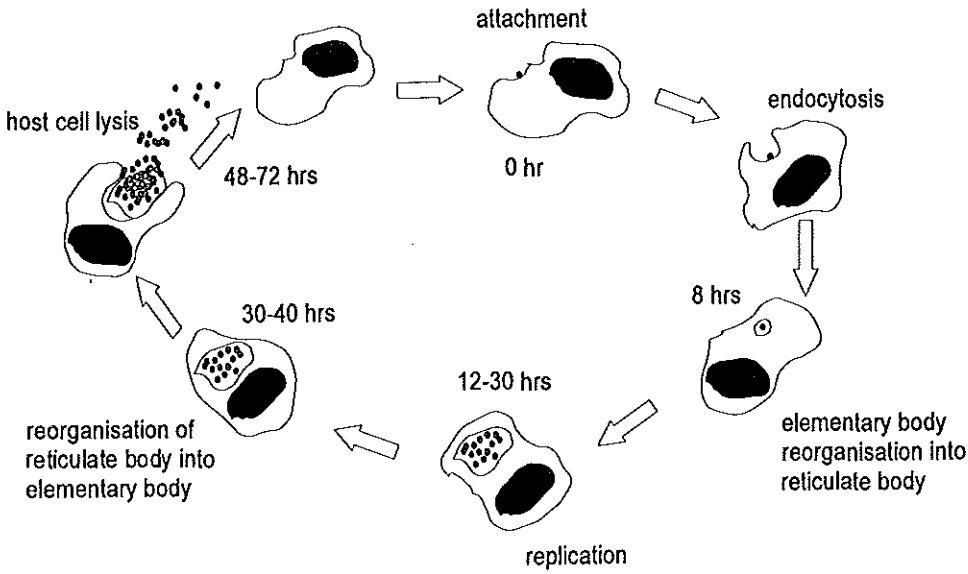

Figure 1. Chlamydia growth cycle.

C.trachomatis is involved in several diseases: trachoma (135,172,188), sexually transmitted diseases (148,152,153, 162,163) and lymphogranuloma venereum (156). Many different *C.psittaci* serovars are known (69). *C.psittaci* is a potential cause of zoonosis, related to psittacosis/ornithosis infection, which is primary a respiratory infection (123,157,174). *C.pneumoniae* is a recently discovered species within the genus *Chlamydia* (75). To date, only one serovar of *C.pneumoniae* is known. Before the acceptance of *C.pneumoniae* as the third chlamydial species, the temporary name *Chlamydia pneumoniae* TWAR was used. TWAR is a designation, which was derived from the laboratory codes of the first two isolates TW-183 and AR-39. The first *C.pneumoniae* strain was isolated in 1965 from the conjunctiva of a child. This child was enrolled in a trachoma vaccine study in Taiwan. It was the 183rd chick embryo egg yolk sac isolate of a Chlamydia organism from a series of studies of trachoma The strain was untypable as a trachoma strain. This strain received the laboratory code TW-183. Several years later when the Chlamydia cell culture was developed, it was found that the TW-183 inclusions formed in cell culture were round and dense, different from *C.trachomatis* (Figure 2). It was therefore assumed

to be a *C.psittaci* strain.

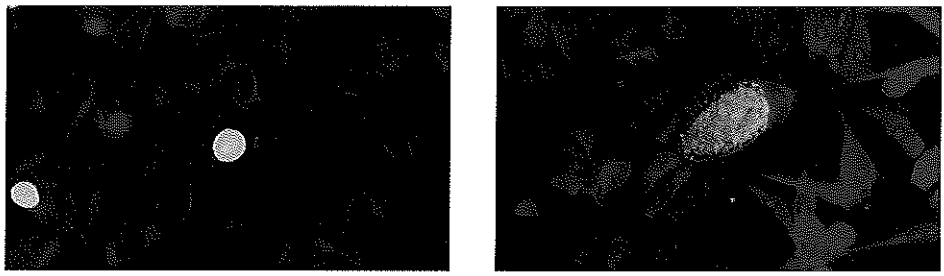

Figure 2, Photomicrograph using fluorescence microscopy (x250) of HeLa 229 infected monolayer with *C.pneumoniae* (left) and *C.trachomatis* (right).

The second strain was isolated from a throat swab taken from a University student in Seattle who had pharyngitis in 1983 (75). Subsequent studies based on immunological, electron microscopy and DNA analysis showed that the TWAR organisms were definitely different from *C.trachomatis* and *C.psittaci* (26,27,32,39,108,109,129). Table 1 shows that *C.pneumoniae* shares less than 10% DNA homology with *C.trachomatis* and *C.psittaci* (39). A high homology of 94-100% between the *C.pneumoniae* strains was observed. Similar results were found among *C.trachomatis* strains, while considerable heterogenicity among *C.psittaci* strains was observed.

Table 1. Percentage of homology in DNA sequence among Chlamydia strains. Three to five isolates of each species were tested (adapted from reference number 39).

	Labeled DNA		
unlabeled DNA	*C.pneumoniae*	*C.psittaci*	*C.trachomatis*
C.pneumoniae	94-100	≤5-8	≤5
C.psittaci	≤5-10	21-100	≤5-11
C.trachomatis	≤5-7	≤5-6	97-100

Ultrastructural studies have shown that *C.pneumoniae* has a pear-shaped elementary body. The cytoplasmic mass is round and there is a large periplasmatic space (Figure 3), while the elementary bodies of *C.trachomatis*

and *C.psittaci* appear to be round, dense and have a narrow or barely discernible periplasmatic space (32).

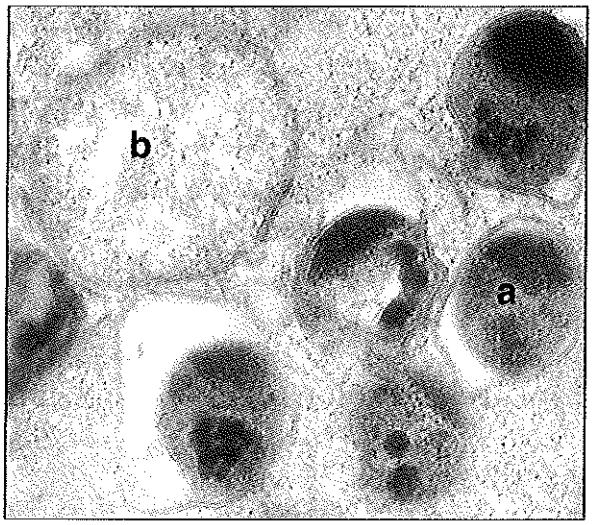

Figure 3. Electron micrograph of *C.pneumoniae* (x80.000) elementary body (a) and reticulate body (b).

LABORATORY DIAGNOSIS

Culture

In contrast to *C.trachomatis*, for which culture has been the diagnostic "gold standard" until recently, *C.pneumoniae* is difficult to culture from clinical specimens and to propagate in cell culture. Serial subpassages of those specimens that are negative on the first attempt are often required. Furthermore, the inclusions are difficult to identify because they are very small, and, once positive, *C.pneumoniae* strains are often lost during passage (33). Only rarely have investigators reported numerous isolates, although some of them were cultured from asymptomatic persons (67,94). Since the discovery of *C.pneumoniae*, several cell culture improvements have been implemented. A few reports are available that compare different cell lines for *C.pneumoniae* culture (35,112,166,206). Furthermore, several modifications of the cell culture protocol have been reported to improve the sensitivity of cell culture (121,122,134,202). The results of Wong *et al.* and Roblin *et al.* indicate that a culture system with Hep-2

cells, which was derived from a human larynx carcinoma, may have increased sensitivity and, simultaneously, eliminate the need for serial subpassages of those specimens that are negative on the first attempt. Despite all these recommendations, the optimal cell line and/or culture protocol may not have been found yet. *C.pneumoniae* strains are rapidly inactivated at room temperature and are susceptible to freezing and thawing (111). Although nasopharyngeal swab samples have been recommended (166), the level of *C.pneumoniae* may be low in swab samples of the pharyngeal epithelium if the organism have invaded deeper layers. Furthermore, the presentation of *C.pneumoniae* in the upper respiratory tract sometimes reflects asymptomatic disease (6,33,94,95); in acute disease the presence of the organism should be ideally be supported by serological results compatible with acute respiratory disease.

Antigen Detection

Several enzyme immunoassays (EIA), initially developed for the detection of *C.trachomatis* in cervical and urethral specimens, may be used for the diagnosis of *C.pneumoniae* infection. These diagnostic tests measure common lipopolysaccharide (LPS) antigen that is present in all *Chlamydia* species (133). However, both the low level of *C.pneumoniae* LPS in respiratory samples and the possibility of cross-reactions may compromise the utility of these assays. *C.pneumoniae* in respiratory smears can be detected using fluorescence antibody staining procedures. These direct fluorescence assays (DFA) have been used in the diagnosis of *C.trachomatis* infection (141,160,178,187). Species-specific monoclonal antibodies have been used for the detection of *C.pneumoniae* in throat swabs. The sensitivity of this technique has been evaluated in several studies and ranges between 20% to 60% (44,72,75,166); however, its specificity may be compromised due to the subjective nature of the test since it depends completely on the skill of the person reading the slides. Fluorescent antibody staining of nasopharyngeal aspirates (175) and sediments of gargled water have also been assessed.(146).

Nucleic acid detection

The development of tests based on nucleic acid amplification technology has been the most important recent advance in the field of *C.pneumoniae* diagnosis. The use of PCR for the diagnosis of *C.pneumoniae* infections has been described extensively (10,16,29,44,60-64,91,159,161,195). Compared with cell culture the PCR is more sensitive and highly specific. However, since this method is capable of detecting one single gene copy, strict quality control is necessary to avoid false-positive results due to DNA contamination (115). On the other hand, false-negative results due to the presence of PCR inhibitors in respiratory specimens may compromise the sensitivity of the test. These false-negative results are difficult to correct since they occur unpredictably and are detectable only if amplification controls such as PCR for human β-globin or spiking duplicate samples with chlamydial DNA are routinely used. Unfortunately, no commercially available diagnostic test has been developed yet, which declays the implementation of PCR for *C.pneumoniae* on a routine basis in a clinical laboratory. A nasopharyngeal specimen seems the preferred type of specimen in patients with respiratory disease. Throat swab samples may not the optimal specimen for PCR, since the organism may be present in small numbers in the epithelium of the throat during invasive infection. Also, asymptomatic carriage of *C.pneumoniae* in the upper respiratory tract may compromise the utility of amplification methods in the diagnosis of lower respiratory infection (6,33,64,94,95).

Serology

Antigens present on the surface of Chlamydiae can be classified as genus-, species-, subspecies- and serotype-specific (2). The serologic tests that have been developed for the diagnosis of acute chlamydial respiratory infection can be divided into two groups based on the antigen used, the lipopolysaccharide (LPS) antigen and the major outer membrane protein (MOMP). The LPS antigen is genus-specific. It contains two Chlamydia-specific epitopes and two epitopes cross-reactive with *Salmonella* RE-type LPS (Figure 4) (15,19,20, 57,92). The MOMP is the most prominent protein of the outer membrane complex of chlamydial elementary bodies which

comprises approximately 50% of all proteins in the outer membrane (23). It has type-, subspecies-, species- and genus-specific epitopes (24,181).

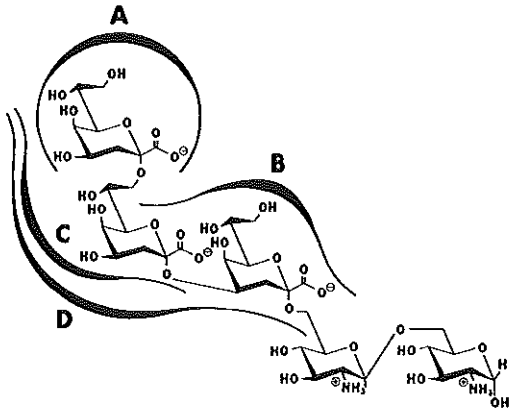

Figure 4. Schematic representation of antibody specificities recognizing distinct sugar epitopes of the carbohydrate backbone of chlamydial LPS. Type A and B antibodies cross-react between chlamydial and *Salmonella* RE-type LPS whereas type C and D antibodies are Chlamydia-specific. (adapted from reference number 19).

Complement fixation The complement fixation (CF) has traditionally been used for the diagnosis of respiratory infections caused by *C.psittaci* (167). This test, however, uses *C.psittaci* LPS, which contains genus-specific antigen determinants. After acceptance of *C.pneumoniae* as the third chlamydial species in 1989, evidence was obtained that most *C.psittaci* infections observed by CF actually are *C.pneumoniae* infections (56,107). Furthermore, the CF test is limited by the fact that it frequently fails to show antibody during infection (125). This lack of sensitivity is thought to be due to the fact that the CF is sensitive in the diagnosis of primary infections of young people only (43,75,107); most reinfections remain unnoticed by CF (43,73,119).

Microimmunofluorescence In the early 1970s, a sensitive microimmunofluorescence (MIF) assay was developed for routine diagnosis of *C.trachomatis* infection (201,203). After the discovery of *C.pneumoniae*, the MIF procedure was applied to diagnose *C.pneumoniae* infection. In the MIF test, purified elementary bodies are used to detect *C.pneumoniae* antibodies, which are mainly directed against the MOMP antigen. Importantly, the MIF allows differentiation of IgG, IgM and IgA antibodies. In literature, the MIF test is still considered to be the gold standard for the serological diagnosis of acute *C.pneumoniae* infection (38,103,180). Debate continues over the serological criteria for diagnosing acute infection, but a

fourfold or greater increase in IgG and/or IgA antibody between acute and convalescent serum sample, or a single IgM titer ≥ 1:16 has been accepted as evidence of acute infection (75). High titers of *C.pneumoniae* IgG (≥1:512) without significant increase has been considered also diagnostic for acute infection. However, this interpretation of high titers in the IgG fraction (≥1:512) is very controversial (85). In addition, the MIF test is rather laborious and highly trained technicians are required to distinguish between a specific and cross-reactive fluorescence pattern. A positive result of the test is indicated by the presence of a bright homogeneous fluorescence pattern, which is based on the reaction of antibody with a species-specific antigen, the MOMP of the elementary body. Antibodies to the LPS, the genus-specific antigen, can be observed as a patchy, scattered fluorescence or as a rough, fuzzy fluorescence pattern without discernible elementary bodies (173,198). The value of the MIF test has recently been disputed (84). Cross-reactions between *C.pneumoniae* and other chlamydial species have been observed (18,104,138, 151,173,198). However, other laboratories have found that, when performed with properly collected paired serum samples and interpreted by an expert, MIF is more sensitive than culture or even PCR of throat specimens (44,72). Some experienced researchers claim that differentiation not only between chlamydial species but also among chlamydial strains is possible (171,201). Further differences in sensitivity and specificity may occur due to difference in antigen preparations. Most studies have used the antigen preparations from the Washington Research Foundation, Seattle, USA or the Institute of Ophthalmology in London, although several were unspecified. The antigens from these two institutes were purified by differential centrifugation using a renografin gradient (93). These elementary bodies contain the MOMP as well as the LPS antigen, while commercially available slides from Lab-systems OY, Helsinki, Finland are coated with *C.pneumoniae* elementary bodies that have been pretreated to remove the LPS fragment in order to increase the species specificity of the test.

Whole Inclusion Fluorescence Another fluorescence assay to measure chlamydial antibodies is the whole inclusion fluorescence (WIF). This test

uses whole inclusions in a cell monolayer as antigen (164,165), that are much easier to read than single elementary bodies. Inclusions contain elementary bodies, reticulate bodies and LPS, which makes this assay a genus-specific reaction (55).

ELISA To date, ELISA tests for the detection of chlamydial antibodies have been developed for the detection of *C.trachomatis* antibodies (31,47, 106,150). In most assays suspensions of serovar L2 are used as antigen, which result in a genus-specific reactivity. Pretreatment of the elementary body with sodium periodate increases the specificity of the test (117,149). No commercially available ELISA has been developed solely for the diagnosis of *C.pneumoniae* infection yet. However, the use of recombinant DNA chlamydial LPS as antigen may be helpful for the diagnosis of respiratory chlamydial infection (20,21,92).

PATHOGENESIS

Details of the pathogenesis are still unknown. The obligate intracellular growth of Chlamydiae within endocytic vesicles likely protects them from many effector functions of the immune system as well as from many antibiotics. As for many obligate intracellular pathogens, entry into phagocytes plays an important role in their pathogenesis. In contrast to extracellular microbes for which ingestion by phagocytes usually results in death, *C.trachomatis* and *C.psittaci* are able to multiply in macrophages (116, 124,176,208). So far there have been few studies of the in-vitro interaction between monocytes or macrophages and *C.pneumoniae*. Limited replication of *C.pneumoniae* has been described in human alveolar macrophages (11). Furthermore, *C.pneumoniae* is able to multiply in a human monocytic cell line (145), in smooth-muscle cells and in endothelial cells (65,101, 184). These reports indicate that *C.pneumoniae* has the ability to maintain chronic, persistent infection, which may, upon systemic access via infected macrophages reach distant sites including he endothelium of the vascular space. Recently it was found that interferon-γ inhibits the intracellular growth of *C.pneumoniae* and may thereby contribute to the

resolution of *C.pneumoniae* infection (30,185).
Animal models have provided evidence that mice intranasal inoculated with *C.pneumoniae* develop acute pneumonia only when given large inocula (100,209,210). Persistent inflammation for up to two months has been observed. Yang *et al.* reported systemic dissemination of *C.pneumoniae* following intranasal inoculation in mice (211).
The strongest evidence establishing *C.pneumoniae* as a respiratory pathogen comes from studies in which the organism has been isolated from patients with acute respiratory symptoms in association with serological results indicative for acute infection. This was first accomplished in 1986, at which time Grayston succeeded to isolate *C.pneumoniae* from eight of 13 students with acute respiratory disease who had serological results compatible with acute infection (75). In the early 1990s, additional reports have confirmed the pathogenicity of the organism (5,25,72). In other studies the clinical relevance of *C.pneumoniae* infection has been less clear. Discrepancies between the results of serology and the presence of the organism by culture or PCR have been observed. In the study of Chirgwin *et al.*, only three of eight culture positive patients had serological evidence of acute infection (33). Hyman *et al.* described how two laboratory technicians were apparently infected with *C.pneumoniae*. From both technicians, *C.pneumoniae* was cultured from throat swabs taken five days after exposure and remained present in one subject up to 20 weeks later. Neither subject developed symptoms, nor serological evidence of infection. These observations suggest that a chronic carriage state had occurred (94). Gnarpe *et al.* isolated the organism from 11/234 throat swabs taken from subjectively healthy persons (67).

EPIDEMIOLOGY

Prevalence

In the last decade, since it was found that *C.pneumoniae* caused acute respiratory disease, important data for the understanding of the general epidemiology of *C.pneumoniae* infections has been published. One of the most unusual aspects of *C.pneumoniae* epidemiology is its high seropreva-

lence worldwide. Several serological studies have demonstrated that *C.pneumoniae* is the most common chlamydial species infecting humans; more than 50% of adults have antibodies to this organism (9,17,41,54,71, 96,98,99,127,168, 197,200). The age and gender specific seroprevalence have been determined (54,71,96,168). The presence of *C.pneumoniae* antibody is rare during preschool age, increases during school age and continues to increase until adult age, and remains at a high level until old age (Figure 5). Similar results has been found in The Netherlands by Stolk-Engelaar and Peeters (182). Since antibodies are often lost after infection (155), the presence and persistence of *C.pneumoniae* antibody in the adult population suggests that infection and reinfection are common during lifetime.

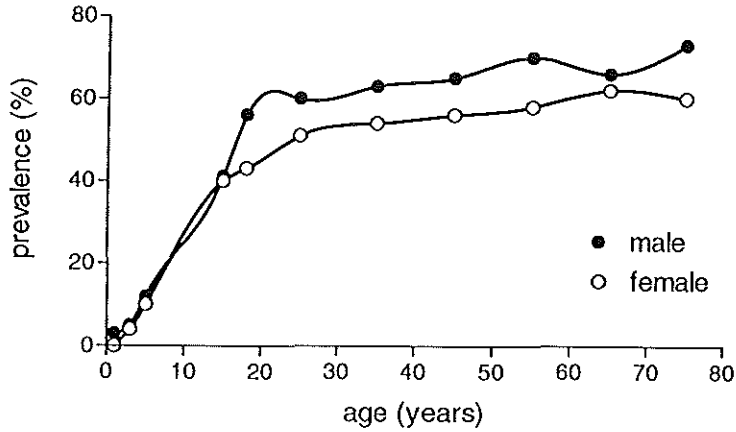

Figure 5. Prevalence of microimmunofluorescence antibody to *C.pneumoniae* by age among 5,242 subjects in Seattle (adapted from reference number 71).

Incidence

Excellent information about age-specific incidence rates of *C.pneumoniae* infection was reported by Aldous *et al.* (1). This study provided age-specific incidences based on fourfold rises in antibody titer observed by MIF (Table 2). The high rates of 6-9% per year for the age groups 5-14 years were consistent with the rapid increase in population prevalence antibody at these ages (54,71,96,168,182). The available clinical information suggested that many antibody conversions were asymptomatic. However, respi-

ratory complaints developed more frequently among persons with serological results compatible with acute infection than among controls. Grayston *et al.* studied the incidence of *C.pneumoniae* pneumonia incidence in a defined population of Seattle (the Group Health Cooperative of Puget Sound) (71). When nearly 2,000 paired serum samples were tested, 10% showed evidence of *C.pneumoniae* pneumonia.

Table 2. Incidence of acute respiratory infection with *C.pneumoniae* in subjects in Seattle, 1975-1979 (adapted from reference number 1).

Age (yr)	number of acute *C.pneumoniae* rises*	number of person-years surveillance	incidence†	CI_{95}#
0-4	0	27	0.0	0.0 - 12.7
5-9	14	151	9.3	5.2 - 15.1
10-14	15	242	6.2	3.5 - 10.0
15-19	2	91	2.2	0.3 - 7.7
>19	6	394	1.5	0.5 - 3.2

* ≥ fourfold rise in antibody titer (MIF)
† rate per 100 person-years at risk
# 95% confidence interval

A high incidence was observed for the age groups 5-14 years, consistent with the previous findings (54,96,168). However, the age curve of incidence of *C.pneumoniae* pneumonia was highest among the elderly (Figure 6).

Epidemics

C.pneumoniae infections are both endemic and epidemic. Grayston *et al.* reported a four-month outbreak of *C.pneumoniae* infections during winter 1990/'91 among university students with respiratory disease in Seattle. The incidence of infection was 22% during the epidemic period, while the incidence rate one year earlier and one year later was only 3% and 0%, respectively (72). Four epidemics of *C.pneumoniae* pneumonia among military conscripts in Finland have been reported. The epidemics occurred in four different garrisons from 1957 to 1985. Each epidemic period lasted approximately six months, and all seasons of the year were involved (107). Additional data are available with compatible results (42,66,76,136).

Periods of low incidence have usually lasted for 3-4 years.

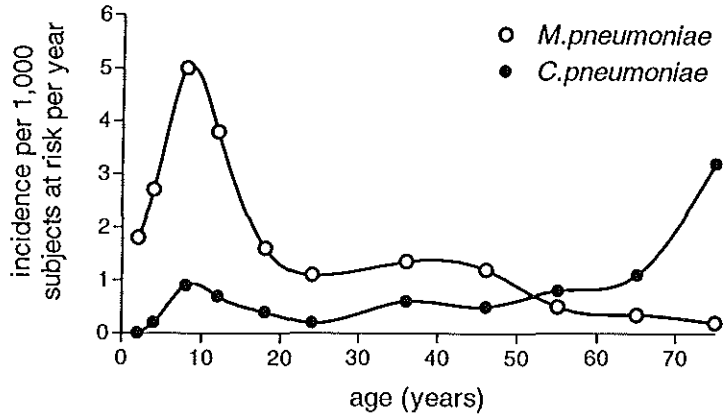

Figure 6. Incidence of pneumonia, by age, for *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*. The population studied was the Group Health Cooperative of Puget Sound in Seattle; the period covered was 1963-1975 (adapted from reference number 71).

CLINICAL MANIFESTATIONS

The first original report of *C.pneumoniae* described a mild pneumonia preceded by pharyngitis (75). However, the clinical manifestation of an acute *C.pneumoniae* infection can range from asymptomatic to life-threatening (5,34,38,94,125). Although pneumonia and bronchitis remain the most frequently recognized illnesses (49,50,70,94,107,125,207), asymptomatic infection or unrecognized, mild symptomatic illnesses are probably the most common result of *C.pneumoniae* infection (6,33,64,94,95). Pneumonia due to *C.pneumoniae* is more common among the elderly (4,12,51, 71,89,97,102, 125,190,196), although *C.pneumoniae* pneumonia was first observed among young adults (42,75,158,192). A prediction of *C.pneumoniae* pneumonia based on its clinical presentation of the patient is not possible. Other causes of atypical pneumonia such as *Mycoplasma pneumoniae*, *Legionella* and respiratory viruses (Influenza, Respiratory Syncytial Virus, Adenovirus) may be associated with similar presentations. Fever, cough and headache are the most common signs, the white blood cell count is reported to be normal in most patients, but the erythrocyte

sedimentation rate is usually raised. The cough may not appear for several days so that the illness appears to be biphasic; the illness may last for weeks or months (103,186). The results of chest radiography in *C.pneumoniae* pneumonia can range from normal findings to signs of extensive pneumonia with bilateral involvement. No radiological findings are unique to pulmonary infection with *C.pneumoniae*. A single subsegmental lesion that fits the usual descriptions of atypical pneumonia is often found (125,128,171). Extensive bilateral appearances may be seen in more severe illness (103, 140). *C.pneumoniae* infection is a rather frequent event during exacerbations among patients with chronic obstructive disease (COPD) (14,36,199), cystic fibrosis (45) and asthma (79-82,142). Pharyngitis often precedes more severe respiratory disease (42,75,192), although pharyngitis has occurred as separate illness associated with *C.pneumoniae* infection (51, 89,196).

The list of clinical diseases due to *C.pneumoniae* is expanding. A increasing number of extra-pulmonary diseases have been associated with *C.pneumoniae* infection; i.e. lymphocytic mengoencephalitis (177), lumbosacral menigo-radiculitis (132), reactive arthritis (22,68), Guillain-Barre syndrome (83), endocarditis (46,126,144) and myocarditis (68,194,205). The history of *Helicobacter pylori* has made people more open-minded to the possible importance of chronic bacterial infection (183). Chlamydial infections have already been associated with a variety of chronic diseases. *C.trachomatis* is a most important pathogen, causing pelvic inflammatory disease (PID), infertility and peri-hepatitis, although approximately 75% of all female genital infections are asymptomatic. Recently an association of *C.pneumoniae* and atherosclerosis and myocardial infarction has been postulated. An increased risk in the development of myocardial infarction has been shown in nine different sero-epidemiological studies (40,120,130, 131,154,169,170, 191,193). Furthermore, the organism has been found in atherosclerotic lesions by others (3,13,28,74,110,113,114,147). Although the association of *C.pneumoniae* and atherosclerosis in these studies has been well established, the meanings of this association has to be elucidated. Recently, Gupta *et al.* have demonstrated for the first time that patients with high titers of circulating antibodies against *C.pneumoniae* had

a four-times-higher risk of adverse cardiovascular events than patients with no detectable *C.pneumoniae* antibodies (77). In this study, the presence of antibodies only indicates exposure to *C.pneumoniae*. No differentiation between active and past infection was possible. A single, three-day course of azithromycin treatment given to some patients with the highest antibody levels virtually eliminated their increased risk. Similar results were reported by Gurfinkel *et al.* (78). In this placebo-controlled pilot study, patients with unstable angina or non-Q-wave myocardial infarction were randomly assigned either roxithromycin or placebo for 30 days. Large-scale trials are needed to confirm these preliminary observations.

TREATMENT

Tetracycline and erythromycin antibiotics, which continue to be treatments of choice for genital *C.trachomatis* infections, are also effective in vitro against *C.pneumoniae*. Newer quinolones, the azalide antibiotic azithromycin and the new macrolide clarithromycin are at least comparable to the previously mentioned antibiotics (8,37,53,86). In fact, azithromycin in particular has been recognized as an effective single dose treatment for *C.trachomatis* infection (88,143,179). However, reports are available indicating that these antibiotics do not appear to be very effective for the treatment of *C.pneumoniae* infection. Several culture-positive patients remained positive despite therapy with prolonged courses of antibiotics known to be effective against *Chlamydia* species (48,51,87). These reports suggest that persistent infection for months with *C.pneumoniae* may follow acute infection. Infection with *C.pneumoniae* may be very difficult to eradicate with use of currently available antibiotics even if there is a clinical response to therapy. Current antibiotic treatment for *Chlamydia* species is not bactericidal, but bacteriostatic. Reducing the reproductive ability of the organisms, and consequently the number of infective organisms, does not necessarily implicate that the immune system may take care of the remaining bacteria. Therefore, new antibiotics that are cidal to *C.pneumoniae* are sought in order to eradicate *C.pneumoniae* from the site of infection.

IMMUNITY

Little is known about immunity following *C.pneumoniae* infection. Ekman *et al.* reported a decreased severity of disease in military conscripts following *C.pneumoniae* reinfection (42). Comparable results in middle-aged persons were reported by Thom *et al.* , although the number of subjects were small (190).

TRANSMISSION

Epidemics of *C.pneumoniae* infection in closely confined populations have learned that the attack rate was 60-80 per 1,000 men and spread of infection seemed to be relatively slow (107). The case to case interval was 20-30 days (105,137). Little is known about the mode of transmission of the organism. Survival of *C.pneumoniae* in small particle aerosols is possible, especially at high relative humidities, but even then the survival is relatively short (52,189). Contact with surfaces contaminated with infectious *C.pneumoniae* may be another important mode of transmission (52).

CONCLUSION

More than ten years after the first publication suggesting that *C.pneumoniae* cause acute respiratory infection a great deal has been learned about *C.pneumoniae* infection. However, the diagnosis of *C.pneumoniae* infection remains difficult and needs highly skilled personnel. Furthermore, the interpretation of the results, obtained from current laboratory methods for the detection of acute infection may be compromised by chronic, asymptomatic or persistent infection. To date, the role of *C.pneumoniae* in chronic diseases is not clear. In many studies, data provide only circumstantial evidence for a causative role. Large-scale intervention studies with precise diagnosis and more effective treatments are necessary to provide causative evidence for *C.pneumoniae* as an inducer of the slowly progressive inflammation associated with chronic infection.

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CHAPTER 2

AIMS OF THE STUDY

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Chlamydia pneumoniae infection occurs worldwide, resulting in a 40% to 90% prevalence of serum antibody to the species in various populations. Many of these infections are mild or even asymptomatic. However, *C.pneumoniae* has been associated with both epidemic and endemic occurrences of acute respiratory disease and is believed to be responsible for 6-20% of all community-acquired pneumonias. *C.pneumoniae* has also been associated with chronic pharyngitis and, more recently, with chronic cardiovascular disease. Since the clinical presentation of acute *C.pneumoniae* infection is not specific, laboratory methods are required for a definitive diagnosis of *C.pneumoniae* infections. In the last decade, several diagnostic tests have been developed for the diagnosis of acute *C.pneumoniae* infection. These methods include several direct and indirect techniques. However, many of these tests are not commercially available at this moment, and are, therefore, difficult to apply in routine clinical practice. Also, many questions concerning interpretation of the laboratory results still remain, especially in relation to chronic infections. Thus, the true impact of *C.pneumoniae* on the spectrum of human disease remains difficult to ascertain. Only few studies on *C.pneumoniae* infection in The Netherlands are available. To design an effective strategy to diagnose acute respiratory *C.pneumoniae* infections, a better understanding of the current technology used for *C.pneumoniae* diagnosis is required.

In general, the aims of the present studies is contribute to the development of laboratory techniques and to improve our understanding of their limitations and interpretation, and to assess the role of *C.pneumoniae* in patients with respiratory tract disease in The Netherlands. The following specific issues were addressed:

a. Microimmunofluorescence serology is the gold standard at this moment for the diagnosis of acute *C.pneumoniae* infection. In literature, there is no general consensus on the sensitivity and specificity of the MIF in comparison with other diagnostic tests. What is the relevance of MIF serology, and, is it possible to improve current technology for use in routine clinical practice ?

b. *C.pneumoniae* culture is laborious and has been reported to be insensitive in a clinical setting. Is it possible to improve the sensitivity of culture for detection active *C.pneumoniae* infections, and, what is the additional diagnostic value of the polymerase chain reaction for detecting *C.pneumoniae* infection ?

c. What is the diagnostic value of a recent developed, commercially available ELISA for the detection of chlamydial antibodies in terms of sensitivity and specificity to diagnose acute *C.pneumoniae* infection ?

d. Patients with chronic obstructive pulmonary disease (COPD) are predisposed more frequent to severe respiratory disease. What is the role of *C.pneumoniae* in patients with COPD in The Netherlands ?

e. What is the role of *C.pneumoniae* as an etiologic agent of severe community-acquired pneumonia in the Netherlands and how is transmission of *C.pneumoniae* infection established ?

CHAPTER 3

AGE-RELATED INTERFERENCE WITH *CHLAMYDIA PNEUMONIAE* MICROIMMUNOFLUORESCENCE SEROLOGY DUE TO CIRCULATING RHEUMATOID FACTOR

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ABSTRACT

Microimmunofluorescence (MIF) serology is commonly used in the diagnosis of chlamydial infections. In the MIF assay, *Chlamydia pneumoniae* elementary bodies were used to detect *C.pneumoniae* immunoglobulin G (IgG) and IgM antibodies in paired serum samples from 286 patients with respiratory illnesses. In 69 patients, MIF serology was compared with *C.pneumoniae* cultures. All *C.pneumoniae* cultures remained negative. However, 205 (71%) of 286 patients were *C.pneumoniae* antibody positive and 64 (22%) had MIF test results indicating recent infection; 11 showed a fourfold increase in IgG titer, 18 had IgG titers of ≥1:512, and 41 had IgM titers of ≥1:16. In 35 (55%) of 64 patients, a recent-infection diagnosis was based on *C.pneumoniae* IgM antibodies only. However, 78% of *C. pneumoniae* IgM-positive patients had circulating rheumatoid factor (RF) by rheumatoid arthritis latex assay. RF positivity increased with age. After absorption with anti-human IgG, all *C.pneumoniae* IgM-positive sera became *C.pneumoniae* IgM negative in the MIF assay. Twenty-five patients with active rheumatoid arthritis but without respiratory illness were also tested; 14 were *C.pneumoniae* IgG positive and *C.pneumoniae* IgM positive as well. Absorption of IgG from these RF-containing sera invariably resulted in disappearance of reactivity in the MIF IgM assay. We conclude that with age the serologic diagnosis of recent *C.pneumoniae* infection becomes increasingly prone to false-positive results unless sera are routinely absorbed prior to MIF IgM testing.

INTRODUCTION

Chlamydia pneumoniae is a common cause of pneumonia and other respiratory tract infections. About 10% of pneumonia cases have been associated with *C.pneumoniae* infection (4,5). Although tissue culture techniques have been developed, serology is most commonly used in the diagnosis of respiratory chlamydial infections. In the early 1970s, a sensitive microimmuno-fluorescence (MIF) assay for specific chlamydial antibodies was developed which proved to be suitable for routine serodiagnosis. In

the MIF test, purified *C.pneumoniae* elementary bodies are used to detect specific antibodies in the immunoglobulin M (IgM), IgG, and IgA serum fractions. The MIF test for IgM antibody to *C.pneumoniae* is very helpful in the diagnosis of recent respiratory chlamydial infections. However, in our studies to ascertain the prevalence of *C.pneumoniae* antibody and the incidence of *C.pneumoniae* infection in The Netherlands, we found the MIF *C.pneumoniae* IgM assay to be highly sensitive to the presence of rheumatoid factor (RF) in serum.

Little is known about the presence of RF in respiratory infections and its effect on diagnostic IgM tests. In the present report, we describe the prevalence of RF in MIF *C.pneumoniae* IgM-positive patients with respiratory illnesses and the effect of IgG absorption on the MIF IgM test for *C.pneumoniae*.

MATERIALS AND METHODS

Patients and materials

The study population included three different groups of patients. The first group consisted of 217 patients from whom paired serum samples had been collected and stored at -85°C after routine complement fixation assays for respiratory viral infection (influenza A and B, parainfluenza, respiratory syncytial virus, adenovirus) or infection due to *Mycoplasma pneumoniae, C.psittaci,* or *Coxiella burnetii.* Patient ages ranged from birth to 84 years, with a median of 53 years. The second group consisted of 69 prospectively studied patients who were consecutively admitted to the St.-Antonius Hospital with serious respiratory symptoms and/or chest radiographs which indicated pneumonic infiltrates. The median age was 64 years, with a range of 21 to 83 years. From each of these 69 patients we obtained three consecutive serum samples on admission and 10 and 30 days later. Also, pharyngeal and sputum cultures for *C.pneumoniae* were collected upon admission of the patients to the hospital. Pleural effusions were cultured for some patients. All specimens were transported in 2SP medium and immediately frozen to -70°C (6). The third group consisted of 25 patients attending the outpatient clinic with active rheumatoid arthri-

tis (RA) but with no signs or symptoms of respiratory illness. Sera from each of these patients were collected and stored at -85°C prior to use in the MIF assay.

MIF assay

The MIF assay described by Wang and Grayston was used to measure *C.pneumoniae* antibodies (15). The antigen was purified, formalinized elementary bodies of the TW-183 strain obtained from the Washington Research Foundation (Seattle, Wash.). Thirty-well antigen slides (Cel-Line Associates, Inc., Newfield, N.J.) were prepared as previously described (4). Specific chlamydial antibodies were detected by using fluorescein isothiocyanate-conjugated anti-human IgM and IgG (Dako A/S, Glostrup, Denmark). Serological diagnosis of a recent *C.pneumoniae* infection was based on the following criteria: the presence of *C.pneumoniae*-specific IgM (titer, ≥1:16) in either acute or convalescent serum, a fourfold or greater *C.pneumoniae*-specific antibody titer rise between acute and convalescent sera, or a *C.pneumoniae*-specific IgG titer of ≥1:512 (4,6). In the absence of *C.pneumoniae*-specific IgM, sera with IgG titers of <1:32 were considered negative.

RF assay and pretreatment of RF-containing sera

RF was detected by agglutination using latex particles sensitized with human IgG to detect levels greater than
1 IU of RF per ml of serum (Mercia Diagnostics, Guildford, Surrey, United Kingdom). All sera positive for RF by the latex method were absorbed with a goat anti-human IgG antibody reagent (GULLSORB; Gull Laboratories, Inc., Salt Lake City, Utah) as prescibed by the manufacturer, centrifuged (9,500 x *g*), and retested for the presence of *C.pneumoniae*-specific IgM antibody in the MIF assay. The absorption procedure with the GULLSORB reagent selectively removes >95% of IgG from serum without decreasing its IgM titer significantly. Alternatively, in some sera positive for RF by the latex method we used ion-exchange chromatography for IgG removal.

C.pneumoniae cultures

For culture of *C.pneumoniae* from patient materials, HL cells (kindly provided by J.H.T. Wagenvoort, Erasmus University, Rotterdam, The Netherlands) were seeded into 24-well plates (Costar Europe Ltd.) and 16-mm-diameter flat-bottom tubes and incubated at 35°C with 5% CO_2 in a fully humidified cabinet (3). At 24 h later, monolayers were examined for confluency and pretreated with DEAE-dextran (30 μg/ml). For each experiment, a patient's specimen was inoculated into four microtiter wells and one flat-bottom tube. Microtiter plates and flat-bottom tubes were centrifuged at 900 x *g* at 25°C for 60 min and subsequently incubated with fresh medium containing cycloheximide (0.6 μg/ml) (Sigma Chemical Company, St. Louis, Mo.). After 3 days, the microtiter plates were aspirated and fixed with methanol. The fixed monolayers were rinsed with phosphate-buffered saline once and stained by the fluorescent-antibody technique using *Chlamydia* genus-specific mouse monoclonal antibody (kindly provided by J.M. Ossewaarde, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands) as previously described (3, 6). The inoculated flat-bottom tubes were passed onto fresh monolayers and reincubated as described before. This procedure was repeated once more. In each culture series, *C.pneumoniae* TW-183 was included in parallel as a positive control. Positive cultures were stained with *C.pneumoniae*-specific mouse monoclonal antibodies (Washington Research Foundation, Seattle, Wash.). Rabbit anti-mouse Ig labeled with fluorescein isothiocyanate (Dako A/S, Glostrup, Denmark) was used as a conjugate. Evans blue (0.05%; Sigma Chemical Company) was used as a counterstain.

RESULTS

We used MIF assays to detect *C.pneumoniae* IgG and IgM antibodies in stored paired serum samples from 217 patients with respiratory illnesses. In sera of 69 similar patients, MIF assays and *C.pneumoniae* cultures were compared prospectively. Overall, 204 (71%) of 286 patients were *C.pneumoniae* antibody positive and 64 (22%) had MIF test results indicating recent infection due to *C.pneumoniae*, i.e., 44 (20%) of 217 and 20

(29%) of 69 of the retro- and prospectively studied patients, respectively. Of these 64 patients, 11 had a fourfold or greater rise in *C.pneumoniae* antibody, 18 had *C.pneumoniae* IgG titers of ≥1:512, and 41 had *C.pneumoniae* IgM titers of ≥1:16. We found no evidence of cross-reactivity between *C.pneumoniae* and the other pathogens tested for by complement fixation assay. Three patients had significantly increased influenza A antibody titers, as well as *C.pneumoniae* antigen titers. However, 13 other influenza A-infected patients had no serologic evidence of recent *C.pneumoniae* infection. Twenty-nine patients (10%) had circulating RF by RF latex assay (Table 1). Interestingly, all patients with *C.pneumoniae* IgG and RF showed positive results in the MIF IgM assay. Of the IgM-positive patients, 78% had detectable circulating RF. However, after absorption with anti-human IgG all IgM-positive sera became *C.pneumoniae* IgM negative by MIF assay, including those that were negative for RF by latex agglutination. After addition of *C.pneumoniae*-specific IgG to some absorbed sera, *C.pneumoniae* IgM positivity by MIF assay was reconstituted as well. For some sera positive for RF by the latex method, we used ion-exchange chromatography for IgG removal. These results were comparable to those of the anti-human IgG method.

Table 1. Association between RF and *C.pneumoniae* serology

C.pneumoniae MIF test result	No. of patients tested	No. (%) RF positive by latex assay
Seronegative	82	4 (4.9)
Seropositive	204	25 (12.3)[a]
Past infection	140	0 (0)
Recent infection based on:		
IgG titer of ≥1:512	14	0 (0)
Fourfold titer rise	9	0 (0)
IgM titer of ≥1:16	26[b]	22 (84.6)
Fourfold titer rise + IgM titer of ≥1:16	2	1 (50.0)
IgG titer of ≥1:512 + IgM titer of ≥1:16	4	2 (50.0)

[a] P =0.06 compared with seronegative patients.

[b] Nine other patients had *C.pneumoniae* IgM titers of ≥1:16 but could not be tested for RF because of insufficient serum samples.

The prevalence of RF strongly correlated with age (Table 2; P=0.0005). Four patients who had positive RF results but were negative for *C.pneumoniae* IgG were negative in the MIF IgM test as well. After correction for RF interference in the MIF assay, 29 (10%) of 286 patients still had positive MIF results, indicating recent infection due to *C.pneumoniae*. These included 8 (12%) of the 69 in the prospectively studied patient group. However, all cultures for *C.pneumoniae* remained negative.

Table 2. Age-related prevalence of RF and associated false-positive *C.pneumoniae* IgM serology in patients with respiratory illnesses

Age group (yr)	No. of patients	No. (%) RF positive	No. RF positive[a] and *C.pneumoniae* IgM false positive
≤30	45	0 (0)	0
31-50	75	6 (8.0)	5
51-70	99	15 (15.2)	14
71-90	57	15 (26.3)	13

[a] Patients were considered RF positive if they were positive by latex agglutination or became *C.pneumoniae* IgM negative after IgG absorption.

We also tested 25 patients with active RA. None of the patients had signs or symptoms of respiratory illnesses when they were tested. All 25 patients with RA had circulating RF. Fourteen patients were *C.pneumoniae* IgM positive, and 11 were negative. All *C.pneumoniae* IgM-positive patients with RA had detectable *C.pneumoniae* IgG, and all *C.pneumoniae* IgM-negative patients were *C.pneumoniae* IgG negative as well. Treatment of the *C.pneumoniae* IgM-positive sera with anti-human IgG abolished their reactivity to *C.pneumoniae* in the MIF assay.

DISCUSSION

The recent recognition of *C.pneumoniae* and its association with respiratory infections in humans has prompted a reevaluation of atypical pneumonia cases in patients. Many of these cases turned out to be *C.pneumoniae* infections on retrospective reexamination by MIF (4,6). The diagnostic

criterion was either a significant increase in specific antibody titer in paired serum samples, a very high specific IgG antibody titer, or the presence of IgM antibodies against *C.pneumoniae* (4,6). Little is known about the specificity of the MIF test using *C.pneumoniae* elementary bodies. Some researchers have reported that the *C.pneumoniae* MIF test is highly specific (5,6,11). Others, however, have noted cross-reactions between *C.pneumoniae* and other chlamydial species (13,14). A strong immunological cross-reaction between a genus-specific chlamydial glycolipid and the lipopolysaccharide of enteric bacteria was also demonstrated (10). In this study, we found no cross-reactions between *C.pneumoniae* and other respiratory pathogens routinely tested for serologically. However, we found that RF may interfere in the detection of *C.pneumoniae* IgM antibodies and thus may lead to false-positive results. RF is an autoantibody, usually of the IgM class, that reacts with antigenic sites located in the Fc region of IgG. RF can frequently be detected in sera from patients with RA (60 to 90%) and occasionally in small quantities in sera from healthy individuals (1 to 5%). RF may also be induced during certain infectious diseases, such as those caused by cytomegalovirus, Epstein-Barr virus, rubella virus, *Treponema pallidum*, *Toxoplasma gondii*, *Candida albicans*, hepatitis B virus, measles virus, mumps virus, and borreliae, and during bacterial endocarditis and several parasitic infections (5 to 60%) (1,9). Also, advanced age is correlated with an increased prevalence of circulating RF. The prevalence of RF in healthy adults may be as high as that observed in our patients (1,12).

Stimulation by immune complexes is one of the major mechanisms responsible for RF formation. This appears to be a T-cell-dependent phenomenon. T-helper cells are triggered by the antigen and activate B cells that respond to this antigen, as well as to the antibody part of the complex (2,9). Another important mechanism responsible for RF production may be nonspecific polyclonal activation of B cells. Many bacterial and viral components, such as Epstein-Barr virus and bacterial lipopolysaccharide and peptidoglycan, may contribute to the stimulation of RF through this mechanism (2,9). Fc receptors expressed by virus-infected cells and those found in the cell walls of staphylococci and streptococci may constitute another trigger for RF production (2,8). In all of these nonrheumatic in-

stances of RF production, it is thought likely that RF plays an important role in immune regulation of infectious deseases. RF may cross-link antigen-antibody complexes and thereby increase their size and ability to fix complement. This may increase the antimicrobial activity of IgG antibodies (bacteriolysis) and also enhance removal of microbial products (9). Recent studies have indicated that idiotypic interactions between RF and other antibodies exist and therefore suggest that RF is a multispecific antibody that could be part of the normal immunoregulatory system (2,8, 9). In many different immunofluorescence techniques and enzyme-linked immunosorbent assay systems, false-positive IgM results have been shown to be due to RF (7). In this study group, we found a high prevalence of RF, which was strongly correlated with age. We showed that RF can cause false-positive *C.pneumoniae* IgM results in the MIF test when *C.pneumoniae* IgG is present. However, RF interference can readily be prevented by treatment of serum with anti-human IgG antibody. In this study, evidence was found that even low levels of RF, undetectable by latex assay, may cause false-positive results in the MIF IgM test. Thus, we found 16 patients whose RF results were negative by latex agglutination but whose *C.pneumoniae* IgM reactivity in the MIF assay disappeared after pretreatment. Therefore, routine absorption of IgG prior to IgM testing is recommended to prevent false-positive *C.pneumoniae* IgM MIF results. Also, such absorption may, at least theoretically, prevent false-negative *C.pneumoniae* IgM MIF results due to competition with IgG antibodies for epitopes on the *C.pneumoniae* antigen. Further studies are required to determine the specificity of the MIF assay with *C.pneumoniae* elementary bodies.

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CHAPTER 4

EFFECT OF CELL TYPE, TEST ORGANISM AND DEAE-DEXTRAN ON THE PERFORMANCE OF CELL CULTURE FOR *CHLAMYDIA PNEUMONIAE*

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ABSTRACT

Chlamydia pneumoniae is difficult to culture from clinical specimens and propagate in vitro. In this study, *C.pneumoniae* isolates TW-183, AR-39, AR-388 and CWL-029 were used to compare growth of *C.pneumoniae* in HL, HeLa 229, Hep-2 and BGM cells. Using three-fold serial dilutions the sensitivity was calculated for each cell type. Although some strain to strain variation was observed, Hep-2 and BGM were the most sensitive host cells for the isolation of *C.pneumoniae.* Since DEAE-dextran was recently reported to decrease the sensitivity of HL cells for *C.pneumoniae* infection, we also studied the effect of DEAE-dextran on the infectivity of *C.pneumoniae* for different cell types. Pretreatment of cell monolayers with DEAE-dextran resulted in a average reduction in the number of inclusions compared to untreated control monolayers. Also, inclusions were smaller in size if DEAE-dextran was used. However, BGM cells were less vulnerable to such inhibition. We conclude that Hep-2 and BGM are more sensitive than HL or HeLa 229 for the isolation of *C.pneumoniae,* and that DEAE-dextran treatment significantly inhibits subsequent *C.pneumoniae* growth.

INTRODUCTION

Chlamydia pneumoniae, the newly recognized third chlamydial species is emerging as a common human respiratory pathogen. Serologic evidence of *C.pneumoniae* infection has been repeatedly found in approximately 10% of patients with community-acquired pneumonia (1,3,10). However, *C.pneumoniae* has proved to be difficult to isolate from clinical specimens and to propagate in cell culture. Although several improvements has been implemented, the sensitivity of cell culture and the ability to propagate *C.pneumoniae* in cell culture remains less than satisfactory. A few reports are available that compare different cell lines for *C.pneumoniae* culture (2,7,8,12) . Many of these evaluations have involved a limited number of strains. In the present study, we compared the growth of four *C.pneumoniae* strains in four different cell lines, and studied the effect of DEAE-

dextran on the performance of *C.pneumoniae* culture.

MATERIALS AND METHODS

Cell culture

HL (Washington Research Foundation; Seattle, Washington, USA), HeLa 229 (ICN Biomedicals Inc., Costa Mesa, CA, USA), Buffalo Green Monkey (BGM) (PAMM, Veldhoven, The Netherlands) and Hep-2 cells were grown as monolayers in plastic culture flasks at 35°C with 5% CO_2 in a fully humidified cabinet. (Costar, Europe, Ltd.) The cells were cultured in Eagle Minimal Essential Medium (EMEM; Life Technologies Ltd., Renfrewshire, Scotland) containing fetal calf serum (10%; Life Technologies Ltd., Renfrewshire, Scotland), non-essential amino-acids (1%; ICN Biomedicals Inc., Costa Mesa, CA, USA), gentamicin (10mg/l;Pharmachemie, Haarlem, The Netherlands), vancomycin (25mg/l;Eli Lilly, Indianapolis, IN, USA) and amphotericin-B (4mg/l;Bristol Myers Squibb, Epernon, France) (11).

C.pneumoniae strains

Stock cultures of TW-183, AR-39, AR-388 (Washington Research Foundation; Seattle, Washington, USA) and CWL-029 (American Type Culture Collection, Rockville, MD, USA) were used in this study. Host cells were seeded into 75cm^2 cell culture flasks (Costar Europe Ltd.,The Netherlands) and incubated at 35 °C with 5% CO_2 in a fully humidified cabinet. Cell monolayers were examined on the day of inoculation for confluency. After inoculation with *C.pneumoniae*, cell culture flasks were centrifuged at 900xg at 25 °C for 60 minutes and subsequently incubated with fresh medium containing cycloheximide (0.6 mg/l; Sigma Chemical Company, St Louis, Mo., USA). After 3 days, the flasks were aspirated and monolayers were suspended in 5 ml Sucrose Phosphate Glutamate buffer (SPG) using a cell scraper (Costar Europe Ltd.). All stock solutions were stored at minus 80 °C prior use.

C.pneumoniae culture

Cells were seeded into 24-well cell culture plates (Costar Europe Ltd.) and incubated at 35 °C with 5% CO_2 in a fully humidified cabinet. All cell

monolayers were examined on the day of inoculation for confluency. After inoculation, 24-well plates were centrifuged at 900 x g at 25 °C for 60 minutes and subsequently incubated with fresh medium containing cycloheximide (0.6 mg/l). After 3 days, the 24-well plates were aspirated and fixed with methanol (Merck, Darmstadt, Germany). The fixed monolayers were rinsed with phosphate-buffered saline once and stained by the fluorescent-antibody technique using *Chlamydia* genus-specific mouse monoclonal antibody (kindly provided by J.M. Ossewaarde, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands), as previously described (10). Rabbit anti-mouse Ig labeled with fluorescein isothiocyanate (Dako A/S, Glostrup, Denmark) was used as a conjugate. Evans Blue (0.05%; Sigma Chemical Company, St Louis, Mo.) was used as a counterstain.

RESULTS

When serial three-fold dilutions of *C.pneumoniae* strains were inoculated into the various host cells, the highest yields were detected in BGM and Hep-2 cells, followed by those in HeLa 229 and HL. Strain-to-strain variation was especially observed in HeLa 229 (Figure 1). No difference in morphology was observed by immunofluorescence between the four different cell lines tested (Figure 2). Pretreatment with DEAE-dextran decreased the number of inclusions (Table 1), as well as the size of inclusions. However, the BGM cells were less vunarable to the DEAE-pretreatment. Again, strain-to-strain variation was observed.

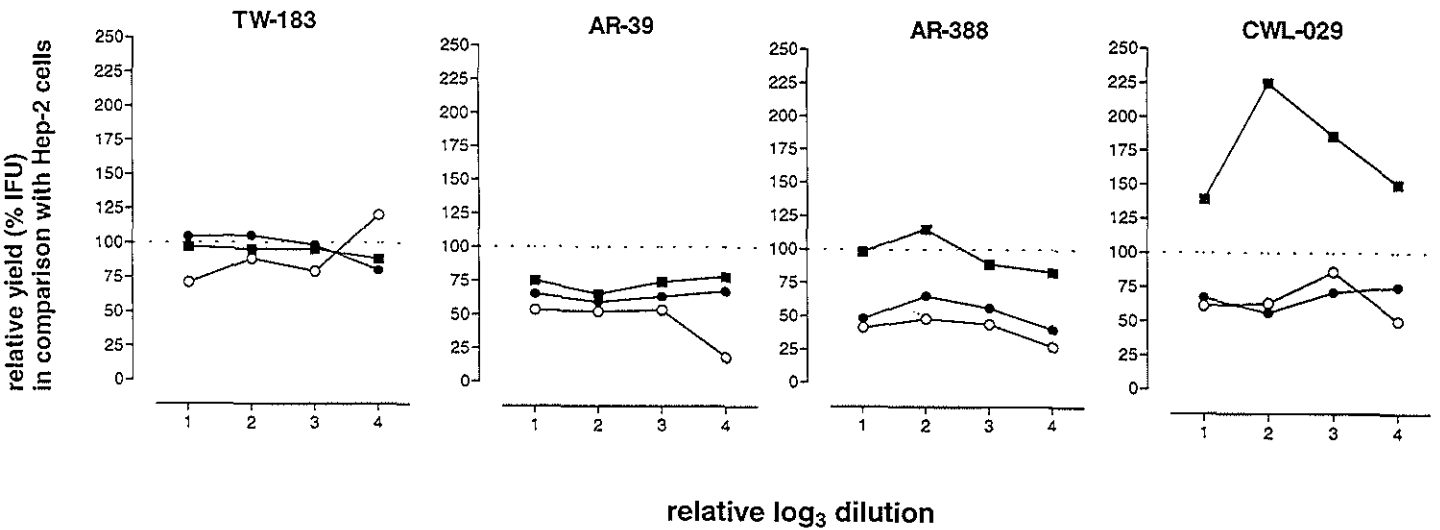

Figure 1. Relative *C.pneumoniae* yield (% inclusion forming units (IFU) in comparison with Hep-2 cells using HL (○), HeLa-229 (●) and BGM (■) cell lines following inoculation with serial dilutions of indicated strains. (average of three separate experiments for each cell line and *C.pneumoniae* strain tested)

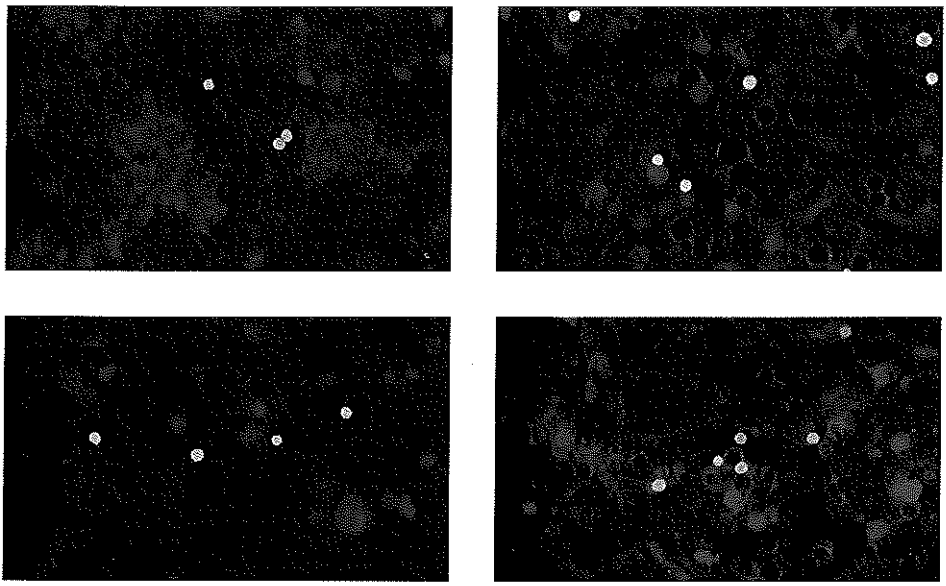

Figure 2. Photomicrograph of host cells (red) infected with *C.pneumoniae* TW-183 (green) and incubated for 72 hours. Upper left: HL cells, upper right: HeLa-229, lower left: Hep-2 cells and lower right: BGM cells. (magnification 200x)

Table 1. Inhibition of *C.pneumoniae* growth by DEAE-dextran

DEAE-dextran pretreated host cell	% growth inhibition of *C.pneumoniae* strains[a]				
	TW-183	AR-39	AR-388	CWL-029	average[b]
HL	35	51	56	29	43±13
HeLa 229	14	47	14	72	37±28
BGM	23	15	8	13	15±6
Hep-2	26	35	42	45	37±8

[a] Average of three separate experiments for each cell line and *C.pneumoniae* strain tested
[b] mean ± sd

DISCUSSION

Culturing *C.pneumoniae* from respiratory specimens is considered to be very difficult. Chirgwin *et al.* were able to isolate *C.pneumoniae* from nasopharyngeal specimens of 15 patients (1). Only 2 of the 15 specimens were positive on initial inoculation; the remainder required two or three passages in HeLa 229 cells. The inclusions were difficult to identify because they were very small; however, once positive, *C.pneumoniae* strains were often lost during passage. Cles and Stamm found HL cells to be more sensitive than HeLa 229 cells for the isolation and propagation of *C.pneumoniae* (2). The results of Wong *et al.* and Roblin *et al.* indicate that a culture system with Hep-2 cells, which was derived from a human larynx carcinoma, may have increased sensitivity and, simultaneously, eliminate the need for serial subpassages of those specimens that are negative on the first attempt (8,12). However, the Hep-2 cell line is known to have been contaminated with HeLa cells. The American Type Culture Collection recommends that cell lines with HeLa 229 markers should not be chosen for study when the specific organ or tissue of presumptive origin is of importance (5). Our results suggest that BGM and Hep-2 are the most sensitive cell lines tested so far for the isolation and propagation of *C.pneumoniae*. In addition, BGM cells seems to be less vulnerable to detachment after confluent growth (data not shown). An enhancing effect of DEAE-dextran pretreatment of cell lines has previously been reported for the culture of Chlamydia trachomatis (4,6,9). However, more recent study has indicated that culture of *C.pneumoniae* with DEAE-dextran pretreated cells may have an adverse effect on the infectivity and growth of *C.pneumoniae* (8). These results were confirmed by our data.

Further clinical studies with BGM cells and/or Hep-2 cells should be undertaken to determine their diagnostic value using patient specimens. In such studies, nucleic acid amplification of *C.pneumoniae* should be incorporated to assess the relative diagnostic sensitivity of culture vs non cultural assays to diagnose *C.pneumoniae* infection.

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CHAPTER 5

WIDELY USED, COMMERCIALLY AVAILABLE *CHLAMYDIA PNEUMONIAE* ANTIGEN CONTAMINATED WITH MYCOPLASMA

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ABSTRACT

We detected mycoplasma contamination in a widely used commercially available *Chlamydia pneumoniae* antigen preparation. Contamination was studied by using a mycoplasma group specific 16S rRNA PCR and sequence analysis. Several lots of the purified *C.pneumoniae* antigen from the Washington Research Foundation (Seattle, WA) appeared to be contaminated with the same *Mycoplasma* species, which appeared to be closely related to *Mycoplasma arginini.* Antigen slides prepared for the detection of Chlamydia antibodies by MRL Diagnostics (Cypress, CA) were contaminated with the same *Mycoplasma* species. Chlamydia antigen slides from Labsystems OY, Helsinki, Finland and two Chlamydia complement fixation reagents (Virion International Distribution Ltd., Chan, Switzerland and Behring Werke, Marburg, Germany) were not contaminated. We conclude that commercially available *C.pneumoniae* antigens may contain mycoplasma antigens as well. Although the impact of such mycoplasma contamination on the results of chlamydia serology may not be significant, we recommend routine screening of all antigen preparations obtained by tissue culture prior to their distribution and use.

INTRODUCTION

Serodiagnosis of *Chlamydia pneumoniae* , *Chlamydia trachomatis* and *Chlamydia psittaci* infection is usually based on Complement Fixation (CF), micro immunofluorescence (MIF) or enzyme-immunoassay (EIA) (8,18,19) . The antigens used in these tests are purified from Chlamydia-infected cell cultures. Many cells used for cell culture are contaminated with mycoplasma all over the world (7). Since we had interpretation difficulties with the MIF for the detection of *C.pneumoniae* specific antibodies due to non-specific background fluorescence, we investigated the mycoplasma contamination of these commercially available Chlamydia antigens by using a mycoplasma group specific 16S rRNA PCR. Sequence analysis was done to identify the species found by PCR.

MATERIALS AND METHODS

Chlamydia antigens

Purified *C.pneumoniae* elementary bodies were obtained from the Washington Research Foundation (WRF) (Seattle, WA, USA; lot numbers 6, 7 and 17). Slides coated with Chlamydia antigen were obtained from MRL Diagnostics; Cypress, CA, USA and from Labsystems OY, Helsinki, Finland (lot number R047994 and 83 PB 1, respectively). These slides are marketed for the specific purpose of serological diagnosis of chlamydial infection. *C.psittaci* complement fixation (CF) reagents were obtained from Virion International Distribution Ltd., Chan, Switzerland and from Behring Werke, Marburg, Germany, (lot numbers 293616.08 and 413588B, respectively)

Mycoplasma PCR

Chlamydia antigen slides from MRL Diagnostics and from Labsystems OY were washed as described by Gilroy *et al.* (4). Briefly, three antigen slides were used per isolation. Sterile distilled water (50μl per slide) was added to the slide using a aerosol resistant tip. The surface was washed and scraped with the tip to dislodge the material and suspend it in the water. Purified *C.pneumoniae* antigen (approximately 10^9 organisms per ml) and *C.psittaci* CF antigen was diluted 1/100 and 1/10 with sterile distilled water, respectively. 100μl diluted antigen suspension or 100 μl recovered chlamydial antigen from slides were pretreated as described by Boom *et al.* (1). Mycoplasmal DNA was amplified by PCR and analysis of the amplified samples were performed as described by van Kuppeveld *et al.* (16). A mycoplasma group specific primer set was used, which amplifies a 280-bp fragment with the following primers: The forward primer GPO-3 (5'-GGGAGCAAACAGGATTAGATACCCT-3') and the reverse primer MGSO (5'-TGCACCATCTGTCACTCTGTTAACCTC-3'). PCR products (40 μl) were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Southern blot hybridization was performed with [γ-^{32}P]ATP-end-labeled internal oligonucleotide GPO-2 (5'-CTTAAAGGAATTGACGG GAACCCG-3'). The sensitivity of the genus-specific mycoplasma PCR using *Mycoplasma pneumoniae* was approximately one colony forming

unit (17). All isolated Chlamydia antigen preparations were amplified also with a Chlamydia specific PCR to monitor the DNA isolation. All *C.pneumoniae* containing antigens were amplified by a *C.pneumoniae* -specific PCR and analysis of the amplified samples were performed as described by Campbell *et al.* (2). All *C.psittaci* CF antigens were amplified by using a PCR which amplifies both *C.pneumoniae* and *C.psittaci,* as described by Tong *et al.* (15). PCR products (40 μl) were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide. Twenty percent of the PCR reaction tubes were used for negative controls. All pipetting was done using aerosol resistant tips and the recommendations of Kwok *et al.* were used to prevent carryover contamination (6).

Mycoplasma hybridization assay

The Gen-Probe Mycoplasma tissue culture hybridization assay (Gen-Probe Inc.,San Diego, CA) was used as prescribed by the manufacturer.

DNA sequencing and phylogenetic tree construction

The 373 DNA Sequencing System (Perkin Elmer, Foster City, USA) was used for sequencing the amplified fragments according to the manufacturer's protocol. Sequence homology searches were done by use of the European Molecular Biology Laboratory nucleotide sequence database (EMBL; Heidelberg, Germany) and the GenBank nucleic acid database (National Center for Biotechnology Information; Bethesda, MD, USA). The GenBank nucleic acid database was used for the sequence retrieval of other frequent contaminants of tissue cultures and other bacteria.

The CLUSTAL W program (European Molecular Biology Laboratory, Heidelberg, Germany), release 1.5, was used to calculate the homology of the sequences and to construct multiple alignments of the sequences (14). The PHYLIP program (Phylogeny Inference Package; University of Washington, Seattle, USA), version 3.5c, was used to construct a phylogenetic tree (9). The Maximum Likelihood method was used to estimate the phylogenetic distances between the sequences (3).

Serology

MIF assay using purified *C.pneumoniae* antigen from the Washington

Research Foundation (Seattle, WA) as described before was used to measure *C.pneumoniae* specific IgG-, IgM- and IgA antibodies (18). In addition, a commercially available MIF assay was used to compare *C.pneumoniae* specific IgG-, IgM- and IgA antibody titers (Labsystems OY, Helsinki, Finland). Sera were obtained from patients with clinical and serological evidence of recent respiratory tract infection due to *Mycoplasma pneumoniae* (n=32) or *Chlamydia pneumoniae* (n=156).

RESULTS

Four batches of *C.pneumoniae* antigen of the Washington Research Foundation were analyzed. Lot numbers 7 and two batches of 17, which were provided in 1992, 1994 and 1995 respectively, were all mycoplasma PCR positive. Lot number 6, which was provided in 1991 was mycoplasma PCR negative. The microscopic slides from MRL for the detection of chlamydial antibodies were also positive for mycoplasma, as determined by PCR, while the microscopic slides from Labsystems for the detection of chlamydial antibodies and the *C.psittaci* CF antigen products proved to be mycoplasma PCR negative. The specificity of the amplified products was confirmed by hybridization with the internal probe GPO-2. Detectable chlamydial DNA was present in all samples, as determined by Chlamydia-specific PCR, while all negative controls remained negative. All mycoplasma PCR positive antigens were confirmed by the Gen-Probe Mycoplasma hybridization assay. However, a total of five MRL slides were needed to obtain a weak positive signal in this hybridization assay.

A DNA fragment of 280bp was PCR amplified using the primers GPO-3 and MGSO. After sequencing the product in both directions, a consensus sequence was constructed. The length of the remaining sequence was 200 nucleotides. This fragment contained the two variable regions V3 and V5 of the 16S rRNA gene. Comparison of the nucleic acid sequences of all mycoplasma positive *C.pneumoniae* antigen lot numbers of the WRF, including the mycoplasma positive *C.pneumoniae* antigen slides from MRL Diagnostics showed a high degree of similarity (Table 1).

The nucleic acid sequence of the *Mycoplasma* species, found in the WRF and MRL products were further aligned with those of a group of nine

Table 1. Homology values (%) between 16S rRNA sequences of bacterial contaminants found in three different *C.pneumoniae* antigen batches of the WRF and the MRL Chlamydia antigen slides and of some other bacterial species.

	WRF17a	WRF17b	MRL	*M.orale*	*M.fermentans*	*M.arginini*	*A.laidlawii*	*M.pneumoniae*	*C.pneumoniae*	*C.psittaci*	*C.trachomatis*
WRF7	99	98	98	86	85	93	74	74	65	66	65
WRF17a	-	97	96	87	86	92	74	73	65	65	65
WRF17b		-	97	87	86	92	74	73	65	66	65
MRL			-	88	88	93	76	75	67	68	67
M.orale				-	83	87	70	69	63	63	62
M.fermentans					-	88	76	76	66	66	65
M.arginini						.	76	75	68	68	67
A.laidlawii							-	77	69	68	67
M.pneumoniae								-	68	67	67
C.pneumoniae									-	93	93
C.psittaci										-	97

note: Homology values were determined with the CLUSTAL W program.

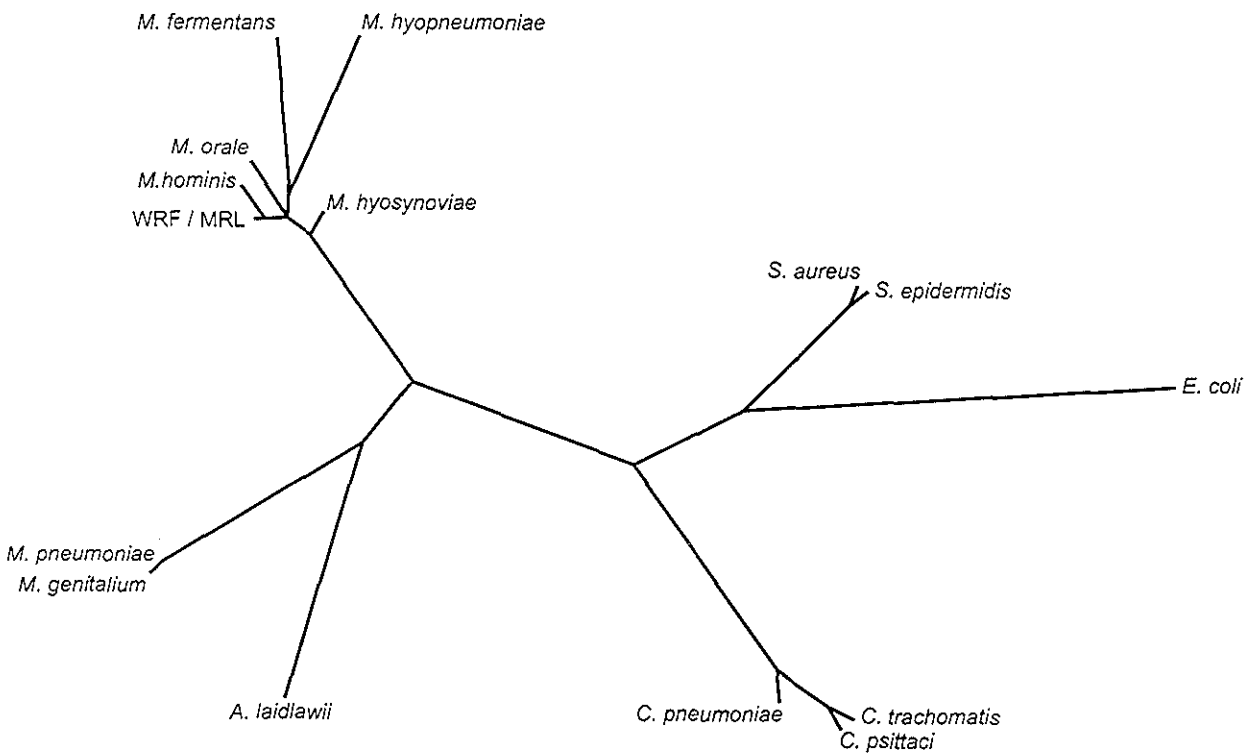

Figure 1. Phylogenetic tree of the WRF - and MRL mycoplasma contaminant and other bacteria based on 16S rRNA sequences. Phylogenetic tree was calculated by the Maximum Likelihood procedure of the PHYLIP program.

known *Mycoplasma* spp, i.e. *M.hyosynoviae, M.hyopneumoniae, M.hominis, M.orale, M.arginini, M.fermentans, M.pneumoniae, M.genitalium* and *A.laidlawii.* Six other bacterial 16S rRNA sequences were aligned as well, i.e. *C.pneumoniae, C.psittaci, C.trachomatis, S.aureus, S.epidermidis* and *E.coli.* To investigate the relationships among the group *Mycoplasma* species, found in the WRF antigen, the mycoplasma found in the MRL antigen slides and other bacteria, a phylogenetic tree was constructed. Analysis of the phylogenetic tree revealed that the mycoplasma sequences from the WRF- and MRL antigen products were highly similar to those published for *M.arginini* (Figure 1).

188 serum samples were used to determine the impact of mycoplasma contamination of these preparations on their used in serology. No fluorescence pattern was found compatible with cross reactivity in the *C.pneumoniae* MIF assay when 32 serum samples from patients with recent *Mycoplasma pneumoniae* infection were used. Also, no significant difference were observed in the *C.pneumoniae* specific IgG-, IgM- and IgA titers if the mycoplasma contaminated - and clean *C.pneumoniae* antigen were used and their results compared (data not shown).

Another confounding factor studied was the possibility wether such mycoplasma contamination would alter the expression of antigens in the *C.pneumoniae* membrane. MIF results were compared from patients with recent *C.pneumoniae* infection using the mycoplasma contaminated - and clean *C.pneumoniae* antigens. 156 serum samples were compared. Again, no significant difference was found in *C.pneumoniae* specific IgG-, IgM- and IgA titers (data not shown).

DISCUSSION

The class Mollicutes consists of a very large group of prokaryotes distinguished phenotypically separated from other bacteria by their lack of cell walls. The largest group is formed by the genus *Mycoplasma*, of which more than 90 species have been described. Because of their small size (300-800nm) and flexibility, mycoplasma can pass easily through 220 and 450 nm filters used in cell culture laboratories (10). Cell cultures are widely used in both diagnostic tests and in biomedical research settings,

as well as in the production of antigens and vaccines by industry. Approximately 15% of all continuous cell lines are estimated to be contaminated with mycoplasma worldwide (7). At this moment, five species account for more than 95% of these infections. The two most prevalent contaminating human strains are *Mycoplasma orale* and *Mycoplasma fermentans,* two bovine Mollicutes, *Mycoplasma arginini* and *Acholeplasma laidlawii* and a porcine Mollicute, *Mycoplasma hyorhinis* (13). Mycoplasmal infection may induce various degrees of cytopathologic effects (CPE) in cells. However, infection often remains inapparent. These inapparent infections may result in unreliable experiments as seen in the study of Tan *et al.* (12). The operon described in their study has been found to be of mycoplasmal origin and not of chlamydial origin as initially reported. Also, mycoplasma infection can be responsible for the distribution of unsafe biological products including vaccines and antigen preparations for serological diagnosis (5,11).

Serological diagnosis of Chlamydia infections is often based on CF or MIF results (18,19). The antigens used in these tests are commonly purified from Chlamydia-infected cell lines. Messmer *et al.* reported that mycoplasma contamination of *Chlamydia pneumoniae* isolated from clinical specimens often occurs (7). Their findings suggests that the *C.pneumoniae* isolates were contaminated by isolation of respiratory specimens, or by propagation in contaminated cell lines.

In this study, mycoplasma contamination was observed in commercially available antigens marketed for the detection of Chlamydia specific antibodies. Several lot numbers of the purified *C.pneumoniae* antigen from the WRF were contaminated with identical mycoplasmas, as determined by PCR and sequence analysis. Also, the MRL antigen slides for the detection of chlamydial antibodies was PCR positive for the same mycoplasma. One of the explanations of this result can be found in the sharing of cell lines and/or the use of the same organism for the production of the antigen. Both companies use the AR-39 organism and HeLa 229 cell line for the production of the *C.pneumoniae* antigen. The organism MRL used, was provided by the WRF.

Analysis of the phylogenetic tree revealed that the mycoplasma sequences from the WRF- and MRL antigen products were closely related to- but not

identical to *M.arginini.* This indicates the possibility of a new *Mycoplasma* species, which was not previously submitted to the GenBank and EMBL databases.

Several methods were used by MRL Diagnostics, Labsystems and the WRF to detect mycoplasmal contamination prior to culture the chlamydial antigens. The mycoplasma detection at MRL diagnostics was done by using the GenProbe Mycoplasma screening assay (Dr. W. Hogrefe, personal communication). Failure to detect mycoplasmal contamination by hybridization assay was probably due to the small number of mycoplasma organisms present in the samples, as seen in the present study. From every new Chlamydia antigen lot the cell culture system from Labsystems was tested with fluorochromic dye (DAPI). Also, the cells were tested twice a year by using the Mycoplasma PCR ELISA from Boehringer Mannheim (Dr. M. Ristola, personal communication). The Washington Research Foundation tested only the stock HeLa 229 cells by DAPI staining twice a year (Dr. C.C. Kuo, personal communication). Despite these prevention strategies, contamination with mycoplasma has apparently occurred frequently. These results demonstrate that the PCR may currently be the preffered tool for the detection of mycoplasma contamination in cell cultures and chlamydial strains.

Although this particular mycoplasma contamination had no significant impact on *C.pneumoniae* serodiagnosis by MIF, caution should be taken. Inapparent cell line contamination with human *Mycoplasma* species is always possible which can have a major impact on the serodiagnosis of many bacterial - and viral infections, and otherwise confound research efforts.

We conclude that mycoplasma contamination of commercially available antigens for the serodiagnosis of *C.pneumoniae* infections has occurred, and therefore recommend routine screening of all antigen preparations obtained by tissue culture prior to their distribution and use.

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CHAPTER 6

EVALUATION OF PCR, CULTURE AND SEROLOGY FOR THE DIAGNOSIS OF *CHLAMYDIA PNEUMONIAE* RESPIRATORY INFECTIONS

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Submitted for publication

ABSTRACT

We prospectively studied 156 patients with a diagnosis of community-acquired pneumonia requiring admission. Several respiratory specimens were obtained for the detection of *C.pneumoniae* using cell culture and PCR. From each patient, three serum samples were obtained. Serological diagnosis of a *C.pneumoniae* infection was determined by microimmunofluorescence (MIF), complement fixation (CF) and recombinant LPS ELISA. The diagnosis of *C.pneumoniae* community-acquired pneumonia was established in 45 patients (29%). Twenty-three patients (15%) had serological results compatible with acute *C.pneumoniae* infection; nine (39%) of these subjects were *C.pneumoniae* PCR positive. Twenty-two patients (14%) had positive PCR results without serological evidence of an acute *C.pneumoniae* infection. An attempt was made to calculate the sensitivity and specificity of the MIF, rDNA LPS ELISA and PCR for the diagnosis of *C.pneumoniae* community-acquired pneumonia. Several gold standards were defined. Generally, the sensitivity of the rDNA LPS ELISA and MIF were comparable, while the sensitivity of the CF test was shown to be very low, particularly in this elderly group of patients. Independent of the gold standard used, the best PCR results were obtained using nasopharyngeal specimens. However, the predictive value of a positive *C.pneumoniae* PCR in cases of community-acquired pneumonia remains unknown and may be low. Although a widely accepted gold standard is still lacking, the rDNA LPS Chlamydia assay may currently be the preferred tool for diagnosing acute respiratory *C.pneumoniae* infections in routine clinical practice. However, MIF remains the method of choice to determine the prevalence of *C.pneumoniae* infections in a given community.

INTRODUCTION

Most respiratory infections caused by *Chlamydia pneumoniae* are mild or asymptomatic (1,9,25,41) . Similar to mycoplasma infections, *C.pneumoniae* can cause recurrent or secondary lower respiratory tract infections, even though antibody due to previous infection are detectable in serum

(1). Infection with *C.pneumoniae* occurs worldwide, resulting in a 40 to 90% prevalence of serum antibody to the species (3,17,33,47). *C.pneumoniae* has been associated with both epidemic and endemic occurrences of acute respiratory disease and is believed to be responsible for 6-20% of all community-acquired pneumonias (1,13,20,27,37,40). Diagnosis of *C.pneumoniae* infection is preferably based on the isolation of the organism from respiratory specimens, PCR, and/or serology (19,50). However, isolation of *C.pneumoniae* by cell culture remains difficult and has unknown sensitivity. Subsequently, culture tests are not available in routine laboratories. Also, PCR requires specially trained, experienced personnel and is not yet commercially available. Therefore, serology is currently the tool most often applied for routine diagnosis of acute *C.pneumoniae* infection. The commercially available serological tests include the complement fixation (CF) test, the microimmunofluorescence (MIF) test and the ELISA. The CF test has been traditionally used for diagnosing chlamydial respiratory infections (31,38). This assay utilizes an enriched LPS antigen derived from *Chlamydia psittaci* for the detection of Chlamydia genus-specific antibodies. CF assays are technically demanding and no information is obtained about the immunoglobulin classes involved in the reaction. The gold standard in *C.pneumoniae* serology at this moment is the MIF test (12,29,39). *C.pneumoniae* elementary bodies (EBs), which are the infective cell forms of *C.pneumoniae*, are used as antigen in the MIF test. This test is purposed to be species specific and can differentiate between IgG, IgM and IgA antibodies (38,43). We evaluated a commercial available rDNA LPS ELISA, microimmunofluorescence, PCR and culture for the diagnosis of *C.pneumoniae* community-acquired pneumonia. In addition, we sought to evaluate the usefulness of the rDNA LPS assay to determine *C.pneumoniae* seroprevalence.

PATIENTS AND METHODS

Patients

The study population included four different groups of patients. The first group consisted of 1,104 blood donors visiting the Red Cross Blood Transfusion Center, Tilburg, The Netherlands. From each blood donor, one

serum sample was stored at minus 80 °C prior to use in this study. Donor's ages ranged from 18 to 68 years with a median of 40 years. The second group consisted of 271 patients with chronic obstructive pulmonary diseases (COPD) attending the outpatient clinic of pulmonary diseases from the St. Antonius Hospital, Nieuwegein, The Netherlands. Patient's ages ranged from 43 to 88 with a median of 67 years. The third group consisted of 156 prospectively studied patients that were consecutively admitted to the same St. Antonius Hospital with community-acquired pneumonia (CAP). The diagnosis was based on clinical signs and symptoms and new or progressive radiographic changes consistent with pneumonia (10). Patient's ages ranged from 20 to 93 with a median of 68 years. The fourth group consisted of 40 other patients with a recent *Mycoplasma pneumoniae* infection, as indicated by rising complement fixation antibody titers or by positive IgM immunofluorescence reaction (kindly provided in part by Dr. R.J.A. Diepersloot, Diakonessen Hospital, Utrecht, The Netherlands).

Clinical specimens

Serum samples were collected using standard procedures and stored at minus 80 °C prior to processing. From each patient with CAP, nasopharyngeal and throat specimens were collected using sterile cotton-tipped aluminum shafted swabs and suspended in 1.5 ml Chlamydia transport medium (2SP). A throat wash sample was obtained using 10 ml phosphate buffered saline (PBS). Sputum samples were collected using standard procedures. The first serum sample was obtained within the first 24 hours of enrollment. A second (convalescent) serum sample was obtained from all patients 10 days after enrollment. From 142 patients (91%), a third serum sample was obtained after 30 days.

Laboratory assays for *C.pneumoniae*

Culture: Culture for *C.pneumoniae* was done as described previously (43). Briefly, Buffalo Green Monkey cells (BGM) (PAMM, Veldhoven, The Netherlands) (44) were seeded into 24-well tissue culture plates (Costar Europe Ltd.) and incubated at 35 °C with 5% CO_2 in a fully humidified

cabinet. All cell monolayers were examined on the day of inoculation for confluency. For each experiment, a patient's sample was inoculated into two microtiter wells (24-well microtiter plate) and one flat-bottom tube. After inoculation, microtiter plates were centrifuged at 900 x g at 25 °C for 60 minutes and subsequently incubated with fresh medium containing cycloheximide (0.6 mg/l) (Sigma Chemical Company, St Louis, Mo.). After 3 days, the 24-well plates were aspirated and fixed with methanol (Merck, Darmstadt, Germany). The fixed monolayers were rinsed with PBS once and stained by the fluorescent-antibody technique using Chlamydia genus-specific mouse monoclonal antibody (kindly provided by J.M. Ossewaarde, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). The inoculated flat-bottom tubes were passed onto fresh monolayers and reincubated as described before. This procedure was repeated once more. In each culture series, *C.pneumoniae* TW-183 was included in parallel as a positive control. Positive cultures were stained with *C.pneumoniae* specific mouse monoclonal antibodies (Washington Research Foundation, Seattle, Wa). Rabbit anti-mouse Ig labeled with fluorescein isothiocyanate (Dako A/S, Glostrup, Denmark) was used as a conjugate. Evans Blue (0.05%; Sigma Chemical Company, St Louis, Mo.) was used as a counterstain.

PCR: Two-hundred μl nasopharyngeal or throat specimen or 1.0 ml throat wash and broncho-alveolar lavage were transferred to a sterile tube and centrifuged for 30 min at 15,000 x g. Sputum samples were suspended in 1.5 ml 2SP transport medium. One-hundred μl suspended sputum sample was transferred to a sterile tube and centrifuged for 30 minutes at 15,000 x g. The sediment was incubated with a solid carrier (celite) and a guanidinium-thiocyanate (GuSCN) containing lysis buffer. Nucleic acids (NA) were bound to the carrier which was rapidly sedimented by centrifugation. The sedimented complexes were washed once with a GuSCN-containing washing buffer, once with 70% ethanol and once with acetone. Complexes were dried and NA was subsequently eluted in 55μl aqueous solution at 37°C for 30 minutes. The isolated NA was removed from the solid carrier by centrifugation (5). Amplification by PCR and analysis of the amplified products were performed as described by Campbell *et al.* (8). Briefly, PCR amplification was performed with 8 μl isolated DNA in a 100

μl reaction mixture containing 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCL (pH 9.0) 0.01% Tween-20, 0.2 mM deoxynucleoside triphosphates, 50 pmol primers and 0.2U Thermoperfect *Taq* polymerase (Integra Biosciences A.G., Wallisellen, Switzerland). A *C.pneumoniae* species specific primer set was used, which amplifies a 437-bp fragment with the following primers: The forward primer HL-1 (5'-GTTGTTCATGAAGGCCTACT-3') and the reverse primer HR-1 (5'-TGCATAACCTACGGTGTGTT-3'). PCR products (20 μl) were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. DNA was transferred from agarose to Hybond plus nylon filters (Amersham International plc, Amersham, United Kingdom) by electrophoretic transfer. The PCR products were analyzed with a *C.pneumoniae* specific probe HM-1 (5'-GTGTCATTCGCCAAGGTTAA-3'). The ECL 3'-oligolabeling and detection system was used for the detection of the PCR products (Amersham International, plc, Amersham, United Kingdom). A 10-fold serial dilution of a *C.pneumoniae* stock solution (10^9 target DNA copies per ml) was incorporated in each amplification. One negative control (pooled cervical specimens suspended in 2SP) was incorporated every 10 amplifications. All PCR positive reactions were confirmed by repeat DNA isolation and amplification of the original specimen. Separate rooms were used for the different steps of the PCR and the recommendations of Kwok *et al.* were used to prevent DNA carryover contamination (32).

Serology: *Microimmunofluorescence:* Microimmunofluorescence (MIF) assay, as described before was used to measure *C.pneumoniae* specific IgG-, IgM- and IgA antibodies (43). Briefly, purified *C.pneumoniae* elementary antibodies (strain AR 39; Washington Research Foundation, Seattle, USA) were used to detect IgG, IgM and IgA antibodies to *C.pneumoniae*. Prior to the IgM determinations, IgG absorption was performed (43). Prevalence of *C.pneumoniae* specific IgG-, IgM- and IgA antibodies was based on the presence of IgG at titers ≥ 1:32, IgM≥1:16 and IgA ≥1:32, respectively. Diagnosis of an acute *C.pneumoniae* infection was based on the following criteria: The presence of *C.pneumoniae* specific IgM in either acute or convalescent serum, or a four-fold or greater titer increase in *C.pneumoniae* specific IgG- and/or IgA antibody between acute and convalescent serum (43).

Complement fixation: The complement fixation (CF) test was used to measure Chlamydia genus-specific antibodies using *Chlamydia psittaci* antigen (Virion International Distribution Ltd, Chan, Switzerland). A ≥4-fold rise in titer was considered a definitive etiologic evidence for infection. *ELISA*: Chlamydia-specific IgG, IgM and IgA antibodies were detected using a recombinant LPS ELISA (rDNA LPS ELISA; Medac GmbH, Hamburg, Germany). This ELISA includes a chemically pure structure of a recombinant LPS which contains a genus-specific epitope of the human pathogens *Chlamydia* spp. (6,7,24). Initial serum dilutions for IgG, IgM and IgA were 1:100, 1:50 and 1:50, respectively. Prior to the IgM determinations IgG absorption was performed (43). Sera with OD values exceeding 2.5 were retested using a 1:4 predilution. A two-fold serially diluted standard serum was used to calculate the $\log_2$ titer of the patients' samples. The IgG-, IgM- and IgA cutoff values were calculated as prescribed by the manufacturer. Prevalence of anti-Chlamydia IgG-, IgM- and IgA anti-bodies was based on the following criteria : ≥calculated cutoff x 1.10, ≥calculated cutoff x 1.15 and ≥calculated cutoff x 1.10, respectively. Serological diagnosis of an acute Chlamydia infection was based on the ELISA results using the following criteria : A three-fold or greater increase in Chlamydia-specific IgG- or IgA antibody titer, or a two-fold or greater change in specific IgM titer, or a two-fold increase in specific IgG antibody titer in combination with a two-fold increase in specific IgA antibody titer (46).

RESULTS

Seroprevalence

The prevalence of Chlamydia-specific IgG antibodies in the healthy blood donor group and COPD patient group was 29.4% and 53.1%, respectively, as determined by rDNA LPS ELISA ($p<0.0001$) (Table 1). Significant higher seroprevalence was observed if the *C.pneumoniae* MIF was used (Table 1). The seroprevalence by MIF was the same for healthy blood donors and COPD patients (71% and 72%, respectively). Using the MIF as the gold standard, the sensitivity and specificity of the rDNA LPS ELISA to determine serological evidence of an *C.pneumoniae* infection in the past

thus depended on the population tested (Table 1). In the healthy blood donor group the sensitivity and specificity was 33.5% and 80.5%, respectively, while in the patients with COPD, the sensitivity and specificity was 64.1% and 75.0%, respectively. Similar trend was observed when calculating the negative predictive value (NPV) and positive predictive value (PPV) (Table 1).

Table 1. Prevalence of chlamydial reactive antibody determined by MIF and rDNA LPS ELISA.

		prevalence in % observed by		rDNA LPS ELISA			
Study population	n	MIF	rDNA LPS ELISA	sensitivity in %	specificity in %	NPV[a] in %	PPV[a] (%)
Blood donors	1104	71.2	29.4	33.5	80.5	32.9	80.9
COPD patients[b]	271	72.0	53.1[c]	64.1	75.0	44.9	86.8

[a] NPV = negative predictive value, PPV = positive predictive value using MIF as gold standard
[b] chronic obstructive pulmonary disease
[c] $p<0.0001$ as compared to blood donors

Acute *C.pneumoniae* infections

A diagnosis of *C.pneumoniae* community-acquired pneumonia was possible in 45 patients (29%). Twenty-three patients (15%) had serological results (MIF and/or rDNA LPS ELISA) indicating acute *C.pneumoniae* infection. Nine of these subjects (39%) were *C.pneumoniae* PCR positive. In addition, 22 (14%) patients had positive PCR results without serological evidence of acute *C.pneumoniae* infection. In 14/15 (93%) patients with MIF results indicative for acute *C.pneumoniae* infection the diagnosis was supported by rDNA LPS ELISA and/or PCR test results. Six patients had serological results indicative of an acute infection only by rDNA LPS ELISA (Figure 1). Only one patient had positive *C.pneumoniae* culture results which was confirmed by PCR , MIF and ELISA (not shown in Figure 1). Of those that responded serologically only two patients had complement fixation results indicative for an acute chlamydial infection.

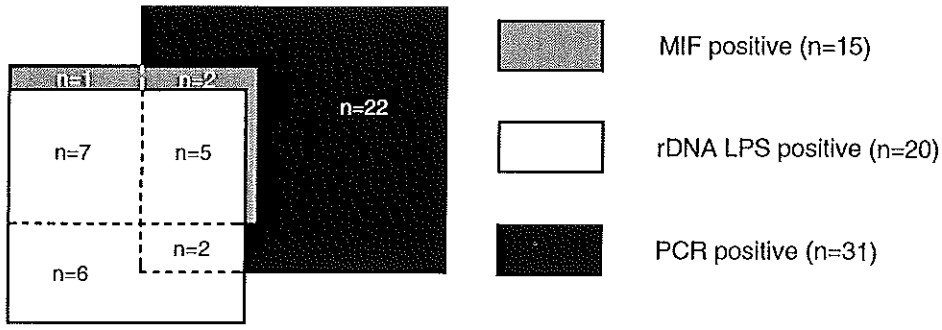

Figure 1. Schematic presentation of partially overlapping positive diagnostic test for acute *C.pneumoniae* respiratory infection in 156 patients with community-acquired pneumonia.

Different approaches were performed to calculate the sensitivity and specificity of the MIF, rDNA LPS ELISA and PCR. When using the MIF as the gold standard for the diagnosis of an acute *C.pneumoniae* infection, the sensitivity and specificity of the rDNA LPS ELISA and PCR was 80.0%, 94.3%, 46.7% and 83.0%, respectively (Table 2). A second gold standard was incorporated to define more liberally the true positive patient population. A patient was true positive if MIF results were indicative for an acute *C.pneumoniae* infection, or one or more respiratory specimens were PCR positive. Using this expanded gold standard, the sensitivity of the MIF and rDNA LPS ELISA were comparable (38.5% and 35.9%, respectively). Increased sensitivity was now observed for the PCR (79.5%) but in this case all respiratory specimens had to be processed (Table 2). Two additional expanded gold standards, one more stringent and the other more liberal but both serology-based, were incorporated in the evaluation to determine the impact of the varying gold standard (Table 2). Clearly, the sensitivity of the PCR assay was substandard in this latter evaluation. The positive predictive value (PPV) became clinically irrelevant.

Table 2. Sensitivity, specificity, negative predictive value and positive predictive value of the rDNA LPS ELISA, MIF and PCR to determine acute *C.pneumoniae* infections in patients with community-acquired pneumonia requiring admission.

gold standard	positive (%)	test evaluated	sensitivity in %	specificity in %	NPV[a] in %	PPV[a] in %
MIF	15 (9.6)	ELISA	80.0	94.3	97.8	60.0
		PCR	46.7	83.0	93.6	22.6
expanded gold standard 1[b]	39 (25.0)	MIF	38.5	100.0	83.0	100.0
		ELISA	35.9	94.9	81.6	70.0
		PCR	79.5	100.0	93.6	100.0
expanded gold standard 2[c]	12 (7.7)	MIF	100.0	97.9	100.0	80.0
		ELISA	100.0	94.4	100.0	60.0
		PCR	41.7	81.9	94.4	16.1
expanded gold standard 3[d]	23 (14.7)	MIF	65.2	100.0	94.3	100.0
		ELISA	87.0	100.0	97.8	100.0
		PCR	39.1	83.5	88.8	29.0

[a] NPV = negative predictive value, PPV = positive predictive value
[b] PCR / culture positive and / or MIF results indicating acute *C.pneumoniae* infection
[c] MIF and rDNA LPS ELISA results indicating acute *C.pneumoniae* infection
[d] MIF and / or rDNA LPS ELISA results indicating acute *C.pneumoniae* infection

Table 3. Effect of specimen type on the performance of PCR to determine acute *C.pneumoniae* infections in 156 patients with community-acquired pneumonia requiring admission.

gold standard	positive (%)	specimen evaluated	sensitivity in %	specificity in %	NPV[a] in %	PPV[a] in %
MIF	15 (9.6)	throat	20.0	89.9	91.2	17.6
		throat wash	6.7	95.6	90.3	14.3
		nasopharynx	40.0	89.9	93.3	30.0
expanded gold standard 1[b]	39 (25.0)	throat	43.6	100.0	83.9	100.0
		throat wash	17.9	100.0	77.9	100.0
		nasopharynx	51.3	100.0	85.8	100.0
expanded gold standard 2[c]	12 (7.7)	throat	16.7	89.4	92.7	11.8
		throat wash	8.3	95.7	92.4	14.3
		nasopharynx	33.3	88.7	94.0	20.0
expanded gold standard 3[d]	23 (14.7)	throat	17.4	90.0	86.1	23.5
		throat wash	8.7	96.1	85.5	28.6
		nasopharynx	36.4	90.9	89.6	40.0

[a] NPV = negative predictive value, PPV = positive predictive value
[b] PCR / culture positive and / or MIF results indicating acute *C.pneumoniae* infection
[c] MIF and rDNA LPS ELISA results indicating acute *C.pneumoniae* infection
[d] MIF and / or rDNA LPS ELISA results indicating acute *C.pneumoniae* infection

Similar calculations were made to determine the impact of specimen type on the sensitivity, specificity, negative predictive value (NPV) and PPV of the PCR (Table 3). Independent of the four different gold standards used, the highest PCR sensitivity and specificity could be obtained using nasopharyngeal specimens (Table 3). Surprisingly, all sputum samples tested remained negative (data not presented in Table 3).

The serological results of the first two serum samples were used to determine the impact of a third serum sample obtained after 30 days. Only 67% and 75% of the patients with serological results indicative for an acute infection were diagnosed using MIF and rDNA LPS ELISA, respectively if only the first and second serum sample was applied.

Since *Mycoplasma pneumoniae* is a common agent in CAP and is a well known polyclonal B-cell activator (4), an attempt was made to investigate the possibility of cross-reactivity in the rDNA LPS ELISA due to acute *M.pneumoniae* infection. Acute and convalescent serum sample of 40 patients with serological evidence of *M.pneumoniae* infection were used for this evaluation. None of these patients had serological results compatible with acute *C.pneumoniae* infection observed by MIF and rDNA LPS ELISA. However, a high prevalence of Chlamydia-specific IgG, IgM and IgA antibodies by rDNA LPS Chlamydia ELISA was found in these patients (75, 18 and 48%, respectively). IgM positive results were found in seven patients. However, all seven patients had an IgM titer that was rather low (OD values ranged between 1.2 and 3.0 times the cutoff value, with a median of 1.5) and did not change in titer between the acute and convalescent phase of their disease. To further elucidate the nature of this IgM reactivity in these patients, their serum samples were absorbed with a highly concentrated *M.pneumoniae* suspension prior to their processing in the ELISA. No significant reduction in Chlamydia-specific IgM antibody titer was found after such absorption (data not shown). Furthermore, in the 156 patients with acute CAP, *M.pneumoniae* complement fixation antibody titers were determined. These data together with those from patients with *M.pneumoniae* infection were analyzed. A positive correlation was found between the mycoplasma titer and the chlamydial IgG-, IgM- and IgA seroprevalence, as determined by rDNA LPS ELISA (Table 4).

Table 4. Correlation between *Mycoplasma pneumoniae* antibody titer found by complement fixation antibody assay and Chlamydia-specific antibodies determined by rDNA LPS ELISA.

reciprocal *Mycoplasma pneumoniae* complement fixation antibody titer	number of patients tested	number of patients seropositive by rDNA Chlamydia LPS ELISA (%)		
		IgG[a]	IgM[b]	IgA[c]
<4	35	19 (54)	2 (6)	18 (51)
4-16	69	35 (51)	8 (12)	37 (54)
32-128	69	43 (62)	11 (16)	40 (58)
≥256	17	15 (88)	5 (29)	12 (71)

[a] correlation coefficient = 0.902; p=0.098, Chi^2 for trend: p=0.020
[b] correlation coefficient = 0.975; p=0.025, Chi^2 for trend: p=0.020
[c] correlation coefficient = 0.954; p=0.049, Chi^2 for trend: p=0.201

DISCUSSION

More than ten years after the first publication suggesting that *Chlamydia pneumoniae* causes acute respiratory infection in 1985 (21), the diagnosis of this infection is still difficult to make. In the early 1970s, a sensitive microimmunofluorescence (MIF) assay was developed which proved to be suitable for routine diagnosis (48,49). In the MIF test, purified elementary bodies are used to detect specific Chlamydia antibodies in the IgM, IgG and IgA serum fractions. Some researchers claim that the *C.pneumoniae* MIF test is highly specific (21,22,35). Others, however, have noted cross reactions between *C.pneumoniae* and other chlamydial species (30,34,36). This may be explained by the fact that the elementary body of each *Chlamydia* species will possess both genus specific and species specific antigenic sites. A species specific positive result is based on the reaction with the major outer membrane protein (MOMP) of the elementary body, while chlamydial lipopolysaccharide is responsible for a genus specific reaction (42). Interpretation may, thus, be difficult. Also, rheumatoid factor can cause false-positive *C.pneumoniae* IgM results (43). Acute *C.pneumoniae* infections can be diagnosed also by culture and PCR. However, these

techniques requires highly trained, experienced personnel and is not yet commercially available. Recently, chemically pure chlamydial LPS has been applied in developing a commercially available recombinant-DNA LPS ELISA (6,7,24). One of the disadvantages of using LPS is the inherent serologic cross reactivity among the *Chlamydia* species. However, a major advantage in the rapid development of anti-LPS antibodies early in infection (28). Also, the rDNA LPS ELISA results are observer-independent and can easily be standardized for routine diagnosis, while the MIF is more subjective and requires highly qualified personnel to interpret the fluorescence results. Little is known about the sensitivity and specificity of this ELISA to determine the *C.pneumoniae* seroprevalence. In this study, we found that the rDNA LPS ELISA may not be reliable to determine *C.pneumoniae* seroprevalence, i.e. searching for patients with serological evidence of a *C.pneumoniae* infection in the past. The seroprevalence in the two different patient populations indicates that the half-life of the anti-LPS chlamydial antibodies is much lower as compared to the MOMP antibodies (this study). Chlamydial LPS-antibody seroprevalence was higher in patients with COPD history compared to blood donors. This may be partly explained by differences in exposure between the two study groups. Another explanation may be found in the coexistence of other respiratory diseases in older adults with COPD, which may, upon exposure to *C.pneumoniae*, predispose them to clinical disease and thus result in increased *C.pneumoniae* seroprevalence (11,23).

Another approach was to evaluate the MIF, complement fixation test, rDNA LPS ELISA, PCR and culture for the diagnosis of community-acquired pneumonia caused by *C.pneumoniae*. However, the major problem in validating these tests is the definition of the gold standard. Using the MIF as the gold standard, the sensitivity, specificity and, especially the positive predictive value of the rDNA LPS ELISA was somewhat disappointing. Even more discouraging results were observed the by complement fixation (CF) test. The sensitivity of the CF test for the detection of chlamydial infection was shown to be very low, particularly in this elderly group of patients, where most *C.pneumoniae* infections are reinfections that may not induce complement fixing antibodies. In the present study only two patients had CF test results indicating acute chlamydial infec-

tion. Similar results were found earlier (13). The diagnostic value of the CF test is therefore questionable and further use of the CF test for the diagnosis of acute *C.pneumoniae* is dissuaded. Discouraging results were also observed by PCR. This finding may in part, be explained by a low sensitivity of the MIF test. Only severe, deeper localized infections may induce a reaction of the immune system. This is one of the reasons that the utility of the MIF in the etiologic diagnosis of *C.pneumoniae* infection has been questioned earlier by several investigators (30,36). Another approach to search for true positive patients has been published by Gaydos (18) and Falck (16). They used an expanded gold standard which was based on MIF serology and detection of the organism in respiratory specimens. A patient was considered to be true positive if serological evidence of an acute *C.pneumoniae* infection was observed by MIF, or respiratory specimens were positive for *C.pneumoniae* by PCR or culture. Using this expanded gold standard, low sensitivities were found for the MIF and the rDNA LPS ELISA. If all respiratory specimens were incorporated in this evaluation the highest sensitivity was achieved by PCR. On the other hand, the use of PCR and culture in the etiologic diagnosis of *C.pneumoniae* community-acquired pneumonia may be questioned. Asymptomatic *C.pneumoniae* infection, often without serological response have been reported earlier (2,14,19 ,25,26), which may affect the specificity of the test. Therefore we designed a more conservative expanded gold standard, which validated a true positive patient if serological results were obtained indicating acute chlamydial infection by MIF and rDNA LPS ELISA. This will increase the probability of true *C.pneumoniae* community-acquired pneumonia. Again, MIF and rDNA LPS ELISA performed equally well and were significantly better as compared to PCR. The last expanded gold standard definition was based on serological results indicating acute chlamydial infection by either MIF or rDNA LPS ELISA. Increased sensitivity was observed when the rDNA LPS ELISA was applied. Six patients had serological results indicative of an acute infection only by rDNA LPS ELISA. This may be explained by difference in sensitivity which was reported earlier (46).

The choice of respiratory specimen may have a major impact on the sensitivity of the *C.pneumoniae* PCR. Independently of the four different gold

standards used, the highest sensitivity and specificity could be obtained using nasopharyngeal specimens. Surprisingly, none of the sputum samples tested became positive. These findings may indicate colonization of the organism in the upper respiratory tract, rather than invasive infection of the lower respiratory tract.

Only one patient was culture positive. This may be partly explained by the cell line used. Previous investigation has indicated that BGM cells are highly sensitive for the isolation and propagation of *C.pneumoniae* (44). However, the diagnostic value using patient samples was not evaluated before. Another possibility may be the cell culture protocol. Also, inapparent cell line contamination with mycoplasma may confound research efforts (45).

Ekman *et al.* demonstrated that the timing of the convalescent serum sample is of great importance, as the MIF test may not show an increase in titer until 4 weeks after infection (13). These results are in accordance with our data; when the results of the third serum sample was disregarded, 33% and 25% of the patients with an established *C.pneumoniae* etiology were not diagnosed, using MIF and rDNA LPS ELISA, respectively. Also, the use of a single serum sample to diagnose an acute chlamydial infection may reduce the sensitivity and specificity of the assays. Falck *et al.* reported high *C.pneumoniae* MIF IgG antibody titers in patients with persistent *C.pneumoniae* infection without clinical signs of infection for periods of six months up to a year (15). Also, persistent IgM for more than a year has been found earlier (46). A chronic, asymptomatic *C.pneumoniae* infection may be responsible for this phenomenon.

The increased prevalence of Chlamydia-specific antibodies in patients with a recent *Mycoplasma pneumoniae* infection found by the rDNA LPS Chlamydia ELISA, may be explained by polyclonal B-cell activation. Polyclonal B-cell activation due to *M.pneumoniae* infection has been reported earlier by Biberfeld *et al.* (4). The positive correlation found between the mycoplasma complement fixation antibody titer and the Chlamydia-specific IgM prevalence may have a significant impact on the specificity of the rDNA LPS Chlamydia, especially when using a single serum sample. None of these patients had significant changes in their anti-LPS-IgM titers. The use of paired serum samples from each patient, taken with at

least a 10-day interval, will negate this problem.

In conclusion, the study shows that the choice of the gold standard remains difficult, and, as expected, has a major impact on the sensitivity and specificity of the tests validated in this study. Further studies are necessary to determine the value of the PCR using upper respiratory specimens to diagnose lower respiratory infections. In addition, the rDNA LPS Chlamydia test may currently be the preferred serological tool for diagnosing acute respiratory *C.pneumoniae* infections in routine clinical practice. The presence of only *C.pneumoniae* DNA in upper respiratory specimens as demonstrated by PCR, without a concomitant serological response, may indicate prolonged, harmless colonization of the respiratory tract rather than pointing to the agent causing pneumonia.

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CHAPTER 7

DIAGNOSIS OF *CHLAMYDIA PNEUMONIAE* INFECTION IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE BY MICROIMMUNOFLUORESCENCE AND ELISA

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ABSTRACT

Incidence rates of *Chlamydia pneumoniae* infection, obtained by prospective serial serology were determined in patients with chronic obstructive pulmonary diseases (COPD). Chlamydia-specific IgG-, IgM-, and IgA antibodies were detected with a recombinant DNA LPS ELISA as well as with a microimmunofluorescence (MIF) assay using *C.pneumoniae* elementary bodies. From 271 consecutive COPD patients who visited the outpatient clinic of the department of pulmonary diseases (211 males, 60 females, age range 34-88 yrs, mean age ± SD: 66±10 yrs), blood samples (n=1058) were taken every 2-7 months; the observation period ranged between 3 and 19 months (mean ± SD: 15±4). The prevalence of chlamydial IgG was 72% with the MIF and 53% with the rDNA LPS ELISA. More than 90% of the COPD patients had no significant changes in their Chlamydia specific IgG, IgA and IgM titers in either test during the observation period. Seven (3%) patients had MIF results indicating acute *C.pneumoniae* infection during their surveillance period, of whom five were confirmed by rDNA LPS ELISA. Eleven (4%) additional patients were infected during observation, as determined by rDNA ELISA only. These patients had significantly elevated *C.pneumoniae*-specific IgG and IgA MIF titers, as compared with the patients without infection. All 18 patients with serological evidence of acute infection during their surveillance period were retested in a commercial MIF test that can distinguish between *C.pneumoniae*, *C.trachomatis* and *C.psittaci* / LPS specific antibodies, but no evidence of *C.trachomatis* or *C.psittaci* infection was found. The incidence of chlamydial infection was 2.2 and 5.3/100 person-year, when diagnosed by MIF and rDNA LPS ELISA, respectively. We conclude that the rDNA LPS Chlamydia assay may currently be the most sensitive serological tool for diagnosing recent respiratory *C.pneumoniae* infections, Also, *C.pneumoniae* infection occurs rather frequently in COPD patients.

INTRODUCTION

Infection with *C.pneumoniae* occurs worldwide, resulting in a 40 to 90% prevalence of serum antibody to the species in various populations (3,9, 18,25). *C.pneumoniae* has been associated with both epidemic and endemic occurrences of acute respiratory disease and is believed to be responsible for 6-20% of all community-acquired pneumonias (1,6,11,16,21,23). The role of *C.pneumoniae* in the pathogenesis of recurrent exacerbations of chronic obstructive pulmonary diseases is, however, unknown. Serology is currently the tool used most often for routine diagnosis of recent *C.pneumoniae* infection. The standard serological technique at this moment is the microimmunofluorescence (MIF) test. *C.pneumoniae* elementary bodies (EBs), which are the infective cell forms of *C.pneumoniae*, are used as antigen in the MIF test. This test is claimed to be species-specific and can differentiate between IgG, IgM and IgA antibodies (22,24). We evaluated a commercial rDNA lipopolysaccharide (LPS) ELISA (Medac GmbH, Hamburg, Germany) for the detection of Chlamydia-specific antibodies in patients with chronic obstructive pulmonary disease (COPD) and compared it with the MIF assay.

PATIENTS AND METHODS

Patients

The study population consisted of 271 patients (211 males and 60 females) with chronic obstructive pulmonary diseases (COPD) attending the outpatient clinic of pulmonary disease unit of the St.-Antonius Hospital, Nieuwegein, The Netherlands. Patient's age ranged from 34 yr to 88 yr with a median of 67 years. From each patient, blood samples were taken every 2-7 months (median 4 months). The surveillance period ranged from 3 to 19 months (median 15 months). All serum samples from each patient were tested in one run to prevent misinterpretation due to day-to-day variation in the assays.

Serology

The microimmunofluorescence assay (MIF) was used to measure specific

IgG-, IgM- and IgA antibodies to *C.pneumoniae* (Cp) elementary bodies (Washington Research Foundation, Seattle, WA) (24). In addition, a commercially available MIF test (Labsystems OY, Finland) was used to distinguish between *C.pneumoniae, C.trachomatis* and *C.psittaci* / LPS specific antibodies. The prevalence of Cp specific IgG-, IgM- and IgA antibodies was based on the presence of antibodies at titers ≥ 1/32, ≥1/16 and ≥1/32, respectively. Diagnosis of a Cp infection during surveillance was based on the presence of Cp-specific IgM in any serum sample, or a four-fold or greater titer increase in Cp-specific IgG- and/or IgA antibody between two consecutive serum samples (24). All two-fold dilutions were made by a pipetting robot (MARK 5; DPC, Los Angeles, CA). Chlamydia-specific IgG-, IgM- and IgA antibodies were also detected by a recombinant DNA (rDNA) LPS ELISA (Medac GmbH, Hamburg, Germany). This ELISA include a chemically pure structure of a recombinant LPS which contains a genus-specific epitope of the human pathogens *Chlamydia* spp. (4,5,13). Each serum sample was mixed thoroughly prior to processing according to the manufacturer's instructions. Initial serum dilutions for IgG, IgM and IgA were 1/100, 1/50 and 1/50, respectively. Sera with OD values exceeding 2.5 were retested using a 1/4 predilution. A two-fold serially diluted standard serum was used to calculate the $\log_2$ titer of the patients' samples. The IgG-, IgM- and IgA cutoff values were calculated as prescribed by the manufacturer. The prevalence of Chlamydia-specific IgG-, IgM- and IgA antibodies was based on the following criteria : ≥calculated cutoff x 1.10, ≥calculated cutoff x 1.15 and ≥calculated cutoff x 1.10, respectively. Serological diagnosis of an acute Chlamydia infection was based on the ELISA results using the following criteria : A three-fold or greater increase in Chlamydia-specific IgG- or IgA antibody titer, or a two-fold increase in specific IgM titer, or a two-fold increase in specific IgG antibody titer in combination with a two-fold increase in specific IgA antibody titer. All pipetting was done by the same pipetting robot.

RESULTS

The inter-assay variability of the Chlamydia IgG-, IgM- and IgA rDNA LPS ELISA was calculated by testing a positive serum sample ten times

in one run. The intra-assay variability was calculated by testing a positive sample in ten different runs. A standard curve was used to calculate the titer of the patient sample. The inter- and intra-assay variability ranged from 2.8% to 6.7% and from 10.6% to 12.2%, respectively.
A total of 1058 serum samples from 271 COPD patients were tested in this study. The *C.pneumoniae* IgG seroprevalence observed by MIF was 72% when the first serum sample of each patient was tested. A seroprevalence of 53% was found when the Chlamydia IgG rDNA LPS ELISA was applied to the same sera. Corresponding results for IgG were found in 182 (67%) of the COPD patients; 70/89 (79%) patients with discordant IgG results were MIF positive and rDNA LPS ELISA negative. There was very little discordance in IgM-class responses; 9/10 (90%) patients with discordant IgM results were rDNA positive and MIF negative. 89/271 (33%) of the sera showed discordant IgA results, again, 52/89 (58%) patients had positive MIF results with negative rDNA LPS ELISA results (Table 1).

Table 1. Occurrence of anti-Chlamydia antibodies among 271 COPD patients as determined by the microimmunofluorescence (MIF) in comparison with the rDNA LPS ELISA.

		IgG MIF		IgM MIF		IgA MIF	
		+	-	+	-	+	-
rDNA ELISA	+	125	19	0	9	51	37
	-	70	57	1	26	52	131

No significant changes in Chlamydia-specific IgG-, IgM- and IgA antibody during the surveillance period were found in 263 (97%) and 248 (92%) patients, as observed by MIF and rDNA LPS ELISA, respectively. The antibody titers of these individuals were constant over time with very limited shifts observed between consecutive samples (Figure 1).

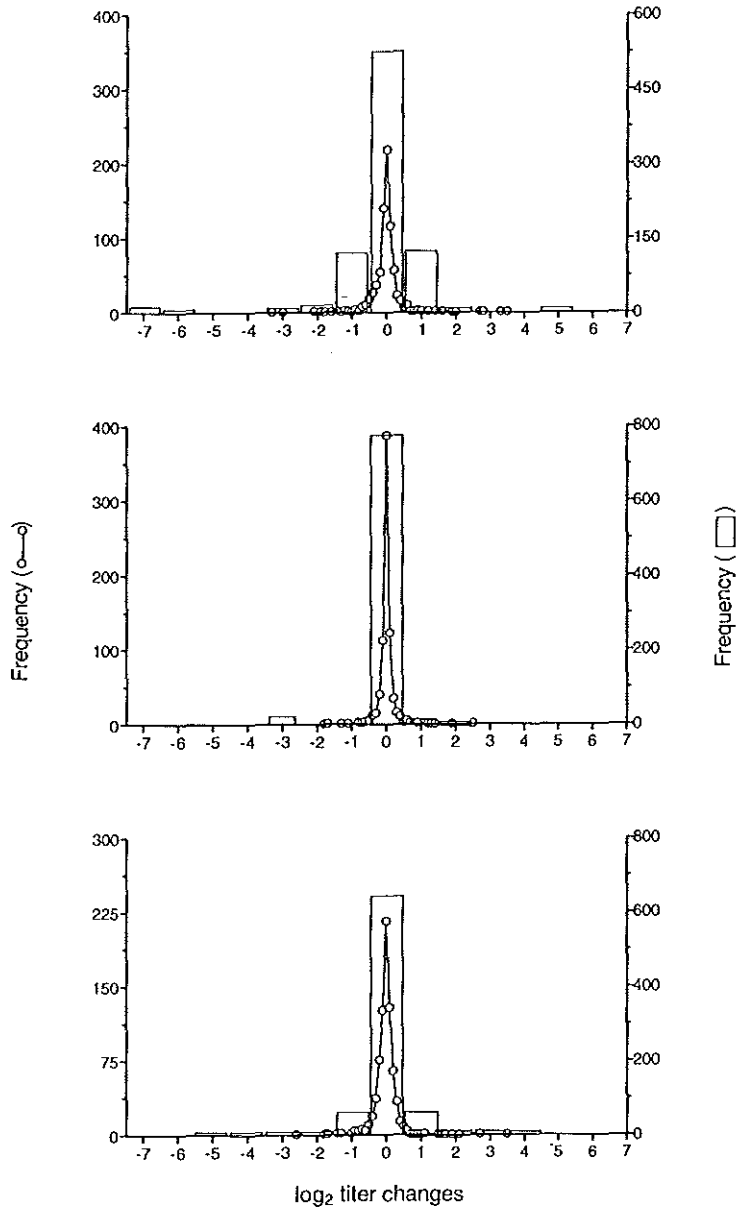

Figure 1. Frequency distribution of log_2 titer changes in anti-chlamydia IgG (upper panel), IgM (center panel) and IgA (lower panel) antibodies in 271 COPD patients between consecutive surveillance moments, as determined by *C.pneumoniae* microimmunofluorescence (□) and rDNA LPS Chlamydia ELISA (o----o).

Seven (3%) patients had MIF results indicating *C.pneumoniae* infection during their surveillance period. Five of these patients were confirmed by rDNA LPS ELISA. Eleven additional patients were infected during observation, according to the rDNA LPS ELISA only. These patients had significantly elevated *C.pneumoniae* specific IgG and IgA MIF titers, as compared with the patients without infection (p = 0.007 and 0.008 respectively). Also, 6/11 (55%) of these patients had MIF IgG titers ≥512, which was significantly more than 19/253 (8%) patients without evidence of infection (p=0.0001; Table 2).

Table 2. Differences in *C.pneumoniae* MIF results between Chlamydia infected patients, as determined by rDNA ELISA only and patients without any serological evidence of Chlamydia infection during the observation period.

patient category	number of patients	patients with IgG titers ≥512 (%)	log_2 MIF titer (mean ± SD) IgG	IgM	IgA
patients with infection during observation as indicated by rDNA LPS ELISA only	11	6 (55)*	7.7 ± 2.8‡	3.0 ± 0.0	5.9 ± 1.8‡
patients without evidence of infection from either MIF or rDNA LPS ELISA	253	19 (8)	5.7 ± 1.9	3.0 ± 0.1	4.6 ± 1.0

* p=0.0001, as compared with the patients without any serological evidence of chlamydial infection during their surveillance period (Fisher's exact test).
‡ p≤0.008, as compared with the patients without any serological evidence of chlamydial infection during their surveillance period (Mann-Whitney-U test).

The serological profile of all 18 patients with evidence of infection during their surveillance period are presented in Table 3. All 18 patients were also tested in the commercially available MIF test from Labsystems. This MIF assay uses three different antigen spots per well, to distinguish between *C.pneumoniae*, *C.trachomatis* and *C.psittaci* / LPS specific antibodies. None of the patients yielded serological results indicating recent *C.trachomatis* or *C.psittaci* infection. In addition, no significantly difference in serological *C.pneumoniae* results were found, as compared with the "in house" MIF. (data not shown).

Table 3. Serological profile of patients with evidence of recent *C.pneumoniae* infection during their observation period, as determined by rDNA ELISA and / or MIF.

Patient number	sex*	age	evidence of *C.pneumoniae* infection based on: MIF‡ IgG	MIF‡ IgM	MIF‡ IgA	rDNA LPS ELISA‡ IgG	rDNA LPS ELISA‡ IgM	rDNA LPS ELISA‡ IgA
1	f	68	+	-	+	+	-	+
2	m	70	+	-	+	+	-	+
3	m	83	+	-	-	+	-	+
4	m	77	+	-	-	+	-	-
5	m	70	-	+	-	-	+	-
6	m	47	-	+	-	-	-	-
7	f	70	-	-	+	-	-	-
8	f	69	-	-	-	+	-	+
9	m	68	-	-	-	+	-	+
10	m	72	-	-	-	+	-	-
11	m	75	-	-	-	+	-	-
12	m	68	-	-	-	+	-	-
13	f	74	-	-	-	-	+	-
14	f	72	-	-	-	-	+	-
15	m	68	-	-	-	-	+	-
16	f	60	-	-	-	-	+	-
17	f	73	-	-	-	-	-	+
18	m	71	-	-	-	-	-	+

* (f) female, (m) male

‡ (+) serological evidence of acute infection, (-) no serological evidence of acute infection

Two patients had Chlamydia IgM rDNA LPS ELISA results indicative for *C.pneumoniae* infection at entry of the study. Three patients had positive Chlamydia IgM rDNA LPS ELISA results during their entire surveillance period (observation period ranged from 13 to 18 months). In two of these

patients, no significant titer change in IgM reactivity was observed over time. One patient had a significant IgM titer fall between the first and the last serum sample. None of these patients had MIF IgM reactivity. Four other patients had Chlamydia IgM rDNA LPS ELISA results close to the cutoff value during their entire surveillance period. Again, none of these patients had MIF IgM reactivity. The incidence of *C.pneumoniae* infection using the MIF and rDNA LPS ELISA was 2.2 and 5.3/100 person-year respectively.

DISCUSSION

Serology is the most commonly used diagnostic tool for the detection of respiratory chlamydial infections in routine clinical practice. In the early 1970s, a sensitive microimmunofluorescence (MIF) assay was developed which proved to be suitable for routine diagnosis (26,27). In the MIF test, purified elementary bodies are used to detect specific *C.pneumoniae* antibodies in the IgM, IgG and IgA serum fractions. In literature, the MIF test is still the gold standard for the serological diagnosis of an acute *C.pneumoniae* infection. However, interpretation of specific- and non-specific fluorescence patterns requires experience and skill, and the interpretation of high titers (≥1/512) is very difficult and subjective. In addition, cross-reactions between *C.pneumoniae* and other chlamydial species have been reported (17,19,20). Chemically pure antigens have been isolated for the detection of antibodies against chlamydial LPS and this has been developed into a commercially available recombinant DNA LPS ELISA (4,5,13). In this study, we have observed that this rDNA LPS ELISA developed for the detection of Chlamydia-specific IgG- IgM- and IgA antibodies achieved a high degree of reproducibility. Partial concordance in seroprevalence was found with our in house *C.pneumoniae* MIF assay. Most of the discordant results between the two tests can be explained by differences in seroprevalence. With the rDNA LPS ELISA, a lower seroprevalence was found as compared with the in house MIF. This may be explained by a lower half-life of the anti-LPS chlamydial antibodies as compared with the MOMP antibodies detected by MIF. However, the serological data obtained by MIF and the rDNA LPS ELISA were identical in more than 95% of

the patients. On the other hand, the rDNA LPS ELISA seems to be more sensitive for the detection of acute respiratory chlamydial infection as compared to the traditional MIF. More patients had serological results indicating infection during the surveillance period when using the rDNA LPS ELISA. Many of these patients had elevated MIF IgG- and IgA antibody titers that did not significantly changed over time. This phenomenon confirms the statement of Grayston *et al.* that *C.pneumoniae* MIF IgG titers ≥512 may indicate current or recent infection (12). On the contrary, several patients with elevated MIF IgG antibodies had no serological results indicating chlamydial infection during the surveillance period when tested by the rDNA LPS ELISA. These constantly high IgG antibody titers may indicate a chronic chlamydial infection. Falck *et al.* reported high *C.pneumoniae* MIF IgG antibody titers in patients with persistent *C.pneumoniae* infection without clinical signs of infection for periods of six months up to a year (8).

In three patients, IgM reactivity by rDNA LPS ELISA persisted during the entire observation period. Four other patients had low levels of anti-Chlamydia IgM rDNA LPS antibody close to the cutoff value of the ELISA during their entire surveillance period. A chronic, asymptomatic *C.pneumoniae* infection may have been responsible for this phenomenon (8). This observation may have a significant impact on the ability to make a diagnosis of an acute respiratory chlamydial infection with the rDNA LPS Chlamydia IgM ELISA, especially when only a single serum sample is available. The use of paired serum samples, taken with at least a 1-2 week interval will remedy this problem, by requiring at least a two-fold titer change in chlamydial IgM for a diagnosis of an acute Chlamydia infection.

The rDNA LPS Chlamydia assay can be used to diagnose acute respiratory *C.pneumoniae* infection despite the genus-specific status of the test. This can be explained by the difference in prevalence of infection by the three *Chlamydia* species. *C.pneumoniae* respiratory infections occur much more frequently than *C.trachomatis* and *C.psittaci* respiratory infections. We now have data that indicates that the current MIF assays using EB's cannot relied upon to accurately distinguish between the three chlamydial species (data not published).

The major issue of this manuscript is the validity of the tests used, especi-

ally the new rDNA LPS ELISA. However, the major problem in validating such a new test is the definition of the gold standard. Culture and/or PCR may be used to calculate the sensitivity and specificity of serological tests. However, asymptomatic infection, often without serological response have been reported (2,7,14,15). Also, little is known about commensalism and the possibility of cariership. Most clinicians are interested in diagnosing acute *C.pneumoniae* infections. A significant increase of Chlamydia-specific antibodies between acute and convalescent serum sample is a relevant indication of an acute infection.

The incidence rate found in this COPD patient group using the MIF was comparable with the incidence found by Grayston (10). Increased incidence rate was found when using the rDNA LPS ELISA.

In conclusion, the study shows that the rDNA LPS Chlamydia assay may currently be the most sensitive serological tool for diagnosing recent respiratory *C.pneumoniae* infections. Also, *C.pneumoniae* infection may contribute to the morbidity seen in these patients.

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CHAPTER 8

SURVIVAL OF *CHLAMYDIA PNEUMONIAE* FOLLOWING CONTACT WITH VARIOUS SURFACES

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ABSTRACT

In this study the survival and recovery of *Chlamydia pneumoniae* (Cp) strains TW-183, AR-39, AR-388 and CWL-029 was measured after inoculation on glass, stainless steel, Formica^{R} laminate, paper, fabrics and human skin. Inoculum in throat washes from healthy volunteers was applied to each surface. Samples were taken immediately after inoculum application and at specified intervals thereafter to determine infectivity. Infectious Cp was recovered from glass for up to 4 hours, from paper and fabrics up to 3 hours, from formica^{R}-laminate for up to 2 hours, from stainless steel for up to 60 minutes and human skin for up to 30 minutes. Desiccation of the inoculated area had no significant effect on the recovery of infectious Cp. Further experiments demonstrated that infectious Cp could be transferred to hands touching these contaminated surfaces and could be recovered from these hands for up to 3 minutes. Addition of albumin, surfactant or phosphatidylcholine had no significant effect on the survival of Cp. These results suggest that contact with contaminated surfaces may be a potential mode of transmission of Cp.

INTRODUCTION

Laboratory evidence of *Chlamydia pneumoniae* (Cp) infection has been demonstrated in approximately 10% of community-acquired pneumonia cases (3,9,28). However, the organism has also been associated with bronchitis,(5,8) asthma,(12) reactive arthritis (18) and a number of other diseases (14,15,24). Recent studies provide evidence that Cp infection may play a causal role in coronary artery disease,(10,22,26). Many *C.pneumoniae* infections are mild or asymptomatic and may therefore remain undiagnosed (1,17,20). Specific antibodies against *C.pneumoniae* were found in 50-95% of the adult population in many different countries all over the world (2,7,9,30). The prevalence of Cp-related antibodies in The Netherlands is approximately 80% between the age of 20 and 80, while no significant difference was found between men and women. These data suggests that most people are infected and reinfected with Cp during

lifetime (9,30). Current evidence suggest that Cp infections spread slowly and the case to case interval is long. Epidemics have been described in Norway, Sweden, Finland and the USA (4,8,11,19). Although Cp infection is presumed to be spread from person to person, the exact route of Cp transmission has not been established. Survival of Cp, albeit brief, in small particle aerosols has been demonstrated (27). In this study we determined whether Cp could remain infectious in respiratory secretions contaminating various surfaces and whether hand contact with these contaminated surfaces would result in transfer of infectious Cp.

MATERIALS AND METHODS

Cell culture

Buffalo Green Monkey cells (BGM) (PAMM, Veldhoven, The Netherlands) were grown as monolayers in plastic culture flasks at 35°C with 5% CO_2 in a fully humidified cabinet. (Costar, Europe, Ltd.) The BGM cells were cultured in Eagle Minimal Essential Medium (EMEM; Life Technologies Ltd., Renfrewshire, Scotland) containing fetal calf serum (10%; Life Technologies Ltd., Renfrewshire, Scotland), non-essential amino-acids (1%; ICN Biomedicals Inc., Costa Mesa, CA, USA), gentamicin (10mg/l; Pharmachemie, Haarlem, The Netherlands), vancomycin (25mg/l;Eli Lilly, Indianapolis, IN, USA) and amphotericin-B (4mg/l;Bristol Myers Squibb, Epernon, France) (29).

Cp-strains

Stock cultures of TW-183, AR-39, AR-388 (Washington Research Foundation; Seattle, Washington, USA) and CWL-029 (American Type Culture Collection, Rockville, MD, USA) were used in this study. BGM cells were seeded into 75cm^2 tissue culture flasks (Costar Europe Ltd.,The Netherlands) and incubated at 35 °C with 5% CO_2 in a fully humidified cabinet. Cell monolayers were examined on the day of inoculation for confluency. After inoculation with Cp, tissue culture flasks were centrifuged at 900xg at 25 °C for 60 minutes and subsequently incubated with fresh medium containing cycloheximide (0.6 mg/l; Sigma Chemical Company, St Louis, Mo., USA). After 3 days, the flasks were aspirated and

monolayers were suspended in 5 ml Sucrose Phosphate Glutamate buffer (SPG) using a cell scraper (Costar Europe Ltd.). Tenfold dilutions were made of each stock solution, and the titer of Cp was calculated by counting the inclusions present in the last dilution. The titer was expressed as inclusion forming units per ml (IFU/ml). All stock solutions were stored at minus 80 °C prior use. The final concentration of each sample was 5 x 10^8 IFU/ml.

Cp-culture

BGM cells were seeded into 24-well tissue culture plates (Costar Europe Ltd.) and incubated at 35 °C with 5% CO_2 in a fully humidified cabinet. All cell monolayers were examined on the day of inoculation for confluency. After inoculation, 24-well plates were centrifuged at 900 x g at 25°C for 60 minutes and subsequently incubated with fresh medium containing cycloheximide (0.6 mg/l). After 3 days, the 24-well plates were aspirated and fixed with methanol (Merck, Darmstadt, Germany). The fixed monolayers were rinsed with phosphate-buffered saline once and stained by the fluorescent-antibody technique using anti-Chlamydia genus-specific mouse monoclonal antibody (kindly provided by J.M. Ossewaarde, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). as previously described (28). Rabbit anti-mouse Ig labeled with fluorescein isothiocyanate (Dako A/S, Glostrup, Denmark) was used as a conjugate. Evans Blue (0.05%; Sigma Chemical Company, St Louis, Mo.) was used as a counterstain.

Magic^R Lite Chlamydia Immunoassay

The Magic ^{R} Lite Chlamydia Immuno-assay (Ciba Corning Diagnostics Corp., Medfield, MA, USA) was used for the measurement of antigen recovery at each interval. The principle of the test was based on a genus-specific chemiluminometric antigen detection assay which uses constant amounts of acridinium ester labeled monoclonal anti-Chlamydial antibody and polyclonal anti-Chlamydial antibody covalently coupled to paramagnetic particles (21). The test records antigen concentrations as relative light units (RLU) per second. All data were expressed as % compared to RLU found in the stock solution which was used for

inoculation.

Throat washes from healthy volunteers

10 ml phosphate-buffered saline was used to collect throat washes from healthy volunteers. Ten throat washes were pooled and stored at minus 20 °C prior use.

Recovery and survival of Cp from various surfaces

The surfaces used were Formica^R laminate, glass, stainless steel, fabrics, paper and human skin (fingertips). 5μl inoculum (2.0 x 10^7 IFU/ml) in pooled throat washes from healthy volunteers was applied to each surface. Recovery and survival of Cp was detected as follows: Formica^R laminate, glass, stainless steel and human skin were sampled with a wet swab that was subsequently suspended in 1 ml SPG. Fabrics and paper were rinsed with 1 ml SPG. 100 μl sample was used to inoculate cell monolayers to determine the infectivity as described above. The Magic^R Lite Chlamydia Immunoassay was used to calculate the recovery of Cp antigen. 200 μl sample was used for the detection of Cp antigen as described by manufacturer. Samples were taken immediately after inoculum application and at specified intervals thereafter. Temperature and humidity were monitored and kept within 22-28°C and 40-60% relative humidity.

Effect of additives on the survival of Cp from various surfaces

Human serum albumin (50mg/l; The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), surfactant (34 mg/l; Kindly provided by J.F. van Iwaarden, Laboratory of Veterinary Biochemistry, University of Utrecht, The Netherlands) and phosphatidylcholine (1,2 mg/l; Sigma Chemical Company, St. Louis, Mo., USA) was added to the inoculum prior to contamination of various surfaces.

Recovery of infectious Cp from human skin after transfer from environmental surfaces

Again, 5 μl inoculum (2.0 x 10^7 IFU/ml) was administered to glass surface. Samples were taken from hands after touching the contaminated surface and were cultured as described above.

RESULTS

Antigen recovery

The recovery of Cp antigen from various surfaces after contamination with Cp are given in Table 1. The average Cp antigen recovery from glass, stainless steel, formica^{R}-laminate and paper at 15 minutes was 44, 42, 60 and 61%, respectively, whereas on fabrics and human skin, Cp antigen was recovered with an average of 81% and 22%, respectively. The antigen recovery was significantly lower on human skin compared to other surfaces tested. ($p \leq 0.05$). The Cp antigen recovery rate remained very constant over time on all surfaces. Desiccation of the inoculated area had no significant effect on the Cp antigen recovery. No significant differences were found between the four Cp-isolates.

Table 1. *Chlamydia pneumoniae* antigen recovery from various surfaces.

Surface	*Chlamydia pneumoniae* antigen recovered after					
	15 min	30 min	60 min	120 min	180 min	240 min
Glass	44±5*	48±13	35±9	46±13	34±8	28±5
Stainless steel	42±2	42±4	50±4	41±6	33±5	37±3
Formica^{R}-laminate	60±4	59±10	72±2	61±7	60±4	57±4
Paper	61±5	66±7	60±7	41±10	31±7	52±11
Fabrics	81±3	65±2	65±7	60±6	54±7	57±6
Human Skin	22±3	19±3	20±3	19±3	17±3	n.t.#

n.t. = Not Tested

*mean % ± SE antigen recovered from indicated surface using Magic Lite^{R} Chlamydia assay. Assay reports antigen concentrations as relative light units (RLU) per second. All data were expressed as % compared to RLU found in the stock solution which was used for inoculation. (Average of 3 separate experiments with 4 different Cp strains).

Survival of Cp following contact with various surfaces

The survival of Cp on Formica^{R} laminate and human skin is depicted in Figure 1. Infectious Cp could be recovered from Formica^{R}-laminate and human skin for up to 120 and 30 minutes, respectively. Desiccation of the inoculated area had no significant effect on the survival of Cp. After 15 minutes, the recovery of infectious Cp on Formica^{R} laminate was 1.8×10^{4}

IFU, whereas on human skin, the survival was only 2.9 x 10^3 IFU (68 and 31% compared to t=0, respectively). In contrast, Cp antigen recovery from these surfaces was stable over the time period sampled, as determined by the Magic^R-Lite Chlamydia assay. (Table 1).

Infectious Cp could be recovered from glass, paper, fabrics and stainless steel for up to 240, 180, 180 and 120 minutes, respectively. After 15 minutes the recovery of infectious Cp was 1.7 x 10^4, 2.1 x 10^4, 3.2 x 10^4 and 1.4 x 10^4 IFU respective surfaces (81, 78, 86, and 71% respectively, as compared with t=0). The Cp survival on stainless steel was significant shorter compared to glass and formica^R laminate tested. ($p \leq 0.05$, data not shown).

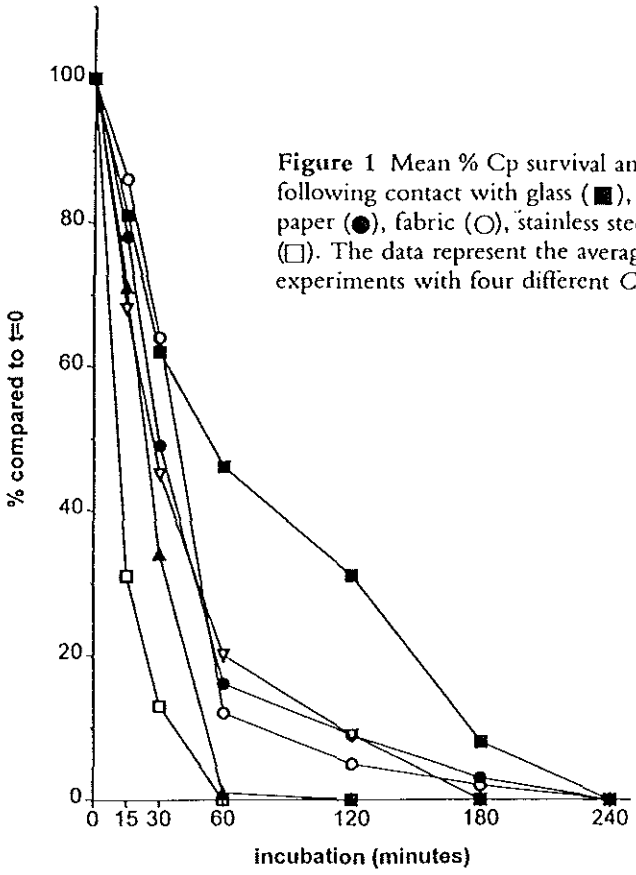

Figure 1 Mean % Cp survival and antigen recovery following contact with glass (■), Formica^R laminate (▽), paper (●), fabric (○), stainless steel (▲) and human skin (□). The data represent the average of four separate experiments with four different *C. pneumoniae* strains).

No significant difference on survival and antigen recovery was found between the four Cp strains tested; also, addition of albumin, surfactant or phosphatidylcholine had no significant effect on the survival and recovery of Cp on these surfaces (data not shown).

Recovery of Cp from human skin after transfer from environmental surface

Cp could be recovered from hands that had touched glass contaminated with Cp. However, recovery of infectious Cp from hands was restricted to the first three minutes following contact with the contaminated area. The average recovery of infectious Cp from fingertips after one and three minutes was 580 (6%) and 96 IFU (1%), respectively. No infectious Cp nor Cp antigen was found if hands were washed with water and soap prior to sampling.

DISCUSSION

Chlamydia pneumoniae is considered an important pathogen associated with approximately 10% of pneumonia cases and other respiratory infections (3,9,28). Many *C.pneumoniae* infections are mild or asymptomatic and may therefore remain undiagnosed (1,17,20). However, the recent association of *C.pneumoniae* with coronary artery disease has prompted a reevaluation of the pathogenesis of *C.pneumoniae* infection in patients (10,22,26). The high prevalence of Cp-specific antibodies among Dutch adults indicates the possibility of reinfection. However, little is known about the mode of transmission of this organism. Survival of Cp in small particle aerosols is possible, especially at high relative humidities, but relatively short (27). Survival of Cp following contact with environmental surfaces has been described once by other investigators (6). In contrast, many survival studies have been performed with respiratory viruses. The feasibility of the spread of rhino viruses and RSV has been well demonstrated (16,25). Investigators demonstrated that rhinovirus transmission occurred mostly by hand contact with contaminated surfaces, occasionally after large-particle exposure and not at all after small particle exposure (16). Transmission of RSV infection is also possible trough indirect contact

with contaminated surfaces (13).

In this study of Cp survival and transmission a theoretical linkage of several steps was examined, starting with Cp antigen recovery from various surfaces. The antigen recovery remained very constant over time on all surfaces. Desiccation of the inoculated area had no significant effect on the antigen recovery. A significantly lower recovery on human skin was observed, as compared to other surfaces tested. One of the possible explanations of this phenomenon could be increased adherence of the organism to the surface of human skin.

Survival of Cp on various surfaces was possible for long periods of time and was transferable to hands. Desiccation of the inoculated area had no significant effect on the survival of Cp. Survival of Cp on human skin and stainless steel appears to be poorer than on other surfaces tested. One of the reasons for this significantly reduced survival on human skin could be the presence of lactic acid which causes the acid pH of the skin. Also, the presence of skin surface lipids and excretory products of the resident microbiological flora may limit the survival of Cp on human skin (23). Addition of albumin, surfactant or phosphatidylcholine, however, had no effect on the survival time of Cp. The reason for the short survival on stainless steel is unexplained. Antimicrobial activities of ferro-ions may be one of the explanations for the accelerated inactivation of Cp.

Infectious Cp could be recovered from hands following contact surfaces inoculated with Cp, although recovery of infectious Cp was restricted to the first three minutes following contact with the contaminated area. No significant differences were found between the four Cp-strains used in this study.

Falsey *et al.* compared the survival of Cp following contact with human skin, tissue and formica^{R}-laminate (6). The survival of Cp on human skin was comparable with our results. Discrepant results were found with Formica^{R}-laminate. Falsey *et al.* reported a survival period up to 30 hours, which is significant longer compared to the 3 hours survival in this study. One of the reasons of this significant difference may be differences in methodology. We applied 5 μl Cp stock suspension to each surface tested, in contrast to the 100 μl which was used by Falsey *et al.* Also, in our study, Cp stock solutions were diluted in human pooled throat washes

prior to sampling, as compared with SPG, which was used by Falsey *et al.* In our opinion, the use of small volumes for sampling, diluted in human pooled throat washes better approaches reality.

The results in this study indicate that *C.pneumoniae* may survive sufficiently long in the environment to allow transfer of infectious *C.pneumoniae* to hands in contact with contaminated surfaces. As for many respiratory viruses this may be the most efficient route of transmission of *C.pneumoniae.*

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CHAPTER 9

SEROLOGIC EVIDENCE FOR A ROLE FOR *CHLAMYDIA PNEUMONIAE* INFECTION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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ABSTRACT

Antibodies to *Chlamydia pneumoniae* were repeatedly measured in the serum of 271 patients with chronic obstructive pulmonary disease (COPD) over a mean period of 14 months and the results were compared similar data from a cohort of 405 blood donors. The prevalence of IgG-, IgA- and IgM against *C.pneumoniae* was higher among COPD patients and increased with age. The incidence of *C.pneumoniae* infection was also higher in COPD patients and likewise increased with age. Thus, COPD and C.pneumoniae may be linked.

INTRODUCTION

The aim of the present study was to prospectively evaluate the antibody response to *C.pneumoniae* in patients with chronic obstructive pulmonary disease (COPD) observed over an extended period of time and to compare these results with those found in a blood donor population in order to further define the role of *C.pneumoniae* infection in COPD.

PATIENTS AND METHODS

The study population consisted of two different groups. The first group included 271 patients (211 males) with COPD attending the outpatient pulmonary clinic of the St.-Antonius Hospital, Nieuwegein, The Netherlands. Patient's age ranged from 34 yr to 88 yr with a median of 67 years. From each patient, blood samples were taken every 5 ± 2 months (mean ± sd). The surveillance period ranged from 3 to 19 months (mean ± sd: 14 ± 4 months). The second group consisted of 1,104 blood donors (730 males) visiting the Red Cross Blood Transfusion Center, Tilburg, The Netherlands. From each blood donor, one serum sample was stored at minus 85 °C prior to use in this study. From 405 blood donors (263 males), blood samples were taken every 9 ± 5 months. Donor's ages ranged from 18 yr to 68 yr with a median of 40 years. The surveillance period ranged from 2 to 22 months (mean ± sd: 16 ± 5 months). All serum samples from each

patient were tested in one run.
Chlamydia-specific IgG, IgM and IgA antibodies were detected using a recombinant LPS ELISA (rDNA LPS ELISA; Medac GmbH, Hamburg, Germany). This ELISA includes a chemically pure structure of a recombinant LPS which contains a genus-specific epitope of *Chlamydia*. (2,3,9). Prior to the IgM determinations IgG absorption was performed (11). Sera with OD values exceeding 2.5 were retested using a 1:4 predilution. A two-fold serially diluted standard serum was used each run to calculate the $\log_2$ titer of the patients' samples. The IgG-, IgM- and IgA cutoff values and prevalence of Chlamydia IgG-, IgM- and IgA antibodies were calculated as prescribed by the manufacturer. Diagnosis of a recent Chlamydial infection during surveillance was based on the ELISA results using the following criteria : A three-fold or greater increase in Chlamydia-specific IgG- or IgA antibody titer, or a two-fold increase in specific IgM titer, or a two-fold increase in specific IgG antibody titer in combination with a two-fold increase in specific IgA antibody titer (12). A commercially available microimmunofluorescence assay was used to distinguish between *Chlamydia pneumoniae*, *Chlamydia trachomatis* and *Chlamydia psittaci* / LPS specific antibodies (Labsystems OY, Helsinki, Finland). Diagnosis of an acute infection during surveillance was based on criteria as described before (12).

RESULTS

The first serum sample from 271 COPD patients and 1,104 blood donors was used to determine the chlamydial IgG seroprevalence. A significantly higher prevalence was found among the COPD patients, as compared to the blood donor group (53% and 29%, respectively; $p<0.0001$). No difference in prevalence was found between male and female blood donors (30% and 29%, respectively). In contrast, the seroprevalence observed in male COPD patients was significantly higher as compared to that of female COPD patients (57% and 40%, respectively; $p=0.03$).
The IgG seroprevalence among COPD patients was age-dependent. An increased seroprevalence was found among the elderly patients (45% among patients <60 years and 70% for those ≥80 years, chi-squared test

for trend p=0.03). A similar age-dependent trend was observed for Chlamydia-specific IgA and IgM. In contrast, seroprevalence was no age dependent in the blood donor group. Two COPD patients and three control subjects had Chlamydia-specific IgM persisting during their entire follow-up period without significant change in their titers. Another COPD patient had high-titerd chlamydial IgM at the start of the study which remained positive for more than 16 months with a subsequent decrease at the end of the surveillance period (OD 2.1 and 1.4, respectively) This patient was also positive for IgG- and IgA that did not changed significantly during his follow-up period.

Serological results indicating recent chlamydial infection during the surveillance period was observed in 16 (6%) COPD patients and 8 (2%) blood donors (p=0.01; Table 1). In one COPD patient and one control subject two separate episodes of Chlamydia infection were detected over a period of 16 months and 19 months, respectively. The incidence of chlamydial infection was higher in the COPD patient group, as compared to the blood donor group (5.3/100 person-year and 1.7/100 person-year, respectively; p=0.006). In five (29%) infection episodes observed among COPD patients and in five (56%) infection episodes in blood donors, an IgM response indicative for an acute chlamydial infection occurred (p=0.23). All patients with serological results indicating recent infection during their surveillance period were also tested with a commercially available MIF test, intended to distinguish between *C.pneumoniae*, *C.trachomatis* and *C.psittaci* / LPS specific antibodies. None of the episodes could be ascribed to *C.trachomatis* or *C.psittaci* infection.

C.pneumoniae infection among COPD patients was positively correlated with age. No recent infections were observed among COPD patients less than 60 years old. A higher rate of infection was observed in the elderly patients (9.3/100 person-years among patients 70 years or older; CI_{95}: 4.8-16.1). Interestingly, the incidence of infection among control subjects was inversely correlated with age. Most infections occurred among control subjects with ages less than 40 years (2.8/100 person-years; CI_{95}: 1.2-5.8).

The chlamydial seroprevalence among COPD patients did not changed significantly over the observation period. However, several patients initially positive at the start of the study became seronegative during their

Table 1. Incidence of *Chlamydia pneumoniae* infection in chronic obstructive pulmonary disease patients and blood donors.

	COPD patients			blood donors		
	male	female	total	male	female	total
number of patients tested	211	60	271	263	142	405
initially IgG seropositive (%)	57	40	53	11	9	10
infection during surveillance						
number of patients involved	10	6	16	5	3	8
number of episodes observed	11	6	17	6	3	9
incidence of infection*	4.4	8.1	5.3	1.7	1.7	1.7
(CI_{95})	(2.2 - 7.8)	(3.0 - 16.8)	(3.1 - 8.3)	(0.6 - 3.7)	(0.4 - 4.8)	(0.8 - 3.2)
diagnosis based on response in:						
IgG class	5	-	5	-	1	1
IgM class	2	3	5	1	1	2
IgA class	2	1	3	1	-	1
IgG and IgA classes	2	2	4	2	-	2
IgG, IgM and IgA classes	-	-	-	2	1	3

* infections per 100 person-years

observation period, and, on the other hand, several patients initially negative became seropositive later.

An attempt was made to calculate the duration of the presence of IgG antibodies in serum against chlamydial LPS. For each serum sample an index was calculated by dividing the OD of the first serum sample by the cutoff OD value of the assay. All initially IgG seropositive COPD patients at the start of the study - without subsequent evidence indicating recent infection during their surveillance period - were included in this evaluation. These seropositive patients were divided into three different groups, according to the level of their initial IgG index. The first group consisted of 48 patients with an initial index value ≥ 3.0, the second group consisted of 36 patients with initial index values between 2.0 and 2.9, and the third group consisted of 54 patients with IgG index values between 1.1 and 1.9). For all three groups, the seroprevalence and the mean index values were thus calculated at the start of the study, after 4-8 months surveillance, after 10-14 months surveillance and after 16-20 months surveillance. Similar calculations were done for Chlamydia-specific IgA antibodies against LPS. All patients with high titers of Chlamydia-specific IgG at the start of the study remained seropositive during the entire surveillance period (Figure 1). Also, no significant change in their mean ELISA index was observed. In the patient group with intermediate titers of IgG at the start of the study only 3/36 (8%) patients became seronegative. Again, no significant change in their indexes was observed. However, in patients with low levels of Chlamydia-specific IgG, 16/54 (33%) had become seronegative at the end of the observation. Again, no significant change in their mean index was observed (Figure 1). Similar results were found when the titers of Chlamydia-specific IgA were evaluated (Figure 1).

DISCUSSION

In the present study, the prevalence of Chlamydia LPS-specific IgG- and IgA antibodies in patients with COPD was remarkably higher than among blood donors. Similar results have been reported previously (1). Cook *et al.* reported an increased risk of *C.pneumoniae* infection among patients with COPD, which they ascribed to the frequent use of steroid therapy in such

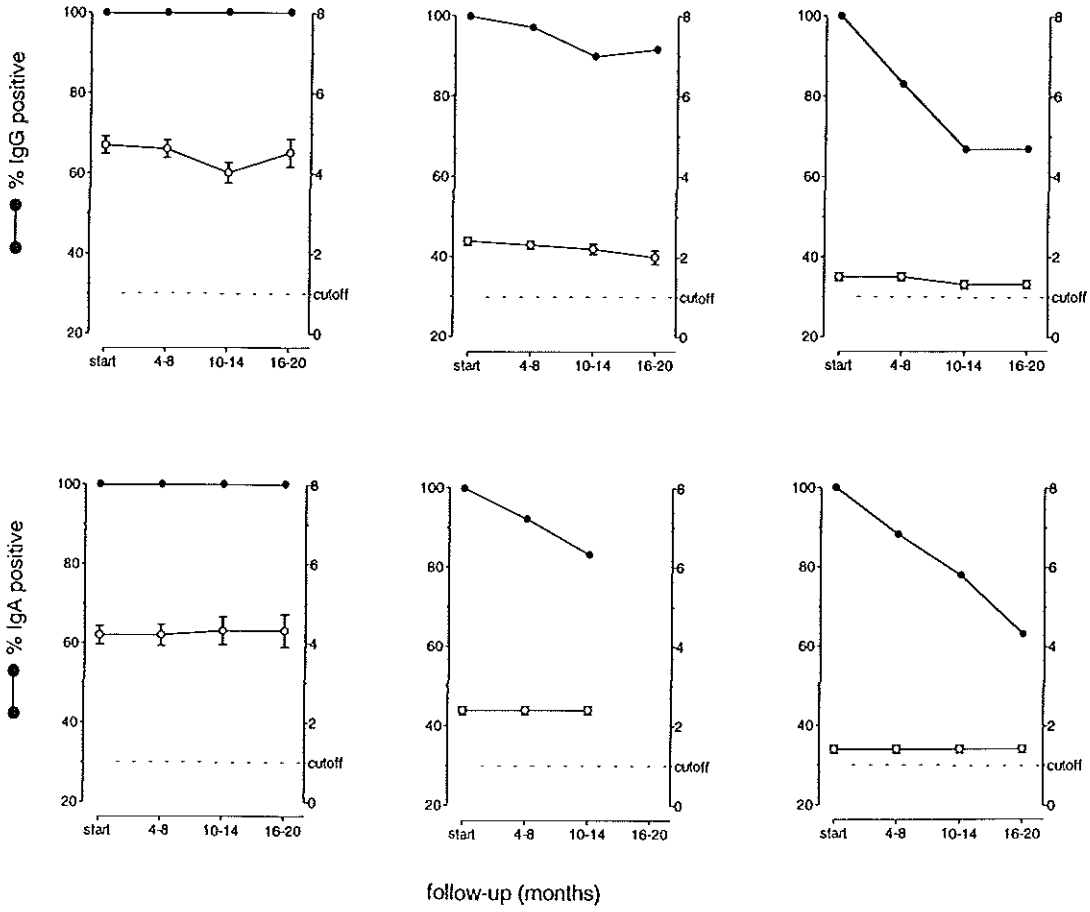

Figure 1. Kinetics of Chlamydia-specific IgG- and IgA antibody titers in seropositive COPD patients without evidence of intercurrent infection during their surveillance period. Data are the percentage of patients seropositive for Chlamydia-specific antibody (●····●) and their mean antibody titer (O····O ; mean ± se of ELISA index) at indicated intervals during follow-up. Note that all patients were initially seropositive and had either high (left panel, N = 48), intermediate (middle panel, N = 36) or low (right panel, N = 54) levels of Chlamydia-specific IgG (upper panels) or IgA (lower panels) at the start of observation.

patients (5). The prevalence of anti-Chlamydia antibodies in COPD patients increased with age. No such correlation with age was observed in the blood donor group. This may be explained by the coexistence of more severe chronic diseases as well as by the frequency and severity of respiratory infections among older adults with COPD (4,8). Blasi *et al.* also found an association between age and seroprevalence in these patients using a MIF assay (1). However, their observation of a significant correlation of *C.pneumoniae* IgG antibody titer among IgG positive COPD patients with age was not confirmed by our results (data not shown). Von Hertzen *et al.* found an increased IgA seroprevalence and elevated IgA titers in COPD patients as compared with control subjects without COPD, current asthma or symptoms of chronic bronchitis (13). They reported that the IgA antibody titers in COPD patients remained constant during exacerbations and suggested the possibility of a chronic inflammatory process induced by *C.pneumoniae* in the bronchi. In such a process continuous antigen shedding occurs that may act as a chronic stimulus for IgA production.

For unknown reasons the seroprevalence was higher in male COPD patients than in female patients. Such a gender association has been reported before in patients with respiratory complaints by Saikku (10). The presence of Chlamydia-specific antibodies changed over time. However, we found that IgG- and IgA anti-chlamydial LPS antibodies may remain detectable in serum for many years. The antibody titer reached during infection seems to have predictive value for their persistence. The decline in IgG- and IgA antibody titer is apparently very slow. This has also been reported by Haidl *et al.* for antibodies against the major outer membrane protein (MOMP) of *C.pneumoniae* (7). Although IgM antibodies are usually considered to have a short half-life and, thus, indicative for recent infection, six subjects in our study had IgM titers that persisted during their entire observation period. A chronic *C.pneumoniae* infection that may entirely go unnoticed may be responsible for this phenomenon. No serologic evidence was found indicating acute *C.trachomatis* or *C.psittaci* infection among our patients and blood donors. The differences in age distribution and other risk factors between the COPD patients and blood donors limits the interpretation of our findings. Nevertheless, the incidence of *C.pneumoniae* infection was higher in patients with COPD as

compared to healthy blood donors. Grayston *et al.* used the MIF assay for the detection of *C.pneumoniae* infection in patients with severe pneumonia. They reported a similar association between age and incidence of infection (6). The difference in incidence of infection between patients with COPD and control subjects may be partly explained by differences in exposure between the two study groups. Another explanation may be found in the coexistence of other respiratory diseases in older adults with COPD, which may, upon exposure to *C.pneumoniae*, predispose them to more frequent and severe respiratory *C.pneumoniae* infection (4,8). A relatively high incidence of IgM reactivity, indicative for acute *C.pneumoniae* infection was found among female patients with COPD and control subjects. This may be explained by the fact that fewer female patients with COPD and control subjects initially had chlamydial antibodies, suggesting that more patients were at risk of primary infection by *C.pneumoniae* in which an IgM response would be expected.

Overall, Chlamydia-specific immunoglobulin from all classes were found more frequently in the elderly (especially male) patients with COPD than in healthy blood donors, suggesting an association between COPD and *C.pneumoniae* infection. The higher incidence of significant shifts in Chlamydia-specific antibody titers further stress this association. Although such shifts in antibody titers are commonly ascribed to reinfection, they may also be a reflection of the dynamics of a chronic infection with *C.pneumoniae* in COPD patients.

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CHAPTER 10

DIAGNOSIS OF *CHLAMYDIA PNEUMONIAE* INFECTION IN PATIENTS WITH COMMUNITY-ACQUIRED PNEUMONIA: SERO-RESPONDERS VERSUS SERO-NON-RESPONDERS

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ABSTRACT

We prospectively studied 156 patients with a diagnosis of community acquired pneumonia requiring admission. Diagnosis of a *C.pneumoniae* infection was determined by culture, PCR, microimmunofluorescence and recombinant LPS ELISA. Other bacterial and viral etiologies were determined using standard laboratory procedures. The etiology was established in 104 patients (67%). *C.pneumoniae* was found in 45 (29%) patients. Other common pathogens, in descending order of frequency, were *H.influenzae*, 24 patients (15%) , *S.pneumoniae*, 23 patients (15%) and influenza viruses, 13 patients (8%). All patients with laboratory results indicative for an acute *C.pneumoniae* infection were divided into two different groups: sero-responders and sero-non-responders. A patient was defined as a sero-responder if serological results were obtained indicative for an acute *C.pneumoniae* infection. Patients with *C.pneumoniae* PCR positive results without serological evidence of an acute *C.pneumoniae* infection were defined as a sero-non-responder. Twenty-three responders and 22 non-responders were identified. Mixed infections with other agents, most commonly with *S.pneumoniae*, were found often, especially in sero-responders (61%), as compared to sero-non-responders (23%; p=0.017). No outstanding differences between sero-responders and sero-non-responders could be observed, although the severity of the disease tended to be higher among sero-responders. The severe clinical picture associated with *C.pneumoniae* pneumonia and the frequency of occurrence emphasizes the importance of highly sensitive and specific techniques to diagnose *C.pneumoniae* pneumonia.

INTRODUCTION

Determination of the exact etiology for community-acquired pneumonia (CAP) is heavily dependent on the laboratory techniques used to diagnose the disease and to identify the causative agent. The discovery of new pathogens including *Chlamydia pneumoniae* has altered the relative frequency of etiological agents. In fact, in 1982, Macfarlane *et al.* (29)

reported a high proportion of *Streptococcus pneumoniae* (76%), whereas, more recently, the proportion due to *S.pneumoniae* was found to be less than 20% (4,13,39). In these latter studies, intracellular pathogens such as *C.pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumoniae* accounted for 20-30% of CAP. However, the relative contribution of *C.pneumoniae* is difficult to assess since most studies have relied on single laboratory assays, that have not been validated in a clinical setting. The aim of this prospective study was to further evaluate the role of *C.pneumoniae* as an etiologic agent in patients admitted for CAP in the Netherlands by systematically applying serological, cultural and DNA-amplification assays on each patient entered into the study.

PATIENTS AND METHODS

Patients

During a 21-month period from September 1992 to June 1994, we prospectively studied 156 patients with a diagnosis of community-acquired pneumonia (CAP) who were admitted to the St Antonius hospital, Nieuwegein, The Netherlands. CAP was considered to be present if a new pulmonary infiltrate was found on the chest radiograph, and a clinical and radiological course that confirmed the diagnosis (11). Chest radiographs from study patients were reviewed by two independent physicians for the location of pulmonary infiltrate and the presence of pleural effusion.

Nasopharyngeal and throat specimens were collected using sterile cotton-tipped aluminum shafted swabs and suspended in 1.5 ml Chlamydia transport medium (2SP). A throat wash sample was obtained using 10 ml phosphate buffered saline. Sputum, blood cultures, broncho-alveolar lavages and pleural fluid were obtained using standard procedures. Sputum samples were considered representative of infection if more than 25 white blood cells and less than 10 squamous cells per 100 x field were observed (3,11). Predominant growth of pathogens was taken to indicate the agent that caused the pneumonia. The first serum sample was obtained within the first 24 hours of enrollment. A second (convalescent) serum sample was obtained from all patients 10 days after enrollment. From 142 patients (91%), a third serum sample was obtained 30 days after enrollment.

Demographic data, clinical signs and symptoms, underlying diseases, antibiotic treatment prior to enrollment and comorbid conditions were recorded. Patients were categorized by the severity of illness as described by Fine *et al.* (17). Baseline laboratory data included a complete blood count, serum electrolytes, liver function tests, erythrocyte sedimentation rate (ESR), C-reactive protein concentration (CRP) and arterial blood gases. Routine microbiologic tests included blood culture, sputum gram stain and culture, broncho-alveolar lavage (BAL) cultures, pleural fluid culture (if possible), and serological tests on acute and convalescent serum samples for complement fixing antibodies to *Mycoplasma pneumoniae*, influenza and para-influenza viruses, Respiratory Syncytial Virus (RSV), Adenovirus and *Coxiella burnetii*. For *M.pneumoniae* a passive hemagglutination test was also included. For *Legionella pneumophila* an indirect immunofluorescence test (IgG and IgM) was applied.

Laboratory assays for *C.pneumoniae*

Culture Culture for *C.pneumoniae* was done as described previously (41). Briefly, Buffalo Green Monkey cells (BGM) (PAMM, Veldhoven, The Netherlands) (42) were seeded into 24-well tissue culture plates (Costar Europe Ltd.) and incubated at 35 °C with 5% CO_2 in a fully humidified cabinet. All cell monolayers were examined on the day of inoculation for confluency. For each experiment, a patient's sample was inoculated into two microtiter wells (24-well microtiter plate) and one flat-bottom tube. After inoculation, microtiter plates were centrifuged at 900 x g at 25 °C for 60 minutes and subsequently incubated with fresh medium containing cycloheximide (0.6 mg/l) (Sigma Chemical Company, St Louis, Mo.). After 3 days, the wells were aspirated and fixed with methanol (Merck, Darmstadt, Germany). The fixed monolayers were rinsed with phosphate-buffered saline once and stained by the fluorescent-antibody technique using anti-Chlamydia genus-specific mouse monoclonal antibody (kindly provided by J.M. Ossewaarde, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). The inoculated flat-bottom tubes were passed onto fresh monolayers and reincubated as described before. This procedure was repeated once more. In each culture series,

C.pneumoniae TW-183 was included in parallel as a positive control. Positive cultures were stained with anti-*C.pneumoniae* specific mouse monoclonal antibodies (Washington Research Foundation, Seattle, Wa). Rabbit anti-mouse Ig labeled with fluorescein isothiocyanate (Dako A/S, Glostrup, Denmark) was used as a conjugate. Evans Blue (0.05%; Sigma Chemical Company, St Louis, Mo.) was used as a counterstain.

PCR Two-hundred μl nasopharyngeal or throat specimen or 1.0 ml throat wash and broncho-alveolar lavage were transferred to a sterile tube and centrifuged for 30 min at 15,000 x g. Sputum samples were suspended in 1.5 ml 2SP transport medium. One-hundred μl suspended sputum sample was transferred to a sterile tube. The sediment (or suspended sputum sample) was incubated with a solid carrier (celite) and a guanidinium-thiocyanate (GuSCN) containing lysis buffer. Nucleic acids (NA) were bound to the carrier which was rapidly sedimented by centrifugation. The sedimented complexes were washed once with a GuSCN-containing washing buffer, once with 70% ethanol and once with acetone. Complexes were dried and NA was subsequently eluted in 55μl aqueous solution at 37°C for 30 minutes. The isolated NA was removed from the solid carrier by centrifugation (5). Amplification by PCR and analysis of the amplified products were performed as described by Campbell *et al.* (9). Briefly, PCR amplification was performed with 8 μl isolated DNA in a 100 μl reaction mixture containing 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCL (pH 9.0) 0.01% Tween-20, 0.2 mM deoxynucleoside triphosphates, 50 pmol primers and 0.2U Thermoperfect *Taq* polymerase (Integra Biosciences A.G., Wallisellen, Switzerland). A *C.pneumoniae* species specific primer set was used, which amplifies a 437-bp fragment with the following primers: The forward primer HL-1 (5'-GTTGTTCATGAAGGCCTACT-3') and the reverse primer HR-1 (5'-TGCATAACCTACGGTGTGTT-3'). PCR products (20 μl) were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. DNA was transferred from agarose to Hybond plus nylon filters (Amersham International plc, Amersham, United Kingdom) by electrophoretic transfer. The PCR products were analyzed with a *C.pneumoniae*-specific probe HM-1 (5'-GTGTCATTCGCCAAGGTTAA-3'). The ECL 3'-oligolabeling and detection system was used for the detection of the PCR products (Amersham International, plc, Amersham, United

Kingdom). A 10-fold serial dilution of a *C.pneumoniae* stock solution (10^9 target DNA copies per ml) was incorporated in each amplification. One negative control (pooled cervical specimens suspended in 2SP) was incorporated every 10 amplifications. All PCR positive reactions were confirmed by repeat DNA isolation and amplification of the original specimen. Separate rooms were used for the different steps of the PCR and the recommendations of Kwok *et al.* were used to prevent DNA carryover contamination (28).

Serology *ELISA* Chlamydia-specific IgG, IgM and IgA antibodies were detected using a recombinant LPS ELISA (rDNA LPS ELISA; Medac GmbH, Hamburg, Germany). This ELISA includes a chemically pure structure of a recombinant LPS which contains a genus-specific epitope of the human pathogens *Chlamydia* spp. (6,7,21). Initial serum dilutions for IgG, IgM and IgA were 1:100, 1:50 and 1:50, respectively. Prior to the IgM determinations IgG absorption was performed (41). Sera with OD values exceeding 2.5 were retested using a 1:4 predilution. A two-fold serially diluted standard serum was used to calculate the $\log_2$ titer of the patients' samples. The IgG-, IgM- and IgA cutoff values were calculated as prescribed by the manufacturer. Prevalence of anti-Chlamydia IgG-, IgM- and IgA antibodies was based on the following criteria : ≥calculated cutoff x 1.10, ≥calculated cutoff x 1.15 and ≥calculated cutoff x 1.10, respectively. Serological diagnosis of an acute Chlamydia infection was based on the ELISA results using the following criteria : A three-fold or greater increase in Chlamydia-specific IgG- or IgA antibody titer, or a two-fold change in specific IgM titer, or a two-fold increase in specific IgG antibody titer in combination with a two-fold increase in specific IgA antibody titer (43).

Microimmunofluorescence Microimmunofluorescence (MIF) assay, as described before was used to measure *Chlamydia pneumoniae* specific IgG-, IgM- and IgA antibodies (41). Briefly, purified *C.pneumoniae* elementary antibodies (strain AR 39; Washington Research Foundation, Seattle, USA) were used to detect IgG, IgM and IgA antibodies to *C.pneumoniae*. Prior to the IgM determinations, IgG absorption was performed (41). Prevalence of anti-*C.pneumoniae* specific IgG-, IgM- and IgA antibodies was based on the presence of IgG at titers ≥ 1:32, IgM≥1:16 and

IgA ≥1:32, respectively. Diagnosis of an acute *C.pneumoniae* infection was based on the following criteria: The presence of *C.pneumoniae* specific IgM in either acute or convalescent serum, or a four-fold or greater titer increase in *C.pneumoniae* specific IgG- and/or IgA antibody between acute and convalescent serum (41).

Criteria for causation

CAP was considered to be caused by *C.pneumoniae* if a serological response indicative for an acute *C.pneumoniae* infection was observed, or any respiratory specimen was culture positive, or yielded a confirmed positive signal by *C.pneumoniae* PCR (32). All bacteria were considered as definitive agents when they were isolated from blood culture or pleural fluid. In the absence of a positive blood or pleural culture, any pathogen isolated from a sputum sample representative of infection (see methods) or other respiratory specimen, was considered as a probable causative agent. A ≥4-fold rise in titer between acute and convalescent serum sample was considered a definitive etiologic evidence for infection. For *L.pneumophila* a positive IgM immunofluorescence was also taken as definitive proof of causation.

Data processing and statistical analysis

Data were coded on a standard case record form, entered into a database and analyzed using the Statistical Package for the Social Science (SPSS for Windows95; version 7.0) (38). Chi-square and Fisher exact tests were used to compare demographic and clinical characteristics. Probability values were two-tailed (33).

RESULTS

Patient characteristics

Over the 21-month period, 180 patients with the suspicion of CAP were enrolled. After review, 156 cases fulfilled the criteria of community acquired pneumonia. Reasons for exclusion of 24 patients were absence of a new infiltrate on chest radiograph (19), a non-infectious process associated with malignancy (3) and bronchiolitis obliterans (2). In the final study

group, 6 (4%) were repeat enrollees. Of the 156 patients finally included, 100 (64%) were male patients. The median age of male and female patients was 69 yr (range 24 to 88 yr) and 65 yr (range 20 to 93 yr), respectively. Underlying diseases or risk factors for the development of respiratory disease were found in 120 (77%) of the patients. In 78 patients (50%) the etiology was established, when the *C.pneumoniae* diagnosis was disregarded. A typical pathogen was observed in 48 patients, while in 38 patients an atypical pathogen was identified. In 17 patients (11%) multiple pathogens associated with their disease were observed. Sixty-four patients (41%) received antibiotics prior to enrollment (Table 1). Four male patients (2.6%) died during admission. None of these observations were significantly different between male and female patients (data not shown).

Table 1. Patient characteristics, etiology (excluding *C.pneumoniae* diagnosis) and mortality of 156 hospitalized patients with community-acquired pneumonia.

patient characteristics	
gender (m/f)	100/56
age	
median	68
range	20-93
underlying diseases / risk factors, number (%)	
COPD	86 (56)
bronchus deformation	22 (14)
immunocompromised	7 (5)
malignancy	5 (3)
smoking	39 (28)
none	36 (23)
etiology established (%)	78 (50)
typical pathogen identified	48 (31)
atypical pathogen identified*	38 (24)
antibiotic used prior to enrollment	64 (41)
mortality	4 (3)

* excluding *C.pneumoniae*

Chlamydia pneumoniae diagnosis

The diagnosis of acute *C.pneumoniae* infection was established in 45 patients (29%). Twenty-three patients (15%) were positive by any serological test, 31 patients (20%) were positive by PCR and one patient was positive by culture. All patients with acute *C.pneumoniae* infection were divided into two different groups: sero-responders and sero-non-responders. 23 patients were defined as sero-responders because their serological results were compatible with acute *C.pneumoniae* infection. Twenty-two patients had *C.pneumoniae* PCR positive signals without serological evidence of an acute *C.pneumoniae* infection and were classified as sero-non-responders. Only 9/23 (39%) sero-responders were *C.pneumoniae* PCR positive. Vice-versa only 9/31 (29%) patients with a positive *C.pneumoniae* PCR also responded serologically. 15/23 (65%) sero-responders had MIF results indicative for an acute *C.pneumoniae* infection and 20/23 (87%) of the sero-responders had serological results indicative of an acute infection by rDNA LPS ELISA. Only one patient had positive *C.pneumoniae* culture results in association with positive PCR and serology. All sero-non-responders remained negative by *C.pneumoniae* culture.

Interestingly, significantly more *C.pneumoniae* infections were diagnosed in patients who already had specific IgG observed by rDNA LPS ELISA at admission as compared to those that were seronegative in this assay at enrollment (21% and 38%, respectively, p=0.021). This difference was most clearly demonstrated in patients subsequently classified as sero-responders (10% and 31%, respectively, p=0.020). However, such difference was not observed when the MIF was used to discriminate between initially seropositive and seronegative patients (27% and 30%, respectively; p=0.842).

Additional specificity analysis of the *C.pneumoniae* PCR was performed in order to better estimate the specificity of the PCR. Thirty cervical scrapes from female patients attending the sexual transmitted disease clinic (20 *C.trachomatis* PCR negative samples and 10 *C.trachomatis* PCR positive samples), suspended in *Chlamydia* transport medium (2SP) were tested using the *C.pneumoniae* PCR. None of these specimens were positive by *C.pneumoniae* PCR. Additionally, 40 nasopharyngeal aspirates from neonates taken directly after birth were tested; again, all specimens remained

negative in our *C.pneumoniae* PCR.

Etiologic agents

If *C.pneumoniae* was included, 104/156 (67%) patients had an etiologic diagnosis. Surprisingly, *C.pneumoniae* was the most common pathogen identified (45 (29%) patients). Other common pathogens, in descending order of frequency, were *H.influenzae* (24 patients (15.4%)), and *S.pneumoniae* (23 patients (14.7%)). In 14/23 (61%) sero-responders, *C.pneumoniae* infection was associated with other etiologies. In six patients, *S.pneumoniae* was also diagnosed. Other frequent concomitant microorganisms were *H.influenzae, M.pneumoniae* and *L.pneumophila.* Although co-infection was observed among sero-non-responders as well, association with other agents were found significantly more often in sero-responders, as compared to sero-non-responders (61% vs 23%, p=0.017) (Table 2).

Clinical manifestations of *C.pneumoniae* CAP

No differentiation between *C.pneumoniae* and other respiratory pathogens could be made using the major physical and laboratory findings of patients at the time of presentation (Table 3). Fever, dyspnoea, cough and chest pain were the most prevalent signs in patients with *C.pneumoniae* etiology alone (83%, 85%, 76% and 48%, respectively). Headache was noted in 31% of these subjects, and mental changes were noted in 38%. Pharyngitis and sinusitis were observed in only 20% and 30% of the cases, respectively. No clear difference in the clinical presentation was observed between sero-responders and sero-non-responders, although the severity of the disease tended to be higher among sero-responders. Among laboratory parameters, an elevated white blood cell count was found in 77% of the subjects, where an elevated erythrocyte sedimentation rate and C-reactive protein was observed in 88% and 83%, respectively. The heart rate on admission was normal in 65% of the subjects. Again, no significantly difference was observed between sero-responders and sero-non-responders. No radiological findings were characteristic of pneumonia caused by *C.pneumoniae*. Unilateral involvement was apparent in 85% of the subjects. Most pneumonic infiltrates were located in the lower lobes (69%). Pleural effusion was found in only 12% of the subjects.

Table 2. Serologic response in 45 patients with community-acquired pneumonia due to *Chlamydia pneumoniae* in relation to other causative micro-organisms.

C.pneumoniae etiology combined with	total patients (n=45)	patients classified as: sero-responders (n=23)	patients classified as: sero-non-responders (n=22)
no other pathogen (%)	26 (58)	9 (39)	17 (77)
other pathogen	19 (42)	14 (61)[a]	5 (23)
Typical pathogen	12	8	4
S.pneumoniae	7	5	2
S.pneumoniae and *H.influenzae*	2	1	1
H.influenzae	2	2	0
H.influenzae and *S.aureus*	1	0	1
atypical pathogen	6	6	0
M.pneumoniae	3	3	0
L.pneumophila	2	2	0
para-influenza virus	1	1	0
typical and atypical pathogen	1	0	1
para-influenza virus, *M.pneumoniae* and *H.influenzae*	1		1

[a]p=0.017 as compared to sero-non-responders

DISCUSSION

Community-acquired pneumonia (CAP) is a serious infection that results in numerous hospitalizations each year. Although diagnostic procedures and treatment improves, CAP still represents a major challenge in terms of morbidity and mortality (13,30). *Chlamydia pneumoniae* has been associated with both epidemic and endemic occurrences of acute respiratory disease and is believed to be responsible for 6-20% of all CAP (1,15,20, 24,37,40). In our study, etiology was established in 67% of the subjects. *C.pneumoniae* was the etiologic agent in 45 hospitalized patients (29%)

Table 3. Major physical and laboratory findings of hospitalized patients with community-acquired pneumonia.

	etiology*				
	typical pathogen	atypical pathogen			unknown etiology
		excluding *Chlamydia pneumoniae* patients	*Chlamydia pneumoniae*		
			sero-responders	sero-non-responders	
number of patients	28	24	9	17	52
physical findings					
Blood pressure systolic blood pressure <100 or diastolic blood pressure <60 (%)	3 (11)	4 (17)	0 (0)	2 (12)	4 (8)
heart rate (bpp) (mean ± sd)	108±22	103±20	106±23	105±26	99±19
patients with fever (%)	18 (64)	17 (71)	7 (78)	13 (76)	34 (65)
temperature (°C) (mean ± sd)#	38.8±0.7	39.0±0.6	39.1±0.5	39.0±0.8	38.9±0.7
sputum production (%)	19 (68)	13 (54)	5 (56)	12 (71)	36 (74)
laboratory findings					
ESR elevated (%)	25 (93)	19 (79)	8 (89)	15 (88)	44 (88)
mean ± sd (mm/h)#	62±30	48±28	73±34	67±33	53±27
C-reactive protein elevated (%)	21 (100)	22 (100)	9 (100)	15 (100)	35 (92)
Mean ± sd (mg/l)#	224±137	101±67	173±129	184±87	152±100
WBC elevated	25 (89)	13 (54)	6 (67)	14 (82)	40 (77)
mean ± sd ($x10^9$/l)#	19.8±7.9	14.2±3.6	15.3±3.6	19.1±5.8	16.4±5.2
antibiotics used prior to enrollment (%)	6 (21)	14 (58)	3 (33)	7 (41)	21 (40)
days of illness before admission (mean ± sd)	7±11	5±5	8±10	9±10	7±7
Severity of illness at enrollment (%)					
mild	5 (18)	7 (29)	2 (22)	7 (41)	24 (46)
moderate	11 (39)	9 (38)	1 (11)	5 (29)	14 (27)
severe	12 (43)	8 (33)	6 (67)	5 (29)	14 (27)

* excluding patients with mixed etiology
\# calculated for patients with elevated findings only

with CAP. The high rate of *C.pneumoniae* infection among the subjects studied suggests that *C.pneumoniae* was circulating at a relatively high level in the area during enrollment. The high rate of a *C.pneumoniae* infection diagnosis may also, in part, be explained by the many tests we used to trace a *C.pneumoniae* infection. Theoretically, increased sensitivity can be obtained by using multiple diagnostic tests simultaneously. In many previous studies, *C.pneumoniae* was diagnosed solely on the basis of serological tests (26,31,34). Other investigators have based their conclusions on the results of culture, antigen detection or PCR in combination with serology (8,13,18). In the present study, three different serological assays, culture and PCR were used to determine the incidence of *C.pneumoniae* etiology among patients with CAP. The true sensitivity and specificity of these tests have never been unequivocally determined due to the difficulty in validating these tests. The major problem is the definition of the gold standard. Culture and/or PCR may be used as gold standard to calculate the sensitivity and specificity of serological assays. However, asymptomatic infection with *C.pneumoniae*, often without serological response have been reported (2,10,19, 22,23).

Since many of the respiratory tract pathogens are also commensals of the upper respiratory tract, the isolation of *C.pneumoniae* or a positive PCR reaction from sputum or other upper respiratory tract specimens does not necessarily implicate it as the causative organism in patients with pneumonia (12). In contrast, in the assessment of pneumonia, serological methods are widely accepted for the etiological diagnosis of infection due to virus and atypical bacterial pathogens. A significant increase in specific antibodies is strongly indicative for an acute infection (32). Therefore, we divided all patients with laboratory results compatible with acute *C.pneumoniae* infection into two different groups: sero-responders and sero-non-responders. Discrepancies between the two serologic tests were observed. The utility of the MIF in the etiologic diagnosis of *C.pneumoniae* infection has been questioned by several investigators (27,36). However, in our study 93% of the patients with MIF results indicative for an acute *C.pneumoniae* infection were confirmed by rDNA LPS ELISA and/or PCR. Some patients were positive by rDNA LPS ELISA alone. This may be explained by difference in sensitivity (43). Many patients were positive in the

C.pneumoniae PCR. However, only 29% of these PCR positive patients were confirmed by chlamydial serology, a discrepancy that requires further elucidation. The use of specimens obtained from the site of infection using transthoracic needle aspiration may be a promising method to obtain uncontaminated pulmonary samples (16,35). Furthermore, in future studies control subjects has to be evaluated together with pneumonia patients during the entire enrollment period to determine the diagnostic value of the PCR.

No immunity following *C.pneumoniae* infection could be observed. In contrary, increased *C.pneumoniae* pneumonia has been observed in patients with detectable IgG antibodies against chlamydial LPS at enrollment. These findings could not be confirmed by the presence of IgG antibodies against the *C.pneumoniae* major outer membrane protein (MOMP). This may be explained by a faster appearance of the anti-LPS chlamydial antibodies in acute infection as compared to the MOMP antibodies detected by MIF. Ekman *et al.* (14) reported an decreased severity of disease following *C.pneumoniae* reinfection, however, this was not evident in our study (data not shown).

C.pneumoniae alone can cause CAP requiring hospitalization in adults. Mixed infections in association with other agents were found significantly more often in sero-responders, as compared to sero-non-responders. This may indicate that *C.pneumoniae* pneumonia mixed with other etiologies may enforce increased involvement of the immune system due to more severe tissue damage. Mixed infections were found most commonly with *S.pneumoniae.* These findings were in accordance with the results of Kauppinen *et al.* (25,26) and Falguera *et al.* (16).

The prediction of an infecting organism based on the clinical presentation of the patient and chest radiographic appearances was not possible. Also, the use of basic laboratory techniques could be used only to determine the severity of the disease. No outstanding differences between sero-responders and sero-non-responders could be observed, although the severity of the disease tended to be higher among sero-responders.

The severe clinical picture associated with *C.pneumoniae* pneumonia and the frequency of occurrence emphasizes the importance of highly sensitive and specific techniques to diagnose an acute *C.pneumoniae* infection. At

this moment, a definitive *C.pneumoniae* diagnosis can be obtained only by serology. The rDNA LPS ELISA seems to be more sensitive for the detection of acute respiratory chlamydial infection as compared to the traditional MIF. However, reliable results can be obtained only if two or more consecutive serum samples are obtained and is therefore not of relevance when considering empirical therapy. The use of PCR may provide additional information during the acute phase of infection especially if clinical specimens obtained from the site of infection are available.

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CHAPTER 11

CHLAMYDIA PNEUMONIAE IN CHILDHOOD COMMUNITY-ACQUIRED PNEUMONIA

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ABSTRACT

Little is known about the role of *C.pneumoniae* in childhood pneumonia. We prospectively studied 45 children with a diagnosis of community-acquired pneumonia (CAP). Diagnosis of a respiratory infection by *C.pneumoniae* was made by PCR and recombinant LPS ELISA. Other bacterial and viral etiologies were determined using standard laboratory procedures. An etiologic agent was found in 30 children (67%), of which 20 (44%) had acute infection by *C.pneumoniae*. Six patients (13%) were positive by serology and 17 children (38%) were positive by PCR. Other common pathogens were *Mycoplasma pneumoniae*, 10 children (22%) , *Streptococcus pneumoniae*, 3 (7%) and para-influenza virus, 3 (7%). Only 3 of the 17 (18%) children positive by *C.pneumoniae* PCR responded serologically. Vice-versa 3 of the 6 children with serological results compatible with acute infection were PCR positive. Mixed infections of *C.pneumoniae* with other agents such as *M.pneumoniae* were found more often than *C.pneumoniae* infection alone. Acute phase responses of children with infection by *C.pneumoniae* were more elevated compared to those of children with infection by *M.pneumoniae*. Also, the clinical severity of illness at enrollment was significantly higher in children with pneumonia by *C.pneumoniae* than in children with infection by *M.pneumoniae* (p=0.009). We conclude that *C.pneumoniae* is often involved in childhood pneumonia and may be associated with severe disease. Therefore a diagnostic work-up for *C.pneumoniae* should be routinely performed.

INTRODUCTION

Community-acquired pneumonia (CAP) in childhood is typically caused by viruses, *Mycoplasma pneumoniae* and pyogenic bacteria. *Chlamydia pneumoniae* infection frequently occurs in school-aged children as evidenced by a rapid increase in the prevalence of antibodies against *C.pneumoniae* in this age group. The relative contribution of *C.pneumoniae* to childhood CAP is not well established, partly due to the lack of validated laboratory tests for to diagnose acute infection by *C.pneumoniae*. Laboratory diagno-

sis requires specialized studies including serology, culture and DNA-based detection methods. The aim of this prospective study was to further evaluate the role of *C.pneumoniae* as an etiologic agent of community-acquired pneumonia in Dutch children by the systematic application of serological and DNA-amplification assays on each patient entered into the study.

PATIENTS AND METHODS

Patients

During a 21-month period from April 1992 to June 1994, we prospectively studied 45 children with clinical signs and symptoms of community-acquired pneumonia (CAP) who were admitted to the Sophia Children's Hospital of the Erasmus University Medical Center Rotterdam, The Netherlands. CAP was confirmed by a radiologist when consolidation of at least a part of a lung lobe without loss of volume on a chest X-ray was present. All findings were compared to results of previous chest radiographs (if available). Exclusion criteria were pre-existent abnormalities without a significant change, immunodeficiency disorders, aspiration pneumonia, congenital malfunction of the respiratory tract, chronic use of a nasogastric tube, cystic fibrosis and severe retardation. Informed consent was obtained from the parents.

Nasopharyngeal aspirates, nose swabs and blood cultures were collected on the day of admission. Sputum samples were obtained if possible. The first serum sample was obtained within the first 24 hours of admission. A second (convalescent) serum sample was obtained 14 days after enrollment. At days 1, 3, 14 and 28, erythrocyte sedimentation rate (ESR), white blood cell count (WBC) and C-reactive protein concentration (CRP) were determined.

Demographic data, clinical signs and symptoms and antibiotic treatment prior to enrollment were collected from the time of presentation and during admission. Patients were classified by the severity of illness as described by Fine *et al.* (11). At days 1, 3, 14 and 28, physical examination was performed. Routine microbiological tests included blood culture, sputum Gram stain and culture, nose culture, and serological tests on acute and convalescent serum samples of complement fixing antibodies against

Mycoplasma pneumoniae, influenza and para-influenza viruses, respiratory syncytial virus (RSV), adenovirus and *Coxiella burnetii*. A ≥4-fold rise in titer was considered to represent a definitive etiologic evidence for infection with these pathogens. Also, *M.pneumoniae* IgG and IgM antibodies were tested using a immunofluorescence test (Zeus Scientific, Inc., Yersey, USA). A fourfold titer rise or IgM positivity was considered evidence of an acute infection. Detection of *M.pneumoniae* in clinical specimens was performed using a *M.pneumoniae* specific PCR as described by Tjhie *et al.* (25). Nucleic acids were extracted as described by Chromczynski and Sacchi (9).

Laboratory assays for *C.pneumoniae*

Serology Chlamydia-specific IgG, IgM and IgA antibodies were detected using a recombinant LPS ELISA (rDNA LPS ELISA; Medac GmbH, Hamburg, Germany). This ELISA includes a chemically pure structure of a recombinant LPS which contains a genus-specific epitope of the human pathogens *Chlamydia* spp. (5,6,15). Initial serum dilutions for IgG, IgM and IgA were 1:100, 1:50 and 1:50, respectively. Prior to the IgM determinations IgG absorption was performed (27). Sera with OD values exceeding 2.5 were retested using a 1:4 predilution. A two-fold serially diluted standard serum was used to calculate the $\log_2$ titer of the serum samples of the children. The IgG-, IgM- and IgA cutoff values were calculated as prescribed by the manufacturer. Prevalence of anti-Chlamydia IgG-, IgM- and IgA antibodies was based on the criteria as prescribed by the manufacturer : ≥calculated cutoff x 1.10, ≥calculated cutoff x 1.15 and ≥calculated cutoff x 1.10, respectively. Serological diagnosis of an acute chlamydial infection was based on the ELISA results using the following criteria : A three-fold or greater increase in Chlamydia-specific IgG- or IgA antibody titer, or a two-fold change in specific IgM titer, or a two-fold increase in specific IgG antibody titer in combination with a two-fold increase in specific IgA antibody titer (28).

PCR Two-hundred μl nasopharyngeal aspirate was transferred to a sterile tube and centrifuged for 30 min at 15,000 x g. Sputum samples were suspended in 1.5 ml 2SP transport medium. One-hundred μl of the sus-

pended sputum sample was transferred to a sterile tube. The sediment or sputum sample was incubated with a solid carrier (celite) and a guanidinium-thiocyanate (GuSCN) containing lysis buffer. Nucleic acids (NA) were bound to the carrier which was rapidly sedimented by centrifugation. The sedimented complexes were washed once with a GuSCN-containing washing buffer, once with 70% ethanol and once with acetone. Complexes were dried and NA was subsequently eluted in 55μl aqueous solution at 37°C for 30 minutes. The isolated NA was removed from the solid carrier by centrifugation (4). Amplification by PCR and analysis of the amplified products were performed as described by Campbell *et al.* (7). Briefly, PCR amplification was performed with 8 μl isolated DNA in a 100 μl reaction mixture containing 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCL (pH 9.0) 0.01% Tween-20, 0.2 mM deoxynucleoside triphosphates, 50 pmol primers and 0.2U Thermoperfect *Taq* polymerase (Integra Biosciences A.G., Wallisellen, Switzerland). A *C.pneumoniae* species specific primer set was used, which amplifies a 437-bp fragment with the following primers: The forward primer HL-1 (5'-GTTGTTCATGAAGGCCTACT-3') and the reverse primer HR-1 (5'-TGCATAACCTACGGTGTGTT-3') (7). PCR products (20 μl) were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. DNA was transferred from agarose to Hybond plus nylon filters (Amersham International plc, Amersham, United Kingdom) by electrophoretic transfer. The PCR products were analyzed with a *C.pneumoniae*-specific probe HM-1 (5'-GTGTCATTCGCCAAG GTTAA-3') (7). The ECL 3'-oligolabeling and detection system was used for the detection of the PCR products (Amersham International, plc, Amersham, United Kingdom). A 10-fold serial dilution of a *C.pneumoniae* stock solution (10^9 target DNA copies per ml) was incorporated in each amplification. One negative control (pooled cervical specimens suspended in 2SP) was incorporated every 10 amplifications. All PCR positive reactions were confirmed by repeat DNA isolation and amplification of the original specimen. Separate rooms were used for the different steps of the PCR and the recommendations of Kwok *et al.* were used to prevent DNA carryover contamination (21).

Data processing and statistical analysis

Patient's history, physical findings, and results of all investigations were obtained in order to determine the etiology of pneumonia. The data were analyzed using the Statistical Package for the Social Science (SPSS for Windows95; version 7.0) (24). Chi-square and Fisher exact tests were used to compare demographic and clinical characteristics. Probability values were two-tailed (23).

RESULTS

Patient characteristics

Over the 21-month period, 45 children (male/female: 24/21, age median: 5 years, range 3 months to 12 years) were enrolled. Admission to hospital was indicated in 24 children (53%). One child was twice enrolled. In 19 children (42%) the etiology was established, when the *C.pneumoniae* diagnosis was disregarded. Pyogenic bacteria were cultured in 4 children, while in 17 children viruses and other atypical pathogens were found (Table 1). Twelve children (27%) received antibiotics prior to enrollment. None of these observations were significantly different between boys and girls (data not shown).

Chlamydia pneumoniae diagnosis

The diagnosis of *C.pneumoniae* CAP was established in 20 children (44%). Six children (13%) had serological results compatible with acute infection by *C.pneumoniae* and 17 (38%) children were positive by PCR. Only 3 of the 6 children with serological results compatible with acute infection by *C.pneumoniae* were PCR positive. Vice versa only 3 of the 17 (18%) children with a positive PCR responded serologically. Three children (7%) had serological results indicative of an acute infection only by rDNA LPS ELISA. No difference in rate of *C.pneumoniae* pneumonia was observed between boys and girls.

Additional specificity analysis of the *C.pneumoniae* PCR was performed in order to better estimate the specificity of the PCR. Thirty cervical scrapes from female patients attending the sexual transmitted disease clinic (20 *C.trachomatis* PCR negative samples and 10 *C.trachomatis* PCR positive

samples), suspended in chlamydial transport medium (2SP) were tested using the *C.pneumoniae* PCR. None of these specimens were positive. Additionally, 40 nasopharyngeal aspirates from neonates taken directly after birth were tested; again, all specimens remained negative.

Table 1. Distribution of etiologic agents in 45 children with community-acquired pneumonia.

micro-organism identified		number of causative agents (%)
children with unknown etiology	15 (33%)	
children with etiologic agent	30 (67%)	
typical pathogens	4	
Streptococcus pneumoniae		3
Haemophilus influenzae		1
atypical pathogens	39	
C.pneumoniae		20
M.pneumoniae		10
para-influenza		3
Influenza viruses		2
Cytomegalovirus		2
adenovirus		1
Coxiella burnetti		1
Total number of pathogens	43	

Etiologic agents

If *C.pneumoniae* diagnosis was included, 30 of the 45 (67%) children had an etiologic diagnosis. Surprisingly, *C.pneumoniae* was the most common pathogen (20 (44%) children). Other pathogens, in descending order of frequency, were *Mycoplasma pneumoniae* (10 (22%) children), and influenza and para-influenza viruses (5 (11%) children) (Table 2). In 9 of the 20 (45%) children infection by *C.pneumoniae* was associated with other microbial etiologies, especially *M.pneumoniae* and viruses (Table 2).

Table 2. *Chlamydia pneumoniae* etiology in relation to other causative micro-organisms in children with *C.pneumoniae* community-acquired pneumonia.

C.pneumoniae combined with		total
no other pathogen (%)		11 (55)
other pathogen (%)		9 (45)
Typical pathogens	1	
S.pneumoniae		1
atypical pathogens	7	
M.pneumoniae		3
M.pneumoniae and adenovirus		1
M.pneumoniae and cytomegalovirus		1
cytomegalovirus		1
para-influenza		1
typical and atypical pathogens	1	
S.pneumoniae and influenza virus		1

Clinical manifestations of CAP by *C.pneumoniae*

Fever and dyspnoea were the most frequent signs in children with community-acquired pneumonia associated with *C.pneumoniae* (73% and 55%, respectively). Pharyngitis, sinusitis and mental changes were observed in only 5% of the cases. Among laboratory parameters, an elevated white blood cell count was found in 82% of the subjects, whereas an elevated erythrocyte sedimentation rate and C-reactive protein was observed in all children with *C.pneumoniae* etiology (Table 3 and Figure 1). No differentiation between *C.pneumoniae* and other respiratory pathogens could be made using physical and laboratory findings, although most laboratory parameters of the acute phase response of children with *C.pneumoniae* etiology were more elevated than those of children with infection by *M.pneumoniae* (Table 3 and Figure 1). Also, moderate to severe illness at enrollment was significantly more prevalent among children with infection by *C.pneumoniae* than among children with *M.pneumoniae* etiology (Table 3; p=0.009). CAP requiring hospitalization occurred most frequent-

Table 3. Major physical and laboratory findings of children with community-acquired pneumonia.

	etiology				
	Chlamydia pneumoniae	*Mycoplasma pneumoniae*	viral pathogens	mixed pathogens	unknown
number of patients	11	4	4	10	15
physical findings					
respiratory rate (rpm) (mean ± sd)	41±17	27±9	33±10	43±18	38±11
heart rate (bpm) (mean ± sd)	124±33	109±18	112±14	124±19	121±30
patients with fever (%)	8 (73)	2 (50)	4 (100)	10 (100)	13 (87)
temperature (°C) (mean ± sd)*	39.7±0.7	38.4±0.2	39.7±0.6	39.4±1.0	39.9±0.9
fever broken at day: (mean ± sd)	4±3	3±4	2±1	3±2	2±2
sputum production (%)	5 (50)	2 (50)	3 (100)	8 (80)	8 (53)
laboratory findings					
ESR elevated (%)	6 (100)	3 (75)	2 (100)	5 (100)	10 (83)
mean ± sd (mm/h)*	53±21	45±30	30±7	56±27	68±23
C-reactive protein elevated (%)	10 (100)	3 (75)	3 (100)	10 (100)	15 (100)
Mean ± sd (mg/l)*	82±64	23±10	150±142	143±114	153±98
white blood cell count elevated	9 (82)	1 (25)	3 (100)	8 (89)	15 (100)
mean ± sd ($x10^9$/l)*	20.2±7.9	24.6	20.3±9.7	22.7±7.8	23.3±7.3
antibiotics prescribed prior to enrollment (%)	2 (18)	4 (100)	1 (33)	1 (10)	4 (27)
days of illness before admission (mean ± sd)	5±3	12±7	7±3	5±4	6±9
Severity of illness at enrollment (%)					
mild	0 (0)	3 (75)	0 (0)	0 (0)	2 (13)
moderate	9 (82)	1 (25)	3 (100)	7 (70)	8 (54)
severe	2 (18)	0 (0)	0 (0)	3 (30)	5 (33)
hospital admission necessary (%)	8 (73)	1 (25)	0 (0)	6 (60)	9 (60)

* children with elevated findings only

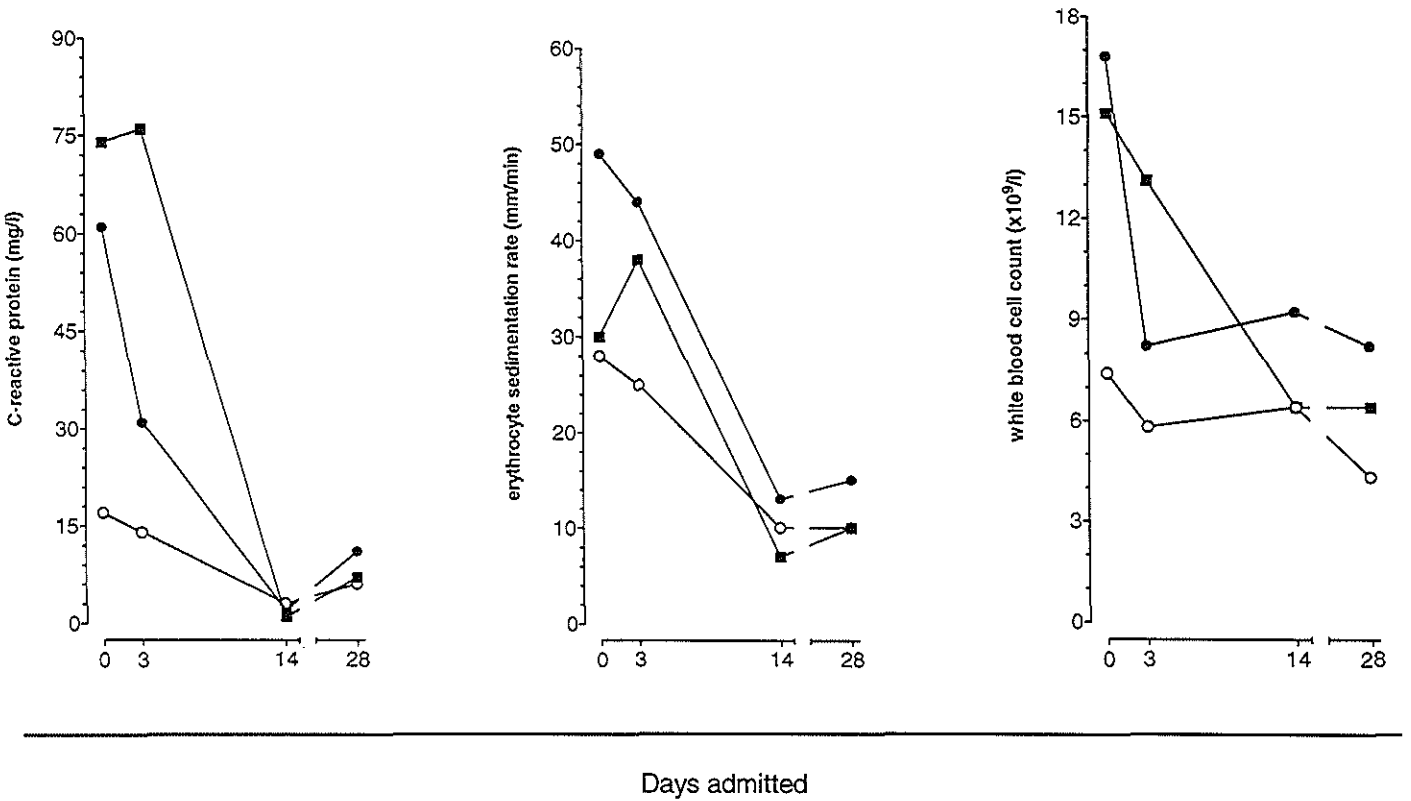

Figure 1. Kinetics of C-reactive protein (CRP) (left), erythrocyte sedimentation rate (ESR) (middle) and white blood cell count (WBC) in children with *C.pneumoniae* etiology (●----●), *M.pneumoniae* etiology (○----○) and viral etiology (■----■). (children with mixed etiologies were excluded) (median).

ly in children with *C.pneumoniae* etiology (73%), while most *M.pneumoniae* and viral pneumonia could be treated on an ambulatory basis (p=0.02). No radiological findings were characteristic of pneumonia caused by *C.pneumoniae*. Unilateral involvement was apparent in 73% of the children. Pleural effusion was found in only 18%.

Only one of the 20 (5%) children with infection by *C.pneumoniae* received erythromycin during enrollment of the study. Fifteen children (75%) received cefuroxim only. However, all children recovered completely. One child was enrolled twice. During the first enrollment, a *C.pneumoniae* PCR positive result was obtained without serological evidence compatible with acute chlamydial infection. No other etiology was found. Treatment was started with cefuroxim, and recovery was established. Two months later, the child was enrolled for the second time with similar complaints. Again, a *C.pneumoniae* PCR positive result was obtained without serological response and any other etiology. Treatment was continued with cefuroxim, and recovery was established again. Similar clinical and laboratory presentation was observed in another child, although no serum samples could be obtained during the second enrollment.

DISCUSSION

The microbiological diagnosis of acute community-acquired pneumonia (CAP) cannot rely on culture alone. For many potential pathogens, such as *Chlamydia pneumoniae*, culture has been hampered by very low sensitivity and tedious subculture procedures. Also, reliable serological results can be obtained only if two or more consecutive serum samples are obtained and is therefore not of relevance when considering empirical therapy. The use of PCR may provide additional information during the acute phase of infection. In this study, the etiology of childhood CAP was established in 67% of the subjects. Infection by *Chlamydia pneumoniae* was found in 20 children (44%) with CAP. The high rate of infection by *C.pneumoniae* among the children with pneumonia suggests that *C.pneumoniae* was circulating at a relatively high level in the area during enrollment. In 6 children (13%), serological results were obtained compatible with acute chlamydial infection. Comparable rates of

chlamydial pneumonia among children were reported earlier (3,8). Several other investigators observed a lower incidence of pneumonia by *C.pneumoniae* (2,18,19) However, the incidence of infection may be underdiagnosed because they used only an acute phase serum sample to confirm the diagnosis. In the present study, only three of the 6 children with serological evidence of an acute chlamydial infection (50%) were *C.pneumoniae* PCR positive. This discrepancy may be due to sampling error since the sampling site (nasopharynx) differed from the site of infection. A negative PCR using upper respiratory specimens may not necessarily imply absence of *C.pneumoniae* at the actual site of infection. Furthermore, If the infection becomes invasive, serological results compatible with acute infection may be expected. A significant increase in specific antibodies is therefore strongly indicative for an acute infection (22). Additionally, 14 children had *C.pneumoniae* PCR positive results without serological evidence of an acute *C.pneumoniae* infection, a discrepancy that requires further elucidation. Apparent lack of an antibody response may be, in part due to an inappropriateness of the serological tests used, resulting in insensitivity. However, asymptomatic carriage of *C.pneumoniae* in the nasopharynx of healthy adults (12,16,17,26) and children (1,8) has been observed. Hyman *et al.* (17) has hypothesized that after *C.pneumoniae* colonizes the respiratory tract, there is an undefined period of asymptomatic carriage. Some people may clear infection without developing clinical symptoms or a serological response. In others, the infection becomes invasive and may result in clinical symptoms and disease which, in most cases, leads to a serological response. Persistent infection following acute illness has been observed in adults by Hammerschlag (14) and Falck (10). These results are in accordance with our findings. The demonstration of two children with *C.pneumoniae* PCR positive results during two consecutive pneumonia periods over a two month period suggests that persistent infection with *C.pneumoniae* following acute respiratory illness is possible. However, clear evidence for such a hypothesis is lacking at this moment. In future follow-up studies control subjects without disease symptoms need to be evaluated together with pneumonia patients during the entire enrollment period to determine the clinical relevance of a positive PCR result.

Hammerschlag *et al.* reported that culture-positive patients may remain positive for many months despite therapy with doxycycline or tetracycline (14). Falck *et al.* demonstrated persistent *C.pneumoniae* infection for periods up to a year in Swedish patients with recurrences of signs and symptoms of respiratory infection despite several prolonged courses of antibiotics known to be effective against chlamydial species (10). In contrast, although only one child with *C.pneumoniae* etiology was treated with adequate antibiotics, all children in our study recovered. It seems possible that patients with pneumonia by *C.pneumoniae* respond to treatment with cefuroxim. This observation has been reported earlier for β-lactam antibiotics (20). However, Hammers-Berggren found that the duration of fever may be longer during treatment with β-lactams than with doxycycline or erythromycin (13). Another distinct possibility is that individuals may clear the infection despite the use of inappropriate use of antibiotics.

C.pneumoniae alone can cause severe CAP in children. Mixed infections were found in 55% of the children with *C.pneumoniae* etiology, most commonly with *M.pneumoniae* and viruses. The prediction of an infecting organism based on the clinical presentation of the patient and chest radiographic appearances was not possible, although the severity of illness was higher in children with *C.pneumoniae* pneumonia as compared to *M.pneumoniae* pneumonia. This is supported by the laboratory findings of these children.

In several children, *C.pneumoniae* was the only apparent explanation for their lower respiratory infection. However, in children with *C.pneumoniae* PCR positive results without serological results compatible with acute *C.pneumoniae* infection, the etiological diagnosis may be questioned. The severity of disease observed in children with *C.pneumoniae* pneumonia and the frequency of occurrence emphasizes the importance of highly sensitive and specific techniques to diagnose acute *C.pneumoniae* infection. Reliable serological results can be obtained only if two or more consecutive serum samples are obtained and is, therefore, not relevant for empirical therapy. The use of PCR may provide more timely information during the acute phase of infection, especially if clinical specimens obtained from the site of infection are available.

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CHAPTER 12

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

SUMMARY

More than 10 years after the first report that *Chlamydia pneumoniae* may be responsible for acute respiratory infection (8), the exact role of *C.pneumoniae* as a cause of disease is still not completely elucidated. Some researchers have postulated that *C.pneumoniae* is not only a respiratory pathogen, but also a co-factor in the development of atherosclerosis, and, thus, may cause acute myocardial infarction (15,17,19). Despite modern laboratory techniques including DNA amplification, ELISA, fluorescence microscopy, the diagnosis of *C.pneumoniae* infection is still difficult to make (12). As signs and symptoms of *C.pneumoniae* infections are not specific, a clinical diagnosis of infection can not be made and laboratory confirmation is required for a definitive diagnosis. However, the clinical value of current laboratory methods for the diagnosis of respiratory infection caused by *C.pneumoniae* has not been fully ascertained. The sensitivity of cell culture seems to be very low, although some research groups achieve excellent results. Complement fixation (CF) has been used extensively for the serological diagnosis of acute *Chlamydia psittaci* infections. After the acceptance of *C.pneumoniae* as the third *Chlamydia* species in 1989, information was obtained indicating that most *C.psittaci* infections found by CF actually are *C.pneumoniae* infections (14). The gold standard in *C.pneumoniae* serology at this moment is the microimmunofluorescence (MIF) test (4,13,20). Purified Chlamydia elementary bodies (EBs), which are the infective cell forms of Chlamydia, are used as antigen in the MIF test. This test is reported to be species specific and able to differentiate between IgG, IgM and IgA class antibodies (18,21). However, the reading of fluorescence patterns requires experience and skill, especially when evaluating high titered serum samples in which a trailing effect may be observed. Also, chronic infections may complicate the interpretation of the classical serology. Recently, PCR has been used successfully to identify *C.pneumoniae* specific DNA and has enhanced our ability to detect this species in clinical specimens (6).

Evaluation of current and newly developed laboratory methods to diagnose acute respiratory *C.pneumoniae* infections plays a central role in the studies

reported in this thesis. In chapter two the aims of our efforts described in this thesis are presented. In general, the aim of the present studies was to contribute to the development of laboratory techniques and to improve our understanding of their limitations and interpretation, and to assess the role of *C.pneumoniae* in patients with respiratory tract disease in The Netherlands.

In chapter 3 we found that with age the serologic diagnosis of acute *C.pneumoniae* infection observed by MIF becomes increasingly prone to false positive results. Rheumatoid factor (RF) is responsible for this phenomenon. RF can frequently be detected in sera from patients with rheumatoid arthritis and, occasionally, in healthy adults. Advanced age is correlated with an increased prevalence of circulating RF. Additionally, RF can be found frequently during certain infectious diseases. Chapter 3 describes that RF interference can readily be prevented by routinely treatment of serum with anti-human IgG antibody, a pretreatment that thus enhances the specificity of the MIF assay.

In chapter 5, mycoplasma contamination was observed in several commercially available antigen preparations used for the serological diagnosis of acute *C.pneumoniae* infections by MIF. This mycoplasma strain appeared by DNA sequence analysis to be strongly related to *Mycoplasma arginini*, a veterinary mycoplasma. Approximately 15% of all continuous cell lines are estimated to be contaminated with mycoplasma worldwide (16). These contaminations often remain inapparent. Although this particular mycoplasma contamination had no significant effect on *C.pneumoniae* diagnosis by MIF, caution should be taken. Inapparent cell line contamination with human *Mycoplasma* species is always possible and can have a major impact on the serodiagnosis of bacterial - and viral infections, or otherwise confound research efforts. Recommendations have been proposed in this chapter for the routine screening of all antigen preparations obtained by cell culture prior to their distribution and use.

In chapter 4 a modified cell culture technique is described. Hep-2 and buffalo Green Monkey (BGM) cells appeared to be more sensitive for the isolation of

C.pneumoniae as compared to HL and HeLa 229 cells. In addition, BGM cells were less prone to detachment after reaching confluent growth. Also, omission of the routine DEAE-dextran pretreatment of cell monolayers significantly enhanced *C.pneumoniae* growth.

In chapter 6 serology was compared with cell culture and an in-house PCR for the diagnosis of acute chlamydial lower respiratory tract infections. Serology included complement fixation (CF), MIF and a recent developed, commercially available, ELISA (rDNA LPS ELISA). This assay utilizes a chemically pure chlamydial lipopolysaccharide developed by recombinant DNA technique. In addition, we sought to evaluate the usefulness of the rDNA LPS ELISA in determining the seroprevalence of *C.pneumoniae* antibody. Several respiratory specimens were obtained for the detection of *C.pneumoniae* using cell culture and PCR. An attempt was made to calculate sensitivity and specificity of these diagnostic tests using several gold standards. Generally, the rDNA LPS ELISA was more sensitive than MIF in detecting new *C.pneumoniae* infections. The timing of the convalescent serum sample proved to be of great importance, as the serological response was delayed in some patients. Acute and convalescent serum sample with a minimum interval of ten days may be used to diagnose acute *C.pneumoniae* infection, although increased sensitivity was observed when a third serum sample (obtained after 30 days) was tested as well. False positive results which were found in patients with *Mycoplasma pneumoniae* infection may compromise the specificity of the rDNA LPS ELISA, especially if only a single serum sample is used for diagnosis. The sensitivity of the complement fixation test for the detection of chlamydial infection was shown to be very low, particularly in this elderly group of patients, where most *C.pneumoniae* infections probably are reinfections that may not induce complement fixing antibodies. Further use of the CF test should be dissuaded. The results of chapter 4 has indicated that BGM cells are highly sensitive for the isolation and propagation of *C.pneumoniae*. However, the diagnostic value of *C.pneumoniae* culture of patient samples remained very low. In contrast, many patients were positive in the *C.pneumoniae* PCR-test. Nasopharyngeal specimens. Were found to be best suited for PCR testing. Increased sensitivity of the PCR was observed when nasopharyngeal and throat

specimens were tested simultaneously. However, only 29% of the PCR positive patients were confirmed by chlamydial serology, a discrepancy that requires further elucidation. Due to asymptomatic or chronic infections, the predictive value of a positive PCR in cases of community-acquired pneumonia remains unknown and may be disappointingly low. The presence of *C.pneumoniae* in the nasopharynx of subjectively healthy adults (7,10,11) and children (1,3) has been noted before. Evidence was given that the rDNA LPS ELISA may not be reliable in determining the prevalence of *C.pneumoniae* infection, i.e. establishing the proportion of subjects with *C.pneumoniae* infection in the past. Apparently the half-life of the anti-LPS chlamydial antibodies as detected by rDNA LPS ELISA is shorter as compared to the half-life of the antibodies against the major outer membrane protein detected by the MIF assay.

In chapter 7, incidence rates of *C.pneumoniae* infection, obtained by prospective serial serology were determined in patients with COPD. Chlamydia specific antibodies were detected using the rDNA LPS ELISA as well as the MIF. The rDNA LPS ELISA achieved a high degree of reproducibility and was more sensitive in detecting intercurrent *C.pneumoniae* infection as compared to the MIF. Despite the genus-specific status of the test, no evidence indicating *C.trachomatis* or *C.psittaci* infection was found. However, in several patients, IgM reactivity was observed by rDNA LPS ELISA that persisted during the entire observation period, a phenomenon that may be due to a chronic, asymptomatic, *C.pneumoniae* infection in these patients.

Little in known about the transmission of *C.pneumoniae* from one person to another. The survival of *C.pneumoniae* in aerosols was previously shown to be rather short. In chapter 8, the survival and recovery of *C.pneumoniae* was studied after their inoculation on various solid surfaces and human skin. Infectious *C.pneumoniae* was recovered from innate surfaces for up to four hours, while infectious *C.pneumoniae* could be recovered for up to 30 minutes from human skin. Desiccation of the surface had no significant effect on the recovery op infectious *C.pneumoniae*. Further experiments demonstrated that infectious *C.pneumoniae* could be transferred to hands touching these

contaminated surfaces and could be recovered from these hands. These results suggest that contact with contaminated surfaces may be a potential mode of transmission of *C.pneumoniae*.

In chapter 9, antibodies to *C.pneumoniae* were repeatedly measured in the serum of patients with COPD over a mean period of 14 months and the results were compared with similar data from a cohort of blood donors in order to further define the role of *C.pneumoniae* infection in COPD. Chlamydial IgG, IgM and IgA serum levels were detected using the rDNA LPS ELISA. The differences in age distribution between the COPD patient group and the blood donors limits the interpretation of our findings. Nevertheless, the prevalence of chlamydial antibodies was higher among COPD patients and increased with age. The incidence of *C.pneumoniae* infection was also higher in COPD patients and likewise increased with age. The prevalence of Chlamydia-specific antibodies changed over time. However, the decline in IgG and IgA antibody titer against chlamydial LPS is apparently relatively slow, which suggests that IgG and IgA chlamydial LPS antibodies once induced, may remain detectable for many years. As mentioned before in chapter 7, in several COPD patients, persisting IgM reactivity was observed during the entire observation period. Similar data were found in the blood donors.

The role of *C.pneumoniae* as an etiologic agent of lower respiratory tract infection in adult patients (chapter 10) and in children (chapter 11) was further evaluated by systematically applying serology, culture (chapter 10 only) and DNA-amplification assays on each patient entered into these clinical studies. In both patient groups, a microbial etiology was established in 67% of the patients. Furthermore, *C.pneumoniae* was the most prevalent microbial agent detected (29% and 44% in the adult patients and children, respectively). The high rate of *C.pneumoniae* infection suggests that *C.pneumoniae* was circulating at a relatively high level in the area during enrollment. However, only 49% of the adult patients and 30% of the children positive for *C.pneumoniae* by PCR and/or culture had serological evidence compatible with acute *C.pneumoniae* infection. Since many other respiratory tract pathogens may colonize the upper respiratory tract without clinical

disease, the isolation of *C.pneumoniae* from the throat does not necessarily implicate it as the causative organism in patients with pneumonia. Furthermore, asymptomatic carriage of *C.pneumoniae*, often without antibody responses in subjectively healthy adults (7,10,11) and children (1,3) has been reported earlier. In contrast, in the assessment of pneumonia, a specific antibody response has widely been accepted to establish causation of infection due to viral and atypical bacterial pathogens. A significant increase in specific antibodies is strongly indicative for an acute infection. Therefore, we divided all patients with laboratory results compatible with acute *C.pneumoniae* infection into two groups: sero-responders and sero-non-responders. It was not possible to predict the species of infecting micro-organism based on the clinical presentation of the patient, chest radiographic appearances or routine laboratory work-up alone. The severity of illness of patients with *C.pneumoniae* infection was higher than in patients with a *M.pneumoniae* etiology. No outstanding differences between sero-responders and sero-non-responders could be observed, although the severity of the disease tended to be higher among those that responded serologically. *C.pneumoniae* alone can cause severe pneumonia. However, mixed infections of *C.pneumoniae* with other agents were found significantly more often among sero-responders, as compared to sero-non-responders. This finding suggests that *C.pneumoniae* pneumonia mixed with other etiologies may enforce increased involvement of the immune system as a consequence of more severe tissue damage. Mixed infections were commonly observed with *Streptococcus pneumoniae* in adult patients and with *M.pneumoniae* and viruses in pediatric patients.

CONCLUSIONS

In this thesis, current and new laboratory techniques to study *Chlamydia pneumoniae* infections were evaluated. A newly developed, commercially available enzyme immunoassay with enhanced sensitivity may now considered to be the preferred serological tool for diagnosing acute respiratory *C.pneumoniae* infections in routine clinical practice. The presence of only *C.pneumoniae* DNA in upper respiratory specimens as demonstrated by PCR, but without a concomitant serological response, may indicate a harmless colonization of the respiratory tract rather than pointing to the agent that is

the cause of respiratory disease. The diagnostic value of the PCR alone is, therefore, limited.

The severity of morbidity associated with *C.pneumoniae* pneumonia and the frequency of its occurrence emphasize the importance of highly sensitive and specific techniques to diagnose acute *C.pneumoniae* infection. At this time, a definitive *C.pneumoniae* diagnosis can be obtained only by serology if two or more consecutive serum samples are obtained.

It is clear that the laboratory techniques have not reached their ultimate point of sophistication. Further studies are necessary to understand the pathogenesis of *C.pneumoniae* infections, and, to determine the predictive value of current and new diagnostic tools. A better understanding of laboratory techniques to diagnose *C.pneumoniae* infections is of great importance, especially since *C.pneumoniae* may play a role in chronic systemic processes including atherosclerosis.

RECOMMENDATIONS

Over the past ten years an increasing number of publications on respiratory infections due to *C.pneumoniae* has appeared in the medical journals. With the currently available laboratory techniques and the observations in this thesis, it seems appropriate to propose guidelines to provide physicians with a basis for rational judgement on their diagnostic value. Every test will be discussed separately.

Serology

in general, the use of single serum samples for the serological diagnosis of acute respiratory *C.pneumoniae* infection will have limited sensitivity and specificity. The decline in IgG and IgA antibody titer against chlamydial LPS and, especially, against the major outer membrane protein (MOMP) is very slow; thus, IgG and IgA chlamydial antibodies may remain detectable for many years after an infection. Prolonged IgM and IgA reactivity may also be found in patients with chronic *C.pneumoniae* infection (5). Furthermore, polyclonal B-cell activation due to other causes including *Mycoplasma*

pneumoniae infection may give false-positive result if only a single serum sample is used (2). The use of two - or more consecutive serum samples will prevent such false-positive results. Acute and convalescent serum sample with a minimum interval of ten days can be used to diagnose most acute *C.pneumoniae* infections, although sensitivity can be further increased if a third serum sample is taken after 30 days.

The sensitivity of the complement fixation (CF) test for the diagnosis of acute *C.pneumoniae* infection is very low, particularly in elderly patients. The diagnostic value of the CF is therefore questionable and further use of the CF test for the diagnosis of acute *C.pneumoniae* infection is not recommended.

The microimmunofluorescence (MIF) can be used for the diagnosis of acute *C.pneumoniae* infection, although the reading of the fluorescence patterns requires experience and skill, especially for high titered serum samples in which a trailing effect may be observed. Also, rheumatoid factor interference should be prevented by routinely pretreatment of serum with anti-human IgG antibody (21). Evidence of an acute infection is based on the following criteria : A fourfold or greater rise of titer between acute and convalescent serum sample in *C.pneumoniae* specific IgG and/or IgA antibodies, or a positive ($\geq$16) IgM result in either sample (21).

The recombinant lipopolysaccharide ELISA (rDNA LPS ELISA) is currently the most sensitive serological method commercially available for the diagnosis of acute *C.pneumoniae* infection. The following criteria are used to diagnose acute infection by ELISA: A three-fold or greater increase in Chlamydia specific IgG- or IgA antibody titer, or a two-fold or greater change in specific IgM titer, or a two-fold increase in specific IgG antibody titer in combination with a two-fold increase in specific IgA antibody titer (22). The use of single serum samples is again dissuaded.

Detection of *C.pneumoniae* in respiratory specimens

Little is known about the role of the organism as a commensal in the upper respiratory tract and the possibility of carrier state. Chronic, asymptomatic infections without serological evidence of *C.pneumoniae* infection have been

observed frequently in adults (7,10,11) and children (1,3). Also, persistent infection with *C.pneumoniae* following acute illness were observed by Hammerschlag and Falck (5,9). Therefore, direct detection of *C.pneumoniae* in upper respiratory specimens for the diagnosis of acute *C.pneumoniae* pneumonia is currently controversial. Additional information is necessary to determine the diagnostic value of direct detection methods. The sensitivity of cell culture is, at least in our hands, disappointingly low. In contrast, the sensitivity of the *C.pneumoniae* PCR is much higher, but does not distinguish between acute infection, persistent infection, or colonization.

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SAMENVATTING

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Ondanks de vele onderzoeksinspanningen dat *Chlamydia pneumoniae* acute luchtweginfecties veroorzaakt, zijn nu meer dan tien jaar na de eerste publikatie de meningen van onderzoekers over het belang van *C.pneumoniae* vooralsnog verdeeld gebleven. Sommige onderzoekers concluderen dat het organisme niet alleen verantwoordelijk is voor een groot deel van de luchtweginfecties, maar ook een belangrijke co-factor is bij de ontwikkeling van hart- en vaatziekten. Klachten en ziekteverschijnselen zijn niet kenmerkend voor een *C.pneumoniae* infectie. Daarom is het verrichten van laboratorium onderzoek noodzakelijk. Ondanks moderne laboratorium-diagnostische methoden zoals DNA amplificatie, ELISA, en fluorescentie microscopie, is de diagnose van een acute *C.pneumoniae* infectie echter nog steeds moeilijk. De *C.pneumoniae* celkweek is nog weinig sensitief, hoewel sommige onderzoekers goede resultaten behalen. Complement fixatie (CF) is uitgebreid toegepast voor de serologische diagnose van acute *Chlamydia psittaci* infecties. Na de acceptatie van *C.pneumoniae* als het derde *Chlamydia* species in 1989, constateerde men dat de meeste infecties die met deze CF test opgespoord worden eigenlijk *C.pneumoniae* infecties zijn. De gouden standaard voor de diagnose van acute *C.pneumoniae* infecties is op dit moment de microimmunofluorescentie (MIF) test. De infectieuze vorm van *C.pneumoniae* (het elementair lichaampje) wordt bij deze serologische test gebruikt als antigeen. De MIF is specifieker dan de CF en kan immuno-globuline klassen (IgG, IgM en IgA antistoffen) van elkaar onderscheiden. Helaas blijkt de interpretatie van de fluorescentiepatronen en daarmee de titer bepaling moeilijk en subjectief te zijn. Het voorkomen van chronische infecties kunnen de interpretatie van de klassieke serodiagnostiek verder compliceren. Recentelijk is de PCR techniek toegepast om *C.pneumoniae*-specifiek DNA te amplificeren wat een sterke verbeterde detectie van het organisme in klinische materialen mogelijk maakt.

Onderzoek naar de waarde van bestaande en nieuwe laboratoriummethoden voor het aantonen van acute luchtweginfecties door *C.pneumoniae* is de rode draad door dit proefschrift. In hoofdstuk 2 zijn de specifieke vraagstellingen geformuleerd voor dit proefschrift. De globale doelstellingen van de studies

in dit proefschrift zijn als volgt: Ontwikkeling van laboratorium technieken voor de diagnose van acute luchtweginfecties door *C.pneumoniae* en het daarmee verkrijgen van meer inzicht in de klinische waarde en beperkingen van deze technieken en in de rol van *C.pneumoniae* bij luchtweginfecties in Nederland.

Hoofdstuk 3 laat zien dat met de MIF test fout-positieve IgM resultaten verkregen worden indien in het serum van de patiënt rheumafactor aanwezig is. Vooral op oudere leeftijd komt rheumafactor in het bloed voor en is dit een probleem bij de diagnose van acute *C.pneumoniae* infecties. Rheumafactor wordt frequent gevonden in het bloed van patiënten met rheumatoide arthritis en in sommige gevallen bij gezonde personen, m.n. op oudere leeftijd. Ook bij sommige infectieziekten kan tijdelijk rheumafactor in het bloed aanwezig zijn. In dit hoofdstuk wordt een modificatie van de klassieke MIF test beschreven waardoor deze fout-positieve reacties worden voorkomen.

In hoofdstuk 5 wordt de verassende bevinding beschreven dat verschillende, commercieel verkrijgbare antigeen preparaten, die voor de diagnose van acute *C.pneumoniae* infecties op de markt waren gebracht, besmet waren met een Mycoplasma soort: deze Mycoplasma bleek genetisch sterk verwant te zijn aan *Mycoplasma arginini*. Geschat wordt dat ongeveer 15% van alle cellijnen die in laboratoria gebruikt worden voor celkweken besmet zijn met dergelijke Mycoplasma. Vaak blijven deze besmettingen onopgemerkt, maar het is niet uitgesloten dat daardoor zowel fout-positieve - als fout-negatieve laboratoriumuitslagen ontstaan. Uit onderzoek bleek dat in dit geval besmetting met Mycoplasma geen invloed had op de betrouwbaarheid van de uitslag van de MIF test voor *C.pneumoniae*. Aanbevelingen worden gedaan aan onderzoekers en industrie om periodiek controles uit te voeren op Mycoplasma besmetting en daarbij gebruik te maken van DNA-amplificatie technieken.

Hoofdstuk 4 beschrijft de ontwikkeling van een verbeterde techniek om *C.pneumoniae* te kweken. Gewoonlijk wordt Chlamydia op HeLa 229, HL of McCoy cellen gekweekt. Andere gastheer cellijnen zoals Hep-2 en BGM

bleken meer sensitief dan HL en HeLa 229 cellen. Ook bleek dat BGM cellen tijdens de kweek minder snel beschadigd werden door de groei van Chlamydia. Het achterwege laten van de klassieke voorbehandeling van de cellaag met DEAE-dextraan uit het kweek protocol verhoogde tenslotte de sensitiviteit van de celkweek.

Bestaande serologische technieken voor de diagnose van acute *C.pneumoniae* infecties werden in hoofdstuk 6 vergeleken met de celkweek en met een "in-huis" ontwikkelde DNA-amplificatie (PCR) methode voor de diagnose van lagere luchtweginfecties veroorzaakt door *C.pneumoniae*. De serologie bestond uit de MIF, CF en de recent ontwikkelde recombinant lipopoly-saccharide (rDNA LPS) ELISA. Omdat de kwaliteit van de verschillende testen bleek af te hangen van de gekozen gouden standaard, werden in dit onderzoek verschillende gouden standaarden gebruikt. Over het algemeen was de rDNA LPS ELISA gevoeliger dan de MIF mits men de criteria voor acute infecties hanteerde zoals beschreven in dit hoofdstuk. De CF test was zo ongevoelig, dat gebruik voor de diagnostiek van *C.pneumoniae* luchtweg-infecties afgeraden moet worden. Indien van patiënten twee "gepaarde" serum monsters met een interval tijd van minimaal tien dagen worden onderzocht (zoals gebruikelijk in de serologie), bleek de sensitiviteit van de MIF en rDNA LPS ELISA toch nog niet optimaal. Indien drie serum monsters worden afgenomen over een periode van ± 4 weken bereikt men met deze testen een beter resultaat. Gebruik van een enkel serum monster (in het begin van de ziekte) bleek ook niet te voldoen, o.a. omdat andere ziekteverwekkers zoals *Mycoplasma pneumoniae* fout-positieve reacties in de Chlamydia serologie kunnen veroorzaken. Ondanks de verbeteringen van de celkweek zoals beschreven in hoofdstuk 4, bleek de celkweek bij onderzoek van klinische materialen nog steeds niet gevoelig. Dit laatste in tegenstelling tot de PCR-test; vele patiënten bleken *C.pneumoniae* PCR positief. De gevoeligheid van de PCR-test bleek o.a. afhankelijk te zijn van het soort patiënten materiaal (waarop de test werd uitgevoerd); de nasopharynx bleek de beste locatie om materialen te verkrijgen voor de PCR-test. Opvallend was dat er een grote groep patiënten was die PCR positief was zonder dat er bij hen een serologische aanwijzing van een acute *C.pneumoniae* infectie werd gevonden. Andere onderzoekers hebben eerder iets dergelijks aangetoond,

vnl. Dat men *C.pneumoniae* PCR positief kan zijn zonder dat zij in het bloed een serologische reactie laten zien die bij een acute infectie past; een dergelijke bevinding zou passen bij asymptomatisch dragerschap en/of een chronische infectie met *C.pneumoniae*. De positief voorspelbare waarde van de PCR-test bij patiënten met longontsteking wordt hierdoor bemoeilijkt en is waarschijnlijk laag. Verder is in dit deel van het onderzoek gebleken dat de rDNA LPS ELISA niet bruikbaar is om alle infecties met *C.pneumoniae* (ook die ver in het verleden zijn opgetreden) op te sporen. Uit resultaten van de seroprevalentie onder bloeddonoren en onder patiënten met CARA bleek dat de antistoffen tegen Chlamydia LPS eerder uit het bloed weer verdwijnen dan de antistoffen tegen de buitenmembraan eiwitten (zie onder).

Hoofdstuk 7 beschrijft de bevindingen van een longitudinaal serologisch onderzoek onder 271 patiënten met CARA. Bij dit onderzoek werden zowel de MIF als de rDNA LPS ELISA toegepast. De rDNA LPS ELISA bleek makkelijk uitvoerbaar te zijn en een hoge reproduceerbaarheid te hebben. De rDNA LPS ELISA was bovendien gevoeliger dan de klassieke MIF-test bij het opsporen van acute *C.pneumoniae* infecties tijdens de 14 maanden dat de patiënten vervolgd werden. Ook bleek dat Chlamydia LPS antistoffen van de IgG - en IgA klassen tot jaren na een infectie aantoonbaar kunnen blijven. Bij enkele patiënten werden IgM antistoffen tegen *C.pneumoniae* gevonden gedurende de gehele observatieperiode hetgeen zou kunnen wijzen op een chronische, overigens asymptomatische, infectie met deze bacterie.

Van *C.pneumoniae* is niet goed bekend op welke wijze hij wordt overgedragen van de ene mens op de andere. DE capaciteit om buiten een gastheer een tijd te overleven is hierbij mogelijk van groot belang omdat daarmee overdracht van infectie wordt vergroot. Experimenten t.a.v. overleving en overdracht van *C.pneumoniae* werden in hoofdstuk 8 beschreven. Uit dit deel van het onderzoek bleek dat *C.pneumoniae* langdurig kan overleven op diverse materialen in de omgeving. Zelfs na drie uur blijken nog infectieuze partikels *C.pneumoniae* te bestaan. Uitdroging had geen invloed op de overleving van *C.pneumoniae*. Op de hand van een vrijwilliger (promovendus) bleek 13% van de infectieuze *C.pneumoniae* na 30 minuten nog in leven. Verder onderzoek liet zien dat door contact met gecontamineerde materialen in de omgeving

infectieuze *C.pneumoniae* partikels overgebracht kunnen worden naar de handen. Contact met besmette materialen in de omgeving is derhalve een potentiële route van besmetting met *C.pneumoniae*.

In hoofdstuk 9 werd de rDNA LPS ELISA gebruikt om de incidentie van acute *C.pneumoniae* infecties bij bloeddonoren en patiënten met CARA te onderzoeken. De verschillen in leeftijdsverdeling tussen de twee onderzochte groepen beperkt de interpretatie van de uitkomsten van dit onderzoek enigszins. De incidentie van *C.pneumoniae* infecties en de prevalentie van antistoffen tegen Chlamydia LPS waren hogen onder de CARA patiënten en namen toe met de leeftijd van deze patiënten. Uit verdere analyses bleek dat IgG - en IgA antistoffen tegen Chlamydia LPS waarschijnlijk jaren na infectie aantoonbaar blijven in het bloed van de patiënt. Persisterende IgM reactiviteit, mogelijk ten gevolge van een chronische infectie werd incidenteel zowel bij patiënten met CARA als bij bloeddonoren gevonden.

In hoofdstukken 10 en 11 werden de incidenties van *C.pneumoniae* infecties bij volwassenen (hoofdstuk 10) en bij kinderen (hoofdstuk 11) met longontsteking besproken. Vanaf oktober '92 werd gedurende een periode van ± twee jaar hiertoe patiënten in onderzoek genomen. Uit beide onderzoeken bleek dat *C.pneumoniae* een van de meest voorkomende verwekker van longontsteking is. Deze hoge incidentie is mogelijk te verklaren doordat in beide onderzoeken gedurende dezelfde periode een tijdelijke verheffing van de incidentie van *C.pneumoniae* infecties geconstateerd is. In beide studies werd bij 67% van de patiënten een micro-organisme aangetroffen. *C.pneumoniae* werd aangetoond in 29% van de volwassenen en 44% van de kinderen met longontsteking. Opvallend was dat maar bij 49% van deze volwassenen en bij 30% van deze kinderen serologisch aanwijzingen waren dat zij een acute *C.pneumoniae* infectie doormaakten. De helft of meer van de patiënten bleek alleen positief te zijn in de *C.pneumoniae* PCR-test: omdat bekend is dat typische verwekkers van luchtweginfecties in de bovenste luchtwegen kunnen voorkomen zonder dat zij klinische verschijnselen van infectie oproepen, bewijst een positieve kweek of PCR-test op *C.pneumoniae* van materiaal uit de bovenste luchtwegen nog niet dat het organisme de longontsteking heeft veroorzaakt. Het is al vaker beschreven dat *C.pneu-*

moniae in de keel en nasopharynx van overigens gezonden personen gevonden word. Daarentegen is een significante titerstijging van specifieke antistoffen tegen *C.pneumoniae* een algemeen geaccepteerd criterium voor een acute, invasieve infectie en is daarmee een sterke aanwijzing dat het organisme een causale rol speelt bij het ontstaan van de longontsteking. Daarom verdeelden wij de patiënten waarbij een *C.pneumoniae* infectie werd aangetoond middels kweek, PCR en/of serologie in twee groepen, nl patiënten die een significante serologische reactie lieten zien (z.g. sero-responders) en zij die dat niet hadden (sero-non-responders). Op klinische gronden was het niet mogelijk om de twee groepen van elkaar te onderscheiden; zij waren even ziek. In het algemeen waren patiënten met een *C.pneumoniae* longontsteking ernstig ziek, vaak ernstiger dan patiënten met een longontsteking, veroorzaakt door *M.pneumoniae*. De ziekte leek bij sero-responders ernstiger te zijn. Bij de volwassen groep kwam naar voren dat een serologische reactie vooral werd gezien bij patiënten waarbij ook andere verwekkers werden gevonden. Met andere woorden, een serologische reactie tegen *C.pneumoniae* bij patiënten met longontsteking wijst op de aanwezigheid van meerdere soorten ziekteverwekkers (dubbelinfectie). *C.pneumoniae* blijkt bij volwassenen met een longontsteking vaak samen voor te komen met *Streptococcus pneumoniae*, bij kinderen vaak in combinatie met *Mycoplasma pneumoniae*. Het is mogelijk dat ten gevolge van het gelijktijdig aanwezig zijn van meerdere ziekteverwekkers de schade in het longweefsel groter is waardoor er ook meer kans is op een significante reactie van het immuunsysteem van dergelijke patiënten.

CONCLUSIES

In het eerste deel van dit proefschrift is de waarde en bruikbaarheid van *C.pneumoniae* laboratorium diagnostiek beschreven. De recent ontwikkelde ELISA voor de detectie van Chlamydia specifieke antistoffen tegen het LPS van deze bacterie blijkt goed reproduceerbaar, makkelijk uitvoerbaar en meer gevoeliger dan de MIF test, indien gebruik gemaakt wordt van de in dit proefschrift beschreven criteria voor acute infecties met *C.pneumoniae*. Ondanks het feit dat deze ELISA genus-specifiek is, blijkt in de praktijk deze test de beste keuze te zijn voor het stellen van de diagnose van acute

luchtweginfecties veroorzaakt door *C.pneumoniae.* infecties in de routine diagnostiek. Daarnaast bleek de door ons opgezette PCR reactie zeer gevoelig te zijn, met name indien toegepast op materiaal uit de keelholte. De klinische waarde van de PCR-test is echter vooralsnog beperkt, omdat de aanwezigheid van *C.pneumoniae* DNA in bovenste luchtwegen zonder dat er sprake is van een serologische reactie mogelijk te verklaren is door kolonisatie van de luchtwegen, en dus niet per se op een actieve invasieve infectie (longontsteking) door deze bacterie.

In het tweede deel van dit proefschrift is de klinische betekenis van *C.pneumoniae* infecties in Nederland onderzocht. Gebleken is dat ook in Nederland *C.pneumoniae* infecties veelvuldig voor komen. *C.pneumoniae* kan ernstige vormen van longontsteking veroorzaken bij volwassenen en bij kinderen, waarbij opname in het ziekenhuis noodzakelijk is. Op dit moment kan een definitieve diagnose longontsteking door *C.pneumoniae* alleen serologisch bewezen worden; daarvoor dient men wel gebruik te maken van twee of meer gepaarde serum monsters per patiënt.

Het is duidelijk dat de technische ontwikkeling met betrekking tot de *C.pneumoniae* laboratorium diagnostiek haar eindpunt nog niet heeft bereikt. Verdere ontwikkeling hiervan in relatie tot de klinische problematiek is noodzakelijk, waarbij ook de rol van *C.pneumoniae* bij het ontstaan van hart en vaatziekten in het onderzoek moet worden betrokken.

AANBEVELINGEN

De afgelopen tien jaar zijn een groot aantal onderzoeken gepubliceerd over de diagnose van *C.pneumoniae* infecties. Toch is de waarde van de laboratorium diagnostiek nog steeds niet duidelijk. Dit in acht genomen, en gecombineerd met de gegevens uit dit proefschrift zijn de onderstaande aanbevelingen samengesteld. De klinische waarde en beperkingen van de in dit proefschrift geëvalueerde testen worden apart besproken.

Serologie

In het algemeen blijkt dat het gebruik van één enkel serum monster voor de

diagnose van een acute *C.pneumoniae* infectie onbetrouwbaar is. Het langdurig aanwezig kunnen zijn (jaren) van IgG- en IgA antistoffen tegen Chlamydia LPS en tegen buitenmembraan eiwitten van *C.pneumoniae* in het bloed van gezonde personen beperkt de diagnostische waarde van serologisch onderzoek op één enkel serum monster. Ook een chronische *C.pneumoniae* infectie kan verantwoordelijk zijn voor langdurig aanwezig zijn van antistoffen tegen *C.pneumoniae* in het bloed. Verder is gebleken dat bij *Mycoplasma pneumoniae* infectie fout-positieve uitslagen voor *C.pneumoniae* in testen optreden indien men gebruik maakt van slechts één serum monster. Er dient tenminste twee- of meer gepaarde serum monsters met een minimum interval van 10 dagen onderzocht te worden, waarbij een derde serum na 30 dagen de kwaliteit van het onderzoek verder verhoogt.

De complement fixatie test als diagnostiek van infecties met *C.pneumoniae* is erg ongevoelig, vooral bij de oudere patiënten.

De microimmunofluorescentie (MIF) test is goed bruikbaar voor de diagnostiek van acute *C.pneumoniae* infecties, hoewel de aflezing van de fluorescentie patronen expertise en ervaring vereist. Fout-positieve IgM reacties komen voor door de aanwezigheid van circulerend rheuma factor, maar kunnen voorkomen worden door voorbehandeling van de serum monsters. Van een acute infectie is sprake indien aan de volgende criteria wordt voldaan: minimaal een viervoudige titer stijging van *C.pneumoniae* IgG en/of IgA antistoffen tussen acuut en convalescent serum monster, of een positief IgM resultaat ($\geq$1/16).

De commercieel verkrijgbare recombinant lipopolysaccharide ELISA (rDNA LPS ELISA) is op dit moment de meest sensitieve serologische techniek voor de diagnose van acute *C.pneumoniae infecties* als gebruik gemaakt wordt van de volgende criteria: Minimaal een drievoudige titer stijging van Chlamydia-specifieke IgG- of IgA antistoffen, of een tweevoudige titer stijging van Chlamydia-specifieke IgG- en IgA antistoffen, of tenminste een tweevoudige titer verandering van Chlamydia-specifiek IgM. Het gebruik van één serum monster wordt ook bij deze test afgeraden.

Detectie van *C.pneumoniae* in materialen van de luchtwegen

Het is niet goed bekend of *C.pneumoniae* als commensaal van de hogere luchtwegen kan voorkomen en derhalve sprake kan zijn van *C.pneumoniae* dragerschap. Chronische infecties met *C.pneumoniae* zonder een serologische reactie zijn beschreven bij volwassenen en bij kinderen. Ook langdurig persisterende infecties, waarop klinische verschijnselen van infectie volgen zijn beschreven. Daarom is detectie van *C.pneumoniae* antigeen in de (hogere) luchtwegen op dit moment controversieel. Verder onderzoek is noodzakelijk om de klinische waarde van directe antigeen detectie methoden te valideren. De gevoeligheid van de celkweek is, althans in onze handen, teleurstellend laag, dit in tegenstelling tot de gevoeligheid van de *C.pneumoniae* PCR-test. De PCR-test kan op dit moment echter nog geen onderscheid maken tussen acute infectie, persisterende infectie of dragerschap.

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CURRICULUM VITAE

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