Chlamydia pneumoniae

Assessment of the microimmunofluorescence test for antibody detection and prevalence studies in adult patients with respiratory tract infection

A Ph.D - thesis

by

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Preface

The studies in the present dissertation were carried out at the Neisseria Unit, Department of Respiratory Infections, Meningitis and STIs, Division of Microbiology, Statens Serum Institut, Copenhagen, Denmark, during the years 1997 – 2002.

The objects of the studies were to assess the performance of commercially available microimmunofluorescence (MIF) tests compared to a reference method for the detection of *Chlamydia pneumonia* antibodies, to describe the prevalence of *C. pneumoniae* and other atypical respiratory tract infections in adult patients with symptoms of acute infection and in patients with chronic pulmonary diseases and to evaluate whether *C. pneumoniae* is associated with acute exacerbations of the diseases; finally, to assess the applicability of serological criteria for the diagnosis of acute and chronic *C. pneumoniae* infections.

The thesis is divided into five chapters. Chapter one is an introduction to *C. pneumoniae* based on literature studies. Chapter two describes the objects of the thesis, and chapter three describes the study of the performances of MIF tests and further three minor laboratory studies.
Chapter four describes the clinical studies and finally, chapter five is a general conclusion of the thesis and a discussion of the current knowledge of *C. pneumoniae* infections in Denmark.

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CHAPTER 3: LABORATORY STUDIES

CHAPTER 4: CLINICAL STUDIES

CHAPTER 5: GENERAL DISCUSSION

List of abbreviations.

Bp	Base pair
CF test	Complement fixation test
CI	95% Confidence interval
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
DFA test	Direct Fluorescent Antibody test
EB	Elementary body
EIA	Enzyme immunoassays
FEV^1	Forced expiratory volume in one second
GP	General Practitioner
Hsp	Heat shock protein
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LAB	Labsystems®
LPS	Lipopolysaccharide
MIF	Microimmunofluorescence test
MOMP	Major outer membrane protein
MRL	MRL Diagnostics®
NAA	Nucleic acid amplification
NAP Study	Nordic Atypical Pneumonia Study
OmcA	Outer membrane complex A
OmcB	Outer membrane complex B
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
Pmp	Polymorphic membrane proteins
RB	Reticulate body
RF	Rheumatoid factor
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
TWAR	TW-183/AR-39-like organisms (the original name for *C. pneumoniae*)
VC	Vital capacity
WRF	Washington Research Foundation

CHAPTER ONE: CHLAMYDIA PNEUMONIAE

History of *Chlamydia pneumoniae*

In 1965 *Chlamydia (C.) pneumoniae* was isolated for the first time from the eye of a child during a field trial testing a trachoma vaccine in Taiwan. The isolate was named TW-183 and considered an unidentifiable immunotype of *C. trachomatis*[57]. In 1968-69 the microimmunofluorescence test (MIF) was developed by Wang and Grayston for immunotyping of *C. trachomatis* isolates[163]; it was further developed into a tool for the diagnosing *C. trachomatis* serovar-specific infections[164]. TW-183 remained untypeable, but when included as antigen in the MIF test a larger percentage of sera from 191 persons observed for the prevalence of trachoma had antibodies towards this antigen than towards *C. trachomatis* serovars[61]. During the 1970s the interest in the role of *C. trachomatis* in respiratory tract infections increased. Serological evidence suggested that pneumonia in children < six months of age could be caused by *C. trachomatis*[8]. Several groups studied the possible connection between *C. trachomatis* and respiratory infections in adults. In 1978 Saikku came to the University of Washington in Seattle to investigate sera from several respiratory tract infection outbreaks in Finland. The sera had shown a positive result by the *Chlamydia* CF test and he wished to test them with the newly developed MIF test for the detection of serotype-specific *C. trachomatis* antibodies. No significant levels of antibodies against any of the *C. trachomatis* serovars were found, but when using TW-183 as antigen, IgG antibody titre rises and IgM antibodies was demonstrated implying that a TW-183 organism had caused one of the outbreaks[131]. Subsequently the group in Seattle conducted a study in students who came to the medical clinic at the University with symptoms of acute respiratory tract infections. From the 39th student an isolate (AR-39) immunologically similar of TW- 183 was found. The organism was called TW-183/AR-39-like organism or TWAR. To study the epidemiology of and clinical diseases caused by TWAR, serum collections from various places were analysed by the MIF test. Mordhorst at Statens Serum Institut in Denmark provided one of the most comprehensive collections of sera from patients suspected of having psittacosis in 1981-1983. All sera had positive test results for chlamydial antibodies by the CF test. The results by the MIF test showed that 52-57% had serological evidence of infection caused by TWAR[63].

In 1988 Saikku published the first study in which high levels of TWAR IgG and IgA antibodies were correlated to the risk of having a cardiac episode or chronic heart disease[128]. The year after the organism was officially named *C. pneumoniae* and considered a common cause of acute respiratory infections[64].

Biology

Taxonomy. The order *Chlamydiales* has one family, *Chlamydiaceae* and one genus, *Chlamydia* which consists of four species: *C. trachomatis, C. psittaci* and *C. pneumoniae* that are human pathogens and the ruminant pathogen *C. pecorum*[64;114]. This classification is based on phenotypic, morphologic, genetic and serologic criteria. In 1999 a new taxonomic order was suggested based on phylogenetic analyses of the 16S and 23S rRNA genes[45]. The major criterion in this classification is the divergence of rRNA. The *Chlamydiales* is divided into four families: *Chlamydiaceae, Parachlamydiaceae, Simkaniaceae* and a fourth, currently unnamed family. C*hlamydiaceae* is divided into two genera: *Chlamydia*, which among others contains *C. trachomatis*, and *Chlamydiaphila* in which *C. pneumoniae* and *C. psittaci* are included. So far the order of *Chlamydiales* contains 14 species, including three species that were previously considered to be *C. psittaci* hosted in different animals.
Currently, the chlamydial society is debating about which classification should be the official classification[44;133]. In this thesis the original classification is used.

Characteristics. The genus *Chlamydia* is a heterogeneous group of small bacteria with similarities in morphology, intracellular developmental cycle and antigenic properties. Lacking some of the enzymes needed to be metabolic independent organisms, they are obligate intracellular parasites[94;141].
Sequences for the whole genome of a number of *Chlamydia* strains have become available since 1998, including sequences for *C. pneumoniae* AR39 and *C. trachomatis* serotypes B, D and L2[85;126;142]. Within the genus *Chlamydia*, the genome of *C. pneumoniae* has a 1,230,230 base pair (bp) chromosome coding for up to 1,073 proteins and that of *C. trachomatis* has a 1,042,519 bp chromosome coding for up to 894 proteins[85]. About 200 of these proteins are unique to *C. pneumoniae*.

Life cycle. Common to all *Chlamydia* species is the biphasic life cycle comprising of a smaller extracellular, metabolic inert, infectious form, the elementary body (EB), and a larger intracellular, metabolic active form, the reticulate body (RB)[78].
EBs are small, round or occasionally pear shaped, electron-dense structures approximately 0.3 microns in diameter. The EB is the only infectious stage of the chlamydial developmental cycle. It functions as a tough body whose purpose is to permit chlamydial survival in the environment outside the host cell. The EBs attach to susceptible host cells and are internalised by an invagination of the host plasma membrane to envelop the EB in a tightly associated vesicle[67]. Within the vesicle, they transform into RBs.
RBs are the stage of the chlamydial developmental cycle responsible for intracellular replication. Typically, reticulate bodies have a diameter of 1 micron and they are non infectious. Reticulate bodies are metabolically active, so their cytoplasm is rich in ribosomes, which are required for protein synthesis[67]. The RBs replicate within a membrane-bound vacuole, the chlamydial inclusion. The inclusion enlarges as the numbers of progeny increases. Gradually the RBs revert to the EB form and after app. 72 hours the life cycle is completed. The egression of the mature, infectious EBs occurs by lysis of the host cells[94].

Outer membrane structures.

Analyses of the chlamydial outer membrane complex has shown that *Chlamydia* species have LPS, a major outer membrane protein (MOMP), and two cysteine-rich proteins: outer membrane complex B (OmcB) and outer membrane complex A (OmcA)[30;78]. In addition, *C. pneumoniae*, *C. psittaci* and *C. trachomatis* contain proteins belonging to a family of proteins named polymorphic membrane proteins[141] and heat shock proteins[78].

Lipopolysaccharide (LPS): LPS are highly immunogenic glycolipids. All species of *Chlamydiae* have common LPS group antigen exposed on the surface[32]. However, the LPS may have more than a genus-specific epitope[142]. Surface exposure of LPS may be greater on RBs than on EBs[15;78]. Chlamydial LPS show common epitopes with LPS from some of the enteric bacteria[40].

The major outer membrane protein (MOMP): The protein present in the largest quantity in EBs and RBs is MOMP encoded by the *ompA* gene. In all species, the

MOMP consists of four variable segments / domains (termed VS or VD 1 to VD 4). These variable segments tend to be surface exposed and the MOMP is both structurally and immunologically the dominant protein in the chlamydial outer membrane complex except in *C. pneumoniae* [78]. The typing of *C. trachomatis* is based on the serological differentiation of antigenic epitopes on MOMP into 19 human *C. trachomatis* serovars (A to K, Ba, Da, Ia, Ja, L1 to L3, and L2a)[31;78]. In contrast, the *C. pneumoniae* MOMP is homogenous, is not a major immunogen and may not be surface exposed[89].

Cysteine rich proteins of the chlamydial outer membrane complex. Apart from MOMP, the major proteins in the chlamydial outer membrane complex are the two cysteine rich proteins, OmcA and OmcB, present in greatest amounts in the elementary body[78]. OmcB (=omp2) is a large 60 KDa protein. The OmcB is highly immunogenic, carries genus specific epitopes, but is thought not to be surface exposed[78]. The protein is a major target in the humoral response in infections caused by *C. trachomatis* and may likewise be so for infections caused by *C. pneumoniae*[115;78]. [107]OmcA (=omp3) is a lipoprotein with a molecular weight of 9 KDa, not exposed at the chlamydial surface[67] and may not be immunogenic. Residues 1 - 88 of the *C. trachomatis* gene are between 55 - 59% homologous with the equivalent region of the corresponding *C. psittaci* and *C. pneumoniae* genes[78;107].

Polymorphic membrane proteins (pmps). Each of the three *Chlamydia* species has in its genome a family of distantly related *Pmp* genes[30]. Comparison of the *C. trachomatis* and *C. pneumoniae pmp* gene sequences show a large amount of heterogeneity among members of the family[142]. It is not clear whether the variability of *pmp* gene sequence is due to pressure from the host immune system, or is intrinsic. At least some of the proteins are located a t the surface of the infectious chlamydial elementary body[89]. The surface exposed pmps, OMP4 (98.9 kDa) and OMP5 (97.2 kDa), was described in *C. trachomatis* and *C. pneumoniae* in 1998 and 1999[89;142]. Epitopes of OMP4 and possible the other pmps encoded by the *C. pneumoniae* gene family are likely to be the target for immune response in mice experimentally infected *C. pneumoniae* infections[89].

Heat shock proteins (hsp) are a family of closely related proteins, widely distributed in virtually all organisms, including *Chlamydia* and humans. Hsp show remarkable structural similarity of the order of 40% or more. Chlamydiae are no exception, with the main chlamydial heat shock proteins showing homology with human mitochondrial proteins cpn10, hsp60 or hsp70. Hsp 70 has been shown to be located in outer membrane complexes of chlamydial EBs, though they might not normally be accessible to antibodies[78]. Hsp60s are highly immunogenic[166] and might be involved in autoimmune responses against the human hsps[166].

Clinical diseases.

Respiratory tract diseases. Infections of the respiratory tract are the most common recognized acute *C. pneumoniae* infections. No set of symptoms or signs is unique to acute *C. pneumoniae* infections, however a subacute onset is common[56;59;62]. There may be a biphasic pattern in the clinical manifestations, with resolution of pharyngitis prior to development of bronchitis or pneumonia. [42;65] Generally, the symptoms are mild to moderate severe and most cases do not require hospitalisation. Despite appropriate antibiotic therapy, complete recovery is slow; prolonged illness and relapses are common[42;74;148]. The period from onset to clinic visit is longer than for most other acute respiratory infections and may be more than 14 days[147;148].

Upper respiratory tract infections. *C. pneumoniae* has been isolated from patients with pharyngitis, laryngitis, otitis and sinusitis[42;59;62;79;143;148]. Sore throat, plugged ears, swollen throat, cough, headache and fatigue may be present alone or in combination[60;74;79].

Lower respiratory tract infections. Pneumonia and bronchitis are the two most frequently recognized acute illnesses associated with *C. pneumoniae*. The pneumonia patients often have sore throat and hoarseness followed by a persistent cough. The body temperature may be slightly increased and seldom goes higher than 38-39 °C. The leukocyte count is often normal but sedimentation rate might be elevated. A chest radiograph usually demonstrates a single sub lobular infiltrate though lobular and bilateral involvement may be seen, especially in the severe cases[34;94;108].

Asthma. In the last decade *C. pneumoniae* has been associated with asthma[35;68;69]. It has been suggested that *C. pneumoniae* infection could be an initiator of adult-onset asthma[72]. Some studies have shown that *C. pneumoniae* causes asthma exacerbations[69;109] and that treatment with macrolide may improve the course of asthma[18;71]. However, results from other studies do not support the hypothesis that *C. pneumoniae* is a cause of adult-onset asthma[33;98].

Chronic Obstructive Lung Disease. As *C. pneumoniae* is involved in acute and protracted respiratory tract infections, it seems reasonable that the organism could be involved in the pathogenesis of COPD. In theory *C. pneumoniae* can play a role in initiation, development or exacerbations of COPD. High seroprevalence of *C. pneumoniae* IgG antibodies in COPD patients has been demonstrated[69] and an association between *C. pneumoniae* and acute exacerbations found[20]. In one study only small differences in serum IgG antibodies between patients and controls were found, however the differences in serum IgA prevalences were significant[159].

Other respiratory tract diseases. On the basis of seroepidemiological studies sarcoidosis and lung cancer have been suggested as being associated with *C. pneumoniae*[99;125].

Cardiovascular diseases. Since the correlation between ischaemic heart diseases and elevated levels of *C. pneumoniae* IgG and IgA antibody titres was shown by Saikku in 1988[128], extensive research has been conducted in the attempt to establish, whether *C. pneumoniae* is involved in the development of atherosclerosis and acute cardiovascular events. Several studies have confirmed the association of acute and chronic heart diseases with a raised level of *C. pneumoniae* antibody titres[37-39]. Further some studies have identified *C. pneumoniae* in atheromatous tissues by electron microscopy, immunocytochemistry or PCR[95;139;145]. Culture of *C. pneumoniae* from atherosclerotic plaques has been reported[101]. However, as these data do not establish a causal relationship between the *C. pneumoniae* and onset or progression of atherosclerosis, it is possible, that *C. pneumoniae* is no more than an innocent bystander within atherosclerotic plaques.

Other diseases. Some have reported that reactive arthritis can be caused by *C. pneumoniae*, however it is less common than arthritis caused by *C. trachomatis*[25].

Several reports have associated *C. pneumoniae* with neurological diseases such as Alzheimer's disease, multiple sclerosis and giant-cell arteritis[54;172].

Treatment

Data on treatment of *C. pneumoniae* infection are limited, thus most recommendations are primary founded on empirical observations and experiences from the treatment of *C. trachomatis* infection. However, since *C. pneumoniae* is an intracellular organism, antimicrobial agents must be able to penetrate cells to be active against the bacteria. Macrolides, tetracyclines, chloramphenicol, quinolones and rifampicin have demonstrated antimicrobial activity intracellularly for *C. trachomatis* or other intracellular pathogens[55].

Acute infections. Treatment with azithromycin once daily for five days have been shown to have a satisfactory therapeutic outcome similar to those of amoxicillin/clavulanate or erythromycin given three times a day for 10 days for treatment of community-acquired pneumonia[77]. Eradication rates of 70-100% for *C. pneumoniae* have been demonstrated with levofloxacin, moxifloxacin, clarithromycin, erytromycin and azithromyzin in patients with pneumoniae[75].

Intervention studies. The last few years several intervention studies have been conducted to study the association between cardiovascular diseases and *C. pneumoniae*. Regimens used were among other: oral roxithromycin 150 mg/ for 30 days (The ROXI Trial)[66]; azithromycin 500 mg/d for 3 days, then 500 mg weekly for three months (The ACADEMIC Study)[3]; azithromycin 600 mg/week for 1 year (ACES)[81] and chlarithromycin 500 mg/d for 14 days (CLARICOR)[76]. So far only few studies are concluded and there is yet no general recommendations as to the benefit of treating patients with cardiovascular diseases with antibiotics.

One of the largest studies, WIZARD (Weekly Intervention with Zithromax for Atherosclerosis and Related Disorders) had included 7 700 subject with a history of myocardial infection and the presence of *C. pneumoniae* IgG antibodies of ≥ 16, They were given either azithromycin 600 mg 4 times a day for 3 days then 600 mg once weekly for the next 11 weeks or a placebo[41]. Earlier this year it was announced that, just before the company was scheduled to report the results, the study was terminated on the grounds that azithromycin in this study had failed to significantly reduce heart disease.

Diagnostic methods

A laboratory diagnosis of *C. pneumoniae* infection can be founded on the detection of the bacteria or by serological methods[6].

Sampling. Since the *C. pneumoniae* is an intracellular bacterium it is of major importance for the detection of the organism, that the sample contains epithelial cells. Specimens obtained for detection of *C. pneumoniae* tract infection by use of culture include swabs of the nasopharynx, oropharync, sputum specimens, broncheoalveolar lavage specimens and tissue biopsy specimens[40]. *C. pneumoniae* DNA has been detected by PCR in clinical samples ranging from respiratory specimens to samples of vascular tissue, serum and peripheral blood mononuclear cells[40;139].

There are only few papers published on the optimal sampling site for laboratory diagnosis of acute, respiratory, *C. pneumoniae* infections. One study compared specimens collected simultaneously and found that a positive diagnosis was more frequently obtained with sputum samples than with throat and nasopharyngeal swabs[22].

Culture. *C. pneumoniae* is cultured in eukariotic host cells. Originally the culture was performed in embryonated yolk sacs but during the 1980s a method for in vitro cell culture was developed [92]. The HeLa 229 cell-line was used for the culture of *C. trachomatis* in several laboratories, thus it was used in the first attempts of culturing *C. pneumoniae*[60]. Later it was found that Hep-2 cells and the HL cells had a higher sensitivity for the culture of *C. pneumoniae* and are thus recommended[48;91;93]. The advantage of culture is the specificity, which by use of an appropriate identification method is 100% while the sensitivity is dependent on the standard of the laboratory procedures and of the suitability of the specimens received for testing. *C. pneumoniae* is considered to be difficult to grow and only few laboratories perform culture. A major problem is that *C. pneumoniae* is easily inactivated during transport. A 50% -80% loss of infectivity of strains stored at room temperature in 12-24 hours was documented [48;102]. To ascertain the best possible result of culture, the sample must be forwarded in *Chlamydia* transport

medium containing fetal calf serum, the transport time shall be as short as possible and preferable the sample should be kept refrigerated during transport[40;102].

Polymerase chain reaction (PCR).. PCR is the nucleic acid amplification (NAA) technique used for the diagnosis of acute *C. pneumoniae* infection. There is yet no commercial NAA assay available for routine diagnostics but several protocols, different targets and primers have been published for the detection of *C. pneumoniae* DNA. The targets most widely used are the major outer membrane protein-coding gene and the 16S rRNA genes[26;52;151]. The nested PCR methods in general have a higher sensitivity than the one-step-based PCR methods but also a higher risk of false positive results[22],[16]. Though guidelines have been developed to minimize this risk[97], comparative studies show diverging inter-laboratory results[4;24;97]. The advantage of PCR in the diagnosis of an acute *C. pneumoniae* infection is the rapidity. The test can be performed in one to two days. The risk however, is false positive result due to contamination or false negative results due to inhibition. Thus all positive results should be confirmed by retesting the specimen with a different set of primers[23].

Direct Fluorescent Antibody (DFA) Test. Direct detection of elementary bodies of *C. pneumoniae* in smears is possible by using commercially available, fluorescein-labelled, monoclonal antibodies specific for *C. pneumoniae*[111]. The sensitivity is estimated to be 20%-60% compared to culture. The specificity is said to be 95%-99% but depends on the expertise of the microscopist, as the reading of results is subjective. The one benefit of the DFA test is that the result is available within 30-60 min[119].

Serological methods. Various enzyme immunoassays (EIA) for the detection of *C. pneumoniae* antibodies are commercially available. They use either LPS-extracted EBs or synthetic peptides unique to *C. pneumoniae* as antigen for the detection of *Chlamydia* antibodies[152]. Some are genus-specific others claim to be species-specific but assessment of their diagnostic performances is limited [40;96;122;123;152]. In contrast to the MIF test, the EIA has an objective readout of results and it is easier to handle a large number of samples. For this reason there is ongoing research to attempt to identify a highly immunogenic target species-specific antigen. How-

ever, the CF test and the MIF test are still the serological methods most widely used in serological testing for *C. pneumoniae* infection.

Complement fixation test. The test is based upon the ability of the antigen-antibody complex to bind complement and thereby prevent lysis of erythrocytes. The indicator system is sheep erythrocytes sensitised with rabbit antiserum to sheep erythrocytes. The complement source is fresh guinea pig serum. The complement of the sera examined is inactivated by heat. The test is less technically demanding than the MIF test and is widely used. The antigen used in the *Chlamydia* CF test when performed in our laboratory is an ether-extracted, acetone-precipitated phosphatide antigen, produced in yolk sac membranes infected with *C.trachomatis*[157]. Generally the antigen used for the CF test is considered to be LPS[12;119;152].

Antibodies reacting in the CF test are produced early in primary infection. Hence a serodiagnosis may be made within one to two weeks after infection; in contrast the species-specific antibodies are seen after two to six weeks. Diagnostic criterion of the test is a fourfold increase in titres[123;156]. A single high titre of ≥ 64 can signify acute infection but is not as reliable as the former criterion[60]. The sensitivity of the CF test for acute, primary *C. pneumoniae* infections is by some found to be as high as 90%[42], whereas others find it to be 60%[119;122].

Microimmunofluorescence (MIF) test. In the MIF test, purified EBs fixed on slides are used as the source of antigen. EBs from different *Chlamydia* species can be applied to the same slide. Thus genus-specific as well as species-specific antibodies can be detected. After incubation with patient sera, fluorescein-conjugated anti-human immunoglobulins are added to detect IgG, IgM and IgA antibodies. Appendix 1 is an in-detail description of the three MIF assays further investigated in the present study. The target of the antibodies in the MIF test is antigens present in the EBs. As described above, the MOMP epitopes are the principal *C. trachomatis* antigens, while epitopes reacting with anti-*C. pneumoniae* antibodies may be present on the pmps.

Even after the availability of PCR, the MIF test is considered the gold standard for laboratory diagnosis of acute *C. pneumoniae* infection [40] and has also been used for measuring *C. pneumoniae* antibodies in patients with assumed chronic *C. pneumoniae* infection[127]. However, there are several technical aspects to be con-

sidered regarding the MIF test: It is time-consuming and requires experience to perform; an indirect immunofluorescence test is subjective and the quality of the microscope as well as that of the reagents used is important. It has been demonstrated that the use of a *C. pneumoniae* antigen produced from a local isolate can result in higher detection rates of antibodies in sera and higher titres in the MIF test than when using a standard[17]. The question of possible cross-reaction in the *C. pneumoniae* MIF test is controversial. While some find the test to be highly species-specific[119;162], others have found a strong association between antibody titres to the three chlamydial species[168]. Part of the explanation for this may be that even with a great deal of experience with the test, genus specific antibodies still influence the results[17;87;117;168]. The specificity of the MIF assay can be attributed to the use of purified EBs of *Chlamydia* species rather than RBs that predominately express /genus-specific epitopes[40].

Apart from the problem of chlamydial species cross-reactivity / genus-specific reactivity in the MIF test, there are also concerns, that false-positive MIF test results may be due to cross-reacting antibodies to nonchlamydial antigens[87]. Serological cross-reactions between *Bartonella* and *C. pneumoniae* species have been demonstrated[104].

<u>*Terminology of serodiagnostic criteria.*</u> In a primary infection *C. pneumoniae* IgM antibodies detectable by the MIF test occur after about three weeks and IgG antibodies after 6-8 weeks. In reinfection IgM antibodies may not appear or may appear only at low titres while the IgG titre rises quickly, often within 1-2 weeks. Normally IgM antibodies begin to fall within two months and disappear in four to six months but in a few cases IgM antibodies persist for a longer period. In contrast, IgG antibodies may be detected several years after infection[94].

Originally, Grayston divided the results of the MIF test for *C. pneumoniae* into acute and pre-existing antibody profiles[60]. Acute antibodies profiles are either IgM antibody titres of ≥ 16 and/or a four-fold change of IgG antibody titres or a stable IgG antibody titre of ≥ 512 (table 1). A high and stable IgG antibody level is no longer strictly regarded as a sign of acute infection [35;40;152] but may be indicative for an acute infection together with clinical and epidemiological data. Pre-existing antibody profiles are IgG antibody titres 16-256[60]. In some publications the pre-existing antibody profile has been subdivided into one for chronic infections (with the presence of IgA antibody titres of ≥ 40) and one for past infection (with IgA an-

tibody titres of ≤ 40) [1;128;129]. The role of IgA antibodies in association with the diagnosis of chronic infections has been questioned[162]and is currently not regarded as a validated indicator of persisting infection[40].

Rheumatoid factor. The prevalence of circulating rheumatoid factor (RF) is strongly correlated with age and the presence of RF in sera containing *C. pneumoniae* IgG antibodies may give false positive *C. pneumoniae* IgM test results. In one study 41 of 286 patients had *C. pneumoniae* IgM antibody titres of ≥ 16. Although only 78 % of the patients had detectable circulating RF, none of the 41 sera were shown to have *C. pneumoniae* IgM antibodies after absorption of IgG antibodies. Since circulating RF at a level below the threshold of detection by routine testing for RF can cause false positive IgM antibody results it is necessary to confirm all *C. pneumoniae* IgM antibody positive results after absorption of IgG antibodies[154].

Blocking antibodies. It has been shown that the presence of *C. pneumoniae* IgG antibodies can influence the results of IgA measuring[82]. The study analysed the effect of removing IgG antibodies before testing for IgA antibodies: in most cases no change of IgA antibody titres were seen but in 9 % of samples a significant increase was detected. This increase was correlated to high IgG antibody titres suggesting that the higher binding affinity of IgG or a much higher concentration of IgG than IgA antibodies in sera blocks IgA reactivity in the test.

Epidemiology

C. pneumoniae infections seems to be both endemic and epidemic in the western countries[56;60;94;149]. No evidence of seasonal periodicity in *C. pneumoniae* infection had been observed[146], but recently a correlation between *C. pneumoniae* infection in clinically stable COPD patients and season has been reported[138]. Since *C. pneumoniae* is not often isolated or detected by PCR, much of the current information on the epidemiology of *C. pneumoniae* is derived from seroepidemiological studies using the MIF test.

Transmission. Humans are the only known reservoir of *C. pneumoniae* and transmission is believed to be from man to man by infectious aerosol droplets[47;131] without any animal reservoir[94;131]. *C. pneumoniae* infection spreads slowly with an

incubation time of several weeks; most often outbreak investigations have revealed no direct chain of transmission between cases but outbreaks within families have been described[49;88;112;171].

Seroepidemiological population studies. Data from studies from many parts of the world have shown a worldwide diffusion of *C. pneumoniae* antibodies. Children under the age of 5 years in developed countries seldom have *C. pneumoniae* antibodies, in contrast some evidence has been reported, that seroprevalence in preschool children in developing countries is high[130]. Seroprevalence increases during school age, reaches 40-50% in those 20-30 years old and continues to increase at a slower rate among older age groups[19;58;118;153]. The seroprevalence is equal in both sexes under 15 years of age; however adult men usually show a higher seropositivity rate than adult women. Seroprevalence is higher in immunocompromised persons than in immunocompetent hosts[19].

The first Danish, *C. pneumoniae* seroepidemiological study was conducted during two time periods (1976-79 and 1981-84)[165]. Sera from a total of 1763 persons were collected by Mordhorst from the Influenza laboratory of Statens Serum Institut. The sera were analysed for *C. pneumoniae* antibodies by Wang and Grayston. The purpose was to estimate the "TWAR population antibody prevalence" defined as IgG antibody titres of 16-256 i.e. preexisting antibody profile. For this reason all sera with *C. pneumoniae* IgG antibody titres of ≥ 512 were excluded, though it was stated that less than 10% of the sera had IgG antibody titres of ≥ 512. Figure 1 shows the "TWAR population antibody prevalence" according to age groups. In total, 39% had IgG antibody titres of 16-256. The prevalence and age distribution was similar to those found in other studies[60;63]. A second study by Mordhorst included sera from 1114 patients submitted to the Influenza laboratory from 1975 to 1987 on the suspicion of ornithosis analysed by MIF, all but 22 of the sera had a positive result by CF test[113]. The Grayston criteria of acute infection were applied to assess the incidence of acute *C. pneumoniae* infection. According to these criteria the incidence of *C. pneumoniae* infection among the patients during the whole period was 54%, including to time periods with a much higher incidence. However, the patients in this study were strongly selected as the vast majority had positive results by the CF test. Furthermore IgG antibody titre of ≥ 512 was used as a criterion of acute infection.

Serological and microbiological studies. Until recently most epidemiological data have been based on seroepidemiological studies. Overall, when using the Grayston criteria for an acute infection, these studies have shown that *C. pneumoniae* is a common pathogen in respiratory tract infections in all age groups > the age of five. As more laboratories perform culture of *C. pneumoniae* and with the emergence of PCR it is now possible to compare the serological based findings with data from studies in which the organism has been detected.

C. pneumoniae infection in children. Acute infection verified by culture can occur in children < five years without seroconversion in MIF[21;77;149]. The positive correlation of *C. pneumoniae* serology and culture is low among pre-school children[21;77]. In two Danish studies the prevalence of *C. pneumoniae* among children with acute respiratory tract infections was investigated by detection of *C. pneumoniae* in respiratory tract secretions by culture and PCR[48]. In total 691 children were examined and *C. pneumoniae* was found in < 1%.

C. pneumoniae infection in adults. In acute respiratory infections in adults and older children there is an acceptable positive correlation between IgM antibody titres of ≥ 16 / fourfold IgG antibody titre rise and culture and it seems reasonable to conclude that *C. pneumoniae* can be recognized as a frequent cause of upper and lower respiratory tract infections. Numerous studies have published findings that 5-10% (-20%) of pneumonia cases in adults are attributed to this organism[2;42;56;59;148]. However, the prevalences of *C. pneumoniae* infection found in different studies vary. In several epidemiological studies the presence of IgG antibodies of ≥ 512 has been indicative of acute infection but the positive correlation between a single high serum antibody titre and detection of *C. pneumoniae* by culture or PCR has been poor.

CHAPTER TWO: AIMS OF THE STUDY.

To assess the performance of two commercially available MIF tests compared to a reference method when applied to sera from different categories of patients.

To assess the applicability of serological criteria for the diagnosis of acute and chronic *C. pneumoniae* infections.

To describe the prevalence of *C. pneumoniae* infection in adult patients with acute community-acquired respiratory tract infections by use of microbiological and serological methods.

To describe the prevalence of *C. pneumoniae* infection in patients with chronic respiratory pulmonary diseases and to evaluate whether *C. pneumoniae* is associated with acute exacerbations of the disease by use of microbiological and serological methods.

CHAPTER 3: LABORATORY STUDIES.

Introduction.

Today several commercially available MIF assays as well as in-house assays for the detection of *C. pneumoniae* antibodies are widely used[106;120;122]; they are based on the same principle as the original test[163], but some modifications of the procedures have been introduced. When the present study was initiated, only few comparative studies on the performance of the assays had been conducted[51;120]. One study examined the interlaboratory variation in MIF test results obtained in 13 laboratories analysing 22 sera from 10 patients[120]. The agreement between a reference standard value and the IgM antibody results obtained by the 13 laboratories using 11 different methods was 50-95%; four laboratories failed to discern false-positive IgM titres possibly because of the presence of rheumatoid factor. For IgG antibody analyses the agreement was 68-87%. In another study, one laboratory compared two MIF assays (a reference method and a commercially available assay) and found a significant difference in IgG and IgA antibody titre levels[51].
The objective of the present study was to assess the performance of two commercially available *C. pneumoniae* MIF assays, one from MRL Diagnostics®, Cypress, USA (MRL) and another from Labsystems®, Helsinki, Finland (LAB). The two commercial assays (LAB and MRL) were under evaluation for use in our routine laboratory. A MIF assay based on *C. pneumoniae* antigen from Washington Research Foundation, (now Washington University), Seattle, USA (WRF) was used as a reference method. The WRF assay was chosen as the reference method because this assay had been available for research purposes during decades and because previous Danish studies on the prevalence of *C. pneumoniae* antibodies had been conducted with the WRF assay[76;113]; prior to the main study the reproducibility of *C. pneumoniae* IgG, IgM and IgA antibody detection by the WRF assay was determined. In the main study the performance of the three assays was assessed by analysing sera from patients with acute respiratory tract infection of known aetiology obtained at various intervals after the onset of the disease. Sera from persons enrolled in the Copenhagen City Heart Study were included due to the present interest in the detection of *C. pneumoniae* antibodies in patients with cardiovascular diseases[76;128].

Since the MIF assays are expensive and time consuming to perform, resources will be saved by use of a screening procedure prior to the analyses by the MIF assay. In previous *C. pneumoniae* seroepidemiological studies some authors found a high positive correlation between the detection of antibodies by the CF test and the MIF assay [50;167], whereas others found that positive CF test results were poor predictors of positive MIF results[12;43]. To examine whether the CF test was an appropriate tool for screening sera prior to the MIF assay analyses a study was conducted to assess the correlation between positive test results by the CF test and by the WRF MIF assay.

THE PERFORMANCE OF THREE *CHLAMYDIA PNEUMONIAE* MICROIMMUNOFLUORESCENCE ASSAYS.

Material and methods

Sera. This study included sera from three groups of patients: two with acute respiratory tract infection and one including patients with possible chronic infection but without known acute infection.

Group I consisted of 83 sera from 28 patients enrolled in the Nordic Atypical Pneumonia Study (NAP Study)[116]. The sera were kindly provided by J. S. Jensen, the Mycoplasma Laboratory, Statens Serum Institut with the permission of P. Saikku, University of Oulu, Finland. The 28 patients had serological evidence of infection with *C. pneumoniae* (13 patients), *Mycoplasma pneumoniae* (11 patients), or both agents (4 patients). The original MIF analyses for *C. pneumoniae* antibodies had been performed using antigens from WRF[116].

Group II consisted of 37 sera from 16 patients with acute *C. pneumoniae* (8 patients) or *C. psittaci* (8 patients) respiratory tract infection confirmed by PCR (15 patients) or culture (one patient) in our routine laboratory. Of the 15 patients tested, 14 had developed antibodies detectable in the CF test; moreover, clinical data were available for all patients with *C. pneumoniae* infections and for 5 of 8 patients with *C. psittaci* infections. For the last group of patients, epidemiological data were also available[46]. Culture was performed in Hep2 and McCoy cells; subsequent identification was obtained with genus- and species-specific staining methods[140]. PCR was performed by an in-house method [140].

Group III consisted of 100 sera from 100 persons enrolled in the Copenhagen City Heart Study, which is a prospective, ongoing, population study: 20 persons who developed acute myocardial infarction within three years after the blood samples were drawn and 80 controls matched according to gender and age at entry without subsequent myocardial infarction[5].

MIF assays. The three MIF assays are described in brief below. For a detailed description of the methods see appendix 1. Prior to IgM and IgA antibody testing all sera were treated with GullSORB®, Gull Laboratories, Salt Lake City, USA. All analyses were performed using a Zeiss axioskop 20 microscope with a plan-neofloar 40x/0.75 objective. The procedures followed the recommendations of each manufacturer.

WRF assay: As antigens, purified *C. pneumoniae* elementary antibodies (strain AR 39) were fixed onto the slides. The conjugates used were fluorescein-labelled rabbit anti-human IgM, IgG and IgA (DAKO®, Glostrup, Denmark). The incubation time was 30 min for both sera and conjugates.
MRL assay. For MIF assays (IgM, IgG and IgA) from MRL, the slides were purchased with prefixed antigen dots for *C. pneumoniae* (strain TW183), *C. trachomatis* (8 serotypes(D-K)) and *C. psittaci* (strains 6BC and DD34). For the detection of IgM antibodies, the incubation period with serum was 90 min.; for the detection of IgG and IgA antibodies, it was 30 min. The subsequent incubation time with the conjugate (fluorescein-labelled goat anti-human Ig's (MRL)) was 30 min.
LAB assay. The slides for MIF assays (IgM and IgG) were purchased with prefixed antigen dots for *C. pneumoniae* (strain Kajaani 6), *C. trachomatis* (strain LGV/ L2) and *C. psittaci* (strains OF 6bC and EAE). For the detection of IgM antibodies, the incubation period with serum was 180 min., for the detection of IgG antibodies, it was 30 min. The subsequent incubation time with the conjugate (fluorescein-labelled goat anti-human Ig's (LAB)) was 30 min.

All sera were tested in serial twofold dilutions from the following initial dilutions and until an endpoint was reached. Group I: 1:16 (IgM), 1:64 (IgG), 1:16 (IgA); group II: 1:16 (IgM), 1:64 (IgG); group III: 1:16 (IgG), 1:16 (IgA). An endpoint titre was defined as the highest serum dilution with a positive test result. An overview of the experiments performed is presented in table 2.

Statistics

A comparison of detection rates obtained by the three assays was performed with Cochran's Q test; the likelihood ratio test was used to compare the proportion of concordant results (both assays positive or both assays negative) obtained by the WRF and the MRL assays versus that obtained by the WRF and the LAB assays. For comparison of the antibody titres Friedman's nonparametric 2-way analysis of variance was used. These analyses were performed by SAS®, version 8 (SAS Institute Inc.). For comparison of the prevalence of antibody titres at certain levels within groups, χ^2 or Fisher's exact test was used. The 95% confidence intervals (CI) for detection rates were calculated as follows:

The proportion (p) $\pm 2\sqrt{p(1-p)/n}$ (normal approximation). $P < 0.05$ was considered significant. In general, test results for all sera are used for the statistical analyses. The results of the same analyses when only including the first serum sample per patient are given separately.

Results

Detection rates of *C. pneumoniae* antibodies. The presence of *C. pneumoniae* IgM and IgG antibodies in 120 sera from 65 patients with acute respiratory tract infection (groups I and II) is illustrated in tables 3-6. No differences between the WRF, the MRL and the LAB assays were seen in the detection rates of IgM antibody titres of ≥ 16, or in the detection rates of IgG antibody titres of ≥ 64 (table 7). Concordant results (both assays positive or both assays negative) were demonstrated in ≥ 92% of the sera for IgM antibodies and in ≥ 76% of the sera for IgG antibodies by the MRL and the LAB assays versus the WRF assay (table 8). No significant differences in the percentage of concordant results were seen (IgM: P= 0.16, IgG: P=0.43). Similar results were obtained when only one sample per patient was included (tables 9-10). For IgA antibodies, the results of the MRL assay versus the WRF assay were concordant in 80% of the sera (66 of 83), (data not shown). When a day-to-day variation of ± 1 dilution step was taken into consideration (see page xx), the results for the MRL assay versus the WRF assay were concordant in 98, 88 and 88% of the sera for IgM, IgG and IgA antibodies and for the LAB assay versus the WRF assay in 98 and 87% of the sera for IgM and IgG antibodies.

For the sera from the patients in group III, agreement in detection rates by the MRL assay versus the WRF assay was found in 92% of the sera (90 of 98) for IgG antibodies and in 91% of the sera (91 of 100) for IgA antibodies (table 9-10). When the day-to-day variation was taken into consideration, the corresponding results were 97 and 95% of the sera, respectively.

Endpoint titre levels. Table 11 shows the geometric mean titres found by each of the three MIF assays of all sera with an antibody level considered significant for the diagnosis of *C. pneumoniae*, i.e. IgM antibody titres of ≥ 16, IgG antibody titres of ≥ 64 and IgA antibody titres of ≥ 32. The geometric mean of IgM antibody

titres obtained by the LAB assay was about three times as high as those obtained by the WRF and the MRL assay. The difference in geometric mean titres for IgM antibodies obtained by the three assays was significant. This difference was due to the diverging results by the LAB assay. Comparing the geometric mean titres for IgG antibodies obtained by the three assays in sera from group I and II, the overall difference demonstrated was due to a lower IgG antibody level by the WRF assay and a higher level by the LAB assay. Similar geometric mean titres for IgM and IgG antibodies were obtained when only one sample per patient was included (table 11).

With the WRF assay 2 of 120 sera (1.7%) had *C. pneumoniae* IgG antibody titres of $\geq$ 512 as opposed to 9 of 120 sera (7.5%) with the MRL assay and 7 of 120 sera (5.8%) with the LAB assay (P=0.10, Fisher's exact test), (table 5-6). However, in only five sera were IgG antibody titres of $\geq$ 512 demonstrated by both the MRL and the LAB assay.

The difference in mean endpoint titres of sera positive by two assays can also be expressed in dilution steps. Results by the MRL assay compared to those by the WRF assay were 0.4 dilution steps lower for IgM antibodies (groups I+II); 0.4 dilution steps higher for IgG antibodies (groups I+II+III) and 0.3 dilution steps higher for IgA antibodies (groups I+III). For the LAB assay compared to the WRF assay the mean titres for groups I+II were 1.7 dilution steps higher for IgM and 0.4 dilution steps higher for IgG antibodies. In conclusion, the determinations of endpoint titres were reproducible with less than one dilution step difference for all three methods, except that the mean IgM antibody titre found by the LAB assay was almost two dilution steps higher than that, found by the other two methods.

Discrepant results: The MRL and the WRF assay results were discrepant (positive versus negative) in 9 of 120 (8%) of sera tested for *C. pneumoniae* IgM antibodies (table 3). Two of the three sera with a positive result for IgM antibodies by the MRL assay but not by the WRF assay came from a patient with *C. psittaci* infection verified by PCR analysis. The third serum, from a group I patient was found negative by both the WRF and the LAB assay but subsequent sera from the same patient were found positive for IgM antibodies by all three assays. The six sera with a negative result for *C. pneumoniae* IgM antibodies by the MRL assay but with positive results by the WRF assay came from two group I patients (three sera from each) and all six sera were also positive for IgM antibodies by the LAB assay.

The LAB and the WRF assay results were discrepant in 5 of 120 (4%) sera tested for IgM antibodies (table 4). The five IgM results were all found in the first or the last of three consecutive sera, the two correspondent sera of which were positive for *C. pneumoniae* IgM antibodies by both assays. For these five sera the results by the WRF assay were concordant with those by the MRL assay.
For the majority of sera with discrepant IgG antibody results the discrepancy was between sera with IgG antibody titres of < 64 in one assay and IgG antibody titres of 64 to 256 in the other assay (tables 5-6).

Patients with *C. pneumoniae* infection. A total of 66 sera from 25 patients with either serological evidence of *C. pneumoniae* infection (group I, n = 17) or microbiological evidence of *C. pneumoniae* infection (group II, n = 8) were included.
A total of 51 sera originated from these 17 patients in group I. For IgM antibodies the agreement in detection rates was 98% of the sera (50 of 51) for the LAB versus the WRF assay and 88% of the sera (45 of 51) for the MRL versus the WRF assay, while accordance in endpoint titre levels, defined as the WRF assay titre ± 1 dilution step, were 78% (40 of 51) and 73% (37 of 51), respectively. For the IgG antibody detection rate the agreement was 90% (46 of 51) for both the LAB versus the WRF assay and the MRL versus the WRF assay, while accordance in endpoint titre levels was found in 84% (43 of 51) of sera by both assays. For the IgA antibodies the agreement in detection rates for the MRL versus the WRF assay was 86% (44 of 51) and the accordance in endpoint titre levels was 75% (38 of 51).

Table 12 shows the proportion of patients with *C. pneumoniae* infection in group I and II fulfilling each of the Grayston criteria of acute infection (table 1). Among the patients in group I, IgM antibody titres of ≥ 16 were detected by all three assays in sera from 6 of 8 patients, in sera from the two last patients IgM antibodies were detected by the LAB and the WRF assay but not by the MRL assay. A fourfold rise of IgG antibody titres was detected in one patient by all three assays, in further four patients was the criterion fulfilled by one or two of the assays. IgG antibody titres of ≥ 512 were seen in 5 patients, though only in one of these by all three assays.
Among the patients in group II, IgM antibody titres of ≥ 16 were detected by all three assays in sera from 7 of 8 patients (11 of 15 sera), (table 12). A fourfold rise of IgG antibody titres was detected in one patient by all three assays and in further

one patient by the LAB assay. IgG antibody titres of ≥ 512 were not detected by any of the assays.

For group II, table 13 shows the serological results by all three methods for each patient with confirmed *C. pneumoniae* infection. The accordance in IgM antibody detection rates for the 15 sera from 8 patients were 87% (13 of 15) for the LAB versus the WRF assay and 100% (15 of 15) for the MRL versus the WRF assay, while accordance in endpoint titre levels defined as the WRF assay titre ± 1 dilution step, were 47% (7 of 15) and 67% (10 of 15). For IgG antibodies the accordance in detection rates were 53% (8 of 15) for the LAB versus the WRF assay and 47% (7 of 15) for the MRL versus the WRF assay, while accordance in endpoint titre levels were found in 40% (6 of 15) of sera by the LAB assay and 47% (7 of 15) by the MRL assay, respectively.

In conclusion, the agreement in detection rates of IgM antibodies was between 87 and 100% by the two commercial assays compared to the WRF assay and the accordance in detection rates of IgG antibodies was 90% in sera from group I and about 50% in sera from group II. The accordance in endpoint titre levels between both assays and the WRF assay was somewhat lower than the accordance in detection rates. The agreement between all three methods in fulfilling the criteria of serological diagnosis was high for the detection of IgM antibody titres of ≥ 16 (13 of 15) but low for the detection of IgG antibody titres of ≥ 512 (1 of 5) and for the detection of a fourfold rise of IgG antibody titres (1 of 5).

Patients with *M. pneumoniae* infection. The agreement of *C. pneumoniae* IgG antibody test results was 91% by the MRL versus the WRF assay and 87% by the LAB versus the WRF assay in the 32 sera from 11 patients included in group I. By the MRL and the WRF assay, the test results for *C. pneumoniae* IgA antibodies were in accordance in 29 of 32 sera. In none of the sera was *C. pneumoniae* IgM antibodies, fourfold rise of IgG antibody titres or IgG antibody titres of ≥ 512 detected by any of the assays. IgG antibody titres of 64 to 256 were detected in 46 to 57% of sera by the three assays.

Patients with *C. psittaci* infection. A total of 22 sera from 8 patients with *C. psittaci* infection were analysed (table 14).
Overall, four of eight patients had *C. psittaci* IgM antibodies and/or fourfold rise of IgG antibody titres by the LAB assay. By the MRL assay one of the eight patients had IgM antibodies towards all three species and further one had a fourfold rise of IgG antibody titres.
C. pneumoniae IgM antibodies were detected in four sera from two patients by the MRL assay, but in none of the sera by the LAB and the WRF assay. *C. pneumoniae* IgG antibody titres of 64 to 256 were detected in sera from three, four and six patients by the WRF, the LAB and the MRL assay, respectively.

Cross-reacting antibodies: In patients with *C. pneumoniae* IgM antibodies, *C. psittaci* IgM antibodies were detected in 3 of 6 patients in group I (5 of 15 sera) and in 4 of 9 patients of group II (5 of 16 sera) by the MRL assay. In 5 of the 10 sera there were less than fourfold difference between *C. pneumoniae* and *C. psittaci* IgM antibody titre levels. *C. trachomatis* IgM antibodies were seen in three patients (6 sera), who showed positive test results for *C. pneumoniae* as well as *C. psittaci* IgM antibodies.
In patients with *C. pneumoniae* IgM antibodies, *C. psittaci* IgM antibodies were detected in 5 of 8 patients in group I (13 of 21 sera) and in 2 of 7 patients of group II (2 of 12 sera) by the LAB assay. In 9 of the 15 sera there were less than fourfold difference between *C. pneumoniae* and *C. psittaci* IgM antibody titre levels. *C. trachomatis* IgM antibodies were seen in three patients (4 sera) in whom *C. pneumoniae* IgM were demonstrated, while *C. psittaci* IgM antibodies were demonstrated in one of these patients.

Comparison of results for group I with the NAP study. The results of group I, tested with antigens from WRF in the present study was compared to the results originally obtained in the NAP Study[116] with the same cut off applied to both sets of results. The detection rate of *C. pneumoniae* IgM antibodies of ≥ 16 was 24% (CI: 19 to 29%) versus 31% (CI: 26 to 36%) in the NAP Study and that of IgG antibodies of ≥ 64 was 45% (CI: 40 to 50%) versus 69% (CI: 64 to 74%), but the detection rate of IgA antibodies of ≥ 16 was 64% (CI: 59 to 69%) versus 40% (CI: 45 to 55%). The mean endpoint titres obtained in the present study were 2.0 and 1.5 di-

lution steps lower for IgM and IgG antibodies and 0.3 dilution step higher for IgA antibodies than in the NAP Study.
The proportion of patients fulfilling each of the Grayston criteria of acute *C. pneumoniae* infection by at least one of the two studies is shown in table 15. The results of the two studies were in agreement in 89% of cases regarding the presence of IgM antibodies, while agreement in fulfilment of the two other criteria could only be demonstrated in 30 and 9% of cases.

Conclusion

The overall agreement between results by the WRF assay and by the two commercial assays was excellent for IgM antibody detection rate (98%). The accordance in detection rates for IgG and IgA antibodies in sera from patients with acute infections was acceptable (87 to 88%) and in sera from group III it was excellent (95 to 97%). No significant difference among the detection rates of the three assays was seen for any of the Ig classes.

The reproducibility of *C. pneumoniae* IgG and IgA antibody endpoint titres was high, though IgG antibody titres by the WRF assay were on average 0.4 dilution step lower than those by the two other assays. The IgM antibody endpoint titres found by the LAB assay were almost two dilution steps higher than those found by the WRF and the MRL assay.
A comparison of the results of the MRL versus the WRF assay of the sera from group I + II and group III showed a tendency towards greater variation of endpoint titres among the patients with acute infection than among the group without acute infection (table 3-8).

The agreement between the three assays in the capacity to distinguish between acute infection and no acute infection in patients with *C. pneumoniae* infection was high for the detection of IgM antibody titres of ≥ 16 (13 of 15 patients), but low for the detection of IgG antibody titres of ≥ 512 and for the detection of fourfold rise of IgG antibody titres (1 of 5 patients respectively).
None of the patients with *M. pneumoniae* infection had serological evidence of acute *C. pneumoniae* infection, whereas two patients with *C. psittaci* infection had *C. pneumoniae* IgM antibodies by the MRL and in further two patients a fourfold

rise of *C. pneumoniae* IgG antibodies were demonstrated by both the MRL and the LAB assay.

When comparing the results by the WRF assay in our laboratory to the results obtained in the original study the agreement in the ability to fulfil the criteria of acute infection was high for the criterion of the presence of IgM antibodies but low for the two criteria in which the level of IgG antibodies is decisive.

IGM ANTIBODY TITRE LEVEL AND DURATION OF INCUBATION OF ANTIGEN WITH SERUM.

In the main study the detection rate for *C. pneumoniae* IgM antibodies was the same by all three assays. However, the mean level of IgM antibody endpoint titres was almost two dilution steps higher by the LAB than by the WRF assay, but identical for the MRL and the WRF assay. One of the modifications used in the commercial MIF assays was a change of the duration of incubation of antigen with serum. While Wang and Grayston recommend an incubation time of 30 min., MRL Diagnostics® and Labsystems® recommend 90 min. and 180 min respectively (appendix 1).
To test to what extend the IgM antibody endpoints titres are influenced by the differences in incubation time the following study was conducted using the reference method (the WRF assay) and the MRL assay, which then had been chosen for use in our routine laboratory.

Material and methods

Sera. The study included 25 selected sera from 11 patients included in group I and II in the main study. Three sera had had a negative IgM antibody result by both the WRF and the MRL assay; each of these three sera was the first of three consecutive sera of which the two latter sera had had IgM antibody positive results by both assays. Further one serum had had a negative result by the WRF but not by the MRL assay. All other sera had had positive IgM antibody result by both assays.

MIF assays. All sera were tested for *C. pneumoniae* IgM antibodies in serial twofold dilutions beginning at 1:32. By each MIF assay the dilutions of sera were tested in duplicate: one dilution row with an incubation time for sera and antigen of 30 min. and the other with an incubation time of 90 min. Otherwise, the procedures followed the recommendations of each manufacturer (appendix 1).

Statistics. The correlation between the incubation time and the *C. pneumoniae* IgM antibody titre level was assessed for the WRF and for the MRL assays with a

Sign Test. The results of the analyses when using only one serum from each patient are given separately.

Results

Of the 25 sera tested for *C. pneumoniae* IgM antibodies four sera had a negative test result by both assays including the three sera that originally had had a negative test result and a serum that had had a weak positive result (32 and 16) by the two assays. Further one serum was negative by the WRF assay with both incubation periods; this serum had originally had a negative test result. The analyses were conducted on the basis of the result from 20 sera tested by the WRF and 21 sera tested by the MRL assay. Tables 16 and 17 present overviews of the results by the WRF and the MRL assays, respectively.

The WRF assay. The geometric mean titre of the 20 sera was 256 when the incubation time was 30 min. and 431 with 90 min.'s incubation, (P=0.0002); that equals a 0.75 dilution step rise of IgM antibody titre with a change from 30 to 90 min. of incubation time; 11 sera had a rise of 1 dilution step, 2 had a rise of 2 dilutions steps and the last 7 did not show any change in *C. pneumoniae* IgM antibody titres. The geometric mean titres for the 11 sera (1 serum per patient) were 309 and 481, (P=0.03) corresponding to a 0.64 dilution step difference.

The MRL assay. The geometric mean titre of the 21 sera was 868 when the incubation time was 30 min. and 1592 with a 90 min.'s incubation, (P<0.0001); that equals a 1.0 dilution step rise of IgM antibody titre with a change from 30 to 90 min. of incubation time. For 16 sera a one-dilution step change was seen, 3 sera had a rise of two dilution steps and the last 2 sera did not show any change in *C. pneumoniae* IgM antibody titres. The geometric mean titres for the 11 sera were 1237 and 2806, (P=0.001) corresponding to 1.18 dilution steps difference.

Conclusion

A positive correlation between the duration of incubation time (30 and 90 min.) and *C. pneumoniae* IgM antibody titres was demonstrated using both methods. An increase of titres was seen regardless of titre levels. Whether this finding implies that the detection rate of *C. pneumoniae* IgM antibodies increases with in-

creasing incubation time will have to be assessed in another study, though in this study none of the sera originally tested by the WRF assay with an incubation time of 30 min. with a negative test result had a positive test result with a 90 min. incubation time.

The geometric mean titres for IgM found by the MRL assay both at 30 min and 90 min. were 3-4 times higher than those found by the WRF assay. In the main study no difference between the geometric mean titres of IgM antibodies by the two assays was seen.

A limitation of this study was, that the results were based on single determinations using only one batch of reagents from each assay.

REPRODUCIBILITY OF TEST RESULTS BY THE WRF MIF ASSAY

In order to determine the reproducibility, a study of the intra- and inter-day variation for *C. pneumoniae* antibody titres obtained by the MIF reference method (WRF) was conducted.

Sera

Two sera were tested for *C. pneumoniae* IgG, IgM and IgA antibodies in triplicate on four experimental days by the MIF assay. Thus for each antibody class 24 observations were made. The experiments were carried through by the same person who performed the analyses of the main study. The sera were analysed in serial, twofold dilution steps starting at 1:16.

Statistics

To determine the reproducibility of end-point titres for each serum for each day a two-way analysis of variance was performed. The inter-day variation was estimated with an F-test. The variation of reproducibility was estimated for each Ig class and for the three classes together. Finally a 95% confidence interval (CI) of the titres was calculated. All analyses were performed by SAS®, SAS Institute Inc.

Results

The variation of reproducibility was estimated to 0.41 dilution step for IgM, 0.48 for IgG, and 0.48 for IgA antibody titres. Overall the day-to-day variation of MIF endpoint titres was 0.46 dilution step with 66 degrees of freedom. The CI was 2 x $0.46 = 0.92 \approx 1$ dilution step.

Conclusion and comments

This study showed, that the MIF assay results varied less than one dilution step. The result of this study is in accordance with results from another laboratory experienced in *C. pneumoniae* MIF analyses[106].

CORRELATION BETWEEN RESULTS OF THE CF TEST AND THE WRF MIF ASSAY FOR THE DETECTION OF *C. PNEUMONIAE* ANTIBODIES.

In the early era several studies on the seroepidemiology of *C. pneumoniae* infections were performed using collections of CF antibody positive sera. Since a high proportion of these sera were shown to have detectable IgG antibodies by the MIF assay, the CF test was considered an appropriate tool of screening sera before further testing by the more costly MIF assay[12;113].

To test whether this procedure was appropriate, a study of the correlation between the presence of CF antibodies and IgG antibodies detected by the MIF test was carried through. The reason for choosing results for IgG and not IgM antibodies was that while approximately 30% of sera received in our routine laboratory had detectable *C. pneumoniae* IgG antibodies, IgM antibodies were found in less than 1% of sera (unpublished data).

Materials and methods

Sera. We conducted a study of 1008 consecutive sera submitted to our routine laboratory for the CF test and/or the MIF test from hospitals and GPs from all over the country.

CF test. For the CF test, *Chlamydia* antigen from Statens Serum Institut (Copenhagen, Denmark) was used[157]. All sera were tested in serial threefold dilutions beginning at 1:12[140]. Titres of < 12 were considered negative, titres 12 to 108 weakly positive and titres of > 108 strongly positive.

MIF assay. The WRF assay was used as the reference method in this study. All sera were tested for *C. pneumoniae* IgG antibodies in serial, twofold dilution rows starting at an initial dilution of 1:64. Titres of < 64 were considered negative, titres 64 to 256 weakly positive and titres of ≥ 512 strongly positive.

Results

Of the 1008 sera, 231 had positive results by the CF test and/or the MIF assay (table 18).

Of the 157 sera with a positive CF test result 42 (27%) had detectable *C. pneumoniae* IgG antibodies. Of these 157 sera, 27 had CF antibody titres of > 108, none of these had *C. pneumoniae* IgG antibodies of ≥ 512.

A positive CF test result was found in 42 of 116 sera (36%) with a positive *C. pneumoniae* IgG antibody result by the MIF assay. Thus, the sensitivity of the CF test to predict the presence of *C. pneumoniae* IgG antibodies detectable by the MIF assay was 42 of 116 (36%), while 42 of 157 (27%) of the CF test positive sera were found with the MIF test.

Four sera had a positive result of *C. pneumoniae* IgG titre of ≥ 512, three of these were negative and one was weakly positive by the CF test.

Conclusion and comments

In the present study, the predictive value of a positive CF test result for the presence of *C. pneumoniae* IgG antibodies was 36%, indicating that the CF test is not a suitable tool for screening sera prior to testing for *C. pneumoniae* IgG antibodies. Fonseca et al compared results by the CF test and the MIF assay for 120 sera (40 single and 40 pairs) from patients with a history of acute respiratory tract infections and a CF test result of >16 in the acute samples[50]. In 77% of all sera and in 54% of acute sera were detected *C. pneumoniae* IgG antibodies of ≥ 16. *C. pneumoniae* IgG antibodies of ≥ 64 were detected in 65% of all sera. Thus Fonseca et al found a higher positive correlation between positive results by the two assays than did the present study. This could be due to differences between the study populations of the two studies.

Persson and Boman[122] compared the performance of five serological tests for the diagnosis of acute *C. pneumoniae* infections by analysing paired sera from 240 patients with symptoms of acute respiratory tract infection. Among the five tests was a CF test using the same antigen as the one used in the studies of the present thesis and the MIF test from Labsystems. In that study the sensitivity of the CF test to predict a significant reaction by the MIF assay was 69% (66 of 95 cases). In 90% of cases positive by the CF test (66 of 73 cases) the MIF test was also positive.

The inclusion criteria of the Persson study were paired sera and a history of respiratory tract infection, whereas we studied consecutive sera received in our routine laboratory without any clinical information about the patients. Further Persson

studied the performance of the tests by assessing the degree of agreement between the tests in detecting significant reactions and/or significant levels of antibodies for the diagnosis of *C. pneumoniae* infection, whereas in the present study the performance of the MIF test and the CF test was assessed by comparing the degree of agreement in detecting IgG antibodies and antibodies detectable by the CF test, respectively.

In the studies by Fonseca et al[50] and Persson and Boman[122] as well as in the present study a proportion of sera positive by the CF-test had negative results by the MIF assay. The chlamydial antibodies detected by the CF test persist for a limited period of time, whereas the antibodies detected by the MIF assay emerge and disappear later than the before mentioned antibodies[162]. Therefore, it is reasonably to expect an early phase with seroconversion detected only by the CF test, an intermediate phase with detectable antibodies by both assays and a late phase with antibodies only detectable by the MIF assay.

In conclusion, while the CF test is not suitable as a tool for screening sera before further testing by the MIF assay, it is an excellent supplement for the diagnosis of early phase chlamydial infection.

DISCUSSION

We conducted a study on the performance of three MIF assays (the WRF, the MRL and the LAB assay) for the detection of *C. pneumoniae* IgG, IgM and IgA antibodies[9]. The agreement in detection rates was high for all three methods and all three Ig classes. The reproducibility of *C. pneumoniae* IgG antibody endpoint titres was high; although IgG antibody titres by the WRF assay were on average 0.4 dilution step lower than those by the two other assays. However, this difference is within the day-to-day variation of one dilution step. Similarly, another recent study comparing IgG antibody titres obtained by the WRF assay and the LAB assay found an insignificant difference of one dilution step higher titre levels by the latter assay[106]. The IgM antibody endpoint titres found by the LAB assay were almost two dilution steps higher than those found by the two other methods. Labsystem recommends that when testing for IgM antibodies the incubation time for sera with the antigen should be 180 min.; that is, six times as long as originally recommended by Wang[161;162]. Results of the experiment described earlier in this chapter suggest, that a prolonged incubation time does affect the IgM antibody titre levels, thus differences in IgM antibody titre levels obtained by different assays could be due in part to the differences in incubation times. It is interesting however, that the prolonged incubation time did not seem to affect the detection rate of *C. pneumoniae* IgM antibodies since the percentages of sera with IgM antibodies were the same by the same assays using different incubation periods.

For the MRL assay the mean IgM antibody endpoint titres was 0.4 dilution step lower than those found by the WRF assay; and 0.4 and 0.3 dilution step higher for IgG and IgA antibodies, respectively. Freidank et al found the mean titres for IgG antibodies and IgA antibodies by the MRL assay to be 2.5 and 3.0 dilution steps higher than those obtained by the WRF assay[51]. Freidank et al used anti-human Igs from Medac® in the WRF assay whereas we used anti-human Igs from Dako®. Though only minor differences among IgG antibody levels detected by the three assays were found, a difference between the proportions of sera with antibody titres of ≥ 512 was seen. IgG antibody titres of ≥ 512 were demonstrated by the WRF assay in 1.6% of sera and by the MRL assay in 7.5% of sera. Among sera studied by Freidank 2% had titres of ≥ 512 with the WRF assay, compared to 30% with the MRL assay.

It has been demonstrated that the use of a *C. pneumoniae* antigen produced from a local isolate can result in higher detection rates of antibodies in sera and higher titres in the MIF assay than when using a standard antigen[17]. The three assays studied each use a different *C. pneumoniae* strain as the antigen. The WRF antigens are whole inactivated (formalin fixed) organisms fixed onto the slides with acetone; the two commercial assays do not inform on the details of the pretreatment. However, the detection rates and endpoint titres by these three assays were similar, with IgM antibody endpoint titres found by the LAB assay as the only notable exception.

In the interlaboratory study by Peeling et al[120] the agreement among the participating laboratories for IgM titres was 50 to 95%. In the present study the agreement among the three assays of detecting IgM antibodies in sera from the eight persons with microbiological verified *C. pneumoniae* infection was 87 to 100%. Considering the technical complexity of the MIF test and the subjective nature of reading the titres, it is hardly surprising that the interlaboratory variation of that study is higher than the interassay variation of the present study. Recently Peeling has presented a follow-up study in which 11 laboratories analysed 10 sera before and after a two-days hands-on workshop[121]. Of the eleven laboratories, nine showed a certain improvement in their % agreement with the reference standard after the workshop. Before the workshop 6 of 11 laboratories had 90-100% agreement with the reference laboratory, after the workshop this figure had risen to 9 of 11 laboratories.

The agreement between the serological and the microbiological diagnoses for the patients with confirmed *C. pneumoniae* infection was high, in contrast to that for the patients with *C. psittaci* infection. For most of the patients with psittacosis, sera were obtained early in the course of the disease, and it is possible that if sera had been obtained later, *C. psittaci* antibodies would have been detected. Furthermore, there is a greater diversity between *C. psittaci* strains than between *C. pneumoniae* strains and it is possible that the *C. psittaci* strains used in the LAB and the MRL assay did not react with *C. psittaci* antibodies from Danish patients with infections caused by local strains.

The detection rates of *C. pneumoniae* IgG and IgM antibodies as well as the antibody titre levels, were low among sera from group I of the main study compared to the results in the NAP Study; the detection rate of *C. pneumoniae* IgA antibody as well as the antibody titre level was higher in the present study, than in the NAP study. Although the same antigen for the MIF assay was used in the two studies, other differences in the test procedures occurred, e.g. the use of different conjugates and interpersonal difference in the reading of slides might explain some of the variations of the results obtained. Furthermore, in the present study the sera had been absorbed before the IgA antibody analyses; removal of IgG has been shown to raise IgA titres in sera with a high level of IgG antibodies[82]. Finally the patients in the NAP Study were recruited between 1990 and 1993 and the sera were analysed shortly thereafter, whereas in our study the sera were tested after storage at -20°C for up to eight years. Though the endpoint titre levels found by the NAP study were higher than in the present study, the agreement in fulfilling the criterion of IgM of ≥ 16 was high while the agreement in fulfilling the two other criteria of acute infection were much lower. In both of the latter criteria, the levels of IgG antibodies are important, thus with higher IgG antibody values in general it is to be expected that more sera fulfil the criteria.

The statistical analyses used in this chapter imply independence between samples, a condition that is not entirely met by the present study as 120 sera originated from 44 patients (group I and II). However, the assessment of the performance of diagnostic tools should reflect the clinical situations in which the tools are used. For the sero-diagnosis of an acute infection, it is important to have more than one serum sample per patient, as the diagnosis is based on the emergence, rise or disappearance of different classes of antibodies. Therefore, it is not possible to select one single sample period in the course of infection representative for the whole course. The conflict between the biological reality and the demands of the statistical analyses most commonly used cannot be solved easily. In papers published within the serological field the authors have ignored, that the samples included are not completely independent[106;123].

In conclusion, the three MIF assays investigated showed about the same ability to detect *C. pneumoniae* IgG, IgM and IgA antibodies but some variation was found in the antibody levels demonstrated. In the light of the Grayston criteria for the se-

rological diagnosis of acute *C. pneumoniae* infection, the difference in average endpoint titre levels of IgM antibodies is of no mayor concern, as the emphasis of the criteria rests on the presence of IgM antibodies, not on the level. In contrast the difference in IgG antibody endpoint titres by different assays presents a problem as long as a high level of IgG titres (≥512) is used as an isolated criterion for the sero-diagnosis of acute *C. pneumoniae* infection.

CHAPTER 4: CLINICAL STUDIES.

Introduction

Since detection of *C. pneumoniae* by culture or by PCR is difficult and until recently mastered only by few laboratories, the diagnosis of *C. pneumoniae* infection has been founded on serological criteria. Detection of *C. pneumoniae* IgM, IgG and IgA antibodies by the MIF assay is still the gold standard for detection of past or present *C. pneumoniae* infection[40;150;162] however, the interpretation of test results is still controversial.

Grayston classified the test results for IgM and IgG antibodies as acute and preexisting antibody profiles (table 1)[60]. Acute antibody profiles were four-fold titre rise and/or IgM titres of ≥ 16 and/or IgG titres of ≥ 512. For acute infections a distinction between primary infection and reinfection might be possible. In the latter case the IgM response might be weak or non-existent. The significance of IgG antibody titres of ≥ 512 as an isolated criterion for an acute *C. pneumoniae* infection has been questioned[35;80] and in the present two studies this criterion is assessed and discussed separately. Serologic criteria of reinfection used in the present studies are fourfold rise of IgG antibody titre and IgM titres of < 16. Preexisting or persistent antibodies can be due to either previous or chronic infections. The profile for previous infection was defined as IgG antibody titres of < 512 and IgM antibodies of < 16. Clinical criteria of chronic infection have not been established and there is no clear distinction between the serological profiles associated with previous and chronic infection. The role of IgA antibodies is not clear. Some believe that IgA antibodies have no independent significance[162], while others consider IgA antibodies a marker of chronic infection[128;158]. Founded on results from studies on the role of *C. pneumoniae* in patients with asthma or COPD, a serological profile of chronic *C. pneumoniae* infection has been proposed: IgG titres of ≥ 128 and IgA titres of ≥ 40 without the presence of IgM antibodies[70].

Objective

The objective was to describe the prevalence of *C. pneumoniae* and other "atypical respiratory tract pathogens"[73], namely *Mycoplasma (M.) pneumoniae, Bordetella (B.) pertussis* and *Legionella (L) pneumophila* in two groups of adult patients: A) patients with acute respiratory tract infections and healthy controls seen by a

General Practitioner (GP) and B) patients with respiratory tract infections and COPD patients with or without exacerbation or pneumonia admitted to a department of internal medicine or seen at the associated out-patients clinic for pulmonary diseases. Further an assessment of the applicability of serological criteria for acute and chronic *C. pneumoniae* infection was made.

Methods

The two studies A and B were conducted using the same panel of microbiological and serological analyses for the diagnoses of *C. pneumoniae, M. pneumoniae, B. pertussis* and *L. pneumophila* infections.

Microbiology: Sputum samples and nasopharyngeal swabs were examined for *Chlamydia* species by culture in Hep2 cells and McCoy cells followed by immunospecific staining as previously described [48]. Furthermore all samples were analysed by in-house PCR's for the detection of *Chlamydia* species (*C. pneumoniae, C. psittaci* and *C. trachomatis*), *M. pneumoniae, L. pneumophilla* and *B. pertussis*[140]. In the study B sputum samples were examined for respiratory tract pathogens by microscopy and culture at the local department of clinical microbiology; urine samples were analysed for pneumococcal antigens by the Streptococcus Unit, Statens Serum Institut[140]. All other microbiological analyses were performed in the routine laboratories of our department[140].

Serology: Sera were analysed for genus-specific chlamydial antibodies by a complement fixation (CF) test with chlamydial antigen from Statens Serum Institut[157] using three-fold dilution steps from an initial dilution of 1:12[140], and for *C. pneumoniae* IgM, IgG and IgA antibodies by a microimmunofluorescence (MIF) assay from MRL Diagnostics®(Cypress, USA) using two-fold dilution steps from an initial dilution of 1:16. All MIF analyses were conducted by the same technician. Reading of the MIF test was performed independently by the technician and by the author of the present thesis. Discrepant results were rare and a difference of more than one dilution step was not seen. Sera were analysed for *M. pneumoniae* and *Legionella* antibodies by a *M. pneumoniae* CF test and a *Legionella* antibody test[140].

The serological criteria for acute infections were as follows: *Chlamydia* CF test: four-fold titre rise or titre of ≥ 108; *M. pneumoniae* CF test: four-fold titre rise or titre of ≥ 256; *Legionella* antibody test: four-fold titre rise or titre of ≥ 256; *C. pneumoniae* MIF assay: see table 1. Quantitation of serum IgG concentration was performed by the Department of Clinical biochemistry, SSI using rate-nephelometry on the first serum sample from each patient[135].

Statistics. Statistical analyses were conducted by use of SAS® version 8 (SAS Institute Inc, Cary, NC, USA) and R version 1.0.0 (Statistical program for health science researches, freeware). Prior to the studies, the optimal number of participants for an estimate of the prevalence of *C. pneumoniae* infection in each study group was calculated: With 80% power, a significance level of 5% and an estimated prevalence of 5% among patients with acute respiratory tract infections and of 15 % among the patients with chronic pulmonary disease, the number of patients in each category should be 160. The categories being patients and controls in study A and patients with pneumonia, patients with infectious exacerbation and patients without pneumonia or infectious exacerbation in study B.

For comparison between groups, χ^2 or Fisher's exact test was used. Logistic regression analyses were used to examine whether the levels of *C. pneumoniae* IgG and IgA antibodies were associated with selected clinical and paraclinical parameters. Association between treatment with systemic steroids and the level of serum IgG concentration was assessed with a t-test. The association between the levels of *C. pneumoniae* IgG and serum IgG concentration was assessed by Spearman's rank correlation test. $P < 0.05$ was considered significant. 95% confidence intervals (CI) for prevalences were calculated as:

The proportion (p) $\pm 2\sqrt{p(1-p)/n}$ (normal approximation).

The studies were approved by the Scientific Ethical Committee for Copenhagen and Frederiksberg municipalities. The study of patients seen by GP's was further approved by Multipraksisudvalget and supported by the Municipal Health Service, which covered the cost of extra consultations due to the study. Written informed consent was obtained from all participants.

STUDY A: THE PREVALENCE OF *CHLAMYDIA PNEUMONIAE* IN PATIENTS WITH COMMUNITY-ACQUIRED ACUTE RESPIRATORY TRACT INFECTION

Study population. Patients ≥ 18 years of age with symptoms of acute respiratory tract infection consulting 20 GPs in the Municipality of Copenhagen from November 1999 through September 2000 were enrolled. Controls were persons seeing the GP without having symptoms of respiratory tract diseases, matched according to gender and age. According to the protocol the patients should be seen three times: day 0, 14 and 36. On each occasion blood samples, nasopharyngeal swabs and if possible sputum were collected, sent by mail to Statens Serum Institut and received at the laboratory the next day. On day 0 and day 14 structured questionnaires were completed by the GP, including questions regarding symptoms, clinical diagnosis and antibiotic treatment (appendix 2).
Examination of controls followed the same procedures as those for patients on day 0, but follow-up visits were not included.

Results

Patients. A total of 112 patients were enrolled in the study (44 men, median age 37 years and 68 women, median age 46 years) and 45 controls (18 men, median age 41 years and 27 women, median age 45 years). A total of 69 % of the participants were recruited during the first three months of the study. Of the 112 patients 64 (57%) were seen three times, 20 (18%) were seen twice and 28 (25%) failed to return for follow-up visits. The number of patients and especially the number of controls enrolled by each Practitioner was lower than expected, thus the optimal number of subjects was not enrolled.

The questionnaire survey. The patients were included in the survey as long as the name and the ID of the patient was given. Table 19 shows selected data from the first questionnaire survey comprising 94 (84%) out of 112 patients included. At the first visit the dominating symptoms and signs reported were cough, fever, dyspnoea, catarrhal and hoarseness. The most common clinical diagnoses included bronchitis acuta, non-specified respiratory tract infection, pneumonia, upper respiratory tract infection, catarrhalia, influenza and asthma. Within 8 weeks prior to

enrolment 14 of 90 patients reported having received antibiotics: penicillin (7), macrolides (3) and sulfamethizol (1); for the last three patients no information about choice of antibiotics was given. Antibiotic therapy was initiated among 26 of 90 patients: penicillin (9) and macrolides (17). Table 20 shows selected data from the 77 (69%) of 112 questionnaires that were received from the second visit. At the second visit 43 of 77 patients (56%) reported that they were not completely recovered. Six of 75 patients were treated with local steroids. One patient with non-specified respiratory tract infection was admitted to hospital.

Aetiological diagnosis. Table 21 shows results of microbiological and serological analyses for six patients in whom an aetiological diagnosis was made. Two patients had *C. pneumoniae*, two had *M. pneumoniae* and two had *B. pertussis* infections. The two patients with *C. pneumoniae* infection had IgM antibodies demonstrated by the MIF test, both at levels of ≥ 1024 in all three sera and both had, as the only two patients in the study, a fourfold rise in *C. pneumoniae* IgG antibody titres. One had significant increase in titres by the CF test and *C. pneumoniae* was demonstrated by culture as well as by PCR. From the other patient *C. pneumoniae* was not isolated; this patient had been treated with penicillin four weeks prior to enrolment. In two patients *M. pneumoniae* and in another two patients *B. pertussis* were detected by PCR. One of the patients with a positive *B. pertussis* result by PCR had a fourfold titre rise in the *M. pneumoniae* CF test from titre of ≤ 16 to titre 64 indicating an acute *M. pneumoniae* infection. No other patients had serological test results indicating an acute *M. pneumoniae* infection. None of the patients had serological evidence of *Legionella* infection. The two patients with *M. pneumoniae* infection verified by PCR did not return for follow up visits, thus it is not known whether they developed antibodies detectable by the *M. pneumoniae* CF test later during the course of illness.

Table 22 describes the clinical features of the six patients. None of the six patients were hospitalised, however the four patients with *C. pneumoniae* or *B. pertussis* infection all reported at day 14 that they had not yet recovered. Overall, about 50% of those answering the second questionnaire reported to be recovered at day 14. Two patients, one with *C. pneumoniae* infection and one with *B. pertussis* infection had seen their GP about four weeks prior to enrolment in this study with symptoms of respiratory tract infection, indicating that the symptoms of the pre-

sent infection may have lasted for more than six weeks. One of the two patients with *C. pneumoniae* infection reported symptoms of acute arthralgia during the course of illness.

Antibody profiles. IgM antibodies were seen in 2 of 112 patients (2%). Overall, the prevalence of IgG antibody titres of ≥ 16 was 59% (CI: 50-68%) and the prevalence of IgG antibody titres of ≥ 64 was 40% (CI: 31-49%), (table 23). The distribution curve according to IgG antibody titres showed a peak at titre of 64, (figure 2). A logistic regression analysis in which, risk factors suspected of influencing the IgG antibody titres served as independent variables and the presence of *C. pneumoniae* IgG antibodies of ≥ 64 as the dependent variables. The following independent variables were selected: Being patient or control, age, sex and serum IgG concentration. Age and serum IgG concentration were coded as continues variables; sex and being patient or control were coded as dichotomous categorical variables. No significant correlation between the presence of *C. pneumoniae* IgG antibodies of ≥ 64 and any of these variables was found.

The prevalence of IgA antibody titres of ≥ 16 was 34% (CI: 25-43%) and the prevalence of IgA antibody titres of ≥ 32 was 21% (CI: 13-29%), (table 23). The distribution curve according to IgA antibody titres showed a peak at titre 64 (figure 3). A similar logistic regression analysis as the one above was performed with the presence of IgA antibody titres ≥ 16 as the dependent variable and same independent variables as above. The only significant association found was between the presence of IgA antibodies and advanced age (P = 0.012 corresponding to Odds Ratio of 1.041 per year (CI: 1.009 – 1.074)).

Among the 106 patients in whom an aetiological diagnosis were not achieved *C. pneumoniae* IgM antibodies were not demonstrated and in sera from the 82 patients from whom more than one serum were obtained no significant changes in *C. pneumoniae* IgG or IgA antibody titres were found. 10 patients (9%) had IgG antibody titre of ≥ 512 at their first and at all subsequent visits and thus fulfilled one of the Grayston criteria of acute infection (table 1); none of the remaining patients had developed IgG antibody titres of ≥ 512 at the follow-up visits. Of the 10 patients 4 were men, median age 45 years old (27-67 years old) and 6 were women, median age 45 years old (25-75 years old). The patients with IgG antibody titres of ≥ 512 reported to have had symptoms of acute respiratory tract infection 4-21 days

(mean 8 days), whereas the patients with IgG antibody titres of ≤ 256 had had symptoms for 1-60 days (mean 9 days).
The criterion of previous infection was fulfilled in 53 (50%) and the criterion of chronic infection was fulfilled in 17 patients (16%). Of the latter 17 patients 7 had IgG antibody titres of ≥ 512 and thus fulfilled the Grayston criteria of acute infection and the remaining 10 patients fulfilled the Grayston criterion of previous infection.

Controls. Of the 45 persons recruited as controls, 16 (36%) had no physical complaints. Within the latter group the most common reasons for seeing the GP were to accompany children or to receive health checks/certificates. The reason for the consultation for six controls was pain in head, shoulder or back. The remaining controls had a variety of non- respiratory tract symptoms. One patient had had antibiotic treatment (tetracycline) within 8 weeks prior to enrolment but none received prescriptions of antibiotics in connection with the consultation, at which they were enrolled.
None of the controls had "atypical respiratory tract pathogens" demonstrated by culture or PCR. The controls neither had chlamydial CF titres indicative of acute infections or *C. pneumoniae* IgM antibodies, nor serological evidence of infections with *M. pneumoniae* and *Legionella.*
The prevalence of *C. pneumoniae* IgG antibody titres of ≥ 16 was 62% (CI: 48-76%) and 31% had titre of ≥ 64 (CI: 17-45%), (figure 2 and table 23). The prevalence of *C. pneumoniae* IgA antibody titres of ≥ 16 was 36% (CI: 22-50%) and 15% (CI: 8-22%) for IgA antibody titres of ≥ 32 (figure 3).
One control (2%) had *C. pneumoniae* IgG antibodies at 512, indicating an acute infection according to the Grayston criteria. This control was a healthy woman accompanying her child to the GP. Previous infection as defined by the Grayston criteria was seen in 27 controls (60%). The criterion of chronic infection was fulfilled in 8 controls (18%); among these one had IgG antibody titre at 512 and the remaining 7 fulfilled the criterion of previous infection. None of the eight controls fulfilling the criterion of chronic infection reported to have cardio-pulmonary diseases.

Conclusion

In the present study of adults presenting themselves to the GP with symptoms of acute respiratory tract infections, six patients obtained an aetiological diagnosis: Two patients had *C. pneumoniae* infection (2%), two had *B. pertussis* infection (2%) and two had *M. pneumoniae* infection (2%).

The two patients with acute *C. pneumoniae* infection both had high-level IgM antibody titres and fourfold rise of IgG antibody titres. Both patients had developed *C. pneumoniae* IgM antibodies at the first visit to the GP and both had fever and coughs and one of them reported to have arthralgia. Further 10 patients (9%) and 1 control (2%) had *C. pneumoniae* IgG antibody titres of ≥ 512 and thus met one of the Grayston criteria for acute *C. pneumoniae* infection[60]; the observed difference between patients and controls was not significant (P=0.18, Fishers test). However, it is striking that these patients did neither show any change of antibody titres during the 36 days of observation nor had any positive test results by culture or PCR, although they reported to have had respiratory tract symptoms on the average eight days before enrolment in the study and thus should be in an early phase of acute infection.

No significant differences between patients and controls in the prevalences or the distribution of IgG or IgA antibodies were observed. No correlation between *C. pneumoniae* IgG antibody titres of ≥ 64 and age, sex or serum IgG concentration was seen, in contrast IgA antibody titres of ≥ 16 were correlated to advanced age, but to none of the other parameters.

The overall prevalence of *C. pneumoniae* IgG antibody titres of ≥ 16 for patients and controls together was 59%, including 37% with titres of ≥ 64; 7% had IgG antibody titre of ≥ 512 and 25% fulfilled the criteria of chronic infection shown in table 1.

No significant differences were seen between the percentages of patients and of controls fulfilling the criteria of chronic *C. pneumoniae* infection or between the prevalences of *C. pneumoniae* IgG antibodies.

STUDY B: PREVALENCE OF *CHLAMYDIA PNEUMONIAE* IN HOSPITALISED PATIENTS AND PATIENTS SEEN AT AN OUTPATIENT CLINIC

Study population

From October 1998 through December 1999 patients were enrolled either from the out-patients' clinic for respiratory diseases or within 48 hours after admittance to the Department of Internal Medicine, Bispebjerg Hospital, Copenhagen, Denmark. Inclusion criteria were COPD or symptoms of acute respiratory tract infection. The patients were seen three times: day 0, 14 and 36. On day 0 white blood cell count and CRP were measured, sputum was collected for culture of respiratory tract pathogens, and a chest radiograph was performed. From the hospitalised patients a urine sample was collected. Day 0 and day 36 the lung function was measured and a questionnaire was filled out (appendix 2). The questionnaire included enquiries regarding smoking, treatment with glucocorticoids, treatment with antibiotics within the last eight weeks prior to enrolment in the study and within the study period. Each time blood samples, nasopharyngeal swabs and if possible, sputum specimens were collected. Each patient could only participate in the study once.

Definition and classification of the severity of COPD followed the recommendations from European Respiratory Society[137]: Patients had COPD if the ratio: FEV^1/VC was < 0.88 of predicted in men or was < 0.89 of predicted in women (i.e. >1.64 residual standard deviation below predicted value). The severity of COPD was assessed by the FEV^1 in relation to reference values: FEV^1 x 100/predicted FEV^1: mild COPD ≥ 70%, moderate COPD 50-69% and severe COPD < 50%. In order to minimize the risk of classifying the patients on the basis of a non-permanent reduction in lung function due to acute infection, the highest FEV^1 value of the two lung function measurements (in most cases the second) was used for the classification.

The patients were also classified according to the clinical and paraclinical findings indicative for the presence or absence of actual infection: Group I, pneumonia verified by a chest radiograph. Group II, infectious exacerbation i.e. the patients

fulfilled at least two out of three criteria: Fever > 38° C, white blood cell count > 9.0 10^9/L or CRP > 10 mg/L. Group III comprised patients that did not fulfil the criteria set up for group I and II (without infection).

Results

Study population. A total of 210 patients were enrolled in the study: 76 men, 36-89 years old (median 68 years) and 134 women, 30-88 years old (median 70 years). Of the 210 patients 117 were recruited from the out-patient clinic and 93 were recruited from the department of internal medicine. The first questionnaire was completed for all 210 patients and the second was filled in for 199 patients. Blood samples and nasopharyngeal swabs were collected from 210, 175 and 164 patients from day 0, 14 and 36, respectively. The first months of the study-period the daily enrolment met the expectations, but thereafter many of the eligible patients admitted to the department or seen at the out-patient clinic were already enrolled in the project.

COPD. Out of 210 patients 159 (76%) fulfilled the criterion for COPD. Among these 11% had mild, 20% had moderate and 69% had severe COPD (figure 4). Of the remaining 51 patients, 85% had a FEV^1 below 70% of expected FEV^1, indicating that, though not fulfilling the criterion of COPD, they had a reduced lung function (figure 5). The clinical diagnoses for these 51 patients were stable COPD (16 patients), exacerbation of COPD (15 patients) and other (20 patients). Thus the majority of patients included in the study had COPD or reduced lung function without obstruction at a level sufficient for the diagnosis of COPD. The patients without COPD or other underlying diseases were often discharged from the department so early that enrolment in the project was not possible.

The questionnaire survey. Table 24 shows selected data from the first questionnaire survey from 93 hospitalised patients and 117 patients from the outpatient clinic included in the study. The hospitalised patients had a higher frequency of symptoms related to infections of the respiratory tract than did the patients enrolled from the outpatient clinic. The majority of the former patients had either exacerbation of their COPD or pneumoniae, whereas most of the patients from the outpatient clinic had COPD without exacerbation. Antibiotic treatment within the eight weeks prior to enrolment had been received by 61 and 44%, respectively. A

higher proportion of the hospitalised patients was current smokers and had cardiovascular and other chronically diseases compared to the patients from the outpatient clinic. Table 25 presents selected data from the second questionnaire survey for the 91 patients admitted to the department of internal medicine and the 108 patients seen at the outpatient clinic for whom the questionnaire was filled out. About one-third in both groups received treatment with systemic steroids during the six weeks study period, whereas antibiotics were received by one third and one fifth, respectively of the groups.

Classification according to clinical and paraclinical findings. The number of patients in each group according to clinical and paraclinical findings was group I (pneumonia): 39 (19%), group II (infectious exacerbation): 52 (25%) and group III (without infection): 119 (57%), (table 26). In group I and II a significantly higher proportion of the patients had fever, leucocytosis and elevated CRP than in group III. However, these three parameters were used in the classification of infectious exacerbation. No significant differences were found between the three groups according to systemic treatment of glycocorticoids, smoking within the last year or severe COPD.

Microbiological results. "Atypical respiratory tract pathogens" were found in three patients (table 26). . In a patient with pneumonia, *M. pneumoniae* infection was diagnosed by a positive PCR result and by the following results in the *M. pneumoniae* CF test: titre 2048, titre 4096 and titre 1024 for the three consecutive samples respectively. No other patients had serological evidence of *M. pneumoniae* infection. One patient had psittacosis verified by culture and PCR. The following serological test results were obtained: MIF assay: negative for *C. psittaci* antibodies in all three samples, *C. pneumoniae* IgG antibodies at 512, 512 and 1024 respectively, negative for *C. pneumoniae* IgM antibodies. CF test: negative, titre 108 and titre 36 respectively, which is consistent with infection caused by *C. psittaci* as well as *C. pneumoniae*. The clinical diagnosis for this patient was febrile exacerbation of COPD. In a patient without signs of acute infection *B. pertussis* was detected by PCR. None had microbiological or serological evidence of *L. pneumophila* infection.

From 76 patients microscopy and culture of sputum were performed; 22 samples showed growth of potentially pathogenic bacteria: *Haemophillus influenzae* (10),

Streptococcus pneumoniae (10) and *Moraxella catarrhalis* (2). The bacteria were found in 5 of 39 patients with pneumonia, in 8 of 52 patients with infectious exacerbation and in 9 of 119 patients with no infection (P=0.35). Out of the ten patients with *S. pneumoniae* demonstrated in sputum, only two had had urine analysed for pneumococcal antigen. Of these two patients one had a positive test result. Overall pneumococcal antigen was detected in further 2 of 40 urine samples tested.

Serological evidence of *C. pneumoniae* infection. None of the patients had IgM antibodies. Except in one patient no significant differences between titre levels of IgG or IgA antibodies were seen in the 1., 2., and 3. blood samples. One patient had a rise of IgA titres from 16 to 64 and IgG titres at 256 and 512. The patient with psittacosis was the only one with significant rise of titres in the CF test. All calculations described below were based on results from the first blood sample.

Antibody profiles. Overall, the prevalence of IgG antibody titres of ≥ 16 was 70% (CI: 64-76%) and the prevalence of IgG antibody titres of ≥ 64 was 62% (CI: 55-69%), (table 23). The distribution curve according to IgG antibody titres showed a peak at 256 (figure 6). The prevalence of IgA antibody titres of ≥ 16 was 51% (CI: 44-58%) and 43 % (CI: 36-50%) had IgA antibody titres ≥ 32 (table 23), with the distribution curve according to IgA antibody titres showing a peak at 32 (figure 7). No patients had *C. pneumoniae* IgM antibodies or a fourfold rise of IgG antibody titres. Of the 210 patients 43 (20%) had *C. pneumoniae* IgG antibody titres of ≥ 512 and thus fulfilled the criteria of acute *C. pneumoniae* infection[58;60]. No significant differences were seen between the three groups (table 27) though IgG antibody titres of ≥ 512 were found in 13% of patients in group III and in 28% of patients in group I (P=0.25). In total, 28% of the men and 16% of the women had IgG antibody titres of ≥ 512 (P=0.13). For the 27 patients with test results indicating an aetiological diagnosis other than *C. pneumoniae* infection (by demonstration of possible pathogenic bacteria in sputum, pneumococcal antigen in urine or "atypical respiratory tract pathogen" in nasopharyngeal swabs) 10 (37%) had *C. pneumoniae* IgG antibody titres of ≥ 512; of the 183 patients without an aetiological diagnosis 33 (18%) had IgG antibody titres of ≥ 512. The difference between the

prevalences of IgG of ≥ 512 among the patients with an aetiological diagnosis and the patients without and aetiological diagnosis was not significant (P=0.13).
IgG antibody titres 16-256 indicating previous infection (table 1) was seen in sera from 106 patients (50%). No significant differences were demonstrated between the three groups (table 27).
The criterion of chronic infection (IgG of ≥ 128 and IgA of ≥ 32) was met by 77/210 (37%) of the patients. However, 37 (48%) of the 77 patients had IgG antibody titre of ≥ 512, thus they would be categorized as acute infection according to one of the Grayston criteria. The remaining 40 patients would be categorized as previous infection according to the Grayston criteria.
There were no significant differences in the percentages of patients from the three groups fulfilling the criteria of chronic infection (table 27)
Table 28 shows the results of a logistic regression analysis in which, risk factors suspected of influencing the IgG antibody titres served as independent variables and three levels of IgG antibodies (1. IgG ≤ 64, 2. IgG 128 – 256, 3. IgG ≥ 512) as the dependent variables. The following independent variables were selected: +/- COPD, degree of COPD, outpatient/hospitalised patient, pneumonia (group I)/infectious exacerbation (group II)/without infection (group III), age, sex, smoking (+/- smoking within the last year), antibiotics within last eight weeks, treatment with systemic steroids, serum IgG concentration, leucocytosis, +/- elevated level of CRP. Age, degree of COPD and serum IgG concentration were coded as continues variables. The remaining variables were coded as dichotomous categorical variables. Age and serum IgG concentration showed a positive correlation with the level of IgG antibodies and treatment with systemic steroids showed a negative correlation. No significant correlation was found between treatment with systemic steroids and the level of serum IgG concentration (P=0.115, (t-test)). A similar logistic regression analysis as the one above was performed with the presence of IgA antibody titres of ≥ 16 as the dependent variable and same independent variables as above. None of the parameters showed a significant correlation with the presence of IgA antibodies.

***C. pneumoniae* IgG antibody titres and serum IgG concentration.** The correlation between the amount of total serum IgG and *C. pneumoniae* IgG antibody titre levels was also assessed by Spearman's rank correlation coefficient (figure 8) and

a significant positive correlation was found ($P < 0.001$), indicating that a high level of specific IgG antibodies was associated with a high level of total IgG.

***C. pneumoniae* IgG antibody titres and CRP.** The amount of CRP was assessed according to *C. pneumoniae* IgG titre levels (figure 9) and a non-significant positive correlation was found ($P = 0.08$, Spearman's rank correlation coefficient), indicating that a high level of *C. pneumoniae* IgG antibodies might be associated with an ongoing infection. There was no significant correlation between the concentration of CRP and serum IgG concentration.

Conclusion

The majority of the 210 patients investigated had severe reduction of their lung function. One fifth of the patients had pneumonia, one fourth had infectious exacerbation, while the remaining patients did not fulfil the criteria set up for current infection. None of the patients had microbiological or serological evidence of acute *C. pneumoniae* infection, except that 20% had an isolated *C. pneumoniae* IgG antibody titre of ≥ 512. In three patients other atypical respiratory tract pathogens were detected. The prevalences of *C. pneumoniae* IgM, IgG and IgA antibody titres of ≥ 16 were zero, 72 and 52%, respectively. Half of the patients fulfilling the criteria of chronic infection had IgG antibody titres ≥ 512. No correlation between antibody level and lung function was seen and no significant differences in the prevalences of antibodies or in the percentages of patients fulfilling the criteria of chronic infection among patients with and those without infection was found.

A correlation was found between *C. pneumoniae* IgG antibody level and the following variables: serum IgG concentration, treatment with steroid and age, indicating that a high level of specific IgG antibodies was associated with a high level of serum IgG and advanced age, while treatment with systemic steroids tend to lower the level of specific IgG antibodies.

DISCUSSION

The aims of the two clinical studies were to assess the applicability of the serological criteria for the diagnosis of acute and chronic *C. pneumoniae* infections and to describe the prevalence of *C. pneumoniae* infections in two different groups of adult patients: Patients who had contracted a community-acquired acute respiratory tract infection and patients with a chronic respiratory tract disease who might be prone to have a chronic *C. pneumoniae* infection. The results of the two studies are summarized in table 23.

***C. pneumoniae* as a cause of acute respiratory tract infection.** In study A of patients seeing the GP the prevalence of *C. pneumoniae* infection was 2% when using microbiological methods and/or the presence of IgM antibodies and/or fourfold rise of IgG antibody titres as criteria. In a Dutch study of 557 patients who contacted a GP with symptoms of acute respiratory tract infection in 1994/1995, nasopharyngeal swabs were examined by PCR[105]. The prevalences of *C. pneumoniae* and *M. pneumoniae* infections were 1.1% and 1.3% respectively, thus about the same as found in the present study.

A Finish study of 304 adults with community-acquired pneumonia seeing a GP revealed a prevalence of *C. pneumoniae* infection of 10 % by using the same serological criteria as in the present studies [84]. The inclusion criteria in study A were adults seeing a GP with any kinds of symptoms of acute respiratory tract infection. It is possible that the prevalence of *C. pneumoniae* infection had been higher if the inclusion criteria had been restricted to patients with lower respiratory tract infection.

In an Italian multicenter study of 613 hospitalised children with lower respiratory tract infection 87 (14.1%) had *C. pneumoniae* infection[124]. Serological evidence of acute *C. pneumoniae* infection was found in 52 children, however none were shown to have IgM antibodies by the MIF test, 36 had a fourfold rise of IgG titres and 16 had IgG antibody titres of ≥ 512. Of the 52 children 13 had a positive PCR test result but it is not stated which of the serological criteria these children fulfilled. Thus up to 31% (16/52) of the children with serologic evidence of *C. pneumoniae* infection and up to 18% (16/87) of all the children with *C. pneumoniae* infection had been given this diagnosis only because they had IgG antibody titres of ≥ 512. In study A the prevalence of acute *C. pneumoniae* infection in adult pa-

tients would rise from 2 to 11% if the patients with isolated IgG antibody titres of ≥ 512 were included.

In some prevalence studies using IgG antibody titres of ≥ 512 as an isolated criterion of acute infection, the deduction of the percentage of patients only fulfilling this criterion is not possible; thus the comparison between results found by these studies[43;116] and results found by studies in which the criterion is not used[84]is hampered.

Further, in the Italian study *C. pneumoniae* DNA was detected in 36 children without any serological evidence of acute infection. In a total of 8% (49) of the children *C. pneumoniae* DNA was detected by PCR[124]. During 1992-1995 the prevalence of *C. pneumoniae* in Denmark among children with acute respiratory infections was investigated in two studies by detection of *C. pneumoniae* in respiratory tract secretions by culture and PCR[48]. In the first study 427 children (age 0-11 years) with symptoms of respiratory infections were enrolled at a department of paediatrics. In the second study 264 patients and controls (median age 3 years; 3 months – 24 years) were enrolled at their General Practitioner, in both studies the prevalence of *C. pneumoniae* was < 1%[48].

In the present studies, none of the patients showed a fourfold rise of IgG antibodies and none had a specimen tested positive for *C. pneumoniae* by PCR and/or culture without the simultaneous detection of high levels of IgM antibodies.

Chronic respiratory tract disease. The majority of the patients in study B had either severe COPD or severely reduced lung function without having COPD, thus the failure to find a correlation between the level of obstruction and *C. pneumoniae* IgG antibodies might be due to lack of statistical power caused by the low number of patients with mild and moderate lung disease; however, the results are supported by other studies[138;144], in contrast one study demonstrated a positive correlation between IgG antibody titre and the severity of COPD[158]. In the present study definition and classification of the severity of COPD followed the recommendations from European Respiratory Society[137]; however recently the Global Initiative for Chronic Obstructive Lung Disease (GOLD) has published their recommendations for definition and classification of the severity of COPD (www.goldcopd.com). The latter recommendations would have been used in the present study, had they been launched earlier.

In study B none of the patients had acute *C. pneumoniae* infection when the IgG titres of ≥ 512 as an isolated criterion was omitted. This result is in agreement with other studies that have concluded that acute *C. pneumoniae* infection is rare in COPD patients[7;20;155].

However, 20% of the patients had *C. pneumoniae* IgG antibody titres of ≥ 512 as opposed to 10% of the patients from study A and 2% of the controls (P=0.006), (table 23). The question is whether the high level of *C. pneumoniae* IgG is a sign of acute infection or whether it might be due to a raised antibody level in general in patients with chronic diseases. With no significant differences between *C. pneumoniae* IgG antibody titre levels among the two groups with acute infections and the group with no infection as defined by clinical and paraclinical parameters and with no further evidence of acute *C. pneumoniae* infection, it seemed unlikely that theses patients actually had *C. pneumoniae* infection. Furthermore, IgG antibody titres of ≥ 512 as a criterion of acute infection have previously been questioned by several authors[53;80;87]. Still, the level of CRP and the *C. pneumoniae* IgG antibody levels showed a tendency towards a positive correlation, however this was not statistical significant.

When classifying the patients according to the clinical and paraclinical findings previously indicated, possible confounding factors should be considered. Treatment with glucocorticoids tends to increase the white cell count and lower temperature and the level of CRP. Further such classification based on symptoms and paraclinical findings of infection might not be the ideal tool to identify groups of patients with *C. pneumoniae* infection. Miyashita et al have described the clinical presentation of patients with community-acquired pneumonia caused by *C. pneumoniae*[108]. For the 40 patients, in whom *C. pneumoniae* was the only pathogen found to cause the infection (= culture positive or fourfold rise in antibody titre level), the mean WBC and mean CRP level was just borderline elevated and the mean temperature was 37.9 ± 1.0 °C. However, elevated WBC counts have previously been reported in association with *C. pneumoniae* pneumonia[86].

In study B there was a highly significant positive correlation between *C. pneumoniae* IgG antibody titre levels and the amount of total IgG, indicating that patients with chronic infections (or frequent infections) such as COPD patients have raised levels of specific antibodies as a result of increased total IgG. We have demonstrated the same positive correlation in a small study of patients with endocarditis,

in whom an aetiological diagnosis other than *C. pneumoniae* was demonstrated in the majority of the patients[11].

It could be argued that the reason for not detecting the bacteria was that COPD patients are often treated with antibiotics and glucocorticoids. However, the patients are in general only treated with antibiotics when they have an exacerbation of their symptoms, that is considered to be caused by an infection and even in that case, it is possible to detect the bacteria by PCR analyses days to weeks after the onset of the treatment. In contrast many patients with severe COPD are treated constantly with glucocorticoids. In vitro experiments have shown that glucocorticoids enhance the growth of *C. pneumoniae*[160], so this treatment should not hinder the isolation of the bacteria from the patients.

Preexisting antibody profiles. The serological criterion of chronic *C. pneumoniae* infection (table 1) was fulfilled by 16%, 37% and 18% of the study A patients, study B patients and controls respectively (table 23). This difference was highly significant (P= 0.004). When excluding the sera with IgG antibody titres of ≥ 512, the criterion of chronic infection was no longer significant (P= 0.12). Since there was no difference between the three groups in the prevalences of IgG antibodies, the main difference in the antibody profiles was the raised level of IgG antibodies in the study B patients, i.e. in patients with frequent or chronic infections.

Whether the presence of *C. pneumoniae* IgA antibodies can be used as a marker of chronic infection, is questionable. Although it is supported by some groups[128;158] others do not believe there is any evidence to support this hypothesis[162]. A major problem is the lack of clinical definitions or signs of chronic *C. pneumoniae* infection, that could help in distinguishing the patients suspected of chronic infection from those with previous infection.

In study B there was no differences in the prevalence or in the level of IgA antibodies between the three groups (table 27), however the study B patients had a higher prevalence of IgA than the patients from study A, though no difference was seen in titre levels (figure 3 and 7). This is in contrast to another study of patients with either pneumonia, severe or mild COPD, in whom only small differences in *C. pneumoniae* IgG antibody levels were found, whereas the differences in serum IgA antibody levels were highly significant[158]. One study examined sera from 219 patients with COPD admitted to a department of internal medicine and 100 controls[100] and found a highly significant difference between patients and

controls in the prevalences of *C. pneumoniae* IgG and IgA antibodies, but like the present study the antibody prevalences were not correlated to the severity of airway obstruction. In that study IgG and the IgA antibodies were analysed in two-fold titrations beginning at 1:64 and 1:32, respectively, whereas in the present studies A and B the initial dilution step applied was 1:16 for both antibody classes. In the present studies, the difference in the prevalences of IgG antibody titres ≥ 64 and IgA antibody titres ≥ 32 among patients from study A, B and the controls were significant (table 23). This example illustrates a problem when prevalences in study groups with different distribution curves are compared. The prevalence found is influenced by the initial dilution step chosen, especially, for groups in which the majority of subjects has low level seropositivity as often seen in control groups, whereas the prevalence among groups of patients with a high level seropositivity as seen among the patients in study B is less influenced by a high initial dilution step. Therefore, what is seen as a difference in prevalence might just be a difference in antibody titre levels.

Acute infection with other "atypical respiratory tract pathogens". *M. pneumoniae* were found in two patients (2%) in study A. Although a *M. pneumoniae* epidemic occurred in Denmark during the first months of study B[83] only one patient in this study where shown to have *M. pneumoniae* (<1%), indicating that this infection is rare in older patients with chronic respiratory diseases.

B. pertussis were likewise demonstrated in two patients (2%) in study A and in one patient (<1%) in study B. The number of participants was too low to estimate the actual prevalences of these pathogens in patients consulting the GP. However, it is noteworthy that whooping cough is an illness to be considered among adults. The patient in study B had COPD in a stable phase and acute infection was not suspected. That *B. pertussis* can be found among adult patients has been confirmed by other studies[28;170]. Recently, a Danish study of 201 adult patients with cough for more than three weeks, showed that 7% had *B. pertussis* infection verified by culture or PCR[14].

C. psittaci was demonstrated in one patient in study B and in none of the patients in study A. Ornithosis is a rare disease in Denmark with about 30 cases reported per year. However, it is to be suspected that cases with a milder course of the dis-

ease tend to be overlooked, thus the true incidence might be higher than that reported[29].

None of the patients had serological or microbiological signs of Legionella infection. The concern about contamination of pharyngeal swabs or sputum with *L. pneumophila* from tap water showed to be unfounded as the bacteria was not detected by PCR in any of the over 1.000 samples from the 367 patients participating in the two studies.

Conclusion. The assumption that 5 and 15% of the patients in the two studies respectively, should have an acute *C. pneumoniae* infection was not met and the number of subjects enrolled in the two studies was much lower than planned. However, the material is still sufficiently large to conclude that in Denmark, acute *C. pneumoniae* infection is uncommon among patients with symptoms of acute respiratory infection and very rare among patients with chronic pulmonary disease. However, if the criterion of *C. pneumoniae* IgG of ≥ 512 was used as a marker of acute infection, 20% of the study B patients and 10% of the GP patients would have had an acute *C. pneumoniae* infection. Using the serological criterion of chronic infection, 37% and 16% of the patients, respectively would have had chronic *C. pneumoniae* infection but lacking any other evidence of *C. pneumoniae* infection the diagnosis seemed unlikely; especially in the patients and controls seeing the GP.
Microbiological results were in agreement with serological results as the bacteria was not isolated from any patients that did not have an antibody profile indicating acute *C. pneumoniae* infection.

CHAPTER FIVE: GENERAL DISCUSSION

Epidemiology

During the last decade several Danish studies on the prevalence of either *C. pneumoniae* antibodies and of acute *C. pneumoniae* infection has been performed. Except for the two early studies by Mordhorst et al[113;165] all serological analyses for the detection of *C. pneumoniae* antibodies in the Danish studies mentioned in this chapter were conducted in the Neisseria Unit, SSI and if no other information is given by the WRF assay. The results are comparable since no interassay or interlaboratory variation of test results has to be considered.

Seroepidemiological population studies. In a Danish study of 106 healthy blood donors about 40 years old (± 10 years) the prevalence of *C. pneumoniae* IgG antibody titres of ≥ 64 was 40% and *C. pneumoniae* IgG antibody titres of ≥ 512 were found in 1% [13]. No significant change in *C. pneumoniae* antibody titre levels between the two visits was seen when the donors were retested four months later. This study along with study described in chapter one[165] showed that the prevalence of *C. pneumoniae* IgG antibodies in the Danish population is high, which is consistent with findings from other countries[19;165]. That a high prevalence would be associated with frequent *C. pneumoniae* infections seems to be a reasonable working hypothesis. Thus it should be possible to encircle groups of patients in whom the incidence of *C. pneumoniae* infection is high.

Prevalence of acute *C. pneumoniae* infection.

Seroepidemiological studies have indicated, that an epidemic of *C. pneumoniae* infections occurred in Denmark in 1980-1982[63]. Mordhorst et al, and Farholt and Hansen each described a family outbreak of *C. pneumoniae* infection but beyond this, mainly sporadic cases have been reported[49;112].

Microbiological studies. During 1992-1995 the prevalence of *C. pneumoniae* among children with acute respiratory infections was investigated in two studies by detection of *C. pneumoniae* in respiratory tract secretions by culture and PCR[48]. In total 691 children were examined and *C. pneumoniae* was found in < 1%.

Combined microbiological and serological studies. The present studies A and B: As only two patients in study A and none of the study B patients were shown to have acute *C. pneumoniae* infection, we conclude that the incidence of acute *C. pneumoniae* infection was low among Danish adult patients with acute or chronic respiratory tract infections.
In group II of the main laboratory study of comparing the performance of different MIF assays, eight patients with respiratory tract infection had *C. pneumoniae* infection verified by a positive PCR result. In addition two patients from the study A had acute *C. pneumoniae* infection (table 13 and 21). It is interesting that nine out of ten patients had *C. pneumoniae* IgM antibodies at the first contact with the GP that elicited taking of specimens for *C. pneumoniae* analyses. Since detectable IgM antibodies emerge after about two weeks, it is an indication of symptoms of *C. pneumoniae* infections being slow in developing. This is in contrast to *C. psittaci* infections, that develop more rapidly, these patients are often *C. psittaci* IgM antibody negative when they first seek medical assistance[10].

In a study of 201 patients with cough for more than three weeks the prevalence of *C. pneumoniae* infection was shown to be 3 of 201 (1.5%)[13]. All three were confirmed by PCR and were shown to have *C. pneumoniae* IgM antibodies by MIF. Further six patients had persistent IgG antibody titres of ≥ 512, none of these had a positive PCR result.

In a study of 67 consecutive patients with community-acquired pneumonia admitted to a hospital during 1997–1998 bronchoalveolar lavage was performed on all patients as well as blood samples were obtained[90]. The obtained liquid was subsequently examined by PCR for the detection of *Chlamydia* species. *C. pneumoniae* DNA was not detected in any of the specimens but one patient had *C. psittaci* DNA. Sera were analysed by *Chlamydia* CF test and by MIF test. None of the patients had serological evidence of *C. pneumoniae* infection by the MIF test but two patients had a significant rise of CF titre including the patient with ornithosis.

In aggregate, the three Danish studies, including the present, using a combination of microbiological and serological methods for the assessment of the prevalence of acute *C. pneumoniae* infection demonstrated a very low prevalence (0-2%) of

acute infection. Further, a high correlation between the detection of *C. pneumoniae* DNA by PCR and the detection of *C. pneumoniae* IgM by MIF test was found.

Possible chronic *C. pneumoniae* infection.

Patients with reduced lung function (COPD). In the second clinical study the prevalence of *C. pneumoniae* infections among hospitalised patients and outpatients, the majority of patients included had COPD or reduced lung function, was assessed. COPD is a lifelong disease with significant morbidity. Smoking is the principal factor in the development of COPD, however as most smokers do not develop the disease there might be additional factors participating in the development of pulmonary obstruction[136;160]. Though widely studied, the role of recurrent infections in the aetiopathogenesis of COPD has remained controversial. The emphasis of these studies has been on opportunistic pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* but no clear role has been found for these bacteria in the pathogenesis of COPD[136].

C. pneumoniae differs from the agents conventionally associated with exacerbations in that it is an intracellular organism that does not belong to the normal nasopharyngeal flora. *C. pneumoniae* infections in COPD patients might amplify smoking- associated inflammation and worsen irreversible obstruction.

Theoretically, one could distinguish between acute *C. pneumoniae* infections causing exacerbations of COPD and chronic infections involved in the pathogenesis of the disease. Considerable data suggest that *C. pneumoniae* might be associated with chronic diseases[20;128;159] and serological evidence of *C. pneumoniae* infection have been reported in 7-63% of patients with COPD [20;110].

It is well established that chronic or recurring *C. trachomatis* infections can cause scarring of eyes (trachoma) and of the Falloppian tubes (salpingitis) leading to blindness[169] or infertility[132]; therefore it seems plausible that *C. pneumoniae* might be involved in similar processes in the lungs.

It has previously been reported that 4% of exacerbations may be associated with *C. pneumoniae* infection[20]. However, among the 210 study B patients including 81 with acute infection none had serological signs of acute *C. pneumoniae* infection and in none of the patients were *C. pneumoniae* detected by microbiological methods.

Patients with cardiovascular diseases. Since Saikku in 1988 demonstrated serological evidence of an association of *C. pneumoniae* with chronic coronary heart disease and acute myocardial infarction[128], several studies have confirmed this association[37].

We investigated the prevalence of *C. pneumoniae* antibodies among 830 patients with ischaemic heart disease (IHD) selected from a larger study [76] and matched according to age and gender to the study B patients (unpublished data). IgG antibody titres of ≥ 512 was measured in 7% of the 830 IHD patients (figure 10) in contrast to 20% of the patients in study B ($P< 0.001$), although the same percentage (70-72%) showed the presence of IgG antibody titres of ≥ 16. There was no difference in IgA antibody level between the two groups (figure 11). The criterion of chronic infection was met in 141/631 (22%) of the IHD patients in contrast to 77/210 (37%) in the present study ($P=0.003$). Figure 10 shows the distribution of *C. pneumoniae* IgG antibodies in the patients and controls of the two present studies and the IHD study. Although the prevalence of IgG antibodies in the study B patients were equal to that of the IHD patients and only slightly higher than the GP-patients, the distribution curves according to IgG antibody titres showed a peak at 256, 128 and 64 respectively for the three groups indicating that the level of IgG antibodies is raised in patients with chronic or recurrent infections. This was supported by the significant correlation between specific *C. pneumoniae* IgG antibodies and total serum IgG concentration. In contrast, there were no significant differences in IgA antibody endpoint titres among the study populations as discussed in chapter 4. The IHD study was conducted during the same period and in the same area of the country as the two present studies, thus it is unlikely that the differences were due to local outbreaks of *C. pneumoniae* infection. Another study of patients with cardiovascular diseases showed poor correlation between the detection of *C. pneumoniae* DNA in coronary artery segments analysed by PCR and the detection of *C. pneumoniae* IgG antibodies by MIF[103].

These findings are supported by a study of serum immunoglobulin levels in patients with tuberculosis as a prototype of chronic infection and *Klebsiella* infection representing acute infection[134]. The study demonstrated significantly elevated levels of IgG among the TB-patients compared to the *Klebsiella* patients in contrast, no such difference was found in the IgA levels concluding that these findings reflected the polyclonal hypergammaglobulinaemia found in chronic infections.

In a meta-analyses that pooled 15 prospective studies, Danesh et al found no overall relationship between *C. pneumoniae* IgG seroprevalence and subsequent cardiovascular events after adjusting for age, gender, smoking and socio-economic status[39]. Circulating *C. pneumoniae* DNA has been found in peripheral blood mononuclear cells (PBMC) of patients with cardiovascular diseases[139]. By a meta-analysis of unadjusted casecontrol studies a pooled prevalence of 14.3 % was found in cardiovascular disease patients versus 8.5% in controls corresponding to an odds ratio of 2.03 (CI: 1.34-3.08, $P < 0.001$). However, when the authors added a new study not yet published that adjusted for smoking and season the overall association was no longer significant (OR = 1.6, CI: 0.7-3.5, P=0.22). As described in chapter one, *C. pneumoniae* has been isolated from atherosclerotic plaques, but no correlation between *C. pneumoniae* serology and the detection of DNA in plaques has been found[139]. The question of whether *C. pneumoniae* contributes to atherosclerotic initiation or progression or to cardiovascular events remains so far unanswered.

The diagnosis of *C.* pneumoniae infection

The high prevalence of *C. pneumoniae* infection reported in studies exclusively based on serodiagnostic methods have been questioned in studies based on both serological and microbiological methods.
To assess the rate of *C. pneumoniae* infection both sensitive and specific diagnostic methods are needed. So far, the description of acute *C. pneumoniae* infections has been hampered by a lack of diagnostic methods that fulfil these needs.

Culture. While culture with appropriate identification methods per definition is specific, the sensitivity depends on many factors, including suitable transport conditions for specimens to the laboratory and a skilled handling in the laboratory[48]. A practical obstacle in obtaining specimens for culture is that special *Chlamydia* transportmedia containing fetal calfserum is compulsory and is not always available at the clinical departments. Further only few laboratories have a sensitive culture method since cell lines used for the culture of *C. trachomatis* are not optimal for the culture of *C. pneumoniae*[48].

PCR. As the PCR analyses do not depend on live microorganism, they are less sensitive than culture to variations in the "pre-laboratory" handling of specimen.

Some PCR methods, especially among the nested PCR assays, have a high sensitivity[24]. However, the analysis has a risk of yielding false positive results due to lack of specificity or contamination of samples. Although many in-house PCR methods are in use, neither a standardised method nor commercially available assays for routine diagnostics exist. Earlier this year experience with a commercial PCR assay has been published, though so far it is only recommended for scientific purposes[27]. The specimen collection depends on the type of clinical condition. Suitable specimens for the detection of *C. pneumoniae* DNA include sputum[22], swabs of nasopharynx or throat[22], bronchoalveolar lavage[36] and tissue[145].

Lately several groups have reported on the detection of *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) mainly in patients with cardiovascular disease but also in patients with COPD and blood donors[24]. It remains unclear which cells within the PBMC layer contain *C. pneumoniae*. The two studies of patients with COPD observed prevalences *C. pneumoniae* DNA in PBMC of 24% and 48%, respectively[139]. In none of these two studies were included controls. In a study 15 identical sets with 50 samples each including 23 negative control samples were sent to each of 15 laboratories performing PCR analyses. The samples considered positive contained DNA from cultured *C. pneumoniae* and PBMC from previously PCR positive patients with cardiovascular disease. The levels of sensitivity of the different PCR methods ranged widely and 5 of 17 laboratories reported false positive results[24].

The DNA has also been detected in atherosclerotic plaques however, there is no clear correlation between detection of DNA in plaques and in PBMC nor between DNA in plaques and the presence of *C. pneumoniae* IgG by MIF test[24;139].

In one study the performances of 16 PCR methods in 9 centres in detecting *C. pneumoniae* in carotid vessels was compared[4]. The conclusions were that there was no consistent pattern of positive results among the various laboratories, and there was no correlation between the detection rates and the sensitivity of the assay used.

Without a thorough validation of these methods by the comparison of PCR results with those of a sensitive culture system and at least one other validated PCR method targeting a different gene or a different sequence of the same gene, the PCR results will be less convincing.

CF test. With the genus-specific CF test a large number of sera can be handled with a minimum of manpower and at a low cost. The results of the test can be influenced by the preparation of the antigen used, and by the procedures of the analysis. In the early era of the study of *C. pneumoniae* infections, in several of the major studies of the epidemiology of acute *C. pneumoniae* infections, the CF antigen from SSI was used and generally a high correlation between the CF test results and the presence of *C. pneumoniae* IgM by the MIF test was seen[62;63;113;131;147]. This correlation is also seen in papers published within the recent years[90;122].

Previously in this thesis it was demonstrated that the test was not suited for screening sera prior to IgG antibody analyses by the MIF test. In conclusion, the method is an excellent supplement for the diagnosis of early phase chlamydial infection.

MIF assays. Among the different serological methods the MIF test, though not perfect, is still considered the method of choice for the diagnosis of *C. pneumoniae* infections[40]. Reported differences in the prevalences of *C. pneumoniae* infections in various geographic areas at different periods of time could be due to problems concerning the performance of the MIF test[43;147]. In the present study it was demonstrated that the reproducibility of detection rates between WRF as reference method and the two commercial assays (MRL and LAB) was excellent for the detection of IgM antibodies, for IgG and IgA antibodies in patients without acute infections and acceptable for IgG and IgA antibodies in patients with acute respiratory tract infections. Other factors than the quality of the assays used can influence on results obtained. Apart from the undeniable subjectivity in the reading of MIF slides and differences in laboratory procedures, the results can be influenced by the conjugates used; especially the anti-IgA conjugates are reported to be of varying quality[162].

Prior to IgA and IgM antibody testing it is recommended that IgG should be removed[40]. Those laboratories that did not remove IgG might have reported false positive IgM antibody results due to circulating rheumatoid factor. Since the presence of IgM antibodies is considered a sign of acute infection, it is possible that some studies have reported prevalences of acute *C. pneumoniae* infection that have been inflated due to the inclusion of patients with false positive IgM antibody results.

In aggregate, culture of *C. pneumoniae* is a specific method, however the sensitivity vary between laboratories; it is expensive, requires careful pre-laboratory handling of specimen and is not assessable everywhere. In contrast the PCR method is more robust to pre-laboratory handling of specimen, some PCR assays can be very sensitive, but the specificity vary between laboratories and is difficult to assess without a very sensitive and well defined gold standard, which currently does not exist.

Of the two serological methods described, the CF test is in our laboratory a good and sensitive method for the diagnosis of early phase chlamydial infection but it cannot distinguish between infections caused by the different species. The MIF test is a resource demanding method. Considerable interlaboratory variations of test results have been demonstrated, however we have shown that within the same laboratory only small differences in test results obtained by different assays occur. In our hands it is a good and reliable method for the diagnosis of acute *C. pneumoniae* infection and for the detection of *C. pneumoniae* IgG and IgA antibodies.

Applicability of serological criteria.

The serological criteria of acute and chronic *C. pneumoniae* infection were also discussed in chapter four.

Serological criteria of acute infection. The Grayston criteria of acute infection are based on three criteria (table 1). The validity of the criteria of detection of IgM antibodies of ≥ 16 and of four-fold rise of IgG titres are supported by the frequent fulfilment of these criteria simultaneous with the detection of *C. pneumoniae* by culture or PCR. In our laboratory we very seldom find specimens positive for *C. pneumoniae* by culture or PCR without finding the above criteria fulfilled if adequate serum samples are obtained. In contrast, we see samples fulfilling the serological criteria without being able to detect the organism by PCR or culture. The third criterion of acute infection IgG antibodies of ≥ 512 is dubious. Among the patients included in the present studies in chapter three and four, only one of ten patients with confirmed *C. pneumoniae* infection developed IgG antibody titres of ≥ 512, while 20% of the study B patients had IgG antibodies of ≥ 512 at a constant level through the six week observation period. In contrast only 2% of the controls

and 10% of the patients with acute respiratory tract infection fulfilled this criteria. Among the study B patients it was not possible to detect any differences in the levels of IgG antibody titres between patients with acute infection and patients without acute infection. Moreover as the bacteria was not detected in any of these patients, it seems unlikely that the patients had acute *C. pneumoniae* infection and thus the criterion of IgG antibodies of ≥ 512 has not been useful in the present studies and if ever used as an isolated criterion of acute infection, it should be interpreted with caution.

If reinfection is defined as a rapid rise of *C. pneumoniae* IgG antibodies and/or the detection of the bacteria with microbiological methods without the simultaneous presence of IgM antibodies we did not detect any proof of such condition. On a purely theoretical basis, it can be speculated that some of the patients with a high, stable level of IgG antibodies had a reinfection and that blood samples were drawn to late in the course of infection to detect the change in titre levels.

Serological criteria of chronic infection. (table 1). A major problem is the lack of clinical criteria for defining a chronic *C. pneumoniae* infection. Among the study groups of chapter four the criterion was fulfilled by 16%, 37% and 18% respectively of the GP-patients with acute infection, study B patients and controls. Apart from the issue of the lack of detection of the bacteria discussed above, it is unlikely that 18% of controls and 16% of patients with acute infection of whom none have clinically detected, chronic cardio-pulmonary diseases, should have chronic *C. pneumoniae* infection. Among the study B patients it was not possible to single out any subgroup of patients that significantly differed from the others in the percentages fulfilling the criterion of chronic infection, thus the hypothesis that 37% of the COPD patients should have chronic *C. pneumoniae* infections could not be supported by any clinical or paraclinical data.

In aggregate, for the serodiagnosis of acute *C. pneumoniae* infection the detection of IgM antibodies or a fourfold rise of IgG antibody titres has shown to be trustworthy criteria, in contrast IgG antibody titres of ≥ 512 is neither a sensitive nor a specific criterion. We have not been able to confirm the existence of specific serological criteria for reinfection. Likewise we have not been able to confirm the applicability of the proposed serological criteria of chronic infection. We have found a high correlation between culture and PCR results and that positive microbiological results corresponded well with the detection of *C. pneumoniae* IgM.

Conclusion.

In this thesis it has been demonstrated that test results of WRF as reference method and the two commercially available assays from MRL and LAB were equivalent when sera from different groups of patients were analysed in one laboratory. All three methods had about the same detection rate of antibodies and the mean level of endpoint titres was likewise the same within a variation of one dilution step. The one exception was IgM antibody titres found by LAB, which were on the average about two dilution steps higher than the mean endpoint titres found by the two other assays.

The assessment of the applicability of serological criteria for the diagnosis of acute *C. pneumoniae* infection confirmed that the presence of IgM antibodies of ≥ 16 was sign of acute infection. Moreover most of the patients with acute *C. pneumoniae* infection had developed IgM antibodies prior to seeking medical assistance, thus the presence of IgM antibodies is a useful tool for routine diagnostics also when only one serum sample is obtained. Fourfold rise of *C. pneumoniae* IgG antibody titres likewise seems to be a valid criterion for the diagnosis of acute *C. pneumoniae* infection, but the rise of IgG antibody titres occurs later than IgM antibodies can be detected. In contrast IgG of ≥ 512 is neither a sensitive nor a specific criterion. We have not been able to confirm the existence of specific serological criteria for reinfection. Likewise we have not been able to confirm the applicability of the proposed serological criteria of chronic infection.
The endemic occurrence of acute *C. pneumoniae* infections among adults with and without chronic pulmonary diseases was low; combined with the results of the other Danish prevalence studies of *C. pneumoniae* infections[13;13;48;113], it can be concluded that so far it has not been possible to distinguish groups of patients in Denmark in whom *C. pneumoniae* infections are frequently occurring.
The results from the microbiological and serological study of patients with reduced lung function did not support the hypothesis that *C. pneumoniae* should be involved in acute exacerbations of COPD. Nevertheless, the prevalence of *C. pneumoniae* IgG antibodies was high in all populations studied, but the mean level of endpoint titres was elevated among the patients with chronic diseases. The reasons for the persistence of chlamydial antibodies and the elevated levels

found in certain groups are not well understood. The future challenge will be to find the mechanisms for long-term persistence of *C. pneumoniae* antibodies, to explore why some groups of patients have elevated levels of chlamydial antibodies and to examine to what extend the bacteria cause chronic infection.

Summary

The aims of the Ph.D. study were:
To assess the performance of two commercially available MIF assays for the detection of *C. pneumoniae* antibodies compared to a reference method when applied to sera from different categories of patients.
To assess the applicability of the following serological criteria recommended for the diagnosis of *C. pneumoniae* infection. Acute infection: IgM antibody titre of ≥ 16, four-fold rise of IgG antibody titres or IgG antibody titres of ≥ 512. Chronic infection: IgG antibody titres of ≥ 128, IgA antibody titres of ≥ 40 and IgM antibody titres of ≤ 8.
By combined use of microbiological and serological methods to describe the prevalence of *C. pneumoniae* infection in adult patients with acute community-acquired respiratory tract infections.
By combined use of microbiological and serological methods to describe the prevalence of *C. pneumoniae* infection in patients with chronic obstructive pulmonary diseases with and without acute exacerbations of the disease.

The results obtained by assays from MRL Diagnostica (MRL) and Labsystems (LAB), respectively, were compared to those obtained with a MIF assay based on *C. pneumoniae* antigen from Washington Research Foundation (WRF) as a reference method. In total 220 sera from three different groups of patients were analysed. I: Patients with serological evidence of acute *C. pneumoniae* and/or *M. pneumoniae* infection. II: Patients with *C. pneumoniae* or *C. psittaci* infection verified by PCR. III: Persons enrolled in the Copenhagen City Heart Study among whom one fifth later developed AMI.

The overall agreement between results by the WRF assay and by the two commercial assays was excellent for IgM antibody detection rate (98%). The accordance in detection rates for IgG and IgA antibodies in sera from patients with acute infections was acceptable (87 to 88%) and in sera from group III it was excellent (95 to 97%). No significant difference among the detection rates of the three assays was seen for any of the Ig classes.
The reproducibility of *C. pneumoniae* IgG and IgA antibody endpoint titres was high, though IgG antibody titres by the WRF assay were on average 0.4 dilution

step lower than those by the two other assays. The IgM antibody endpoint titres found by the LAB assay were almost two dilution steps higher than those found by the WRF and the MRL assay.

The applicability of the serological criteria for the diagnosis of acute *C. pneumoniae* infection was assessed. The study confirmed that *C. pneumoniae* IgM antibodies of ≥ 16 was a sign of acute infection. Most patients had developed IgM antibodies before seeking medical assistance, thus detection of *C. pneumoniae* IgM antibodies is a useful tool for routine diagnostics. Fourfold rise of *C. pneumoniae* IgG antibody titres likewise seems to be a valid criterion for the diagnosis of *C. pneumoniae* infection but the rise of IgG antibody titres occurs later during the course of illness than detectable IgM antibodies. The presence of *C. pneumoniae* IgG antibody titres of ≥ 512 was not useful in the diagnosis of *C. pneumoniae* infection. We have not been able to confirm the existence of specific serological criteria for reinfection. Likewise we have not been able to confirm the applicability of the proposed serological criteria of chronic infection.

The endemic occurrence of acute *C. pneumoniae* infections among adults with and without chronic pulmonary diseases was low i.e. < 2%; combined with results of other Danish prevalence studies of *C. pneumoniae* infections, it can be concluded that so far it has not been possible to distinguish groups of patients in Denmark in whom *C. pneumoniae* infections are frequently occurring.

The results from the microbiological and serological study of patients with reduced lung function did not support the hypothesis that *C. pneumoniae* should be involved in acute exacerbations of COPD. Nevertheless, the prevalence of *C. pneumoniae* IgG antibodies was high in all populations studied, but the mean level of endpoint titres was elevated among the patients with chronic diseases. A positive correlation between *C. pneumoniae* IgG antibody titres and concentration of serum IgG was found. This finding might be consistent with polyclonal stimulation of B-lymfocytes; in contrast the results do not support the occurrence of chronic *C. pneumoniae* infection. The reasons for the elevated levels of IgG *C. pneumoniae* antibodies found in certain groups are still not well understood.

Dansk resumé

Formålet med ph.d. – studiet var:
At vurdere ydeevnen af to kommercielle MIF tests til påvisning af *C. pneumoniae* antistoffer sammenlignet med en reference metode ved at undersøge sera fra forskellige patientgrupper.

At vurdere brugbarheden af internationalt anbefalede serologiske kriterier til diagnostik af *C. pneumoniae* infektion. Akut infection: IgM antistof titer ≥ 16, fire-fold stigning i IgG antistof titer eller IgG antistof titer ≥ 512. Kronisk infektion: IgG antistof titer ≥ 128 , IgA antistof titer ≥ 40 og IgM antistof titer ≤ 8.

På grundlag af både mikrobiologiske og serologiske undersøgelser at beskrive forekomsten af *C. pneumoniae* infektioner blandt voksne patienter med akut luftvejsinfektion set i almen praksis.

På grundlag af både mikrobiologiske og serologiske undersøgelser at beskrive forekomsten af *C. pneumoniae* infektioner blandt patienter med kronisk obstruktiv lungesygdom med og uden akut exacerbation af sygdommen.

For at undersøge ydeevnen af MIF testen blev resultater opnået ved assays fra henholdsvis MRL Diagnostica (MRL) og Labsystems (LAB) sammenlignet med resultaterne opnået ved en reference metode baseret på antigen fra Washington Research Foundation (WRF). Der blev i alt undersøgt 220 sera fra tre forskellige patientgrupper. I: Patienter med serologisk tegn på akut *C. pneumoniae* og/eller *M. pneumoniae* infektion. II: Patienter med *C. pneumoniae* eller *C. psittaci* infektion verificeret ved PCR analyse. III: Personer indgået i Østerbro-undersøgelsen, blandt hvilke en femtedel senere udviklede AMI.

Overensstemmelsen mellem resultaterne opnået ved WRF og de to kommercielle metoder var god for påvisning af IgM antistoffer (98%). Overensstemmelsen i detektions rater for IgG og IgA antistoffer i sera fra patienter med akutte infektioner var acceptabel (87 til 88 %) og i sera fra gruppe III var den god (95 til 97%).For ingen af Ig klasserne, var der nogen signifikant forskel i detektions rater opnået ved de tre test metoder.

Reproducerbarheden for *C. pneumoniae* IgG and IgA antistof titer niveau opnået med de tre metoder i var ligeledes høj, dog var IgG antistof titer niveauet påvist ved WRF testen gennemsnitligt 0.4 fortyndingstrin lavere end ved de to andre metoder. IgM antistof titer niveauet påvist ved LAB testen var gennemsnitligt 1.7 fortyndings trin højere end niveauet opnået med WRF og MRL testene.

Anvendeligheden af de serologiske kriterier til at stille diagnosen akut *C. pneumoniae* infektion blev vurderet. Studiet bekræftede, at *C. pneumoniae* IgM antistoffer ≥ 16 var et tegn på akut infektion. De fleste patienter havde udviklet *C. pneumoniae* IgM antistoffer før de søgte læge, det vil sige at påvisning af IgM antistoffer er et brugbart redskab til rutine diagnostik. Fire-fold titer stigning af *C. pneumoniae* IgG synes ligeledes at være et anvendeligt kriterium for at stille diagnosen akut *C. pneumoniae* infektion, men IgG antistof titer stigning forekommer senere i forløbet af sygdommen end dannelsen af IgM antistoffer. Tilstedeværelsen af *C. pneumoniae* IgG antistof titre ≥ 512 viste sig ikke brugbart til at stille diagnosen akut *C. pneumoniae* infektion. Det har ikke været muligt at fastlægge serologiske kriterier til påvisning af reinfektioner eller at vurdere brugbarheden af de foreslåede kriterier til serologisk påvisning af kronisk infektion.

Den endemiske forekomst af akut *C. pneumoniae* infektion blandt voksne med og uden kronisk lungesygdom var lav, i.e. <2%. Når disse resultater sammenlignes med resultater fra nyere danske studier må det konkluderes, at det endnu ikke har været muligt at indkredse patientgrupper i Danmark med høj forekomst af *C. pneumoniae* infektioner.

Resultaterne fra det mikrobiologiske og serologiske studium af patienter med kronisk lungesygdom kunne ikke bekræfte hypotesen om at *C. pneumoniae* er associeret til akutte exacerbationer hos KOL patienter. Forekomsten af *C. pneumoniae* IgG antistoffer var høj i alle studiepopulationer, men det gennemsnitlige titer niveau var forhøjet blandt patienter med kroniske sygdomme. Der fandtes en signifikant positiv korrelation mellem *C. pneumoniae* IgG antistof titre og koncentrationen af serum IgG. Dette kan være foreneligt med polyklonal B-lymfocyt stimulation, hvorimod det ikke har været mulig at sandsynliggøre forekomsten af kronisk *C. pneumoniae* infektion. Baggrunden for at visse patientgrupper har forhøjet *C. pneumoniae* IgG antistofniveau er ikke endeligt afklaret.

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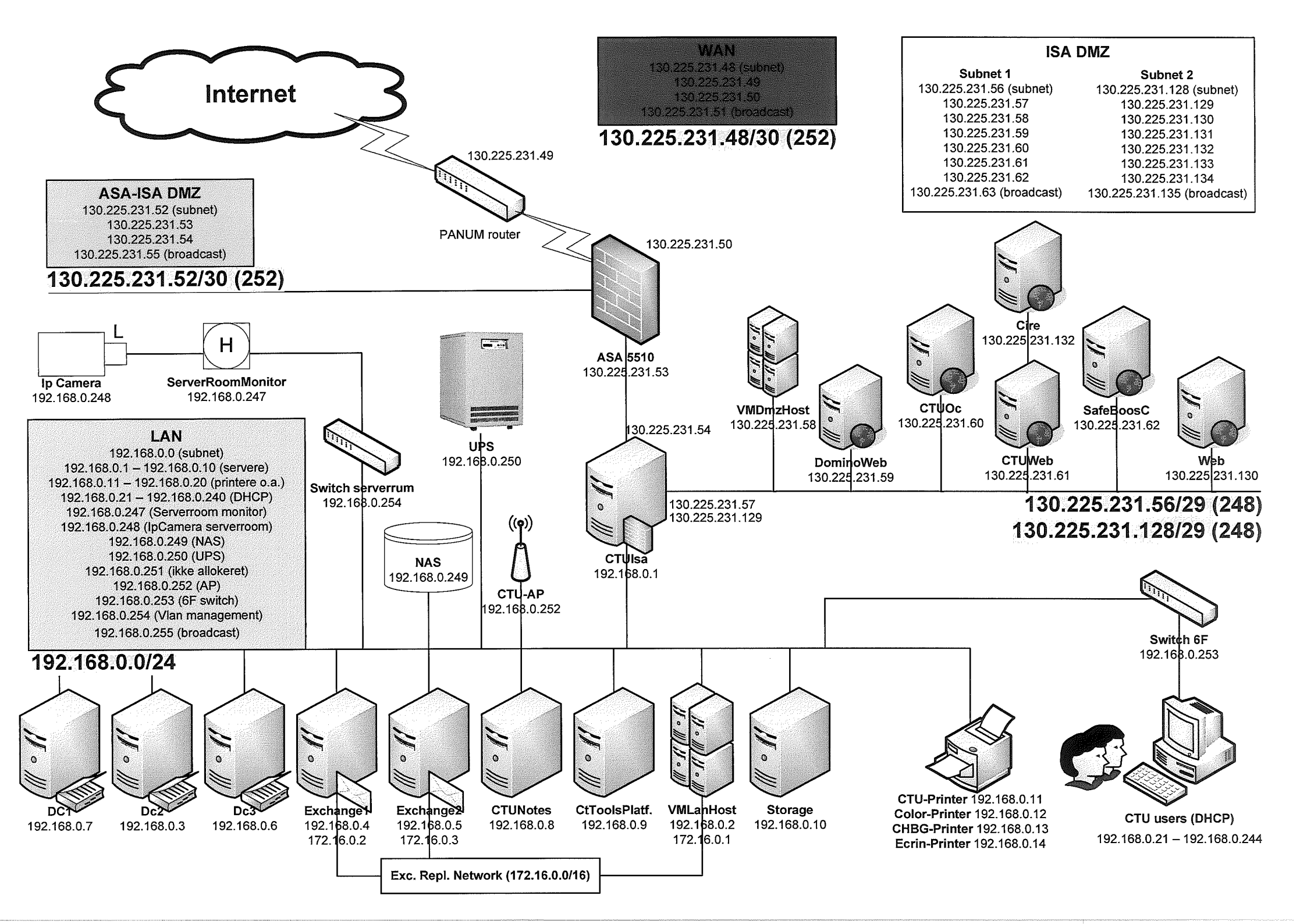
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UPS
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DominoWeb
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CTUOc
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SafeBoosC
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CHBG-Printer 192.168.0.13
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Tables 1-28

Table 1. Interpretation of *C. pneumoniae* antibody profiles determined by the MIF test

	Antibody profiles	**Interpretation**
Grayston et al[60]	Four-fold rise of IgG antibody titres or IgG antibody titre ≥ 512 or IgM antibody titre ≥ 16	Acute infection
Grayston et al[60]	IgG antibody titre 16-256 and IgM antibody titre ≤ 8	Previous infection
Hahn et al[70]	IgG antibody titre ≥ 128 and IgA antibody titre ≥ 40* and IgM antibody titre ≤ 8	Chronic infection

In the present study IgA antibody titre ≥ 32 is considered equivalent to IgA antibody titre ≥ 40

Table 2. Overview of experiments performed in the assessment of three MIF assays for the detection of *C. pneumoniae* IgM-, IgG- and IgA antibodies.

Sera from	Method	Ig antibody class tested		
		IgM	IgG	IgA
Group I	WRF	+	+	+
(N=83)	MRL	+	+	+
	LAB	+	+	ND
Group II	WRF	+	+	ND
(N=37)	MRL	+	+	ND
	LAB	+	+	ND
Group III	WRF	ND	+	+
(N=100)	MRL	ND	+	+
	LAB	ND	ND	ND

ND= Not done

Table 3. *C. pneumoniae* IgM endpoint titres in 120 sera from patients with acute respiratory tract infection (groups I+II): Number of sera at each titre level for the MRL versus the WRF assay

MRL	**WRF**								
	<16	16	32	64	128	256	512	1024	Total
< 16	85		4	2					91
16									0
32	3				2	1	1		7
64			1	1		2			4
128					1		3	1	5
256				1		4	1		6
512					1	2			3
1024						1	2		3
2048									0
4096						1			1
Total	88	0	5	4	4	11	7	1	120

Table 4. *C. pneumoniae* IgM endpoint titres in 120 sera from patients with acute respiratory tract infection (groups I+II): Number of sera at each titre level for the LAB versus the WRF assay

LAB	**WRF**								
	<16	16	32	64	128	256	512	1024	Total
< 16	85		1		1				87
16	1								1
32									0
64			1						1
128	1		3		1				5
256	1			2	1	1			5
512					1	7			8
1024				1		2	2	1	6
2048				1			5		6
4096						1			1
Total	88	0	5	4	4	11	7	1	120

Table 5. *C. pneumoniae* IgG endpoint titres in 120 sera from patients with acute respiratory tract infection (groups I+II): Number of sera at each titre level for the MRL versus the WRF assay

MRL	**WRF**					
	<64	64	128	256	512	Total
<64	47	3	7			57
64	11	6	3			20
128	7	9	12	2		30
256	1	1	1	1		4
512			2	5	1	8
1024					1	1
Total	66	19	25	8	2	120

Table 6. *C. pneumoniae* IgG endpoint titres in 120 sera from patients with acute respiratory tract infection (groups I+II): Number of sera at each titre level for the LAB versus the WRF assay

LAB	**WRF**					
	<64	64	128	256	512	Total
< 64	52	4	6	1		63
64	5	4	6			15
128	7	9	7	2		25
256	1	2	6	1		10
512	1			4	1	6
1024					1	1
Total	66	19	25	8	2	120

Table 7. Detection rates (%) for IgM and IgG antibodies by the three assays in sera from patients with acute respiratory tract infection (group I and II)

	IgM		IgG	
Detection rates	All samples N=120	1. sample N=44	All samples N=120	1. sample N=44
WRF assay	27	25	45	41
MRL assay	24	25	53	43
LAB assay	28	27	48	41
P-value[1]	0.50	0.82	0.21	0.78

[1]P-value: Comparison of detection rates obtained by the three assays (Cochran's Q test).

Table 8. Proportion (%) of concordant results[1] for IgM and IgG antibodies by the MRL and by the LAB assays versus the WRF assay in sera from patients with acute respiratory tract infection (group I and II)

	IgM		IgG	
	All samples N=120	1. sample N=44	All samples N=120	1. sample N=44
MRL assay	92	93	76	82
LAB assay	97	98	79	82
P-value[2]	0.16	0.65	0.43	0.74

[1]Concordant result: Both assays had positive results or both assays had negative results.
[2]P-value: Difference in the proportion (%) of concordant results of the MRL assay versus the WRF assay and of the LAB assay versus the WRF assay (Likelihood ratio test).

Table 9. *C. pneumoniae* IgG endpoint titres in 98 sera from patients without acute respiratory tract infection (group III): Number of sera at each titre level for the MRL versus the WRF assay

MRL	**WRF**								
	<16	16	32	64	128	256	512	1024	Total
< 16	26	3	1						30
16	4	4	4						12
32		1	5	5					11
64	2		6	11	4				23
128				4	6	2			12
256					1	6			7
512						2			2
1024							1		1
Total	32	8	16	20	11	10	1		98

Table 10. *C. pneumoniae* IgA endpoint titres in 100 sera from patients without acute respiratory tract infection (group III): Number of sera at each titre level for the MRL versus the WRF assay

MRL	**WRF**							
	<16	16	32	64	128	256	512	Total
< 16	42	3						45
16	1	1	2					4
32	4	5	4	1				14
64	1	2	11	7				21
128			1	5	3	3		12
256					1	2		3
512					1			1
Total	48	11	18	13	5	5		100

Table 11. Geometric mean endpoint titres obtained by three *C. pneumoniae* MIF assays: Sera from 44 adult patients with and 100 without acute respiratory tract infection.

	With acute infection						Without acute infection	
MIF test:	IgM		IgG		IgA		IgG	IgA
	All[1]	One[2]	All	One	All	One	One	One
WRF	173	155	116	104	75	46	113	59
MRL	159	136	131	128	102	96	114	70
LAB	546	483	145	151	ND	ND	ND	ND
P-value (all assays)	≤ 0.0001	0.018	0.004	0.032	-	-	-	-
P-value (MRL vs WRF)								

P-value: Comparison of antibody titres (Friedman's nonparametric 2-way analysis of variance)
ND = Not done

Number of *C. pneumoniae* antibody positive sera.
[1]With acute infection, all sera from each patient:
IgG ≥ 64; WRF: 54, MRL: 63, LAB 57
IgM ≥ 16; WRF: 32, MRL: 29, LAB 32
IgA ≥ 32; WRF: 47, MRL: 39
Without acute infection, one serum from each patient:
IgG ≥ 64; WRF: 43, MRL: 43
IgA ≥ 32; WRF: 45, MRL: 51

[2]With acute infection, one serum from each patient:
IgG ≥ 64; WRF: 17, MRL: 19, LAB: 17
IgM ≥ 16; WRF: 11, MRL: 12, LAB 11,
IgA ≥ 32; WRF: 13, MRL: 12

Table 12. Number of patients among 25 patients with *C. pneumoniae* infection included in group I and II who fulfilled the Grayston criteria for acute *C. pneumoniae* infection when sera were tested in three different MIF assays

Criteria	No. of patients		WRF	MRL	LAB
	Group I	Group II			
	9	1	-	-	-
IgM ≥ 16[1]	6	7	+	+	+
	2	0	+	-	+
Four-fold rise of	12	3	-	-	-
IgG	1	1	+	+	+
titres[2]	2	0	+	+	-
	2	1	-	-	+
IgG ≥ 512[1]	12	8	-	-	-
	1	0	+	+	+
	2	0	-	+	+
	1	0	-	-	+
	1	0	-	+	-

+ : Fulfilled the criterion, - : did not fulfilled the criterion

[1]At least one sample pr patient fulfilled the criterion

[2]Paired sera were available from 22 patients.

Table 13. Results of serological analyses in eight patients in whom the microbiological findings supported a diagnosis of *C. pneumoniae* infection

Patient	Day	CF	WRF MIF titres		MRL MIF titres						LAB MIF titres					
			C. pneumoniae		*C. pneumoniae*		*C. trachomatis*		*C. psittaci*		*C. pneumoniae*		*C. trachomatis*		C. psittaci	
		Titres	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
1	0	2916	128	64	n	256	64	n	512	n	256	1024	1024	n	128	n
	21	972	128	256	n	256	n	n	512	n	64	512	1024	n	128	n
2	0	108	128	256	n	256	n	n	n	n	n	512	n	n	n	n
	26	108	128	256	n	256	n	n	n	n	n	512	n	n	n	n
	59	108	64	128	n	128	n	n	n	n	n	128	n	n	n	n
3	0	972	n	512	n	128	n	n	n	n	n	1024	n	n	n	n
	19	972	128	512	n	1024	n	n	n	n	n	2048	n	n	n	n
	204	-	256	n	128	n	n	n	n	n	128	256	64	n	64	n
4	0	972	n	512	n	512	512	n	256	128	n	2048	64	n	64	n
5	0	8748	n	n	-	-	-	-	-	-	-	-	-	-	-	-
	64	26244	128	n	128	n	n	n	64	n	n	n	n	n	n	n
	77	8748	256	n	128	n	64	n	64	n	n	n	n	n	n	n
6	0	972	64	64	64	64	n	n	n	n	256	2048	128	n	64	64
7	0	2916	n	256	n	4096	n	256	n	256	n	4096	n	n	n	64
8	0	108	64	1024	n	128	n	n	n	32	n	1024	64	128	64	n
	23	-	128	128	n	32	n	n	n	n	256	n	n	n	64	n

n = titres: IgG < 64; IgM < 16; CF < 12.
-: Analyses not performed.

Table 14. Results of serological analyses in eight patients in whom the microbiological findings supported a diagnosis of *C. psittaci* infection

Patient	Day	CF titre	*WRF MIF titres*		MRL MIF titres						LAB MIF titres					
			C. pneumoniae		*C. pneumoniae*		*C. trachomatis*		*C. psittaci*		*C. pneumoniae*		*C. trachomatis*		C. psittaci	
			IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
1	0	-	n	n	n	n	n	n	n	n	n	n	128	n	64	n
	19	36	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	35	8748	n	n	128	n	n	n	128	n	128	n	128	n	n	n
2	0	324	128	n	64	n	64	n	n	n	64	n	1024	n	n	n
	6	972	128	n	128	n	128	n	n	n	64	n	1024	n	n	n
	12	8748	128	n	n	n	n	n	n	n	n	n	1024	n	n	n
3	0	12	n	n	n	n	n	n	n	n	n	n	n	n	128	n
	5	12	n	n	128	n	n	n	64	n	128	n	128	n	256	n
	6	36	n	n	128	n	n	n	n	n	512	n	512	n	512	n
4	0	n	n	n	n	16	n	n	n	n	128	n	n	n	n	n
	5	36	n	n	64	32	n	16	n	16	128	n	n	n	64	n
	30	324	128	n	64	32	n	16	64	16	64	n	128	n	256	16
5	0	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
6	0	-	n	n	64	n	n	n	128	n	n	n	n	n	n	n
	2	108	n	n	64	n	n	n	64	n	n	n	n	n	n	n
	3	-	n	n	128	n	n	n	128	n	n	n	n	n	n	n
	4	108	n	n	64	n	n	n	64	n	n	n	n	n	n	n
	6	324	n	n	64	n	n	n	128	n	n	n	64	n	128	n
7	0	-	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	2	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
	10	108	n	n	n	16	n	n	n	n	n	n	1024	n	128	128
8	0	-	128	n	128	n	n	n	n	n	n	n	n	n	n	n

n = titres: IgG < 64; IgM <16; CF < 12. -: Analyses not performed.

Table 15. Comparison of the number of patients included in group I with *C. pneumoniae* and/or *M. pneumoniae* infection fulfilling each of the Grayston criteria for acute *C. pneumoniae* infection according to WRF MIF results obtained in the NAP study and in the present study

Criterion	Study	*C. pneumoniae* infection (N = 13)	*C. pneumoniae* and *M. pneumoniae* infection (N = 4)	*M. pneumoniae* infection (N = 11)
IgM ≥ 16	Present	7	1	0
	NAP	7	2	0
Four-fold rise of IgG titres	Present	3	0	0
	NAP	7	3	0
IgG ≥ 512	Present	1	0	0
	NAP	8	3	0

Table 16. WRF assay: *C. pneumoniae* IgM endpoint titres in 25 sera from 11 patients with an incubation period of 30 and 90 min., respectively. Number of sera at each titre level. In brackets are shown the results from one serum sample per patient.

90 min	**30 min.**								
	<32	32	64	128	256	512	1024	2048	Total
<32	5								5
32									0
64									0
128				2 (1)					2 (1)
256			1	3 (1)					4 (1)
512				1 (1)	6 (3)	5 (4)			12 (8)
1024						1			1
2048							1(1)		1 (1)
4096									0
8192									0
Total	5	0	1	6(3)	6(3)	6(4)	1(1)	0	25 (11)

Table 17. MRL assay: *C.pneumoniae* IgM endpoint titres in 25 sera from 11 patients with an incubation period of 30 and 90 min., respectively. Number of sera at each titre level. In brackets are shown the results from one serum per patient.

90 min	**30 min.**									
	<32	32	64	128	256	512	1024	2048	4096	Total
<32	4									4
32										0
64										0
128		1								1
256										
512					1					1
1024						3	1			4
2048						1 (1)	8 (6)	1		10 (8)
4096							1 (1)	3 (2)		4 (3)
8192									1 (1)	1 (1)
Total	4	1	0	0	1	4 (1)	10 (7)	4 (2)	1 (1)	25 (11)

Table 18. Correlation between CF test and WRF MIF test results for 1008 consecutive sera received in a routine laboratory for determination of *C. pneumoniae* antibodies

IgG titres	CF test Number of sera with titres			
	<12	12 - 108	> 108	Total
< 64	777	94	21	892
64 - 256	71	35	6	112
≥ 512	3	1	0	4
Total	851	130	27	1,008

Table 19. Selected data from the first questionnaire survey in study A for patients with acute respiratory tract infection seen by the GP

	Total answers	Yes	
	No.	No.	%
Symptoms:			
Cough	94	75	80
Fever	94	18	17
Dyspnoea	94	13	12
Catarrhal	94	9	8
Hoarseness	94	8	8
Clinical diagnoses:	94		
Bronchitis acuta	94	26	28
Non-specified respiratory tract infection	94	15	16
Pneumonia	94	15	16
Upper respiratory tract infection	94	15	16
Catarrhalia	94	13	14
Influenza	94	4	4
Asthma	94	4	4
Antibiotics within 8 weeks prior to enrolment in study	90	14	16
Initiation of antibiotic therapy	90	26	29
Current smoking	81	37	46
Previous smoker	83	22	27

Table 20. Selected data from the second questionnaire survey in study A for patients with acute respiratory tract infection seen by the GP

	Total answers	Yes	
	No.	no.	%
Symptoms: Acute arthralgia	76	11	14
Treatment with local steroids	58	6	8
Treatment with systemic steroids	58	0	0
Not completely recovered at 2. visit	77	43	56

Table 21. Results of microbiological and serological analyses in six patients in whom the findings supported an aetiological diagnosis

Pt.	Aetiology	Day		*C. pneumoniae*			*Chlamydia*	*M. pneumoniae*
			PCR[1]	IgG	IgM	IgA	CF test	CF test
37	*C. pneumoniae*[2]	0	Pos.	64	8192	≤ 16	108	≤ 16
		14	Neg	64	8192	≤ 16	972	≤ 16
		32	Neg	1024	8192	≤ 16	2916	≤ 16
81	*C. pneumoniae*	0	Neg	≤ 16	2048	16	0	≤ 16
		13	Neg	64	2048	64	0	≤ 16
		32	Neg	256	1024	64	0	≤ 16
40	*B. pertussis*	0	Pos.	≤ 16	≤ 16	≤ 16	0	≤ 16
		17	Neg	≤ 16	≤ 16	≤ 16	0	≤ 16
		45	Neg	≤ 16	≤ 16	≤ 16	0	≤ 16
113	*B. pertussis*	0	Pos.	64	≤ 16	≤ 16	0	≤ 16
		14	Neg	64	≤ 16	≤ 16	0	≤ 16
		41	Neg	64	≤ 16	≤ 16	0	64
19	*M. pneumoniae*	0	Pos	≤ 16	≤ 16	≤ 16	0	≤ 16
145	*M. pneumoniae*	0	Pos	≤ 16	≤ 16	≤ 16	0	≤ 16

[1]Results of PCR are indicted for the microorganism considered the aetiological agent. [2]*C. pneumoniae* demonstrated by culture from throat swab. ND= Not done

Table 22. Clinical features of six patients with acute infection and verified diagnosis

Pt.	Etiology	Age	Gender	Days[1]	Cough	Fever	Phlegm	Acute arthralgia	Antibiotic treatment	Clinical Diagnosis[2]
37	*C. pneumoniae*	37	Female	NR	Yes	Yes	Yes	Yes	Azithromycin 500 mg x 3	NR
81	*C. pneumoniae*	25	Female	7	Yes	Yes	Yes	No	Penicillin 1 mill. x 3	Pneumonia
40	*B. pertussis*	24	Male	8	Yes	No	No	No	Azithromycin 500 mg x 3	Bronchitis Acuta
113	*B. pertussis*	39	Male	7	Yes	No	No	No	No	Catarrhalia
19	*M. pneumoniae*	28	Male	NR	Yes	No	Yes	Yes	No	Bronchitis seq.
145	*M. pneumoniae*	36	Male	5	Yes	No	No	No	Azithromycin 500 mg x 3	NR

[1]Days of symptoms before enrolment in the study. [2]Clinical diagnosis at the time of sampling
NR = Not reported.
The remaining 106 patients had had symptoms in average 6.5 days, 52 reported coughing, 20 fever, 52 pleghm and 17 acute arthralgia.

Table 23. Evidence of acute, previous or chronic *C. pneumoniae* infection in 322 patients with respiratory tract disease and in 45 controls.

Findings			Study A (N=112)		Study B (N=210)		Controls (N=45)		P
			N	(%)	N	(%)	N	(%)	(χ^2-test)
Positive culture/PCR			1	(1)	0	(0)	0	(0)	ND
CF test[1]			1	(1)	1	(<1)[2]	0	(0)	ND
MIF-serology[3]	Prevalence	IgG ≥ 16	66	(59)	149	(70)	28	(62)	0.576
		IgG ≥ 64	45	(40)	131	(62)	14	(31)	0.018
		IgA ≥ 16	38	(34)	108	(51)	16	(36)	0.123
		IgA ≥ 32	24	(21)	91	(43)	15	(33)	0.021
	Acute:	IgM	2	(2)	0	(0)	0	(0)	ND
		Four-fold change of IgG	2	(2)	0	(0)	0	(0)	ND
		IgG ≥ 512	11	(10)	43	(20)	1	(2)	0.006
	Previous:	IgG 16-256	55	(49)	106	(50)	27	(60)	0.78
	Chronic:	IgG ≥ 128 and IgA ≥ 32	17	(16)	77	(37)	8	(18)	0.004
		IgG 128-256 and IgA ≥ 32	10	(9)	40	(19)	7	(16)	0.12

[1]Significant changes in titres.[2]This patient had psittacosis (see text)[3]See table 1 for criteria appliedND: Not done

Table 24. Selected data from the first questionnaire survey of study B for the 93 patients admitted to the department of internal medicine and the 117 patients seen at the outpatient clinic.

	Yes (% of 93 hospitalised patients)	Yes (% of 117 outclinic patients)	P-value χ^2 test
Symptoms: (more than usual)			
Dry cough	39	16	< 0.001
Cough with sputum	57	22	< 0.001
Fever	41	3	< 0.001
Dyspnoea	92	42	< 0.001
Affected general condition	76	28	< 0.001
Acute arthralgia	3	3	0.023
Clinical diagnoses:			
COPD without exacerbation	1	73	< 0.001
COPD with exacerbation	61	17	< 0.001
Pneumonia[1]	38	0	ND
Other chronically diseases:			
Cardiovascular	25	16	0.016
Other diseases	30	19	0.010
Antibiotics within 8 weeks prior to enrolment in study	61	44	0.020
Smoking within the last year	61	53	0.021
Never smoked	3	4	0.017
X-ray indicating pneumonia	32	7	< 0.001
White cell count > 9.0 10^9/L	66	42	0.005
CRP >10 mg/L	69	38	< 0.001

[1]The diagnose was made before the description of the x-ray was available

Table 25. Selected data from the second questionnaire survey in study B for the 91 patients admitted to the department of internal medicine and the 108 patients seen at the outpatient clinic.

	Yes (% of 91 hospitalised patients)	Yes (% of 108 out-clinic patients)	P-value χ^2 test
Treatment with systemic steroids	36	29	0.055
Treatment with antibiotics during study period	33	21	0.023
Not completely recovered	55	43	0.053

Table 26:

Selected results for 210 patients distributed according clinical and paraclinical findings

	Group I Pneumonia n = 39		Group II Infectious exacerbation n = 52		Group III No infection n = 119	
	No.	(%)	No.	(%)	No.	(%)
Fever ≥ 38° C	15	(38)	26	(50)	1	(1)
White cell count > 9.0 10^9/L	31	(79)	46	(88)	33	(28)
CRP > 10 mg/L	32	(82)	48	(92)	26	(22)
Systemic steroid*	11	(28)	22	(42)	31	(26)
FEV^1< 50% of predicted FEV^1*	24	(62)	36	(69)	80	(67)
Smoking[1]*	27	(69)	29	(56)	63	(53)
"Atypical bacteria" in naso-pharyngeal swabs[2]*	1	(3)	1	(2)	1	(1)

[1]Smoking = smoking within the last year

[2]"Atypical bacteria": *M. pneumoniae, C. psittaci* and *B. pertussis*

*No significant differences were found between the three groups.

Table 27. Prevalences of *C. pneumoniae* IgG and IgA antibodies in patients with pneumonia, infectious exacerbation and no infection

	Group I Pneumonia n = 39		Group II Infectious exacerbation n = 52		Group III No infection n = 119	
C. pneumoniae antibody titres	No.	(%)	No.	(%)	No.	(%)
IgG ≥ 16	25	(64)	45	(87)	78	(66)
IgG ≥ 64	21	(54)	39	(75)	71	(60)
IgG 16-256*	14	(36)	32	(40)	60	(51)
IgG ≥ 512*	11	(28)	13	(25)	18	(13)
IgA ≥ 16	17	(44)	34	(65)	57	(48)
IgA ≥ 32 and IgG ≥ 128*	13	(33)	21	(41)	42	(36)

No significant differences were found between the three groups. Nor were there significant differences between groups I+II vs III: $P > 0,05$, χ^2-test

*See table 1

Table 28. Association between IgG antibody levels and selected clinical and paraclinical parameters in 210 patients. Logistic regression analysis with three different IgG antibody levels[1] as the dependent variable.

	P-value	Odds Ratio	(CI: Odds Ratio)
Age	0.0075	1.031	(1.008 –1.055)
Log (serum IgG)	0.0039	2.450	(1.334 – 4.500)
Systemic steroid	0.0008	0.372	(0.208 – 0.664)

[1] IgG antibody levels: 1. IgG ≤ 64, 2. IgG 128 – 256, 3. IgG ≥ 512

The following variables were also analysed, but showed an insignificant correlation with the level of IgG antibodies: +/- COPD, degree of COPD, outpatient/hospitalised patient, pneumonia (group I)/infectious exacerbation (group II)/without infection (group III), sex, smoking (+/- smoking within the last year), antibiotics within last eight weeks, leucocytosis, +/- elevated level of CRP.

Age, serum IgG and degree of COPD were coded as continues variables. The remaining variables were coded as dichotomous categorical variables

Figures 1-11

Figur 1. Age specific prevalence (%) of C. pneumoniae IgG antibody titres 16-256 in the general population in Denmark during 1976-79 and 1981-84, ref. nr. 138, data from C.H. Mordhorst

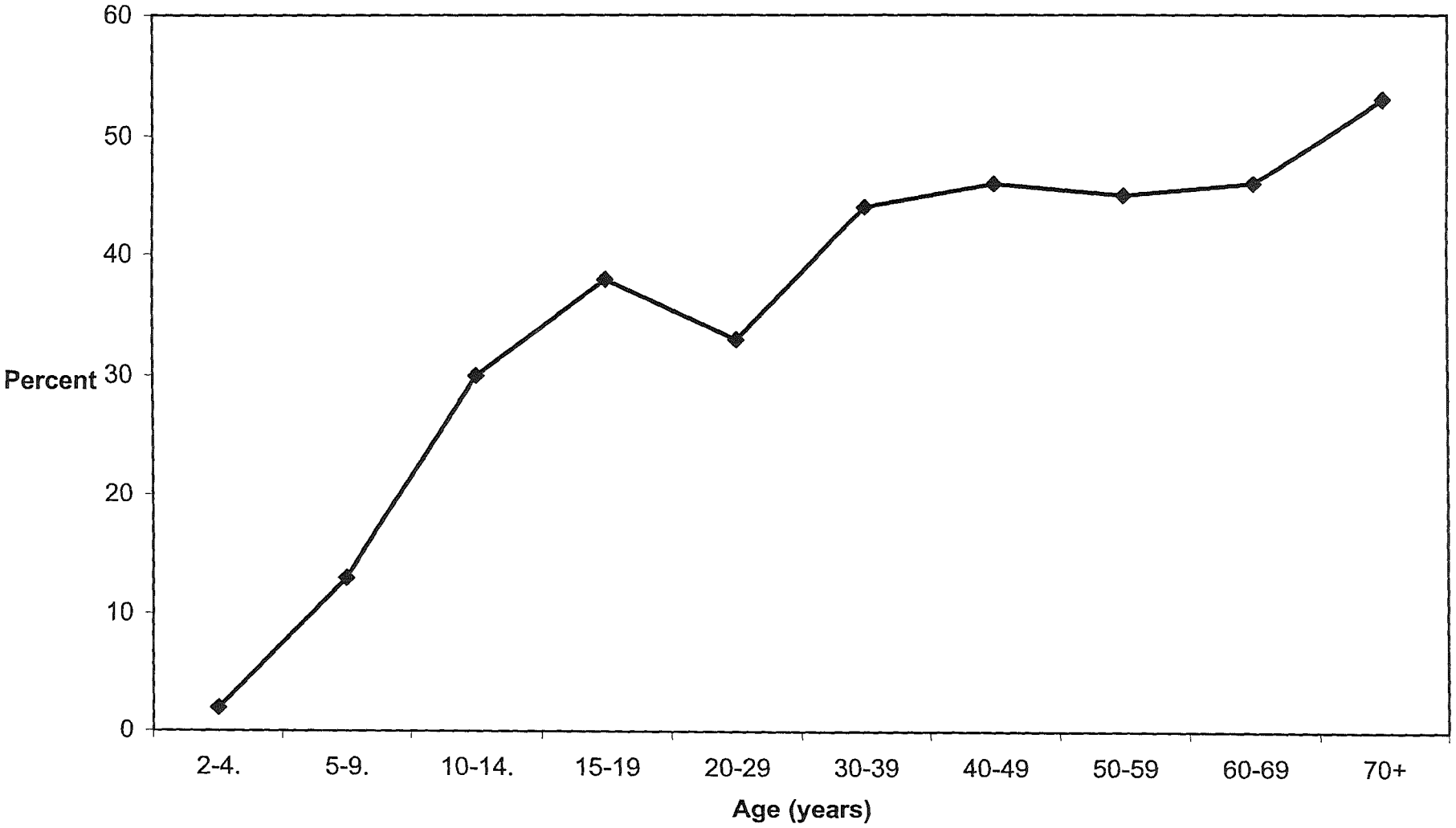


Figure 2. The percentage distribution of *C. pneumoniae* IgG antibody titres in sera from 65 seropositive patients with community-acquired acute respiratory tract infection and 28 seropositive contols

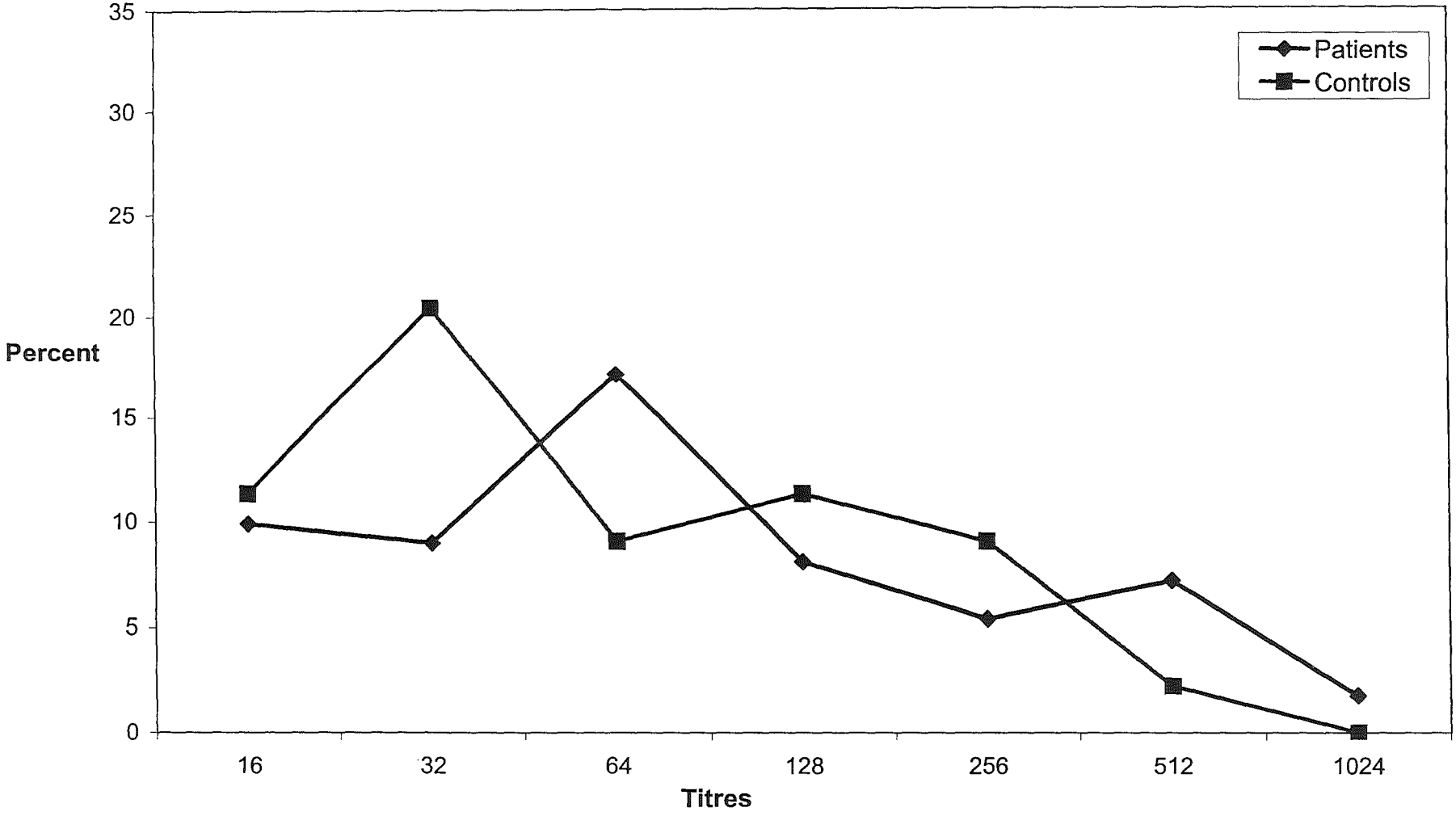


Figure 3. The percentage distribution of *C. pneumoniae* IgA antibody titres in sera from 38 seropositive patients with community-acquired acute respiratory tract infection and 16 seropositive controls

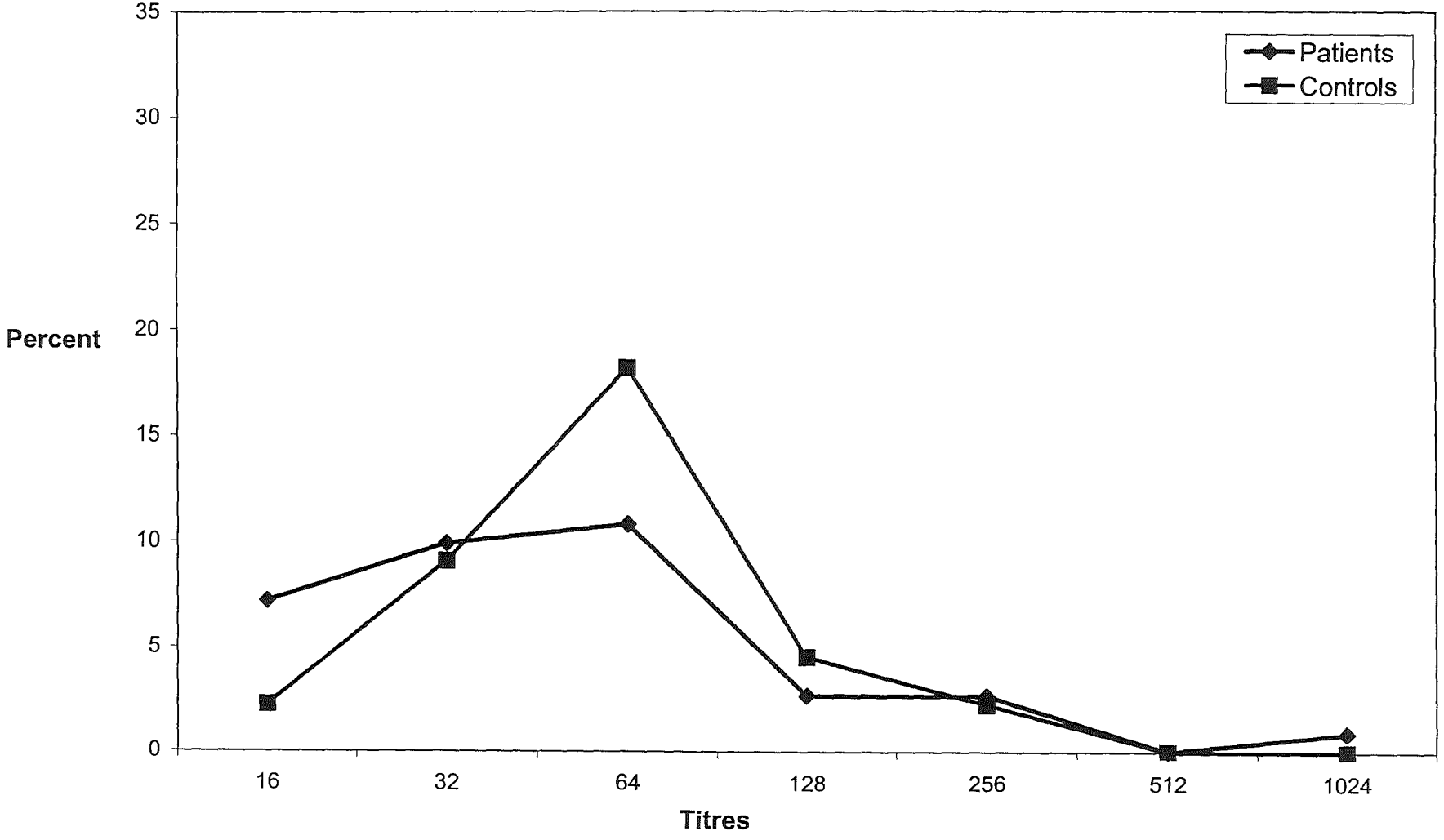


Figure 4.
Cummulative rate of 159 COPD patients according to result of a lungfunction test. Severe COPD: below 50%, moderate COPD: between 50 and 70% and mild COPD: above 70% of expected FEV1

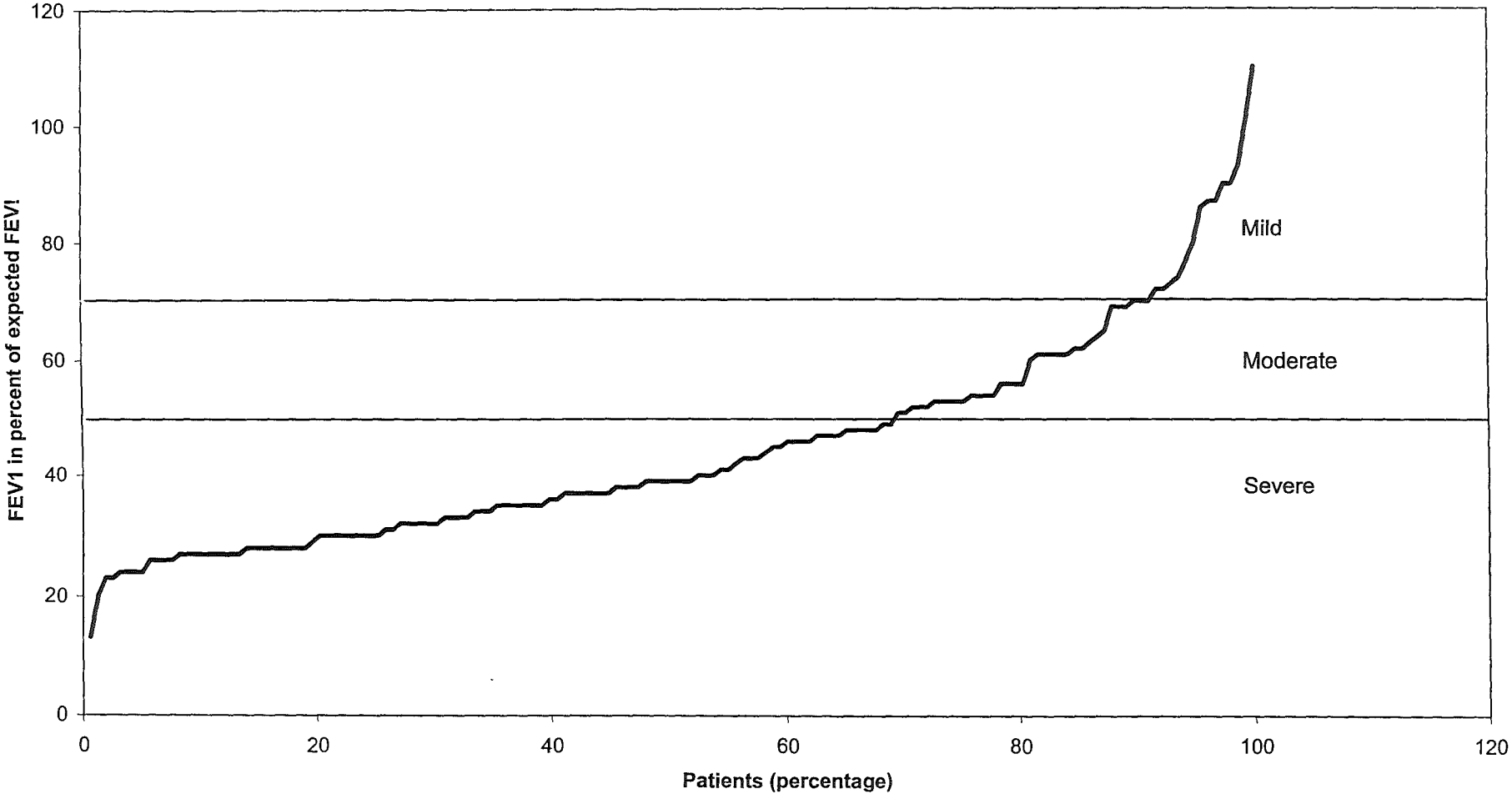


Figure 5. Cummulative rate of 51 patients without COPD according to result of a lungfunction test

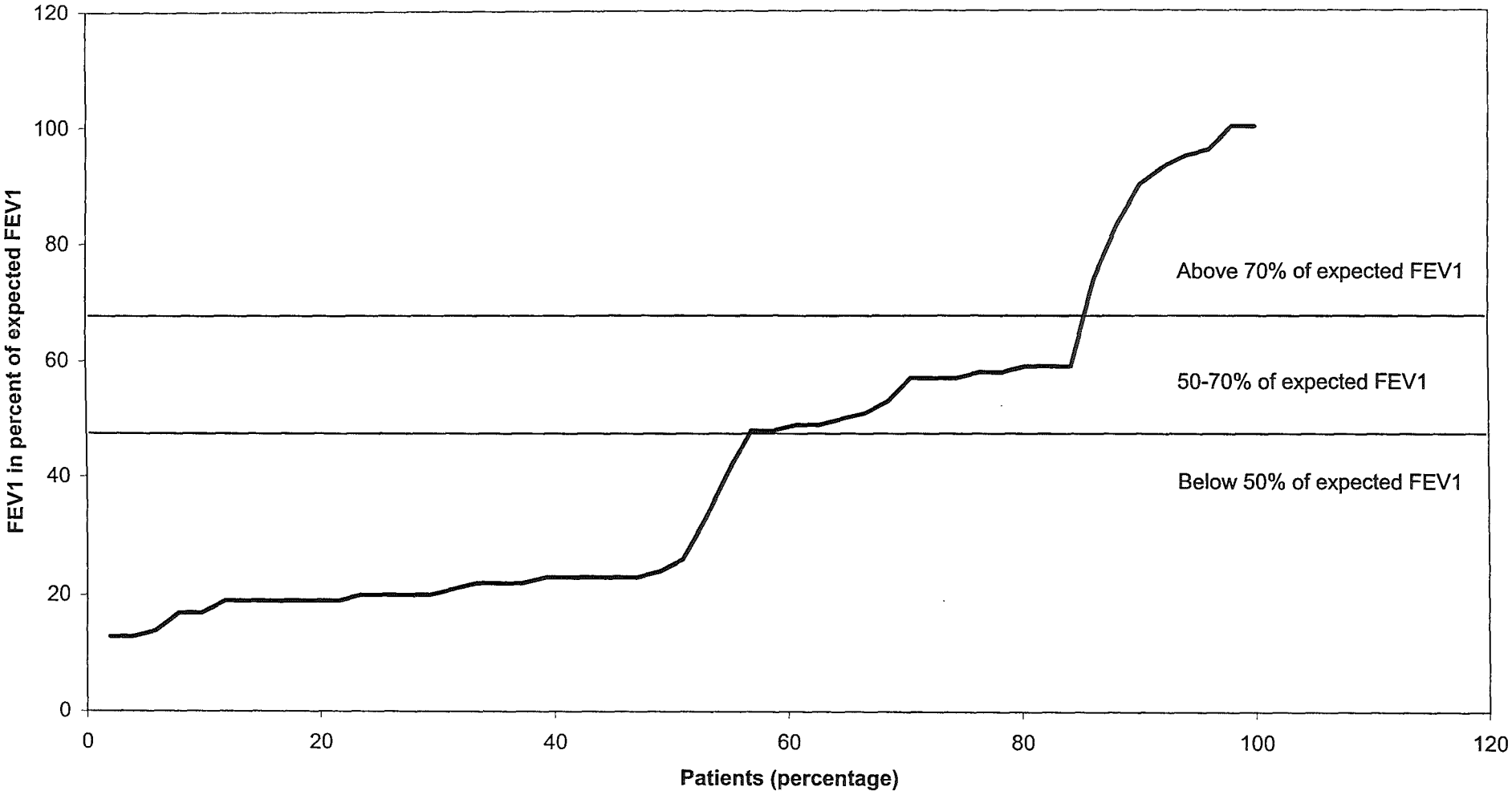


Figure 6. The percentage distribution of C. pneumoniae IgG antibody titres in sera from 149 seropositive patients from study B

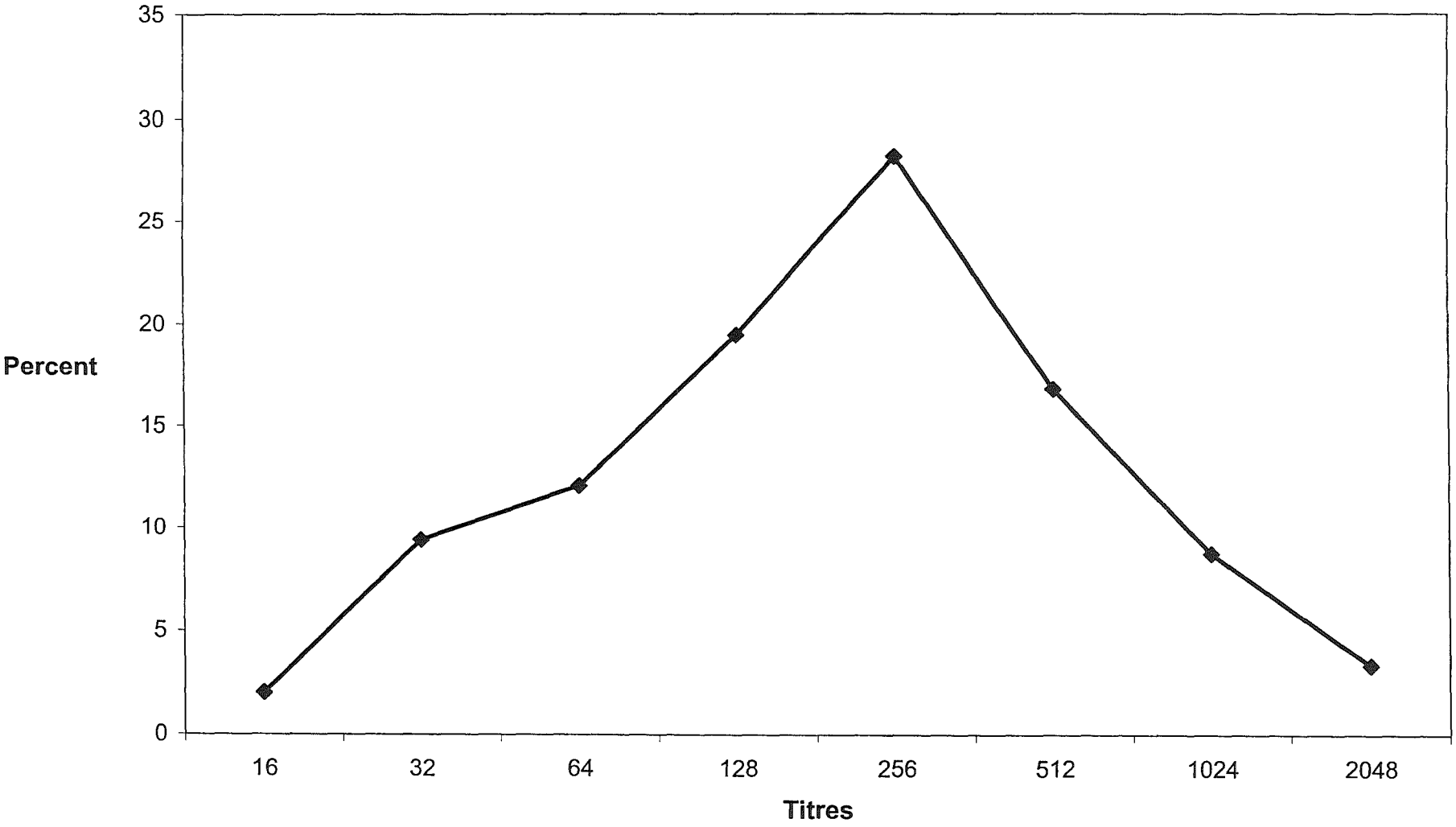


Figure 7. The percentage distribution of C. pneumoniae IgA antibody titres in sera from 108 seropositive patients from study B

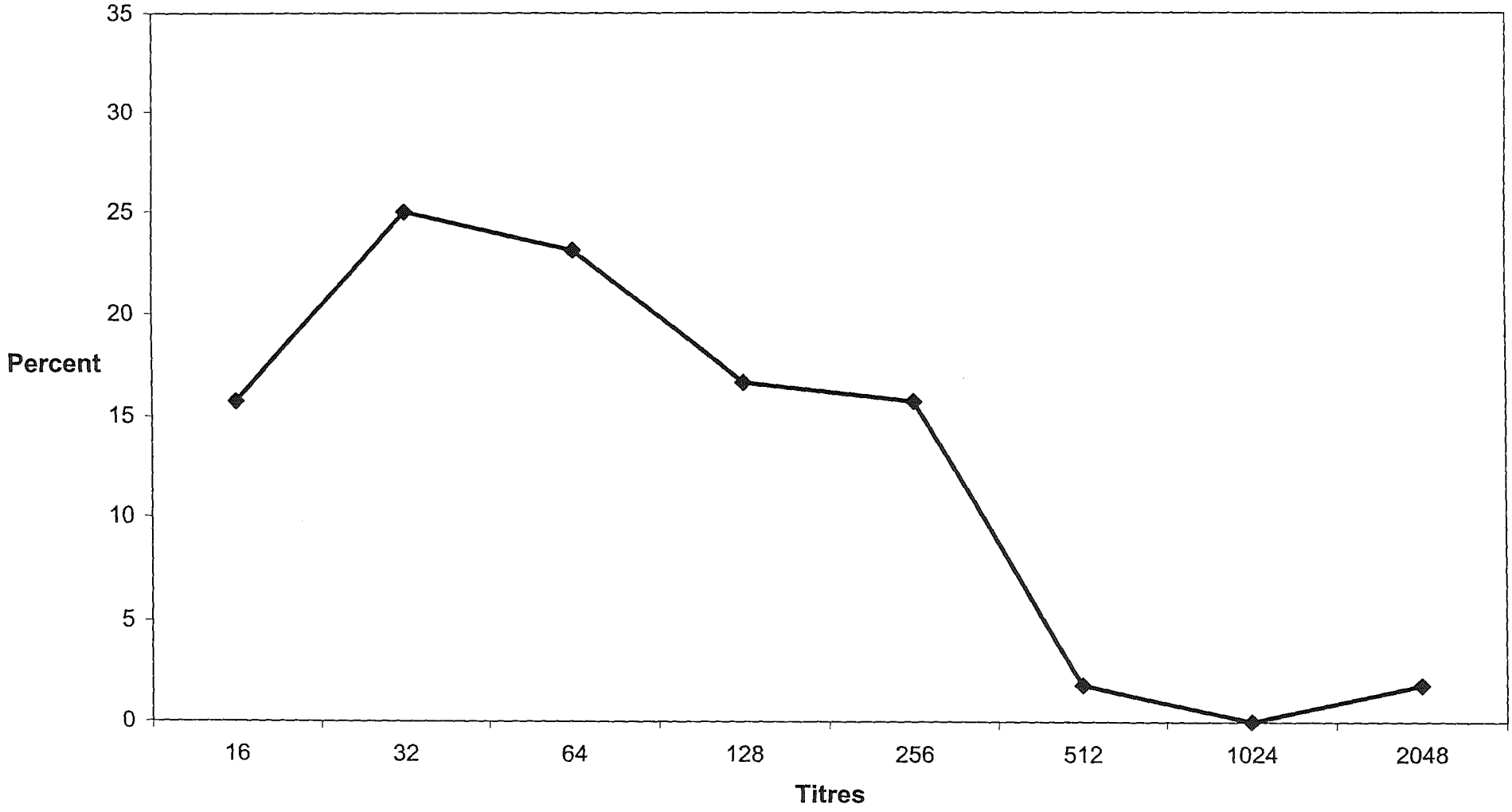
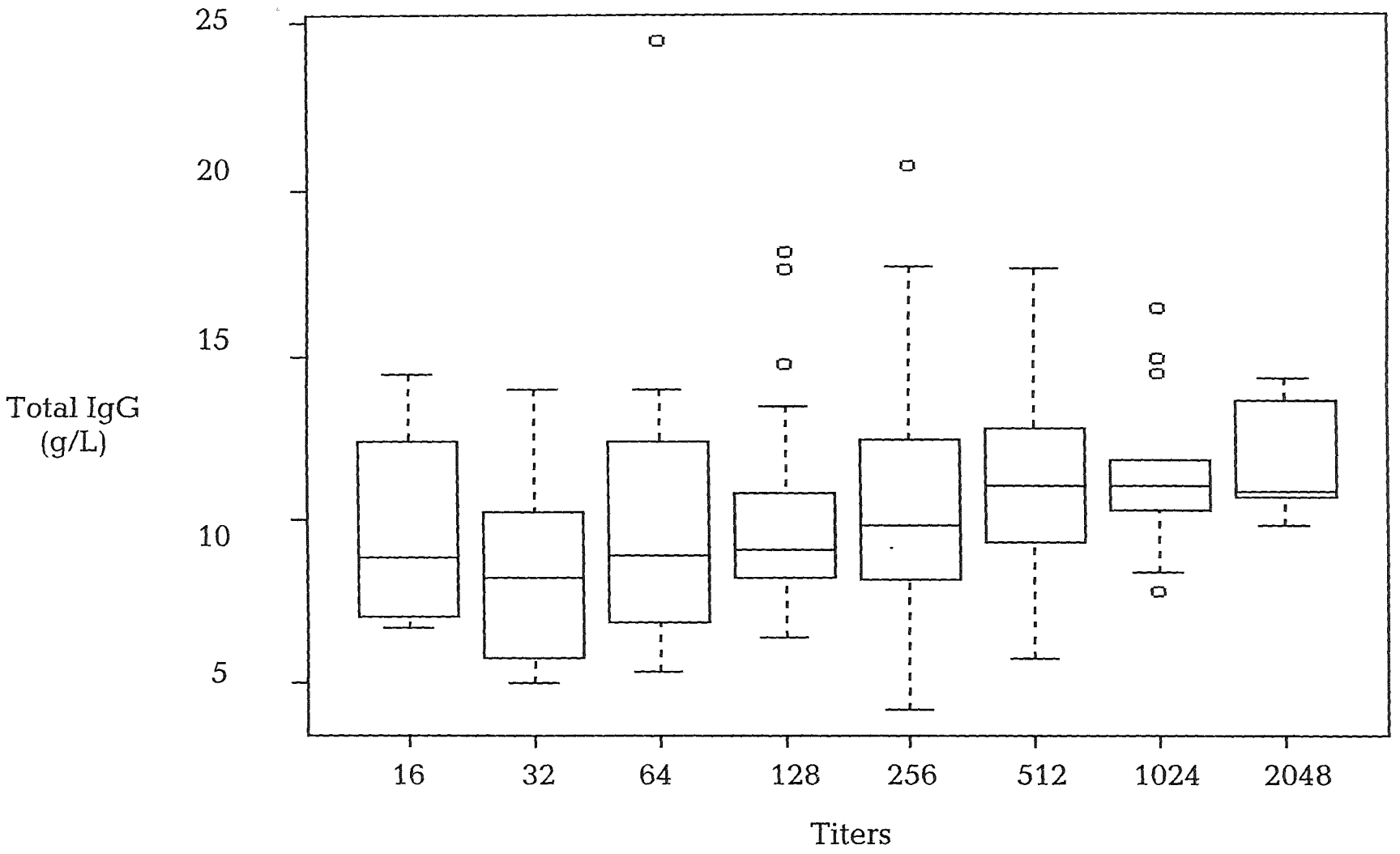
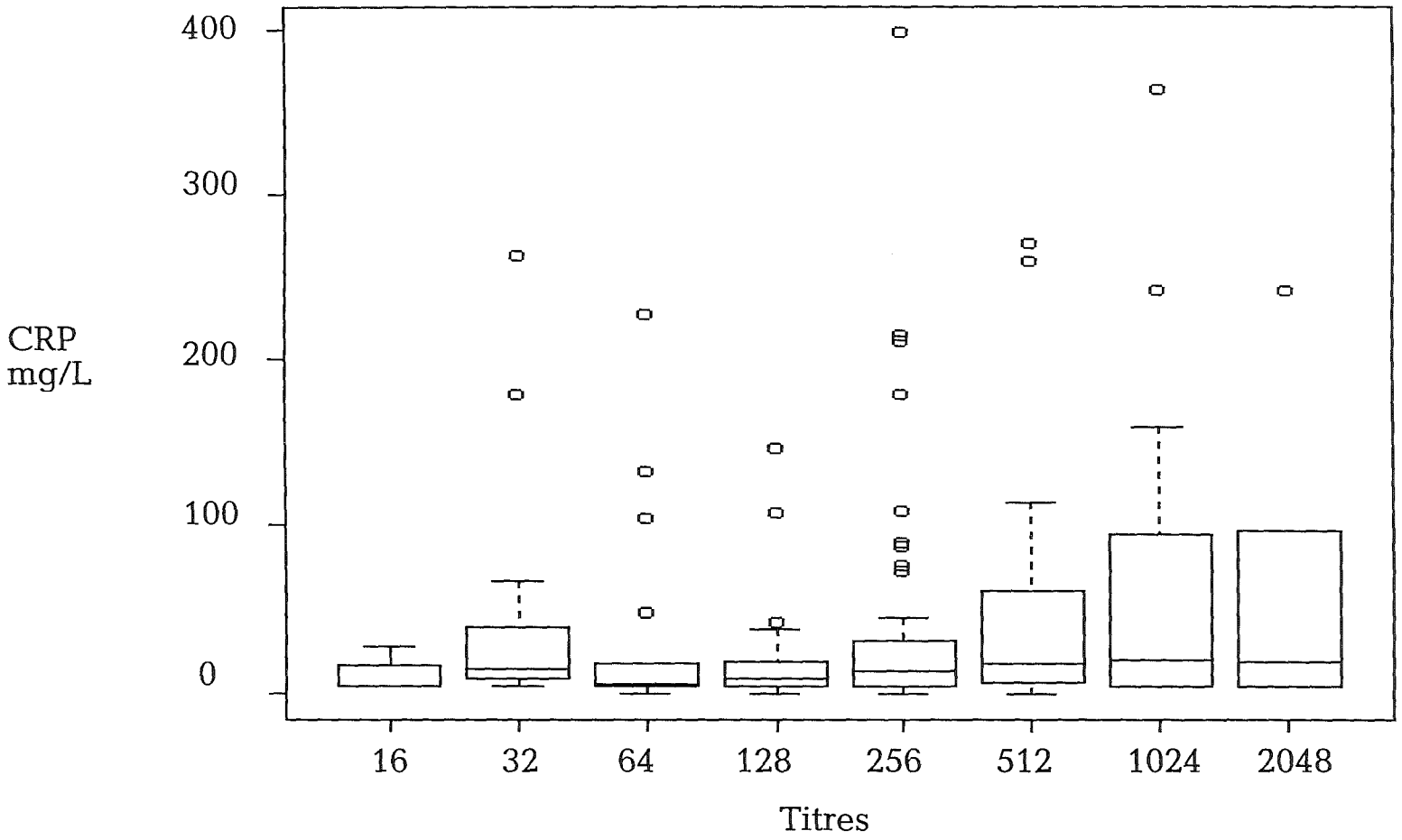


Figure 8. Concentrations of serum IgG in sera from 210 patients of study B distributed according to *C. pneumoniae* IgG antibody titres.



Positive correlation between concentrations of serum IgG and *C. pneumoniae* IgG titres: P< 0.001 (Spearman's rank correlation test).
The box-and-whisker plot: The box indicates the lower and upper quartiles and the central line is the median. The points at the ends of the "whiskers" show the largest / smallest observation if they fall within a distance of 1.5 times interquartile range from the nearest quartile. Outliers are shown separately.

Figure 9. Concentrations of CRP in sera from 210 patients of study B distributed according to *C. pneumoniae* IgG antibody titres



Positive correlation between concentrations of CRP and *C. pneumoniae* IgG titres: P=0.08 (Spearman's rank correlation test).
The box-and-whisker plot: The box indicates the lower and upper quartiles and the central line is the median. The points at the ends of the "whiskers" show the largest/smallest observation if they fall within a distance of 1.5 times interquartile range from the nearest quartile. Outliers are shown separately.

Figure 10. The percentage distribution of C. pneumoniae IgG antibody titres in sera from 149 seropositive outpatients or hospitalised patients, 596 seropositive patients with IHD and 93 seropositive patients seen in General Practice

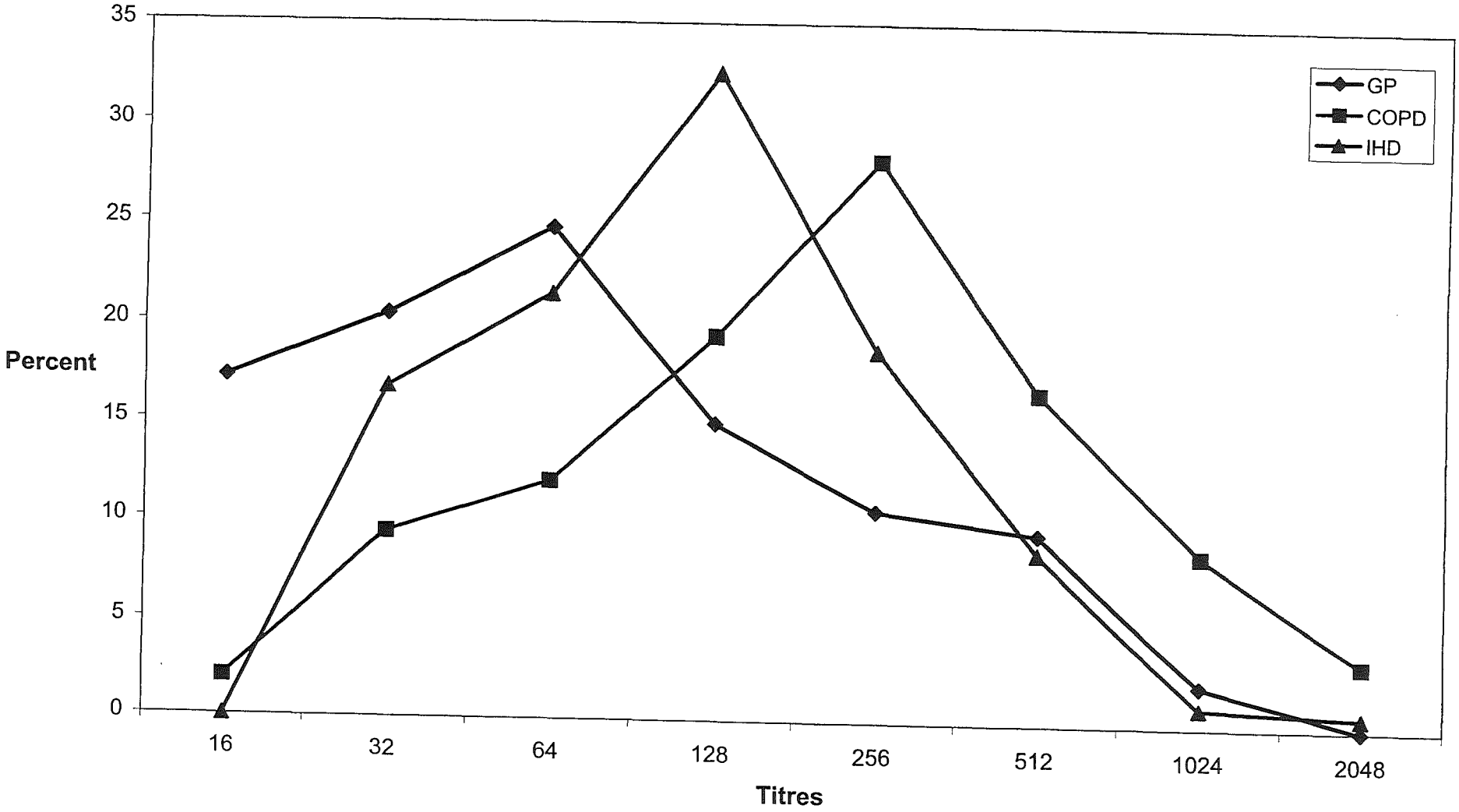
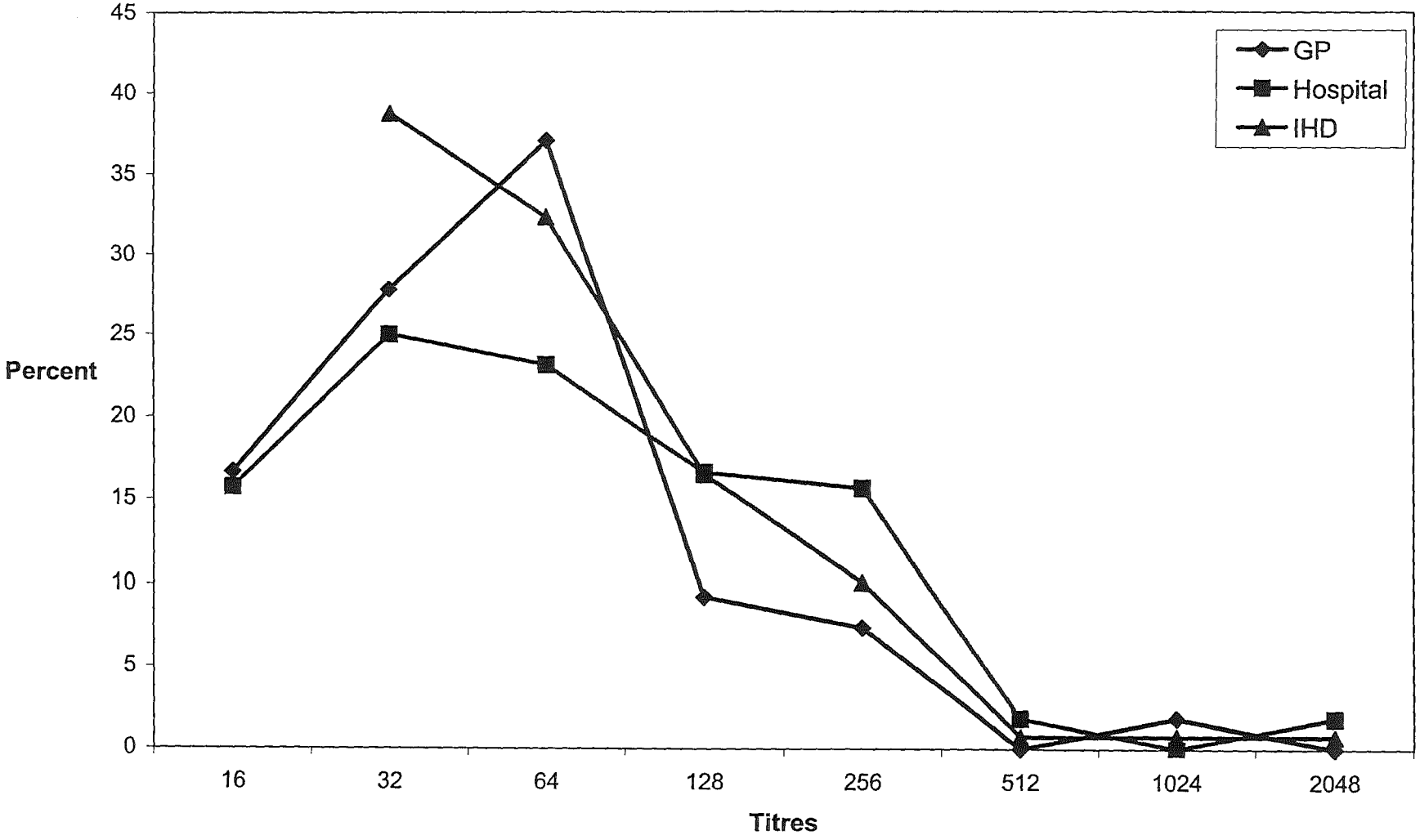


Figure 11. The percentage distribution of C. pneumoniae IgA antibody titres in sera from 108 seropositive outpatients or hospitalised patients, 266 seropositive patients witw IHD and 54 seropositive patients seen in General Practice



Appendix one

Materials and procedures of the MIF tests

Comparison of materials to conduct WRF, MRL and LAB MIF test

Materials	Washington Research Foundation	MRL®	Labsystems®
Antigens	C. pneumoniae elementary bodies (AR 39) treated with 0.02% formalin and mixed 1:1 with 3-5% yolk sac suspension.	Elementary bodies of C. pneumoniae (TW183), C. psittaci (6BC, DD34) and C. trachomatis (eight serotypes (D-K)). The elementary bodies have been treated in order to remove LPS activity and mixed with 3% yolk sac.	Elementary bodies of C. pneumoniae (K Elementary bodies of C. pneumoniae (TW183), C. psittaci (6BC, DD34) and C. trachomatis (eight serotypes (D-K)). ajaani 6), C. psittaci (OF 6bc and EAE) and C. trachomatis (LGV and L2). LPS activity has been reduced in C. pneumoniae and C. trachomatis antigens but not in C. psittaci antigens
Slides	The antigen is placed on the slide as 16 dots (4x4). The slide is airdried and fixed in acetone for 15 min. at room temperature.	Each slide has 12 wells. Each well has a dot with C. pneumoniae, C. trachomatis, C. psittaci elementary bodies and a yolk sac control.	Each slide has 12 wells. Each well has a dot with C. pneumoniae, C. trachomatis and C. psittaci elementary bodies.
Sample diluent	PBS, pH 7.3	PBS, pH 7.2	PBS, pH 7.4, proprietary additives and 15 mM sodium azide.
Mounting fluid	80% glycerol + 20% o.2 M Tris, pH 8.4	glycerol mixed with PBS , pH 7.2.	Glycerol, proprietary additives and 15 mM sodium azide.
Conjugate	FITC conjugated rabbit, anti-human IgM γ-chain F317 DAKO mixed with 0.01 M PBS, 15 mM NaN3, pH 7.2 FITC conjugated rabbit, anti-human IgG γ-chain F315, DAKO mixed with 0.01 M PBS, 15 mM NaN3, pH 7.2	FITC conjugated goat, anti-human IgG γ-chain blended with a goat, anti-mouse IgG, Evan's Blue, protein stabilizator and 0.025 thimerosal FITC conjugated goat, anti-human IgM μ-chain blended with a goat, anti-mouse IgG, Evan's Blue, protein stabilizator and 0.025 thimerosal	FITC conjugated goat, anti-human IgG, 0.001 Evan's Blue counterstain, proprietary additives and 15 mM sodium azide. FITC conjugated rabbit, anti-human IgM, 0.001 Evan's Blue counterstain, proprietary additives and 15 mM sodium azide.
Absorption	GULLSORP™: Goat, antihuman IgG containing 0.1% (w/v) sodium azide.	MRL's pretreatment diluent: Goat anti-human IgG added to PBS containing 0.05 % thimerosol.	Labsystems IgG blocking agent
Controls	Not included	Positive control: Diluted mouse serum. Negative control: Diluted human serum	Positive control: Diluted human serum. Negative control: Diluted human serum

Comparison of procedures to conduct WRF, MRL and LAB MIF test

Procedure:	Washington Research Foundation	MRL®	Labsystems®
Incubation with serum	Twofold serumdilutions with serum in PBS (pH 7.3). The dilutions are added to each dot. The slides are incubated in a moist chamber for 30 min. for both IgG and IgM slides.	Twofold serumdilutions with serum in PBS (pH 7.2). The dilutions are added to each well. The slides are incubated in a moist chamber for 30 min. for IgG and 90 min. for IgM slides.	Twofold serumdilutions with serum in serumdiluent. The dilutions are added to each well. The slides are incubated in a moist chamber for 30 min. for IgG slides and 3 hours for IgM slides.
Washing	The slides are rinsed in five dishes with PBS and three dishes with distilled water. Thereafter drying in a upright position at room temperature.	The slides are rinsed in a stream of PBS and submersed in PBS for 10 min. Dipped in purified water and airdried.	The slides are rinsed in four dishes with PBS and two dishes with distilled water. Thereafter drying in a upright position in 37° C.
Staining	IgG or IgM conjugate is added to the dots. Incubate at 37° C for 30 min. in moist chamber.	IgG or IgM conjugate is added to the wells. Incubate at 37° C for 30 min. in moist chamber.	IgG or IgM conjugate is added to the wells. Incubate at 37° C for 30 min. in moist chamber.
Washing	The slides are rinsed in five dishes with PBS and three dishes with distilled water. Thereafter drying in a upright position at room temperature.	The slides are rinsed in a stream of PBS and submersed in PBS for 10 min. Dipped in purified water and airdried.	The slides are rinsed in four dishes with PBS and two dishes with distilled water. Thereafter drying in a upright position in 37° C.
Mounting	Mounting fluid is added.	Mounting fluid is added.	Mounting fluid is added.
Absorption	Mix serum and Gullsorp Reagent 1:10. May be used immediately.	Mix serum and IgM "pretreatment diluent" 1:10. Wait 5 min., centrifuge and use.	We used Gullsorp Reagent

Appendix two

Questionnaires used in the clinical studies

Appendix 3.

C. PNEUMONIAE HOS PATIENTER I ALMEN PRAKSIS

SPØRGESKEMA 1. konsultation

Navn:_____

CPR.nr._____

Undersøgelsesdato_____

Indgår som patient ☐ kontrol ☐

- Årsag til henvendelse til læge_____
- Kronisk lunge/hjerte/karsygdom Nej ☐ Ja ☐

  Hvis ja, hvilken?

_____

- Anden kronisk sygdom:_____
- Luftvejsinfektion inden for de sidste 6 måneder? Nej ☐ Ja ☐

  Hvis ja, hvornår, hvilke(n) og behandling:_____

_____

_____

- Antibiotika givet inden for de sidste 8 uger? Nej ☐ Ja ☐

  Hvis ja, indikation, antibiotikum, dato påbegyndt, dato afsluttet:

_____

_____

- Rygning aktuelt Nej ☐ Ja ☐
  Rygning tidligere Nej ☐ Ja ☐
  Tidspunkt for rygeophør_____
  Tidspunkt for start på rygning_____

Appendix 3.

C. PNEUMONIAE HOS PATIENTER I ALMEN PRAKSIS

SPØRGESKEMA 1. konsultation

Hvis ja til nuværende eller tidligere forbrug:
Antal: Cigaretter/dag_____
Cerutter/dag _____
Cigarer/dag _____
Pibetobak: Pakker/uge _____

- Alkohol: Antal genstande pr uge _____

- Har deltageren normalt:
  - Tør hoste — Ja ☐ Nej ☐
  - Produktiv hoste (>3 mdr. i 2 år) Ja ☐ Nej ☐
  - Åndenød — Ja ☐ Nej ☐

- Har deltageren:
  - Tør hoste (mere end normalt) — Ja ☐ Nej ☐
  - Produktiv hoste (mere end normalt) — Ja ☐ Nej ☐
  - Åndenød (mere end normalt) — Ja ☐ Nej ☐
  - Feber (>38° C) — Ja ☐ Nej ☐
  - Påvirket almen tilstand — Ja ☐ Nej ☐
  - Purulent ekspektorat — Ja ☐ Nej ☐
  - Akutte ledsymptomer — Ja ☐ Nej ☐

- Antal dage med symptomer:_____

- Fund ved stetoskopi:
  _____

- Klinisk diagnose:
  _____

- Gives behandling? Ja ☐ Nej ☐

  Hvis ja, hvilken (præparat, dosis, varighed): _____

_____

- Indlægges patienten? Ja ☐ Nej ☐

  Hvis ja, indikation:

_____

Skema udfyldt af:_____ _____
Navn, adresse (stempel)

Appendix 4.

C.PNEUMONIAE HOS PATIENTER I ALMEN PRAKSIS

SPØRGESKEMA DAG 36

Navn:_____

CPRnr._____Undersøgelses dato_____Evt udskrivelsesdat_____

- Indgik som patient ☐ Kontrol ☐

- Føler deltageren sig rask/i sin habituel tilstand? Ja ☐ Nej ☐

Hvis nej, er der bedring af symptomer/objektive fund jvf 1 spørgeskema:

- Bedring af: Tør hoste Ja ☐ Nej ☐
  Produktiv hoste Ja ☐ Nej ☐
  Åndenød Ja ☐ Nej ☐
  Feber (>38° C) Ja ☐ Nej ☐
  Påvirket almen tilstand Ja ☐ Nej ☐
  Purulent ekspektorat Ja ☐ Nej ☐

- Har der været akutte ledsymptomer i forbindelse med aktuelle episode: Ja ☐ Nej ☐

- Har patienten fået antibiotika i forbindelse med aktuelle episode? Ja ☐ Nej ☐

Hvis ja, præparat, dosis og varighed_____

- Har patienten fået steroidbehandling i i forbindelse med aktuelle episode? Ja ☐ Nej ☐

Hvis ja, peroral/inhalation: præparat, dosis og varighed_____

Peakflow (bedst af tre) ved undersøgelse nr.: 2: _____ 3: _____ Højde_____

- Hvis mulighed for spirometri i praksis: FEV_1 _____ FVC_____
  Forventet FEV_1:_____

- ***Ellers:*** har patienten tidligere fået udført spirometri? Ja ☐ Nej ☐
  Hvis Ja, Dato:_____ FEV_1: _____ FVC:_____

- Er der taget røntgen af thorax siden 1. kontrol? Ja ☐ Nej ☐
  Hvis ja, røntgen af thorax:_____

Er der foretaget andre parakliniske undersøgelser inklusive blodprøver: Ja ☐ Nej ☐

Hvis ja, skriv resultat _____

Skema udfyld f_____

Navn, adresse (stempel)

Appendix 5

C. PNEUMONIAE HOS INDLAGTE PATIENTER

SPØRGESKEMA DAG 0

Navn:_____

CPR.nr._____ Indlæggelsesdato_____Undersøgelsesdato_____

Indlæggelsesdiagnose_____

Diagnose efter gennemgang af journal_____

- Kronisk lungesygdom Nej ☐ Ja ☐
- Hjertekarsygdom Nej ☐ Ja ☐

Hvis ja, hvilken: Akut myokardie infarkt Nej ☐ Ja ☐ Dato_____
Angina pectoris Nej ☐ Ja ☐ Debut_____
Hypertensio arterialis Nej ☐ Ja ☐ Debut_____

Andet:_____

- Anden kronisk sygdom:_____
- Luftvejsinfektion inden for de sidste 6 måneder? Nej ☐ Ja ☐

Hvis ja, hvornår, hvilke(n) og behandling:_____
_____
_____

- Antibiotika givet inden for de sidste 8 uger? Nej ☐ Ja ☐

Hvis ja, indikation, antibiotikum, dato påbegyndt, dato afsluttet:
_____
_____

- Rygning aktuelt Nej ☐ Ja ☐
  Rygning tidligere Nej ☐ Ja ☐
  Tidspunkt for rygeophør_____
  Tidspunkt for start på rygning_____

Hvis ja til nuværende eller tidligere forbrug:
Antal: Cigaretter/dag_____ Antal år:_____
Cerutter/dag _____Antal år:_____
Cigarer/dag _____Antal år:_____
Pibetobak: Pakker/uge _____ Antal år:_____

Appendix 5

SPØRGESKEMA DAG 0

- Alkohol dagligt? Ja ☐ Nej ☐
- Alkohol hver uge? Ja ☐ Nej ☐
  Hvis ja, antal genstande pr uge _____

Debut?_____

- Har deltageren normalt:
  - Tør hoste Ja ☐ Nej ☐
  - Produktiv hoste (>3 mdr. i 2 år) Ja ☐ Nej ☐
  - Åndenød Ja ☐ Nej ☐

- Har deltageren:
  - Tør hoste (mere end normalt) Ja ☐ Nej ☐
  - Produktiv hoste (mere end normalt) Ja ☐ Nej ☐
  - Åndenød (mere end normalt) Ja ☐ Nej ☐
  - Feber (>38° C) Ja ☐ Nej ☐
  - Påvirket almen tilstand Ja ☐ Nej ☐
  - Purulent ekspektorat Ja ☐ Nej ☐
  - Akutte ledsymptomer Ja ☐ Nej ☐

- Antal dage med symptomer:_____

- Pulsoxymetri:_____littilskud_____L /min

- Peakflow:_____
  Højde_____

- Evt. spirometri: FEV_1: _____ FVC: _____ Forventet FEV_1 : _____
  Efter evt. Beta 2 - agonist: FEV_1: _____ FVC: _____

Skema udfyldt af:_____

C.PNEUMONIAE HOS INDLAGTE PATIENTER

SPØRGESKEMA DAG 36

Navn:_____

CPRnr._____Undersøgelses dato_____

1. Udskrivelsesdato_____

2. Føler deltageren sig rask/i sin habituel tilstand? Ja ☐ Nej ☐

3. Hvis nej, er der bedring af symptomer/objektive fund jvf 1. spørgeskema:

Bedring af:	Tør hoste	Ja ☐	Nej ☐
	Produktiv hoste	Ja ☐	Nej ☐
	Åndenød	Ja ☐	Nej ☐
	Feber (>38° C)	Ja ☐	Nej ☐
	Påvirket almen tilstand	Ja ☐	Nej ☐
	Purulent ekspektorat	Ja ☐	Nej ☐

4. Har der været akutte ledsymptomer i forbindelse med aktuelle episode: Ja ☐ Nej ☐

5. Har patienten fået antibiotika i forbindelse med aktuelle episode? Ja ☐ Nej ☐

   Hvis ja, præparat_____

   dosis_____

   varighed_____

6. Har patienten fået steroidbehandling i i forbindelse med aktuelle episode? Ja ☐ Nej ☐

   Hvis ja, peroralt ☐ inhalation ☐

   præparat_____

   dosis _____

   varighed _____

Skema udfyld af_____

C. PNEUMONIAE HOS AMBULANTE PATIENTER

SPØRGESKEMA DAG 0

Navn:_____

CPR.nr._____
Undersøgelsesdato_____

1. Normalt lungerask Nej ☐ Ja ☐

2. KOL i stabil fase: Nej ☐ Ja ☐

3. KOL i exacerbation: Nej ☐ Ja ☐

4. Hjertekarsygdom Nej ☐ Ja ☐

   Hvis ja, hvilken: Akut myokardie infarkt Nej ☐ Ja ☐ Dato_____
   Angina pectoris Nej ☐ Ja ☐ Siden_____
   Hypertensio arterialis Nej ☐ Ja ☐ Siden_____

Andet:_____

5. Anden kronisk sygdom:_____

6. Episoder med forværring af luftvejssymptomer inden for de sidste 6 måneder? Nej ☐ Ja ☐

   Hvis ja, hvornår:_____

hvilke(n)_____

behandling:_____

7. Antibiotika givet inden for de sidste 8 uger? Nej ☐ Ja ☐

   Hvis ja, indikation_____
   antibiotikum_____
   dato påbegyndt_____
   dato afsluttet:_____

8. Rygning aktuelt Nej ☐ Ja ☐
   Rygning tidligere Nej ☐ Ja ☐
   Tidspunkt for rygeophør_____
   Tidspunkt for start på rygning_____

   Hvis ja til nuværende eller tidligere forbrug:
   Antal: Cigaretter/dag_____ Antal år:_____
   Cerutter/dag _____Antal år:_____
   Cigarer/dag _____Antal år:_____
   Pibetobak: Pakker/uge _____ Antal år:_____

C. PNEUMONIAE HOS AMBULANTE PATIENTER

SPØRGESKEMA DAG 0

9. Alkohol dagligt? Ja ☐ Nej ☐
   Alkohol hver uge? Ja ☐ Nej ☐
   Hvis ja, antal genstande pr uge _____
   Debut?_____

10 Har deltageren normalt:

Tør hoste	Ja ☐	Nej ☐
Produktiv hoste (>3 mdr. i 2 år)	Ja ☐	Nej ☐
Åndenød	Ja ☐	Nej ☐

11 Har deltageren:

Tør hoste (mere end normalt)	Ja ☐	Nej ☐
Produktiv hoste (mere end normalt)	Ja ☐	Nej ☐
Åndenød (mere end normalt)	Ja ☐	Nej ☐
Feber (>38° C)	Ja ☐	Nej ☐
Påvirket almen tilstand	Ja ☐	Nej ☐
Purulent ekspektorat	Ja ☐	Nej ☐
Akutte ledsymptomer	Ja ☐	Nej ☐

12 Antal dage med symptomer:_____

Skema udfyldt af:_____

C.PNEUMONIAE HOS AMBULANTE PATIENTER

SPØRGESKEMA DAG 36

Navn:_____

CPRnr._____Undersøgelses dato_____

1. Har patienten været indlagt for sin lungesygdom siden første kontrol? Ja ☐ Nej ☐

   Hvis ja, dato for indlæggelse_____ dato for udskrivelse_____

2. Føler deltageren sig rask/i sin habituel tilstand? Ja ☐ Nej ☐

3. Hvis nej, er der bedring af symptomer/objektive fund jvf 1. spørgeskema:

Bedring af:	Tør hoste	Ja ☐	Nej ☐
	Produktiv hoste	Ja ☐	Nej ☐
	Åndenød	Ja ☐	Nej ☐
	Feber (>38° C)	Ja ☐	Nej ☐
	Påvirket almen tilstand	Ja ☐	Nej ☐
	Purulent ekspektorat	Ja ☐	Nej ☐

4. Har der været akutte ledsymptomer i forbindelse med aktuelle episode: Ja ☐ Nej ☐

5. Har patienten fået antibiotika i forbindelse med aktuelle episode? Ja ☐ Nej ☐

   Hvis ja, præparat_____
   dosis_____
   varighed_____

6. Har patienten fået steroidbehandling i i forbindelse med aktuelle episode? Ja ☐ Nej ☐

   Hvis ja, peroralt ☐ inhalation ☐

   præparat_____
   dosis _____
   varighed _____

Skema udfyld af_____