Mika Paldanius

SEROLOGICAL STUDIES
ON CHLAMYDIA PNEUMONIAE
INFECTIONS
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Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in the Auditorium of Kastelli Research Centre (Aapistie 1), on March 31st, 2007, at 12 noon

OULUN YLIOPISTO, OULU 2007
Abstract

Chlamydia pneumoniae is a common, widespread pathogen that causes acute and chronic infections. Serological diagnosis of C. pneumoniae infection is primarily based on the microimmuno-fluorescence (MIF) method, but only a fourfold IgG antibody increase between paired sera and the presence of IgM antibodies have generally been accepted as markers of acute infection. At the present, no commonly accepted, reliable serological or other methods for the diagnosis of chronic C. pneumoniae infection exist.

We evaluated C. pneumoniae specific serological tests in different populations, followed the kinetics of C. pneumoniae antibodies in multiple sera obtained from the same individuals, compared anti-human IgA FITC conjugates in MIF test and evaluated C. pneumoniae specific antibody tests before and after coronary events in case-control pairs matched for the time point of serum sampling, place of residence, and treatment.

We showed that reinfection or reactivation is needed for the persistence of elevated IgG and IgA antibody levels. In chronic infections and upon reactivation, chronic processes may be better diagnosable based on IgA persistence than IgG levels because of the rapid disappearance of IgA levels after seroconversions. The cycle of reinfection and reactivation seems to be faster than previously thought in crowded conditions, such as in military service, since we recorded several antibody changes between the arrival and departure sera of military recruits during 6-month service. The presence of antibodies does not provide protection from reinfection.

Commercial anti-human IgA conjugates act differently in MIF tests, and there is marked variation in their ability to detect IgA antibodies. The EIA test used here overestimated the prevalence and persistence of IgA antibodies when compared to MIF. The best compatibility between MIF and EIA antibody levels was seen in the participants with high titers.

Only high IgA MIF titers to C. pneumoniae at the baseline predicted future coronary events. In the present study, seroconversions both in the participants who developed a coronary event and in the controls were detected by MIF and EIA, but mostly in different persons. Seroconversion suggesting reinfection or reactivation of persistent infection may have a role in accelerating chronic processes, because the participants with MIF seroconversion between consecutive sera had a slightly higher risk for coronary events than the controls. EIA seroconversions were more common in the controls than in the cases before the coronary event. The difference in the kinetics of EIA and MIF antibodies warrants future research and supports the use of the MIF method as a golden standard in the measurement of C. pneumoniae IgG and IgA antibody levels and seroconversions.

In their diagnostic practice, laboratories should use, compare, and validate more C. pneumoniae IgA antibody tests in addition to IgG tests. Unspecific findings in C. pneumoniae EIA tests require re-estimation and a new way to interpret the results. Chlamydia experts should speak for MIF and rethink the meaning of IgA antibodies and recommendations in the diagnosis of C. pneumoniae infections.

Keywords: antibodies, asthma, chlamydia infections, Chlamydia pneumoniae, coronary heart disease, serology
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Oulu, March 2007

Mika Paldanius
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAA</td>
<td>abdominal aortic aneurysm</td>
</tr>
<tr>
<td>ARI</td>
<td>acute respiratory infection</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>CF</td>
<td>complement fixation</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>DFA</td>
<td>direct fluorescence antibody assay</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EB</td>
<td>elementary body</td>
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<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
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<tr>
<td>EIU</td>
<td>enzyme immunoassay unit</td>
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<tr>
<td>FA</td>
<td>fluorescent antibody</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>HSP</td>
<td>heat shock protein</td>
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<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
<tr>
<td>IHA</td>
<td>indirect hemagglutination assay</td>
</tr>
<tr>
<td>Kdo</td>
<td>3-deoxy-manno-octulosonate</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Mabs</td>
<td>monoclonal antibodies</td>
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<tr>
<td>MIF</td>
<td>microimmunofluorescence test</td>
</tr>
<tr>
<td>MOMP</td>
<td>major outer membrane protein</td>
</tr>
<tr>
<td>NAAT</td>
<td>nucleic acid amplification test</td>
</tr>
<tr>
<td>OMC</td>
<td>outer membrane complex</td>
</tr>
<tr>
<td>Omp 2</td>
<td>outer membrane protein 2</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pmps</td>
<td>polymorphic membrane proteins</td>
</tr>
<tr>
<td>RB</td>
<td>reticulate body</td>
</tr>
<tr>
<td>rELISA</td>
<td>recombinant enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>RTI</td>
<td>respiratory track infection</td>
</tr>
<tr>
<td>SPG</td>
<td>Chlamydia transportation medium (sucrose-phosphate-glutamate dilution)</td>
</tr>
<tr>
<td>STD</td>
<td>sexually transmitted disease</td>
</tr>
<tr>
<td>TRIA</td>
<td>time-resolved fluoroscopic immunoassay</td>
</tr>
<tr>
<td>TRIC</td>
<td>trachoma-inclusion conjunctivitis</td>
</tr>
<tr>
<td>WIF</td>
<td>whole-inclusion fluorescence</td>
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</tbody>
</table>
List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.


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1 Introduction

In 1935, the Chlamydia complement fixation (CF) test was taken into use, and a number of atypical pneumonia cases were considered to have been caused by C. psittaci infection. In 1943, Joe Smadel suspected that CF-positive infections without exotic bird contact could have been transmitted from man to man. His vision had to brew up for four decades before C. pneumoniae was recognized. The isolation of C. trachomatis from trachoma was done as late as 1955. Both C. pneumoniae and C. trachomatis may have been present in the population for centuries before their discovery and the availability of diagnostic tools (Grayston 2000).

As a causative agent of both acute and chronic infections, C. pneumoniae is an extremely common and widespread pathogen. In addition to atherosclerosis (O'Connor et al. 2001, Leinonen & Saikku 2002), the leading cause of morbidity in the western world, C. pneumoniae has been associated with several other chronic diseases. After the first serological data linking C. pneumoniae to atherosclerosis in 1988 (Saikku et al. 1988), more than 500 reports have supported this finding (Boman & Hammerschlag 2002). At the moment, we still lack a fully satisfactory serological method for the diagnosis of C. pneumoniae infection, although the use of microimmunofluorescence (MIF) test is recommended. The seroconversion in C. pneumoniae specific IgG antibodies and the presence of IgM antibodies have been accepted as markers of acute infection. The role of IgA antibodies as markers of chronic infection is under debate (Dowell et al. 2001). The main problems in C. pneumoniae serology are the difficulties to obtain paired serum samples, the high background prevalence of IgG antibodies in adults, the lack of standardized methods and reagents, and the interpretation of the results.

Objective enzyme immuno assays (EIA) have been developed along with subjective MIF tests, and limited comparative studies between these two methods have been made with conflicting results (Persson & Boman 2000, Hermann et al. 2002, Hermann et al. 2004). Objective EIA tests are easy to use, standardized, and require equipments, whereas subjective MIF is based on the microscopist’s experience and skill in reading fluorescence with evenly distributed elementary bodies. The kinetics of C. pneumoniae antibodies is not well known. The prevalence and persistence of C. pneumoniae antibodies needs to be explained by using sera from same individuals.

The diagnosis of C. pneumoniae infections is mainly based on serological tests, and improvements in this field are therefore needed. In the present study, we compared the
results of EIA *C. pneumoniae* antibody tests carried out in different populations to the results of MIF tests, considered the ‘golden standard’.
2 Review of the literature

2.1 Chlamydia pneumoniae

2.1.1 Taxonomy

The taxonomic classification of *Chlamydia* has been based on limited phenotypic, morphologic, and genetic criteria. Recently, a revised taxonomy of the family *Chlamydiaceae* was suggested. The new, controversial classification is based on a recent analysis of the ribosomal operon of chlamydia-like organisms in the developmental cycle of replication (Everett *et al.* 1999).

*Chlamydiae* are intracellular gram-negative bacteria with unique composition of the cell wall and growth by binary division (Kuo *et al.* 1995). The order *Chlamydiales* has classically one family, *Chlamydiaceae*, one genus, *Chlamydia*, and four species (Herring 1993). At first, *Chlamydiaceae* had only two species, *C. trachomatis* and *C. psittaci*, later on, two other species, *C. pneumoniae* (Grayston *et al.* 1990) and *C. pecorum* (Fukushi & Hirai 1992), were added to the list. The new chlamydial taxonomy divides the family *Chlamydiaceae* into two genera, *Chlamydia* (three species) and *Chlamydophila* (six species) (78). The five new species have been split off from *C. psittaci* and *C. trachomatis*, and the discovered Chlamydia-like bacteria make up new families: *Waddliaceae*, *Parachlamydiaceae*, and *Simkaniaceae* (Table 1). Chlamydia-like bacteria grow in eukaryotic cells, have a similar developmental cycle, and have at least 80% identity in 16S and 23S rRNA to *Chlamydiaceae* (Everett 2000).

Everett’s revised taxonomy of the order *Chlamydiales* has been considered unnecessary. The sequence differences do not warrant a division into genera, since the strains are taxonomically and phylogenetically coherent enough to be regarded as one genus. The well-recognized name *Chlamydia* has been accepted among public health workers, funding agencies, and the public, and any change of the name would cause confusion. The revised taxonomy will only complicate and produce chaos even among scientific colleagues (Schachter *et al.* 2001).
Table 1. A summary of the proposed taxonomy of the order Chlamydiales.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Chlamydiaceae</td>
<td>Chlamydia</td>
<td>Chlamydia muridarum</td>
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<td></td>
<td></td>
<td>Chlamydia suis</td>
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<td></td>
<td></td>
<td>Chlamydia trachomatis</td>
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<td></td>
<td>Chlamydophila</td>
<td>Chlamydia abortus</td>
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<td></td>
<td></td>
<td>Chlamidia caviae</td>
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<td></td>
<td></td>
<td>Chlamydophila felis</td>
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<tr>
<td></td>
<td></td>
<td>Chlamydophila pecorum</td>
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<tr>
<td></td>
<td></td>
<td>Chlamydophila pneumoniae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlamydophila psittaci</td>
</tr>
<tr>
<td>II Simkaniaceae</td>
<td>Simkania</td>
<td>Simkania negevensis</td>
</tr>
<tr>
<td>III Parachlamydiaceae</td>
<td>Parachlamydia</td>
<td>Parachlamydia acanthamoebae</td>
</tr>
<tr>
<td>IV Waddliaceae</td>
<td>Waddlia</td>
<td>Waddlia chondrophila</td>
</tr>
</tbody>
</table>

2.1.2 Historical milestones in C. pneumoniae culture

The isolation of the *C. pneumoniae* (TW-183) organism during the trachoma vaccine trial carried out among children in Taiwan in 1965 marked the beginning of the story of *Chlamydia pneumoniae* (Woolridge et al. 1967). However, the discovery and acceptance of TWAR (the strain name being a combination of the names of the first two isolates: TW-183 and AR-39) organisms as a separate species took over two decades (Grayston 1989). The organism was first isolated from the conjunctiva of a Taiwanese child (Kuo et al. 1986). At that time, the organism could be isolated in the yolk sac of an embryonated egg, and the measurement of antibody responses was not yet possible. On 20th November 1965, the historical swab sample for isolation was obtained from the eye of a child with trachoma, and it was kept frozen until 15th February 1966, when the attempts at isolation were started (Grayston et al. 1990). The name of the isolate, TW-183, was based on the isolation number in the laboratory, and it was considered a *C. trachomatis* strain. In Taipei, at the U.S. Medical Research Unit, the first trial passages in eggs were made. A second, routinely made blind passage demonstrated typical elementary bodies of *Chlamydia*. In the very first passage, all eggs survived for 13 days, but Macchiavellos’s stain of yolk sac smears remained negative (Grayston JT 1999).

In 1968, the chlamydial isolate in a chicken egg yolk sac was obtained from the eye of a child (IOL-207) in Iran. This isolate was later proven to be *C. pneumoniae* (Dwyer et al. 1972). Although the isolation was obtained from a conjunctival source, serological studies proved that this organism was not related to the eye diseases caused by *C. trachomatis*. Cell culture methods were applied to this *Chlamydia* in 1971. The organism (TW-183) was observed to be morphologically more similar (round, dense inclusions in host cells, no glycogen) to *C. psittaci* than to *C. trachomatis*, and it was classified as *C. psittaci*. 
At the beginning of the 1980’s, the first respiratory isolate (AR-39) was obtained from a university student with pharyngitis, and the strain name TWAR was coined from the isolations of the first conjunctival and respiratory strains (TW-183 and AR-39) (Grayston et al. 1986, Kuo et al. 1995). At that time, TWAR was considered a human C. psittaci strain spreading from human to human without a bird or animal host. (Grayston et al. 1986).

### 2.1.3 Historical milestones in C. pneumoniae serology

At the end of the 1960’s, the development of the micro-immunofluorescence (MIF) test replaced the mouse toxicity prevention test used for immunotyping new isolates (Alexander et al. 1967, Wang & Grayston 1970), and during the 1970’s, there was a suggestion of the possible role of C. trachomatis in respiratory infections. The most important step, however, was taken when Dr. Saikku started to apply the MIF technique to Chlamydia CF positive sera obtained from a respiratory infection outbreak in Finland. The Chlamydia organism, TW-183, was shown to be the probable cause of the outbreak in northern Finland (Saikku et al. 1985), and the association of the agent with the respiratory tract (RTI) was discovered. A few years later, in 1988, a serological study by Saikku et al. (Saikku et al. 1988) suggested that C. pneumoniae might be associated with atherosclerosis in patients with coronary artery disease (CAD). After these findings, similar results were obtained in serological studies made among CAD patients in other countries (Kuo et al. 1995).

### 2.1.4 Identification of new Chlamydia species, Chlamydia pneumoniae

The third species of Chlamydia, C. pneumoniae, was established in 1989 based on the findings of immunological separateness, i.e. pear-shaped elementary bodies (EBs) with a periplasmic space and a loose outer membrane and less than 10 % DNA homology with C. trachomatis and C. psittaci organisms (Grayston & Wang 1999). Ultrastructural differences in the elementary bodies, deoxyribonucleic acid analysis and serology were thus the criteria for species identification. The round, almost bubble-like shape of C. pneumoniae EBs has later been described for the IOL-207, Kajaani-6, YK-41, and KKpn-1 strains (Popov et al. 1991, Popov et al. 1992, Miyashita et al. 1993, Komoda et al. 2000). Evidence of new and unique serotypes of C. pneumoniae was provided by Black et.al (Black et al. 1991, Wang SP 1994). As a member of the genus Chlamydia, the strain had to fulfil the following criteria: obligate intracellular parasitism, unique developmental cycle, shared genus-specific lipopolysaccharide (LPS), and complement fixation antigen and comparable guanine-plus-cytosine content (Grayston JT 1989).
Chlamydiae are intracellular gram-negative bacteria that grow inside a vacuole called inclusion, and have a unique developmental cycle with two forms: a smaller extracellular EB and a larger replicating intracellular reticulate body (RB). Infective EBs, typically 0.2-0.6 µm in diameter, attach to susceptible host cells and are phagocytosed (Kuo et al. 1995). In primary differentiation, reorganization into larger RBs (up to 1.5 µm in diameter) occurs after metabolic and morphologic changes of inert EBs. RBs divide by binary fission with the expanding endosome. The chlamydial inclusion becomes visible as a microcolony, and after a period of growth in secondary differentiation, RBs transform again into infective EBs (Fig. 1).

Exocytosis or host cell lysis completes the development by releasing EBs for new infectious cycles. The length of the complete cycle is dependent on the infecting strain, the host cell, and the environmental conditions, varying from 48 to 72 hours in cell culture models (Beatty et al. 1994). In host cells, Chlamydia organisms share energy and nutrients before destroying the cells and infecting adjacent cells. Structural components, such as the virulence factors of Chlamydia strains and host cell factors, affect the interaction between Chlamydia and host cells (Leinonen 1993). In their attachment to and entry into host cells, Chlamydia use mechanisms that promote survival, growth, and division in a cytoplasmic vacuole for forming inclusions. EBs are infectious, but metabolically inactive, whereas RBs act in an opposite way. Actually, the exact mechanisms that regulate the developmental cycle are still unknown (Campbell & Kuo 2004).

In persistent infections, the alterations in the chlamydial developmental cycle are consistent with the prevention of re-organization from RB to EB. Chlamydia enter into the persistent state after treatment with certain cytokines, such as interferon gamma, treatment with antibiotics, and restriction of certain nutrients (Beatty et al. 1994). Metabolic activity is reduced in the persistent state, and C. pneumoniae is refractory to antibiotic treatment most of the time (Hammerschlag 2002). Tobacco smoke is also able to induce persistent C. pneumoniae infection: in vitro, smoke exposure inhibited productive chlamydial infection and prokaryotic cell division and induced aberrant bodies (Wiedeman et al. 2005). In a persistent infection, Chlamydia remain in a viable, but culture-negative form, and the factors that favor the establishment of persistent C. pneumoniae infection are male sex, advanced age, smoking, concomitant disease, glucocorticoid usage, and hereditary factors (von Hertzen 2002).

The general endotoxin in gram-negative bacteria is lipopolysaccharide (LPS), and the chlamydial version of LPS seems to have lower endotoxin activity than, for instance, enterobacterial LPS (Nurminen et al. 1983, Birkelund et al. 1989). Structurally, chlamydial LPS is similar to the rough form of LPS in enterobacteria and includes both cross-reactive and genus-specific epitopes (Brade et al. 1985, Nurminen et al. 1985, Brade et al. 1986, Brade et al. 1987). In addition to the rough–type LPS, the smooth type of LPS (S-LPS) has also been observed by different methods in chlamydia with a monoclonal antibody against S-LPS (Lukacova et al. 1994). The chemical composition of chlamydial LPS includes components of gram-negative LPS, such as D-glucosamine, phosphate, and long-chain β-hydro fatty acids (Kosma 1999). However, not all chlamydia
species have identical LPS structures (Nurminen et al. 1983, Brade et al. 1985, Nurminen et al. 1985, Brade et al. 1986, Ingalls et al. 1995). Oligosaccharides containing an acidic Kdo (ketodeoxyoctonate) region linked to the unique structural features of the lipid A part of the molecule are related to the low endotoxic activity of chlamydial LPS, and very long-chain fatty acids are typical of chlamydia (Leinonen 1993, Kosma 1999).

The first extensive description of the major outer membrane protein (MOMP), ompA, as a surface protein was provided by Caldwell et al. (Caldwell et al. 1981). Species-, subspecies-, and serovar-specific antigenic determinants are located within C. trachomatis MOMP. C. pneumoniae MOMP seems to be different since it does not react in immunoblotting gels, suggesting that immunodeterminants are conformational (Campbell et al. 1990). Species-specific monoclonal antibodies (MAbs) that react with the surface of C. pneumoniae EBs and the outer membrane complex (OMC) have been used in immunoelectron microscopy (Puolakkainen et al. 1995, Knudsen et al. 1999). The polymorphic membrane proteins (Pmps), MOMP, and outer membrane 2 (omp2) are the major proteins of OMC (Mygind et al. 2004). Analysis of the genome sequences of chlamydial species have shown that the C. trachomatis genome encodes nine and C. pneumoniae 21 Pmp paralogs. The demonstration of Porin-B, Omp85, and several additional open reading frames (ORFs) has greatly increased our understanding of chlamydial genomic information (Stephens & Lammel 2001). The exact function of chlamydial Pmps is not known, even though Pnp expression and its variation within and between strains have been studied during experimental infections (Grimwood et al. 2001, Mygind et al. 2004).

A number of prokaryotic proteins have been localized on the membrane of the inclusion. These Inc proteins have been grouped into a family based on the size of their hydrophobic domain and their localization to the inclusion membrane in the host cell (Subtil et al. 2001). The stress-induced proteins, known as heat shock proteins (HSP), are expressed and/or secreted by C. pneumoniae during chronic, persistent infection, and they can stimulate host cells to produce endogenous HSPs (Kol et al. 1998, Kol et al. 1999). HSPs have been classified into six families according to molecular size: HSP100, HSP90, HSP70, three HSP60, HSP40 and small HSPs (Lamb et al. 2003).

### 2.1.6 Epidemiology

The high prevalence of antibodies to C. pneumoniae suggests that most of us are infected at least once in our lifetime (Kuo et al. 1995). Most of the epidemiologic data on C. pneumoniae is based on serological studies (Forsey et al. 1986, Wang SP 1990, Kanamoto et al. 1991, Marton et al. 1992, Montes et al. 1992), and most of the epidemiological information has been obtained with C. pneumoniae specific MIF tests (Wang & Grayston 1970, Wang SP 1990, Aldous et al. 1992, Wang 2000). Globally, epidemiological studies have indicated that C. pneumoniae is a common cause of respiratory tract infections, causing about 10% of all community-acquired pneumonias in adults (Grayston 1992a) and about 5% of bronchitis and sinusitis cases (Grayston & Wang 1999). In the long run, everyone gets infected with C. pneumoniae, and reinfections are common (Grayston & Wang 1999, Grayston 2000).
In children aged under 5 years, antibodies to *C. pneumoniae* are not common in industrialized countries in contrast to developing and tropical countries (Wang SP 1990, Aldous *et al.* 1992). Before 20 years of age, the seroprevalence rate is 50% and continues to rise to about 80% in elderly men and 70% in elderly women (Kuo *et al.* 1995, Grayston 2000). Analysis of banked serum samples has shown that *C. pneumoniae* is not a new pathogen, but has caused frequent infections long before its discovery (Aldous *et al.* 1992, Grayston 1992b, Karvonen *et al.* 1992, Karvonen *et al.* 1993). The summary table 1 shows antibody prevalences obtained from seroepidemiological studies based on the measurement of *C. pneumoniae* MIF antibodies in different countries.

Antibody levels to *C. pneumoniae* do not remain elevated without frequent reinfection (Kuo *et al.* 1995). It has been shown that the antibodies from the first infection are usually lost within about 5 years (Patnode 1990). In clinical studies and in sera obtained from serum banks, reinfections with *C. pneumoniae* have been demonstrated based on follow-up sera collected during several years (Aldous *et al.* 1992). The high seropositivity in older people can thus be explained by frequent infections during their lifetime (Grayston 2000).

In both sexes, seroprevalence is almost equal up till adolescence, but higher among adult men than adult women (Kuo *et al.* 1995). Smoking has been shown to be associated with *C. pneumoniae* infection (Hahn & Golubjatnikov 1992, Karvonen *et al.* 1994b, Laurila *et al.* 1997a, von Hertzen *et al.* 1998, Mayr *et al.* 2000). In addition, smoking may additionally contribute to atherosclerosis by inducing a persistent chlamydial infection (Wiedeman *et al.* 2005). Annual cyclic variation of *C. pneumoniae* infections has been demonstrated. Periods of higher incidence are followed by years with lower incidence (Gnarpe *et al.* 1999, Saikku 1999). During Finnish epidemics (Ekman *et al.* 1993a, Kauppinen & Saikku 1995), 43% of pneumonia cases were caused by *C. pneumoniae*, compared to the usual causation of approximately 10% of all community-acquired pneumonias (Saikku 1992).
3 Clinical picture and treatment of *Chlamydia pneumoniae* infections

3.1 Clinical picture

3.1.1 Acute infections


In *vitro* actively replicating *C. pneumoniae* have been tested against various antibiotics for the selection of antibiotics for prevention and eradication therapies (Gieffers *et al.*}

3.1.2 Chronic infections

C. pneumoniae infections have been linked to several chronic diseases, including cardiovascular, respiratory, neurological and other diseases (Saikku 1993, Johnston 1997, Hahn 1999, Stratton & Sriram 2003, Whitten & Feuer 2004, Lindholdt & Shi 2006). Recent case reports have associated C. pneumoniae with recurrent optic neuritis (Pohl et al. 2006) and acute generalized exanthematous pustulosis (Manzano et al. 2006), and the association between the severity of primary biliary cirrhosis and C. pneumoniae has been demonstrated in cirrhosis patients (Montano-Loza et al. 2006). C. pneumoniae is an obvious contributor, as a primary or co-pathogen, to acute respiratory tract infections and chronic respiratory conditions. It may act both as a trigger and as an exacerbating factor in chronic obstructive pulmonary disease and asthma (Blasi 2004, Blasi et al. 2005, Johnston SL 2005b). A preliminary report of 10 days of telithromycin in adults with acute asthma exacerbations provided a positive signal similarly to a pilot randomized trial of azithromycin treatment for adults with stable, persistent asthma, which alleviated overall asthma symptoms (Johnston SL 2005a, Hahn et al. 2006). The relationship between serologic evidence of past infections with C. pneumoniae and lung cancer suggests that chronic C. pneumoniae infection may also cause inflammation that leads to carcinogenic processes (Laurila et al. 1997b, Jackson et al. 2000b, Koyi et al. 2001, Anttila et al. 2003, Kocazynebek 2003, Littman et al. 2004, Littman et al. 2005). In all these diseases, C. pneumoniae infection may contribute to the progression and acceleration of disease processes, including atherosclerosis, and be an infectious factor among several other risk factors, including smoking, hypertension, obesity, and genetic background (Leinonen & Saikku 2002, Campbell & Kuo 2004, Littman et al. 2005).

mechanisms of how *C. pneumoniae* can initiate and propagate inflammation that leads to atherosclerosis. *C. pneumoniae* infects alveolar macrophages in the lung, and circulating monocytes become infected and disseminate organisms all over the body (Kothe et al. 2000, Rosenfeld et al. 2000, Haranaga et al. 2003, Gieffers et al. 2004). Arterial endothelium can capture the infected monocytes, which migrate through adjacent endothelial cells into the intima. Smooth muscle cells with infected *C. pneumoniae* EBs stimulate proliferation and can affect the atheroma biology. Infection of macrophages leads to plaque destabilization and thrombus formation, which may induce a myocardial infarction (Kalayoglu et al. 2002, Campbell & Kuo 2004).


Eradication of chronic *C. pneumoniae* infection has turned out to be extremely difficult. Even long courses of antibiotics do not guarantee eradication (Kutlin et al. 2002). Despite the longer treatment and the larger size of the recent trials, no reduction either in events or chlamydial markers have been achieved with antibiotics effective against *C. pneumoniae* (Cannon et al. 2005).

Mouse models (de Kruif et al. 2005) have yielded conclusive evidence of the pathogenic role of *C. pneumoniae*, since the findings of different study groups have been both reproducible and mutually consistent (Moazed et al. 1999, Liu et al. 2000, Blessing et al. 2001, Burnett et al. 2001, Rothstein et al. 2001, Chesbro et al. 2003). Short-term antibiotic treatment against *C. pneumoniae* in mice did not show any differences between doxycycline, azithromycin, erythromycin, amoxicillin-clavulinate, telithromycin, and ciprofloxacin in their efficacy to clear active infection when introduced within two weeks (Malinverni et al. 1995, Masson et al. 1995, Tormakangas et al. 2004b). When antibiotics have been administered 2 weeks after infection, no effects on the atherogenic properties of *C. pneumoniae* have been achieved (Rothstein et al. 2001). Thus, antibiotic therapy seems to clear the acute infection, but does not influence infection-induced atherogenesis, if the therapy has been started too late after infection (de Kruif et al. 2005). In rabbits, treatment started within 5 days after infection was inhibited atherogenesis much more effectively than treatment started after 2 weeks, which induced only a slight effect (Muhslestein et al. 1998, Fong 2000). In addition, rat and pig studies have demonstrated the atherogenic properties of *C. pneumoniae* (Herrera et al. 2003, Pislaru et al. 2003).
Efforts at complete eradication of *C. pneumoniae* from human and animal bodies by antibiotic treatment encounter many difficulties, which are based on the unique obligate intracellular structure, the characteristics of this gram-negative bacterium, and its virus-like developmental cycle (de Kruif et al. 2005). Even though large-scale prospective antibiotic intervention trials have not been successful, knowledge of the right therapy and target population for cure are future challenges (Leinonen & Saikku 2002). In the chronic course of *C. pneumoniae* pneumonitis in mice, combination therapy with azithromycin plus rifampin showed favorable effects (Bin et al. 2000). In animal models (Tormakangas et al. 2004a) and in humans trials, antibiotic monotherapy has proven insufficient to eradicate chronic *C. pneumoniae* infection.
4 Diagnosis of *Chlamydia pneumoniae* infections

4.1 Culture

The isolation of *C. pneumoniae* requires cultivation within eukaryotic host cells, and it has therefore been centralized to research and reference units instead of routine laboratories (Grayston *et al.* 1986, Kuo *et al.* 1986, Kuo & Grayston 1988, Grayston *et al.* 1990, Grayston 1992a). The culturing process is quite time-consuming, technically difficult, and too expensive for routine use. However, it is the only reliable method to confirm the viability of the organism in different specimen types (Dowell *et al.* 2001). *C. pneumoniae* can infect a variety of host cells, including epithelial cells, monocytes/macrophages, endothelial cells, smooth muscle cells and small mononuclear lymphocytes, and multiply in them (Gaydos *et al.* 1996, Airenne *et al.* 1999).

The growth of *C. pneumoniae* in culture is poorer and the inclusions are smaller compared to other *chlamydiae*. This may be the reason for the late recognition and still limited knowledge of this pathogen (Grayston *et al.* 1986, Kuo *et al.* 1986). In the basic procedures, chlamydial infectivity is enhanced by centrifugation onto non-replicating host cells. Conditions affect viability and growth in cells lines. At the end of the 1980’s, Kuo and Grayston studied incubation temperatures, centrifugation speeds, chemical treatments of cell monolayers, and the effect of storage temperature on viability. They showed that +35 °C was a better temperature for growth than +37 °C, and that centrifugation speed enhanced growth. *C. Pneumoniae* in transport medium SPG was rapidly inactivated at room temperature (+22 °C), and only 1 % were viable after 24 h. If cultures can be done from samples (swabs, secretions or tissues) within 24 h, the best storage temperature is –4 °C. Bacterial and fungal growth may cause problems in the refrigerator and at room temperatures, and –70 °C freezing preserves samples from contamination and inactivation (Kuo & Grayston 1988).

In 1992, a Dutch group investigated the influence of variation in pH, NaCl concentration, temperature, and concentrations of calcium and magnesium ions on the infectivity of *C. pneumoniae* EBs in HL cells (Theunissen *et al.* 1992). Use of a HL cell line improved the result of isolation and the passage of *C. pneumoniae* compared to McCoy, Hela 229, and BHK-21 cells in another study (Cles & Stamm 1990). Figure 1 shows the host cell line (BV-2) infected with the Finnish *C. pneumoniae* strain Kajaani 6.
Improvement and standardization of cell culture methods and isolation procedures was needed after the discovery of *C. pneumoniae* and the common recognition of this new pathogen. The isolation of *C. pneumoniae* by cell culture is difficult, because only tens of viable strains have been isolated worldwide during two decades (Cles & Stamm 1990, Fenelon *et al.* 1990, Kuo & Grayston 1990a, Kuo & Grayston 1990b, Popov *et al.* 1991, Ridgway *et al.* 1991, Roblin *et al.* 1992, Maass *et al.* 1993, Maass & Harig 1995, Verkooyen *et al.* 1998, Apfalter *et al.* 2000, Puolakkainen *et al.* 2003, Suchland *et al.* 2003, Wehrl *et al.* 2004). In 1995, Maass and Harig studied the growth rates of eight *C. pneumoniae* strains and five host cell lines. The growth-promoting effect of host cell cytostatics, adherent or suspended host cells, optimal incubation time in the first passage, and the use of multiple blind passages were evaluated. Hep-2 or NCI-H 292 was the most sensitive cell line, and incubation time could be varied from 3 to 7 days in the first passage. Additional passages enhanced chlamydia recovery, and subcultures were shown to be necessary for optimal sensitivity in wild-type strains (Maass & Harig 1995). Outside the host cell, the viability of *C. pneumoniae* rapidly decreases. Laboratory strains are more stable than wild-type strains (Maass & Dalhoff 1995).

Specimens (swabs, secretions, or tissues) can be obtained from nasopharynx or oropharynx, sputum, bronchoalveolar lavage, or tissue biopsy, and peripheral blood mononuclear cells (PBMC) can be isolated from blood. Recommendations concerning the transport and processing of specimens have been introduced. The reliability of the culture is also dependent on cell type, culture media, inoculation, incubation, passages, identification of inclusions, and quality assurance during and after assay (Dowell *et al.* 2001). The specimens should be processed in the laboratory within 24 h (storage at +4 °C) or frozen at −70 °C. Tissue culture plates or shell vials are used in the processes of centrifugation and inoculation onto human cell lines. Cycloheximide-supplemented...
medium is used in three-day incubation at +35º C with 5% CO₂ (Fong 2003). Respiratory specimens have been recommended to be passaged two additional times after primary isolation. Tissue specimens require 4-6 additional passages before the results are determined. Tissue culture plates can be used in the routine isolation of \textit{C. pneumoniae} instead of cumbersome shell vials (Maass & Harig 1995). The additional passages increase the risk of contamination. The specificity of the culture leans on the expertise of the laboratory workers to distinguish true chlamydia inclusions from artifacts (Dowell \textit{et al.} 2001). It is recommended by chlamydia experts that a true positive \textit{C. pneumoniae} culture positive sample should produce inclusions, not just EBs, and it can be passaged and detected by PCR (Boman & Hammerschlag 2002).

\subsection*{4.2 Antigen detection}

Specific monoclonal antibodies to the TWAR organism were produced using the hybridoma technique by Kohler and Milstein (Stephens \textit{et al.} 1982, Kuo \textit{et al.} 1986). The development of monoclonal antibodies for \textit{C. pneumoniae} led to the direct fluorescence antibody (DFA) tests (Peeling 1999). DFA from throat smears, nasopharyngeal swabs, and bronchial lavage or aspirate specimens can be used in the laboratory diagnosis of \textit{C. pneumoniae} infection. Monoclonal antibodies either directly or indirectly labelled with fluorescein can be used for the confirmation of \textit{C. pneumoniae}. In the fixation of the smear, acetone is recommended instead of methanol, as it does not destroy the antigenic reactivity of \textit{C. pneumoniae} EBs (Wang & Grayston 1991).

The sensitivity of DFA has been estimated to be 20\% to 60 \% compared to culture, depending on the site where the specimen has been obtained. Direct fluorescent-antibody staining of throat specimens has been shown to be less sensitive and specific than cell culture for identifying \textit{C. pneumoniae} infection. Several artefacts causing non-specific staining may occur in respiratory specimens containing mucus. Because the reading of the results is highly subjective, the researcher must have sufficient expertise and experience to interpret the results reliably (Grayston \textit{et al.} 1986, Montalban \textit{et al.} 1994).

\subsubsection*{4.2.1 LPS-EIA}

Rapid diagnostic methods for the measurement of chlamydial antigen have been developed by manufacturers and have been successfully used for the diagnosis of \textit{C. trachomatis} infections (Mahony \textit{et al.} 1989, Thomas \textit{et al.} 1994). After improvements, the specificity of EIA tests has recently increased. The recent IDEIA PCE Chlamydia test utilizes a genus-specific monoclonal antibody, a polymer conjugate with a high enzyme antibody ratio (Stanley & Lihme 1995) and a liquid ready-to-use amplification system (Pugh \textit{et al.} 1985). Signal amplification is provided by a polymer conjugate consisting of multiple copies of antibody and enzyme molecules (Okadome \textit{et al.} 1999). Recently, a novel EIA method to quantify serum chlamydial lipopolysaccharide (cLPS) in chronic infection was introduced. In this EIA, lipopolysaccharide-binding protein (LBP) is used to capture LPS, LPS-LBP complexes are bound to the solid phase by using monoclonal
anti-LPS antibody, and detection is carried out by the enzyme-labelled polyclonal anti-LBP antibody (Tiirola et al. 2006).

### 4.2.2 Immune complexes

Chlamydial LPS immunocomplexes (LPSICs) have been detected by two antigen-specific EIAs. For LPS capture assay, microtiter plates are coated with IgG2a mouse monoclonal antibody representing an IgG2a subclass and specific to chlamydial LPS. For IgM capture assay, plates are coated with rabbit anti-human IgM (Leinonen et al. 1990). Immune complexes (IC) containing chlamydial protein antibodies have been isolated by modified polyethylene glycol (PEG) precipitation (Schutzer et al. 1990), and the dissociated ICs have been analyzed in MIF and immunoblot tests (Linnanmaki et al. 1993).

#### Table 2. Diagnosis of C. pneumoniae infections by different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFA</td>
<td>Antigen</td>
<td>20%-60%</td>
<td>70%-95%</td>
<td>Subjective</td>
</tr>
<tr>
<td>Culture</td>
<td>Infectious organism</td>
<td>50%-75%</td>
<td>100%</td>
<td>Subjective</td>
</tr>
<tr>
<td>PCR</td>
<td>DNA</td>
<td>10-100 organisms</td>
<td>95%-100%</td>
<td>Objective</td>
</tr>
<tr>
<td>Serology</td>
<td>Antibodies</td>
<td>60%-80%</td>
<td>90%-100%</td>
<td>Subjective/objective</td>
</tr>
</tbody>
</table>

### 4.3 Serology

In the diagnosis of *C. pneumoniae* infection, the role of serological testing has been emphasized, despite of the weaknesses of the available assays. The variation in laboratory processes and the interpretation of the results of in-house assays and commercially available tests confuses inexperienced researchers and clinicians. MIF (microimmunofluorescence) assay is a subjective method, whereas EIA and time-resolved fluorimetry (TRF) are objective ways to measure *C. pneumoniae* specific antibodies (Peeling 1999, Peeling et al. 2000, Persson & Boman 2000, Persson & Haidl 2000, Tuuminen et al. 2000a, Dowell et al. 2001).

The principle of the MIF test is an indirect fluorescent antibody (FA) technique that detects the binding of the antigen and the antibody using secondarily fluorescein-conjugated anti-globulin called conjugate. The chlamydial antigen contains purified 0.05 % formalin-treated EBs, which have been grown in cell culture. The formalin-fixed antigen can be stored in the refrigerator for many years. The homogenous and dense staining of *Chlamydia* antigen is readable under x 400 magnification, and the enhanced adhesion and facilitated location of the antigen on the microscopic slide are achieved by diluting the antigen with egg yolk sac PBS (Wang & Grayston 1970, Wang 2000).

MIF assay was originally developed for *C. trachomatis* immunotyping. The unique strain classification by San-Pin Wang enabled division of the species *C. trachomatis* into 18 serovars (Wang & Grayston 1970, Wang 1999). Grayston’s group used first a
modification of the MIF test in *C. pneumoniae* serology. They dotted purified *C. pneumoniae* strain, TW183, EBs on the slide instead of *C. trachomatis*. In *C. trachomatis* MIF, cross-reactions do occur between serovars of the same species, such as C and A, E and D, and F and G, whereas *C. pneumoniae* seems to have specific staining (Wang 1999).

The genus-specific lipopolysaccharide (LPS) of the *Chlamydia* species and other organisms such as *Bartonella* and *Bordetella pertussis* have been suggested (Maurin *et al.* 1997, Kutlin *et al.* 1998, Jackson *et al.* 2000a) to induce cross-reactions with major outer membrane protein (MOMP). In endpoint determination, only fluorescence with evenly distributed elementary bodies is an acceptable positive reaction, as shown in figure 2. Over-reading of the MIF test, when all clear, bright fluorescence is accepted, leads to false positive results (Kern *et al.* 1993). Cross-reactivity can thus be avoided by ignoring all but homogeneous fluorescence (Grayston JT 1989, Grayston *et al.* 1990, Wang 1999).

The fluorescence without distinctive elementary morphology is non-specific on the *C. pneumoniae* antigen spot. The positive reaction should be studied until negative reactions are seen in dilution (Wang 1999, Wang 2000). A recent study estimated the serological cross-reactivity between different Chlamydia-like organisms and strengthened the assumption of species specificity, because no cross-reactivity with distantly related species was detected (Casson *et al.* 2006), with the exception of the *P. acanthamoebae* strain Hall’s coccus, which reacted with *C. pneumoniae* (titer of 1/128).

**Fig. 2.** *C. pneumoniae* specific immunofluorescence with EBs on a MIF slide.

In primary infection, the IgM antibody response appears at 6 to 8 weeks after the onset of illness. On the other hand, Ekman et al. showed that IgM antibodies are detected by MIF and CF in primary infection at the onset of illness already (Ekman *et al.* 1993a). The IgM test is usually negative in reinfection, and the IgG antibody rise can be measured earlier within one to two weeks (Kuo *et al.* 1995). The development of the serological response takes place slowly in primary infection, and the antibody response may therefore be missed if convalescent-phase sera are obtained earlier than three weeks after
the onset of symptoms (Boman & Hammerschlag 2002). The rheumatoid factor of human serum interferes with IgM measurements, causing false positive results. Immune IgG interferes with assays for IgM antibodies, competing with specific IgM for substrate-binding sites and forming ICs with rheumatoid factor by mimicking immune IgM antibodies (Verkooyen et al. 1992). The removal of interfering IgG antibodies in the measurement of IgA antibodies made the IgA reactivity patterns easier to interpret, in addition to which prozone effects disappeared and titers increased (Linder & Miettinen 1976). In the measurement of IgA antibodies, rheumatoid factors did not seem to interfere with the MIF method (Jauhiainen et al. 1994).

In primary and reinfections, the diagnosis should rely on convalescent serum specimens, although this is not optimal for patient care (Thom et al. 1994, Ljungstrom et al. 1997, Anttila et al. 1998, Dowell et al. 2001). The diagnosis of acute infection based on a single IgG titer should be avoided and interpreted with caution, because elderly people may have persistently high IgG titers after several reinfections and/or past infections, which may have become chronic (Boman & Hammerschlag 2002).

A long-term association between Chlamydia pneumoniae and its host cells is called persistence (Beatty et al. 1994). Persistence of C. pneumoniae antibodies has been described after acute infections: follow-up studies have shown that IgG antibodies may persist for over three years after primary infections (Falck et al. 1996). The reason for the persistence of C. pneumoniae antibodies may be a latent or chronic infection (Hogan et al. 2004). Before stabilizing and decreasing after an acute infection, antibody titers peak at about six months (Patnode 1990), and antibodies persist longer after a reinfection than after the primary infection (Aldous et al. 1992).

The persistence of C. pneumoniae antibodies has not been studied equally much as the persistence of C. trachomatis antibodies. In C. trachomatis infection, IgG may persist for a long time without any sign of infection. Persistence reveals the failure of the host defence mechanisms at the immunological level, which manifests as reduced or suppressed pro-inflammatory or cytotoxic responses (Mpiga & Ravaoarinaro 2006). After C. trachomatis seroconversion, IgG and IgA antibodies peak within a few weeks and persist for years or decrease slowly. Persistence of C. trachomatis titers depends on the reactivity of the individual’s immune system and the sensitivity of the serological test used. Only ever-infected and never-infected individuals can be differentiated by species-specific serological tests (Clad et al. 2000). In C. pneumoniae infection, the serological diagnosis of chronic infection remains an unresolved question, similarly to C. trachomatis infection: IgG antibodies to C. pneumoniae and C. trachomatis may only be a marker of previous exposure to pathogen (Grayston et al. 1990).

The persistence of IgA antibodies has been thought to reflect chronicity, because the half-life of IgA antibodies is shorter than that of long-lasting IgG (Falck et al. 2002). Especially persistently elevated IgA antibodies have been more strongly associated with coronary heart disease and the predictive risk for coronary events than IgG antibodies (Frick et al. 1987, Danesh et al. 2000, Roivainen et al. 2000, Danesh et al. 2002, Huittinen et al. 2002, Huittinen et al. 2003). In the Finnish study, healthy children persistently seropositive for C. pneumoniae had increased aortic intima-media thickness at the age of eleven years (Volanen et al. 2006). In animals and birds, C. psittaci can persist lifelong and cause occasional disease mostly after stress (Grayston 2000) Thus, the role of reactivation of persistent C. pneumoniae infection in the pathogenesis of
Atherosclerosis, asthma and cancer should be studied (Paltiel et al. 1995, Roivainen et al. 2000, Sotgiu et al. 2001, Huittinen et al. 2003). Antibody-negative persons can show specific cell-mediated immunity, which shows that they are not immunologically naive of *C. pneumoniae* (Halme et al. 1998).

In the CF test, chlamydial genus-specific LPS antibodies are measured. The CF test has an objective end-point (red blood cell lysis) and can detect increased antibody levels in primary infection, but cross-reactions with other *Chlamydia* and other bacteria with rough-type LPS interfere with the measurements, and sensitivity remains low in reinfections (Nurminen et al. 1983, Ekman et al. 1993b, Peeling 1999, Persson & Boman 2000). A serodiagnosis could be obtained much earlier by CF compared to the IgM MIF test, because chlamydial LPS antibodies are mostly of IgM type and are produced early in the primary infection. In the MIF test, IgM antibodies appear later, i.e. about 3 weeks after the onset of symptoms in primary infections (Kuo et al. 1995). There are also non-species-specific, whole-inclusion fluorescence (WIF) kits, in which infected cells are fixed on glass slides (Peeling 1999). The inclusion immunofluorescence (I-IFA) test detects antibodies directly against species and genus-specific structures, if present in inclusions. The cell-grown whole inclusions of *Chlamydia* simplify the production of IF antigens (Richmond & Caul 1975, Saikku & Paavonen 1978). The Inclusion Fluorescent Antibody Test (IFAT) has been demonstrated to be the best screening test for identifying specimens with elevated MIF titers (Tapia et al. 2002). The indirect hemagglutination (IHA) test for chlamydial antibodies was introduced to the diagnostics of *C. psittaci* infections. In this test, tanned sheep erythrocytes sensitized with a deoxycholate extract of *C. psittaci* grown in Vero cell monolayers were used for the measurement of chlamydial antibodies (Lewis et al. 1972). The species-specific hemagglutination (HA) of erythrocytes by purified LPS was used to demonstrate that LPS is a genus-specific chlamydial hemagglutinin (Watkins et al. 1987).

For the determination of chlamydial antibodies in routine diagnostics, the following tests have been applied earlier: agglutination test (Lazarus & Meyer 1939), indirect hemagglutination test (Benedict & O’Brien 1958), radioisotope precipitation test (Hahon & Cooke 1965), gel diffusion test (Gerloff & Watson 1967), radial hemolysis test (Collins & Barron 1970), and tests based on immunoelectrophoresis (Caldwell et al. 1975a, Caldwell et al. 1975b, Caldwell & Kuo 1977). Time-resolved fluoroscopic immunoassay (TRIA) was introduced more than 5 years ago, but the antigen used and the diagnostic criteria have not been specified (Wong et al. 1999b, Danesh et al. 2000, Danesh et al. 2002), and the method has only been used in a few laboratories. In the TRIA method, Europium-labelled streptavidin was used as a stable and highly sensitive non-isotopic label to measure fluorescence (Suonpaa et al. 1992).

The most promising new objective tests are enzyme immunoassay (EIA) modifications, which have the strengths of high throughput, objective end points, technically simple processing, and electronic recording of the results. The detection of antibodies to *C. pneumoniae* can be done with LPS-extracted EBs or synthetic peptides. High hopes have been set for objective EIAs, but the limited published evaluations have not been convincing: there have been problems with sensitivity and specificity (Boman & Hammerschlag 2002) and cross-reactions between the chlamydial species (Gnarpe et al. 2000, Persson & Boman 2000, Stralin et al. 2001).
Recommendations have been introduced by the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada) after reviewing the available approaches in serological testing, culture, DNA amplification and tissue diagnostics. In their recommendations, only the use of microimmunofluorescence test is acceptable with the following interpretation: acute infection (IgM ≥ 16 or 4-fold increase in IgG), possible acute infection (IgG ≥ 512), and presumed past infection (IgG ≥ 16) (Dowell et al. 2001).

4.3.1 Comparison of Chlamydia pneumoniae specific serological tests

The choice of the method is important when investigating the associations between C. pneumoniae and diseases (Schumacher et al. 2001, Hermann et al. 2002, Hoymans et al. 2003). A smaller antibody increase as an indicator of acute infection is acceptable in the quantitative and reproducible EIA tests than in MIF. Fourfold rises are necessary for a reliable laboratory diagnosis in the MIF test, (Tuuminen et al. 2000a). Comparative studies and evaluations of MIF and EIA have not led to clarity or approval of their use in the USA by the Food and Drug Administration (Boman & Hammerschlag 2002). The summary Table 2 shows some of the comparative studies of C. pneumoniae MIF and EIA published during the 2000’s.

4.4 Polymerase chain reaction (PCR)

In the field of infectious diseases, nucleic acid amplification tests have been developed over the past 20 years for the detection of micro-organisms, for diagnostic testing, and for research purposes. The small amount of specific DNA and RNA can be detected rapidly by PCR for micro-organisms that are present in small numbers, nonviable, or growing slowly or are present in material not suitable for culture (Apfalter et al. 2005).

In C. pneumoniae infections, nucleic acid-based amplification techniques such as PCR have been applied to the following clinical samples: respiratory specimens (Gaydos et al. 1993, Grayston et al. 1993, Hyman et al. 1995, Dalhoff & Maass 1996), samples of vascular tissue (Esposito et al. 1999, Jantos et al. 1999, Bartels et al. 2000, Berger et al. 2000, Farsak et al. 2000, Gibbs et al. 2000, Boman & Hammerschlag 2002) and peripheral blood samples (Smieja et al. 2002). Despite their superior sensitivity, there are problems in clinical samples; both false-negative and false–positive results can and do occur. The possible errors can be attributed to preanalytical procedures, sample preparation and DNA extraction, assay design, assay set-up, interpretation and confirmation of results, and assay validation (Apfalter et al. 2005).

Numerous PCR modifications have been developed for the diagnosis of C. pneumoniae infection. The complex interactions of the PCR components require careful individual optimization of procedures for all situations (Boman et al. 1999). The development of different PCR-based assays has led to problems with standardization (Maass et al. 1998, Nadrchal et al. 1999, Wong et al. 1999a). Before adopting a new
assay design or evaluation, the advantages and disadvantages of each PCR type should be carefully estimated.

Single-step PCR is less sensitive than nested PCR, in which the 2-step amplification and the use of two sets of primers improve sensitivity, but there is an increased risk of contamination due to the reamplification of the products (Dowell et al. 2001). In multiplex PCR, more than one target sequences are amplified. In multiplex modifications, the potential risk factors are the decreased sensitivity and specificity when the optimal annealing temperatures for individual primers are not identical. In hot-start PCR, increased specificity is achieved when the temperatures are kept sub-optimal, in order to prevent non-specific primer binding. Fluorescent probe-based assays have recently shown their superiority among the PCR methods. The real-time or end point readings are more sensitive, more specific and better able to avoid contamination than the classic single-step modifications (Boman et al. 1999, Dowell et al. 2001, Boman & Hammerschlag 2002).

The detection of C. pneumoniae is complicated, although specific recommendations for PCR assays have been established by the leading researchers in the field (Dowell et al. 2001). There are still unsolved problems, including sensitivity, specificity, and contamination (Kwok & Higuchi 1989, Apfalter et al. 2002, Apfalter et al. 2005). The long list of issues must be taken into account as a source of possible errors in the preanalytical, analytical, and postanalytical steps (Apfalter et al. 2005).
Aims of the study

1. To compare the antibody findings obtained by the golden standard MIF test to those obtained by objective EIA tests in different populations, and to improve the serological diagnosis of *C. pneumoniae* infection and the measurement of *C. pneumoniae* specific antibodies.

2. To follow the kinetics of IgG and IgA antibodies in multiple sera obtained from the same individuals during follow-up for months or years with MIF and EIA.

3. To compare commercial anti-human IgA FITC conjugates in the MIF test.

4. To evaluate the predictive value of *C. pneumoniae* antibody levels obtained by MIF and EIA before coronary events.
6 Materials and methods

6.1 Study populations

6.1.1 Pneumonia patients (I)
Altogether 261 sera from 90 adults and 32 children with pneumonia (collected in different pneumonia studies) were originally tested by an in-house MIF for *C. pneumoniae* antibodies. Paired sera, obtained at a median interval of 21 days (range, 5-224 days), were available from all patients, and third samples were obtained later from 17 patients (range 14-28 days).

6.1.2 Finnish military recruits (II)
The study population consisted of 518 out of 892 male military recruits from two intake groups (altogether 3697 men) in July 2004 and in January 2005 in Kajaani garrison, Kainuu Brigade, in the northern part of Finland. The 892 recruits were enrolled into a project to study respiratory infections in military conscripts with a previous diagnosis of asthma and randomly chosen conscripts without asthma. Agreement and informed consent were obtained from 420 men in July 2004 (including 113 men with asthma) and 472 men in January 2005 (including 108 men with asthma). Sera were obtained at the beginning and at the end of the service. More than half (58.1%) of the 892 recruits served for 180 days, 6.2% served 270 days, and 27.5% served 362 days. The 518 men (127 asthmatic and 391 non-asthmatic) included in this study completed full 180-day service, and paired sera (arrival-departure) were available from 512 military recruits (123 asthmatic and 389 non-asthmatic). The ages of the participants ranged from 17.4 to 29.6 years, their mean age being 19.6 years. The study protocol was approved by the Medical Ethics Committee of Kainuu Central Hospital.
6.1.3 Healthy laboratory personnel (III)

The study subjects belonged to the personnel of the Department of Virology, University of Helsinki, and National Public Health Institute in Oulu. The annual serum samples were collected for occupational health surveillance and as pre-infection sera. A total of 592 serum specimens from 87 persons, 67 women and 20 men, were tested. Informed consent was obtained from all study subjects. In Helsinki, 407 sera were collected from 47 persons. All of them had had at least three serum samples taken during 1958-1990. The follow-up time varied from 3 to 31 years. From 77% of the persons, blood samples were obtained every year. The sera from 55 subjects in Oulu were collected during the period from 1994 to 1999. One serum sample was available from 11 subjects, two sera from four, and at least three sera from 40 subjects (185 sera) for the measurement of C. pneumoniae antibodies. The follow-up time varied from three to six years. The mean age of 35 women and 5 men was 41 years (range 22-57 years) at the beginning of 1994. The personnel were divided into two age groups: < 40 (16 cases) and ≥ 40 (24 cases) years at the baseline in 1994.

6.1.4 Helsinki Heart Study patients (IV)

The Helsinki Heart Study was a randomized, double-blind, coronary primary prevention trial with gemfibrozil, which has been described in detail previously (Frick et al. 1987, Manttari et al. 1987, Saikku et al. 1988, Linnanmaki et al. 1993, Heinonen et al. 1994, Wang 2000). Briefly, 4081 study participants with non-HDL cholesterol > 5.2 mmol/l and no evidence of coronary heart disease or other significant disease were selected from among 23 531 men between 40 and 55 years of age (Manttari et al. 1987). Two hundred and forty one of the 4081 participants suffered a coronary event, either a nonfatal myocardial infarction or coronary death, during the 8.5-year follow-up time. The sera were obtained at baseline, after 2 years, 0 to 3 months before the coronary event, and after the event, if available. Case-control pairs were matched to the time point of serum sampling, region of residence, and treatment. IgG and IgA C. pneumoniae species specific IgG and IgA antibodies were measured from 1336 serum samples by in-house MIF and Labsystems’ EIA tests.

6.1.5 Statistics

The sensitivity of each test for the detection of C. pneumoniae seropositivity at different cut-off points was calculated with the reference MIF conjugate as the golden standard (I). The strength of agreement between the in-house MIF tests with the Kallesladt conjugate versus the other conjugates were analyzed by calculating the kappa values for each titer (I). The differences in proportions were tested using the chi-square test or Fisher’s exact test as appropriate (II, III), and Spearman’s correlation coefficients were used to compare the results obtained by the in-house MIF and Labsystems EIA kits (III, IV). In the fourth
study (IV), the variables were categorized by quartiles. Conditional logistic regression analysis was used to estimate the predictive value of the MIF- and EIA-based test variables in view of the CHD risk.

6.1.6 Ethical considerations

The study protocol of Helsinki Heart Study (IV) was approved by the Ethical Committee of the Faculty of Medicine, University of Helsinki, by the National Board of Health in Finland, and by the Food and Drug Administration in the USA. The study protocol was also accepted by the Trade Unions and the management of public and private sector industries by whom the participants were employed. For the other study populations (I-III), appropriate ethical permissions had been obtained for antibody measurements. Written consent had been obtained from all participants in our serum archive.

6.2 Laboratory methods

6.2.1 C. pneumoniae specific microimmunofluorescence test (MIF) (I-IV)

In-house MIF. *C. pneumoniae* species-specific IgG and IgA antibodies were measured by in-house MIF (Wang & Grayston 1970) using purified EBs of *C. pneumoniae*, K6 strain, and *C. trachomatis*, L2 strain, as antigen. Slides were prepared on the day before the test or on the test day by experienced laboratory workers. Sera were diluted with 0.25% PBS-egg yolk sac (biodrading Benelux B.V., 3640 AG Mijdrecht, the Netherlands), and fluorescein-conjugated FITC anti-human IgG (Kallestadt, Chaska, MO, USA) and IgA (Sigma, St. Louis, USA) with Amidoschwartz as counterstain were used as conjugates. Interfering IgG antibodies were removed before the measurement of IgA antibodies by Gullsorb (Gull Laboratories, Salt Lake City, USA) reagent for easier interpretation. The sera were tested in serial fourfold dilutions from 1/8 (IgG) and 1/10 (IgA) to the end point, and all test series included positive and negative sera. Incubation time was 60 minutes with diluted sera and 30 minutes with conjugates in a moistured chamber at +37 °C. In study II, fourfold serum dilutions were started from 1/32 in IgG and 1/10 in IgA and IgM antibodies. The serum dilutions were incubated in the moistured chamber for one hour at +37 °C for IgG, overnight at +8 °C for IgA, and for three hours at +37 °C for IgM antibodies.

After incubation, the slides were washed with PBS and distilled water. The mounting fluid for setting coverslips on the slides contained glycerol and Veronal buffer (0.1 M 5,5'-barbituric acid and 0.15 M NaCl (pH 8.5)) (3:2). One experienced reader (MP) viewed all the slides using a Leitz Aristoplan fluorescent microscope with a x 40 objective on the same day or the next day after the preparation of slides.

Seven commercial FITC-conjugated, anti-human, α-chain-specific IgA antibodies were used: Dako (Glostrup, Denmark), Kallestadt (Sanofi Diagnostics Pasteur), Zymed
(San Francisco, USA), Jackson (Jackson Immuno Research USA), Caltag (Burlingame, USA), Labsystems MIF kit conjugate (Labsystems, Finland), and Sigma (St. Louis, USA). Optimal dilutions for the anti-human IgA conjugates were determined using ten IgA-positive sera before comparison, and 1:20 dilution in PBS, with 2% Amido Schwartz as counterstain, was used for all conjugates.

Commercial MIF (I). On the commercial MIF kit slides (ThermoLabsystems, Finland), the EB antigens of C. trachomatis and C. pneumoniae were treated to remove genus-specific lipopolysaccharide (LPS), while the EBs of C. psittaci remained untreated.

6.2.2 C. pneumoniae specific enzyme immuno assay (EIA) (I,III,IV)

Labsystems EIA Chlamydia pneumoniae species-specific IgG and IgA antibodies were measured by a commercial EIA (Labsystems, Helsinki, Finland)\(^1\) test, which is based on indirect solid-phase enzyme immunoassay with horseradish peroxidase as a marker enzyme. Ten microliters of diluted specimen (1/101) were pipetted into microplate wells coated with LPS-extracted antigen, and 100 µl of sample diluent was added one hour before incubation at +37 ºC (Persson & Boman 2000). The test procedures were followed step by step according to the manufacturer’s instructions, except for further titrations, which were not rediluted when the results were higher than the kit’s positive control. The IgG-removing reagent was not included for the measurement of IgA antibodies. The results were expressed as enzyme immuno units (EIU) calibrated to the titers of the Labsystems MIF test (n = 130 in IgG and n = 30 in IgA)\(^2\), following counts by the Genesis II software (Labsystems, Vantaa, Finland and Life Sciences International (UK) LTD, Hampshire, England). The following formula for the calculations of EIU was used:

\[
\text{EIU} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{calibrator}} - A_{\text{blank}})} \times n
\]

Values of 45 EIU or more in the IgG tests and 12 EIU or more in the IgA tests were considered positive, except in substudy III, where the value of 30 EIU or more in IgG antibodies was considered a positive result, as the manufacturer recommended in the kit insert at that time.

\(^1\) Currently Ani Labsystems Ltd. Oy, Helsinki, Finland

\(^2\) n = The calibration factor of EIUs to the titers of MIF test

\(^3\) Currently Thermo Electron Corporation, Vantaa, Finland
7 Results

7.1 Anti-human IgA conjugates in *C. pneumoniae* specific antibody tests (I)

We compared seven commercial fluorescein–conjugated anti-human IgA antibodies by using the in-house MIF test, one commercial MIF kit, and one commercial enzyme immunoassay (EIA) kit for the measurement of IgA antibodies. The sera were obtained from adults and children with pneumonia. Interfering IgG antibodies were removed with Gullsorb reagent before the measurement of IgA antibodies. The Kallestadt conjugate in the in-house MIF was considered as a reference method.

A total of 14 seroconversions were demonstrated in MIF among the paired sera from 122 patients (90 adults and 32 children) with pneumonia by at least one of the anti-IgA conjugates, while no increases were found in EIA. All of the seroconversions were measured in adult patients. A fourfold IgA antibody increase with all of the fluorescein-labelled conjugates was only found in one patient. Five (36%) of the significant fourfold increases were measured by at least two conjugates, and single conjugates reacted in nine patients. Six increases were detected by the reference method. The Jackson conjugate recognized two increases, and the other conjugates only one of the six increases indicated by the reference method. The total number of detected increases varied from 1 to 8 in 14 patients. The commercial IgA EIA kit failed to detect any of the significant rises measured in the MIF tests (Table 3). Earlier, an acute phase infection had been diagnosed in six (43%) out of 14 patients. The diagnostic criteria were based on positive diagnostic results in at least two out of five *C. pneumoniae* tests (two different MIF tests and three commercial EIAs for IgG, IgA, and IgM antibodies). The diagnostic rises of titer in all pneumonia patients measured by five different tests are presented in Table 4.
Table 3. IgA increases in the in-house MIF (with six FITC conjugates) and in commercial MIF and EIA kits compared to the Kallestadt reference conjugate

<table>
<thead>
<tr>
<th>PA</th>
<th>RE</th>
<th>KI</th>
<th>JA</th>
<th>SI</th>
<th>Z</th>
<th>LA</th>
<th>DA</th>
<th>CA</th>
<th>EI</th>
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<td>1</td>
<td>1</td>
<td>1</td>
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</tbody>
</table>

PA = Patient. RE = Reference, Kallestadt. KI = Labsystems with commercial antigen. JA = Jackson. SI = Sigma. ZY = Zymed. LA = Labsystems with in-house antigen. DA = Dako. CA = Caltag. EI = Labsystems IgA EIA. TO = Total.

Table 4. Diagnostic titer increases in different antibody classes by five different antibody tests in 122 patients with pneumonia

<table>
<thead>
<tr>
<th>Antibody classes</th>
<th>In-house MIF</th>
<th>Labs. MIF</th>
<th>Labs. EIA</th>
<th>Savyon</th>
<th>Medac</th>
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<tr>
<td></td>
<td>adult</td>
<td>child</td>
<td>adult</td>
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<td>adult</td>
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<td>IgG, IgA, IgM</td>
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<td></td>
</tr>
<tr>
<td>IgA, IgM</td>
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<td></td>
<td>3</td>
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</tr>
<tr>
<td>IgG, IgA</td>
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<td>7</td>
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<td>1</td>
</tr>
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<td>IgM</td>
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<tr>
<td>Total</td>
<td>19</td>
<td>1</td>
<td>17</td>
<td>1</td>
<td>16</td>
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</tbody>
</table>

Diagnostic criteria: In-house MIF: IgM titer of ≥ 10, fourfold IgG, IgA titer increase between paired sera. Labs. MIF: IgM titer of ≥ 10, fourfold IgG, IgA titer increase between paired sera. Labs. EIA: IgM ≥ 1.1 signal/cut-off (S/CO) ratio, 1.5-fold IgG, IgA titer increase or 1.3-fold IgG, IgA titer increase (values > 130 EU in IgG and 50 EU in IgA). Savyon EIA: IgM ≥ 1.1 cut-off index (COI), a COI increase of at least 40% from the first to the second serum samples in the IgG and/or IgM tests. Medac rEIA: For IgM antibodies, OD values exceeding the upper limit of the grey zone were considered as positive. A fourfold IgG or a twofold IgA increase between paired sera.

In table 5, at a cut-off point of 1/10, the IgA seropositivity rates were significantly higher in the commercial EIA (54%) and MIF (46%) tests compared to the in-house MIF test (38%) (95% confidence intervals for the difference between the rates were 13 to 19 for EIA and 5 to 11 for MIF). In the in-house MIF, the agreement was over 90% with all
conjugates at any cut-off point. The lowest values were recorded with the commercial EIA (range, 81% to 90%) and MIF kits (range 87% to 94%). The strength of agreement between the in-house MIF tests with the Kallestadt conjugate versus other conjugates was analyzed by calculating the values for each titer. In the EIA tests, high EIA values (higher absorbances than the positive control) were not retested relative to the end point, as recommended by the manufacturer. This explains the declined strength of the agreement at high titers in the EIA tests. In the MIF test, all kappa values remained good at all IgA cut-off points, with one exception, in which case the values dropped from good to moderate at an IgA cut-off ≥ 160.

Table 5. IgA seropositivity rates (%) at the different titer cut-off points.

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>Re</th>
<th>Si</th>
<th>La</th>
<th>Da</th>
<th>Ja</th>
<th>Ca</th>
<th>Zy</th>
<th>MIF</th>
<th>EIA</th>
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<tr>
<td>IgA ≥10</td>
<td>38</td>
<td>32</td>
<td>31</td>
<td>39</td>
<td>36</td>
<td>29</td>
<td>33</td>
<td>46</td>
<td>54</td>
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<tr>
<td>IgA ≥20</td>
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<td>21</td>
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<td>22</td>
<td>18</td>
<td>21</td>
<td>28</td>
<td>20</td>
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<tr>
<td>IgA ≥80</td>
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<td>10</td>
<td>14</td>
<td>12</td>
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<td>11</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>IgA ≥160</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>6</td>
<td>3</td>
<td>8</td>
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</table>


The sensitivity of the different conjugates varied from 68% to 97% at titers of ≤ 40. The highest sensitivity values were found by the commercial EIA and MIF kits at a cut-off level of 1/10. The Dako conjugate had the best sensitivity at three cut-off points: 1/20, 1/40, and 1/160. The lowest sensitivity values were recorded for the Caltag conjugate at all cut-off levels by the MIF methods.

### 7.2 Asthmatic and non-asthmatic military recruits and their *C. pneumoniae* antibody levels during military service

We followed up 127 asthmatic and 391 non-asthmatic military conscripts for six months in order to explore the kinetics of serum *C. pneumoniae* antibodies and assessed the association between *C. pneumoniae* and asthma during six months of military service in two intake groups.

During the six-month follow-up of the July 2004 military intake group, *C. pneumoniae* IgG antibodies decreased from the arrival sample to the departure sample, dropping below the cut-off titer level (<32) in 36.9% (62/168) of non-asthmatic and 22.2% (14/63) of asthmatic conscripts (p = 0.034). The corresponding values for IgA antibodies were 15.9% (10/63) in asthmatics and 7.2% (12/167) in non-asthmatics (p = 0.046). In asthmatic conscripts, *C. pneumoniae* IgG antibodies persisted more often than in non-asthmatic conscripts (38.1% versus 19.0%, p = 0.03), whereas no difference was seen in IgA antibodies (Table 6).

During the six-month service of the January 2005 intake group, a decrease of IgG antibodies was demonstrated in 5.0% (3/60) of asthmatics and 8.1% (18/221) of non-
asthmatic conscripts (p = 0.582). *C. pneumoniae* IgG (33.3% versus 29.5%, p = 0.558) and IgA antibody persistence (13.3% vs. 10.4%, p = 0.521) was more prevalent, although not significantly so, in asthmatic than in non-asthmatic conscripts.

Among asthmatic conscripts, during the six-month follow-up period, the appearance of *C. pneumoniae* antibodies was demonstrated more often in the January 2005 intake group than in the July 2004 intake group: 15.0% (9/60) vs. 4.8% (3/63), p = 0.056. The difference in the persistence of *C. pneumoniae* IgG and IgA antibodies between the July 2004 and January 2005 intake groups was non-significant.

Among non-asthmatic conscripts, during the six-month follow-up period, the appearance of *C. pneumoniae* antibodies was demonstrated more often in the January intake group than in the July intake group: 13.6% (30/221) vs. 3.6% (6/168), p = 0.001. The IgG antibody levels persisted more often in the January intake group than in the July intake group (29.5% vs. 19.0%, p = 0.019).

Table 6. Changes in *C. pneumoniae* seropositivity between the arrival and departure sera in two intake groups of Kainuu Brigade. Positive titer value in IgG : ≥32. Positive titer value in IgA: ≥10

<table>
<thead>
<tr>
<th>Intake group</th>
<th>IgG antibodies</th>
<th>IgA antibodies</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>asthmatic</td>
<td>non-asthmatic</td>
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<tr>
<td>Appearance of antibodies</td>
<td></td>
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<tr>
<td>July 2004</td>
<td>4.8% (3/63)</td>
<td>3.6% (6/168)</td>
</tr>
<tr>
<td>January 2005</td>
<td>15.0% (9/60)</td>
<td>13.6% (30/221)</td>
</tr>
<tr>
<td>Disappearance of antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>July 2004</td>
<td>22.2% (14/63)</td>
<td>36.9% (62/168)</td>
</tr>
<tr>
<td>January 2005</td>
<td>5.0% (3/60)</td>
<td>8.1% (18/221)</td>
</tr>
<tr>
<td>Persistence of antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>July 2004</td>
<td>38.1% (24/63)</td>
<td>19.0% (32/168)</td>
</tr>
<tr>
<td>January 2005</td>
<td>33.3% (20/60)</td>
<td>29.5% (65/221)</td>
</tr>
<tr>
<td>p-value ²</td>
<td>0.202</td>
<td>0.019</td>
</tr>
</tbody>
</table>

¹ = p-value or Fisher’s Exact Tests for asthmatic versus non-asthmatic
² = p-value or Fisher’s Exact Tests for comparison between the July 2004 and January 2005 intake groups in asthmatics and non-asthmatics

7.3 Kinetics of *C. pneumoniae* specific antibodies in healthy laboratory personnel (III)

For over 30 years in Helsinki and for 6 years in Oulu, the annual prevalence and persistence rates of *C. pneumoniae* antibodies were followed by MIF in healthy
laboratory personnel. A total of 592 serum specimen had been collected from 87 persons, 67 women and 20 men, during 1958-1990 in Helsinki and during 1994-1999 in Oulu. The follow-up time thus varied from 3 to 31 years, and 77 % of the persons had given blood samples every year.

In Helsinki, the IgG antibody prevalence was 69% (280/407) and the corresponding IgA prevalence 41% (168/405), and all IgM antibody titers remained negative. The annual incidences in Helsinki during 1971-1990 varied from 0 to 15.4/100 person-years (Figure 3). IgG titers from 8 to 64 were seen in 60 % (244/407) of sera, while in 9% (36/407) of sera the titers were ≥ 128. For IgA antibodies high titers were only found in 0.5% (2/405) of the sera, and the seropositivity rate was 41.5% (168/405).

![Fig. 3. The incidence rate /100 person-years measured as seroconversions, suggesting acute infections in the Helsinki personnel during 1971-1990](image_url)

In MIF and EIA, the prevalences of IgG and IgA antibodies in Oulu were significantly higher among the persons ≥ 40 years of age than among those < 40 years old (Table 7). In the younger group, all sera were negative for IgA antibodies, whereas in the older group, the prevalence was 26% (33/126) in the MIF and 60% (75/125) in the EIA test.
Table 7. Prevalence, persistence, and increases and decreases of IgG and IgA antibodies among the Oulu personnel and the significance of the differences between the two age groups

<table>
<thead>
<tr>
<th></th>
<th>Age group &lt; 40</th>
<th>Age group ≥ 40</th>
<th>P-value $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N= 16</td>
<td>N= 24</td>
<td></td>
</tr>
<tr>
<td>MIF %</td>
<td>EIA %</td>
<td>MIF %</td>
<td>EIA %</td>
</tr>
<tr>
<td>IgG prevalence</td>
<td>54 (32/59)</td>
<td>63 (37/59)</td>
<td>72 (91/126)</td>
</tr>
<tr>
<td>IgA prevalence</td>
<td>0 (0/59)</td>
<td>7 (4/59)</td>
<td>26 (33/126)</td>
</tr>
<tr>
<td>IgG persistence$^1$</td>
<td>50 (8/16)</td>
<td>63 (10/16)</td>
<td>83 (20/24)</td>
</tr>
<tr>
<td>IgA persistence$^1$</td>
<td>0 (0/16)</td>
<td>13 (2/16)</td>
<td>17 (4/24)</td>
</tr>
<tr>
<td>IgG + IgA</td>
<td>0 (0/16)</td>
<td>6 (1/16)</td>
<td>4 (1/23)</td>
</tr>
<tr>
<td>IgG increase</td>
<td>0 (0/16)</td>
<td>0 (0/16)</td>
<td>17 (4/24)</td>
</tr>
<tr>
<td>IgA increase</td>
<td>0 (0/16)</td>
<td>6 (1/16)</td>
<td>13 (3/24)</td>
</tr>
<tr>
<td>IgG decrease</td>
<td>0 (0/16)</td>
<td>0 (0/16)</td>
<td>29 (7/24)</td>
</tr>
<tr>
<td>IgA decrease</td>
<td>0 (0/16)</td>
<td>13 (2/16)</td>
<td>4 (1/24)</td>
</tr>
</tbody>
</table>

$^1$criteria for persistence: MIF: titer of ≥ 8 in IgG and in IgA antibodies ≥ 3 years. EIA: EIU ≥ 30 in IgG and EIU ≥ 12 in IgA antibodies ≥ 3 years. $^2$Fisher’s Exact Test or Chi-Square.

In Helsinki, 25 seroconversions in 21 persons were detected during the follow-up, and the incidence rate was 6.9 (25 seroconversions per 360 person years of follow-up). The highest annual incidence rate was 15.4/100 person-years in 1987, and high (over 10/100/year) incidence rates were also recorded in 1980, 1981, and 1986. Before the 1970’s, only one or two seroconversions were recorded annually, because of the small number of serum specimens obtained between the years 1958 and 1969. After the seroconversion and after several years of follow-up, in the persons who had an IgG titer of ≥ 64, the titer decreased in four or more years to 32. In 6 % (3/47) of the personnel, multiple seroconversions in cycles of reinfections were seen (range from 2 to 6 years). IgG antibodies persisted for more than 10 years in 15 % (7/47) and IgA antibodies in 4% (2/47) of the persons. Usually, IgA MIF antibodies decreased rapidly in 1 to 2 years after the seroconversions. In one person in Helsinki, an IgG titer of ≥64 persisted for over 26 years. In Oulu, seroconversions suggesting acute infections were measured only in one person per year, except in 1998 and 1999, when two persons per year seroconverted.

The comparison of MIF and EIA by Spearman’s nonparametric correlation coefficients gave better results in IgG (r = 0.76; P < 0.0001) than in IgA (r = 0.57; P < 0.001) tests. The strength of the agreement in Kappa analysis was moderate for IgG (κ = 0.52) and IgA (κ = 0.43) antibodies. The prevalence of IgG antibodies was about 10 % higher in EIA than in MIF, but the persistence rates of IgG antibodies remained similar. In IgA antibodies, the prevalences in the older group were 60% in EIA vs. 26% in MIF, and the persistence was 63% in EIA vs. 17% in MIF. No MIF seroconversions were detected by EIA and vice versa.
7.4 Predictive value of *C. pneumoniae* specific antibody levels and seroconversions before coronary events (IV)

Two serological methods, MIF and EIA, were used for measuring *C. pneumoniae* specific antibodies in a large prospective cohort of dyslipidemic middle-aged men in the Helsinki Heart Study. The predictive power of the antibody levels and seroconversions were estimated by conditional regression analysis and chi-square or Fisher’s Exact Test.

The results of conditional regression analysis indicated that only high IgA antibodies at baseline have predictive value for coronary events with ORs of 1.69 (unadjusted) and 1.66 (age-adjusted). IgG antibodies measured by either MIF or EIA and IgA antibodies measured by EIA did not have any predictive value for coronary events.

In the analysis of all available 1336 sera, Spearman’s correlation between MIF and EIA was 0.77 (p < 0.0001) for IgG and 0.75 (p < 0.0001) for IgA antibodies. The best agreement between *C. pneumoniae* antibody levels in MIF and EIA was found for high antibody titers. During the 8.5-year follow-up, the participants had several seroconversions in IgG and IgA tests (Table 8). Before the coronary event, 19 (9%) of cases vs. 13 (6.2%) controls (p = 0.26) had IgG seroconversion in MIF and 16 cases (8%) versus 28 (13.6%) controls (p = 0.07) in EIA. Before the event, IgA seroconversions were detected by MIF more commonly (p = 0.13) in 19 (n = 19, 9%) cases than in 11 (n = 11, 5.2%) controls (p = 0.13, ns.), but by EIA less often (p = 0.46) in cases (n = 38, 18.9%) than in controls (n = 45, 21.8%).

Table 8. *C. pneumoniae* seroconversions in cases and controls in the Helsinki Heart Study during 8.5-year follow-up

<table>
<thead>
<tr>
<th>Antibody class and method</th>
<th>N</th>
<th>All N (%)</th>
<th>Before event</th>
<th>Cases N (%)</th>
<th>Controls N (%)</th>
<th>P-value¹</th>
<th>After event</th>
<th>Cases N (%)</th>
<th>Controls N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>421</td>
<td>210/211</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>124/125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG MIF</td>
<td>41</td>
<td>19/13</td>
<td>0.26</td>
<td>5/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA MIF</td>
<td>42</td>
<td>19/11</td>
<td>0.13</td>
<td>4/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG and IgA MIF</td>
<td>9</td>
<td>5/2</td>
<td>0.28</td>
<td>2/1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Either IgG or IgA MIF</td>
<td>64</td>
<td>27/20</td>
<td>0.27</td>
<td>5/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIF</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>407</td>
<td>201/206</td>
<td></td>
<td>118/122</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG EIA</td>
<td>60</td>
<td>16/28</td>
<td>0.07</td>
<td>8/9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IgA EIA</td>
<td>100</td>
<td>38/45</td>
<td>0.46</td>
<td>9/8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IgG and IgA EIA</td>
<td>35</td>
<td>11/16</td>
<td>0.35</td>
<td>2/2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Either IgG or IgA EIA</td>
<td>89</td>
<td>31/41</td>
<td>0.24</td>
<td>13/14</td>
<td></td>
<td></td>
<td></td>
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<td>201/206</td>
<td></td>
<td>118/122</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any MIF and any EIA</td>
<td>21</td>
<td>5/12</td>
<td>0.09</td>
<td>2/2</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

¹ Chi-Square or Fisher’s Exact Test
8 Discussion

8.1 Study populations and laboratory methods

The study populations consisted of short- and long-term follow-up samples from laboratory and military personnel and from patients with pneumonia and coronary heart disease. In the Helsinki Heart study (IV), geographical matching of cases and controls was considered necessary, because of the regional differences in the incidence of CHD and the possible differences in the regional exposure to some infectious microbes. All participants were men, and the controls were matched for age, smoking, and place of residence. In the pneumonia study (I), the selected paired serum samples (collected in different pneumonia studies) were originally tested with the in-house MIF test for *C. pneumoniae* antibodies, to guarantee an adequate serological diagnosis of acute infections for a comparison with IgA conjugates. Randomly selected participants (II), 892 military recruits, were selected from two intake groups of 3697 military recruits. The summer (420 men) and winter (472 men) intake groups made it possible to follow the kinetics of *C. pneumoniae* antibodies seasonally. No statistically significant differences were found in age, education, depressive symptoms, or body mass index (BMI) between the summer and winter intake groups. Only smoking was more prevalent in the winter than the summer intake group. Suggestive of chronic *C. pneumoniae*, infection is more common in smokers and ex-smokers than in non-smokers. (Laurila et al. 1997a) The availability of multiple sera obtained from the same individuals (III), i.e. the two sets of laboratory personnel, made it possible to follow the prevalence and persistence of *C. pneumoniae* antibodies for years and even decades. The laboratory population may, however, have an elevated risk for *C. pneumoniae* infections compared to the general population, as they are exposed to infected materials and chemical compounds.

The MIF test, which is the golden standard of chlamydial serology, has been used for the measurement of *C. pneumoniae* antibodies for over twenty years, and the use of the MIF test actually led to the identification of *C. pneumoniae*, an “unusual *C. psittaci* strain”, after an epidemic of mild pneumonia in Finland (Saikku et al. 1985). In the diagnosis of acute *C. pneumoniae* infection, MIF is still the recommended serological method. Antibodies to all three major human chlamydial species can be measured
simultaneously in the MIF test by using purified formalinized elementary bodies from *C. pneumoniae*, *C. trachomatis* and *C. psittaci* as antigens (Dowell et al. 2001).

In our in-house MIF tests, interfering IgG antibodies were removed with Gullsorb reagent before the measurement of *C. pneumoniae* specific IgA antibodies. The removal of IgG antibodies in *C. pneumoniae* IgA tests makes the interpretation of the findings easier, because the prozone effect disappears. In the sera with high IgG titers, IgA titers also seem to increase. The IgA rheumatoid factor does not interfere with the IgA MIF measurements (Jauhiainen et al. 1994). We tested all anti-human IgA conjugates simultaneously at similar dilutions and in similar laboratory conditions. The antigen densities and quality constraints for all experiments were guaranteed by an optimal concentration of elementary bodies. The interpretation of the results was done under the same microscope and by the same experienced reader (M.P) in all of our studies (I-IV). Our MIF slides were always prepared on the preceding or the same day when the sera were diluted. The slides were prepared by experienced laboratory employees able to maintain all conditions equal between the test runs. Thus, our MIF test run by experienced laboratory technologists can be considered as a golden standard in our studies, but when a new laboratory starts to use the MIF test, they may experience problems especially in interpretation. The intrinsic variation in the MIF test might be caused by differences in the dilution protocol (Tuuminen et al. 2000a).

In the measurements of antibodies by commercial EIAs, we followed the manufacturer’s instructions. The levels of enzyme immuno units in IgG and in IgA antibodies given in the kit’s instructions were used as the cut-off values for seropositivity. The significant EIU change was smaller (1.3-fold) between paired sera, when the EIU levels were higher than 130 EIU in IgG and 50 EIU in IgA antibodies (I, III and IV). Smaller increases are acceptable in the quantitative and reproducible EIA tests than a fourfold increase in MIF, and diagnostic seroconversion can be obtained in a week.

The storage of the samples was not a problem, because all the samples in our studies were stored in identical conditions at -20°C. The antibody titers remained stable after long storage and did not suffer from thawing, as the clear MIF pattern of *C. pneumoniae* indicated no deterioration despite storage. The measurements of the MIF and EIA results were done at different times. The specimens used in the EIA test were thawed one more time than those in the MIF test. For epidemiological research, specimens that have been stored frozen and subsequently thawed can be used, since the additional misclassification rate due to thawing is relatively small (Pearce et al. 1996). Contamination of samples with bacteria, which may be problematic in EIA, can be detected in MIF. In the EIA tests, the long storage of serum may cause the formation of lipid aggregates, which may lead to non-specific results.

In seroepidemiological studies, critical assessment of the diagnostic methods used to analyze the serological association between *C. pneumoniae* and atherosclerosis is missing, because the criteria for chronicity and the adjustment for confounding factors vary (Boman & Hammerschlag 2002). In addition, as shown by the present work, the results are too much dependent on the commercial kits and antigens used (Hoymans et al. 2003). As a useful screening criterion to predict an elevated risk for coronary events, the combination of persistently, but not transiently, elevated IgA antibodies to *C. pneumoniae* and slightly elevated hsCRP values and/or elevated IgA antibodies to human hsp60 has been suggested (Saikku et al. 1992, Roivainen et al. 2000, Huittinen et al. 2003). The
diagnosis of active chronic infection is difficult if based only on C. pneumoniae antibodies. The combination of markers for C. pneumoniae infection with the markers of systemic inflammation or autoimmunity may point to active and progressive disease and thus be a more significant risk factor for a coronary event than C. pneumoniae markers alone. However, follow-up sera for the analysis of the persistence of antibodies are not often available.

8.2 Kinetics of C. pneumoniae antibodies in a healthy population (III)

The third study (III) comprised the unique serum material from healthy employees. This material provided us with a possibility to measure the prevalence and persistence of C. pneumoniae IgG, IgA, and IgM MIF antibodies for 31 years in Helsinki and for 6 years in Oulu based on the multiple sera obtained from the same individuals. The comparison of MIF and EIA results was possible for part of the sera.

When analyzing the annual follow-up sera from healthy persons, we showed that repeated reinfections every fourth year in IgG and every second year in IgA are needed for the persistence of high antibody titers (≥ 64), while low IgG and IgA antibody levels could persist for decades. After seroconversion, IgA antibodies disappear rapidly, and their persistence may represent ongoing chronic processes better than IgG antibodies. In addition, the presence of antibodies did not protect from reinfection. Animal models suggest that repeated infections are needed for the development of chronic infections and for the acceleration of the development of atherosclerosis (Blessing et al. 2000, Erkkila et al. 2002). In this study, we showed that the prevalence and persistence rates of IgA antibodies were higher when measured/defined by EIA (62% and 26%) than when measured by MIF (26% and 17%), respectively, whereas the results for IgG antibody prevalences were quite similar. The prevalence rates in our study were at the same level as in the study of Tuuminen et al. The only difference was seen in the levels of IgA EIA antibodies in the age group of < 40 years old (40% versus 7%), which were much lower than those in the age group of 30-39 years in the study of Tuuminen et al. (Tuuminen et al. 2000b). In our study, the seroconversions by MIF were not confirmed by EIA and vice versa. Several studies have shown the difference between antibody findings obtained by EIA and MIF in the measurement of C. pneumoniae specific antibodies (Messmer et al. 2001, Schumacher et al. 2001, Vammen et al. 2001, Halvorsen et al. 2002). Thus far, the validation of commercial EIA tests in acute and chronic infections has not been done properly, and the lack of a true standard in the serological diagnosis of persistent infections hampers the evaluation of markers for chronicity (Jackson et al. 2000b, Dowell et al. 2001).

8.3 C. pneumoniae antibodies in acute infections (I, II)

In the first study, we included sera from different pneumonia studies to get enough paired serum samples from patients with acute C. pneumoniae infections for the testing of the performance of seven anti-human FITC-conjugated alpha-chain-specific IgA antibodies
in the MIF test. The sera had been stored at -20°C for different periods, but that evidently did not have any effect on the findings of the present study, because all sera were tested by all the methods used simultaneously after thawing.

In a comparative study of fluorescein-labeled anti-human IgA conjugates (I), we evaluated the IgA titers and seroconversions detected with six FITC-conjugated anti-human conjugates in the in-house MIF and two commercial kits by using one commercial MIF test and one enzyme immunoassay kit. We showed the variation between different conjugates in adult pneumonia patients: only one of the seroconversions was found by all conjugates, and most of the detected significant titer increases were obtained with one of the conjugates used without the backup of other conjugates. Our results are in agreement with the previous findings reported by the founder of the MIF method, San Pin Wang, who also pointed to the considerable variation in commercial anti-IgA conjugates (Wang 2000).

In the second study, the study population included young male conscripts, whose sera were collected at the baseline and at the end of military service. Military conscripts are susceptible to respiratory infections, and the crowded living conditions also constitute a high risk for primary and reinfections by *C. pneumoniae*.

In the crowded conditions of the military barracks, the levels of *C. pneumoniae* IgG, IgA, and IgM antibodies changed surprisingly rapidly. The numerous fourfold titer changes (increases and decreases) and changes in *C. pneumoniae* seropositivity during military service support the finding that the frequency of *C. pneumoniae* exposures and also reinfections is high in some individuals. In previous Finnish military studies, military conscripts eligible for the study were selected based on symptoms of upper respiratory infections and pneumonia (Kleemola et al. 1988, Ekman et al. 1993a). Kleemola et al. showed that the epidemics in garrisons lasted for about six months and occurred in all seasons (Kleemola et al. 1988). In three conscripts, two episodes of TWAR pneumonia three weeks to three months apart were demonstrated. In these epidemics, a recent TWAR infection was diagnosed by MIF in 88.5% (62/70) of paired sera, which had been screened previously as chlamydia-positive by CF. Ekman et al reported that laboratory evidence for acute *C. pneumoniae* infection was found in half of the military conscripts enrolled into the study (43 of 86) with symptoms of acute respiratory disease. Among these young men, reinfections with *C. pneumoniae* were surprisingly frequent, and the time from the previous infection was as short as we found in our study, indicating a possible ongoing epidemic in Finland (Ekman et al. 1993a).

### 8.4 *C. pneumoniae* antibodies in chronic infections (IV)

The fourth study was a randomized, double-blind coronary prevention trial with gemfibrozil as previously described (Frick et al. 1987). Case-control pairs were matched to the time point of serum sampling, place of residence, and treatment (gemfibrozil/placebo). The follow-up sera during the 8.5-year follow-up and at different time points before and after coronary events made it possible to estimate the predictive power of *C. pneumoniae* antibodies by the MIF and EIA methods.
Our results showed that the best correlation between antibody levels measured by MIF and EIA was achieved among the persons who had high antibody titers. In regression analysis, only IgA MIF antibodies in the baseline sera had a predictive value for future coronary events. Previous studies have shown an association between *C. pneumoniae* antibodies and cardiovascular diseases (Markus et al. 1999, Sander et al. 2001, Huittinen et al. 2003). Cross-sectional case-control studies have shown a significantly increased risk for coronary heart disease among *C. pneumoniae* seropositive persons, whereas prospective studies have shown no or only a marginal risk (Danesh et al. 2000, Danesh et al. 2002, Bloemenkamp et al. 2003).

The use of a single IgA antibody titer as a marker of chronic infection is not recommended (Dowell et al. 2001). However, our results suggest that IgA antibodies have a predictive value before coronary events and a better ability to detect chronic *C. pneumoniae* state than IgG antibodies because of their rapid decrease after reinfections. The participants who had IgG or IgA MIF seroconversions during the 8.5 years of follow-up had a slightly higher risk for coronary events than the controls. The kinetics of *C. pneumoniae* antibodies measured by EIA and MIF in the analysis of seroconversion and the measurement of IgG and IgA antibodies warrant future research.
9 Conclusions

This study showed that MIF – the golden standard of chlamydial serology - is still the most reliable method for measuring *C. pneumoniae* antibodies. There are no diagnostic criteria for chronic infections, but when testing multiple sera from the same individuals, we were able to show that reinfection or reactivation is needed for the persistence of elevated IgG and IgA antibody levels. In chronic infections and reactivations, IgA persistence may reflect chronic processes better than IgG levels because of the rapid disappearance of IgA levels after seroconversion. The cycle of reinfection and reactivation seems to be faster than previously thought in crowded conditions, such as military service, since we recorded several antibody changes between the arrival and departure sera of military recruits during 6-month service. The presence of antibodies does not provide protection from reinfection.

The EIA test used for the measurement of *C. pneumoniae* antibodies overestimated the prevalence and persistence of IgA antibodies compared to MIF. The closest agreement between the MIF and EIA antibody levels was found among the persons with high antibody titers.

Only high IgA MIF titers to *C. pneumoniae* at the baseline predicted future coronary events. In the present study, seroconversions both in the persons who developed coronary events and in the controls were found by MIF and EIA, but mostly in different persons. The seroconversion suggesting reinfection or reactivation of persistent infection may have a role in accelerating chronic processes, because the persons with MIF seroconversion between consecutive sera had a higher (though non-significant) risk for coronary events than the controls. EIA seroconversions were more common in the controls than in the cases before the coronary events. The difference in the kinetics of EIA and MIF antibodies warrants future studies and supports the use of the MIF method as a golden standard in the measurement of *C. pneumoniae* IgG and IgA antibody levels and seroconversions.
References


Appendix


Summary table 2. Comparative studies on *Chlamydia pneumoniae* MIF and EIA during the 2000’s.

<table>
<thead>
<tr>
<th>Continent</th>
<th>Sample size</th>
<th>Age group</th>
<th>Reference</th>
<th>Collection years</th>
<th>Prevalence %</th>
<th>Total</th>
<th>Cut-off titer ≥</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Africa</td>
<td>64</td>
<td>26-80</td>
<td>Njamnshi et al. 2006</td>
<td>2000-2001</td>
<td>55 64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>India</td>
<td>844</td>
<td></td>
<td>Satpathy et al. 2001</td>
<td>1996-1997</td>
<td>77 72</td>
<td>74 16</td>
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</tr>
<tr>
<td>Japan</td>
<td>3748</td>
<td>&gt;15</td>
<td>Miyashita et al. 2002b</td>
<td>1991-2000</td>
<td>68 61</td>
<td>64 16</td>
<td></td>
</tr>
<tr>
<td>the lowest</td>
<td>300</td>
<td>&gt;15</td>
<td>Miyashita et al. 2002b</td>
<td>1996</td>
<td>59 16</td>
<td>73 16</td>
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</tr>
<tr>
<td>the highest</td>
<td>300</td>
<td>&gt;15</td>
<td>Miyashita et al. 2002b</td>
<td>1993</td>
<td>72 16</td>
<td>64 16</td>
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<td>Korea</td>
<td>240</td>
<td>&gt;21</td>
<td>Choi et al. 1998</td>
<td>1996-1997</td>
<td>70 56</td>
<td>62 32</td>
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<td>Singapore</td>
<td>1068</td>
<td>18-69</td>
<td>Koh et al. 2002</td>
<td>1998</td>
<td>75 66</td>
<td>69 16</td>
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<td>Taiwan</td>
<td>433</td>
<td>20-86</td>
<td>Lin et al. 2004</td>
<td>1999-2000</td>
<td>80 64</td>
<td>72 16</td>
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<td>Australia</td>
<td>451</td>
<td>40-89</td>
<td>Coles et al. 2003</td>
<td>1981</td>
<td>32 19</td>
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<td>150</td>
<td>&gt; 15</td>
<td>Van den Abeeke et al. 1992</td>
<td>1990</td>
<td>61 16</td>
<td>73 16</td>
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<td>Czech</td>
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<td>Sodja et al. 1998</td>
<td>1974-1975</td>
<td>80 16</td>
<td>64 32</td>
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<td>Denmark</td>
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<td>Birkebæk et al. 2000</td>
<td>1995-1997</td>
<td>41 36</td>
<td>40 64</td>
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<tr>
<td>Finland</td>
<td>1000</td>
<td>&gt;14</td>
<td>Karvonen et al. 1992</td>
<td>1998</td>
<td>56 16</td>
<td>48 32</td>
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<td>Finland</td>
<td>1188</td>
<td>25-59</td>
<td>Karvonen et al. 1994a</td>
<td>1982, East</td>
<td>45 38</td>
<td>41 16</td>
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<td>the lowest</td>
<td>593</td>
<td>25-59</td>
<td>Karvonen et al. 1994a</td>
<td>1987, East</td>
<td>54 16</td>
<td>59 16</td>
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<tr>
<td>the highest</td>
<td>593</td>
<td>25-59</td>
<td>Karvonen et al. 1994a</td>
<td>1982, East</td>
<td>41 16</td>
<td>49 16</td>
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<td>France</td>
<td>170</td>
<td>&gt;50 (72%)</td>
<td>Badiga et al. 2003</td>
<td>1991</td>
<td>69 57</td>
<td>65 16</td>
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<td>Hungary</td>
<td>257</td>
<td>46±11.9</td>
<td>Rugontális-Kiss et al. 2002</td>
<td>1989-1990</td>
<td>53 16</td>
<td>32 32</td>
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<td>Iceland</td>
<td>936</td>
<td>&gt;10</td>
<td>Einarsen et al. 1994</td>
<td>1998-1990</td>
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<td>Ireland</td>
<td>400</td>
<td>25-64</td>
<td>O'Neill et al. 1999</td>
<td>1991-1992</td>
<td>73 67</td>
<td>70 16</td>
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<td>Italy</td>
<td>369</td>
<td>20-44</td>
<td>Ferrari et al. 2000</td>
<td>1992-1993</td>
<td>59 16</td>
<td>16 16</td>
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<tr>
<td>Sweden</td>
<td>765</td>
<td>18-64</td>
<td>Gnrpe &amp; Gnrpe 1993</td>
<td>1993-1994</td>
<td>66 59</td>
<td>64 32</td>
<td></td>
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<tr>
<td>Turkey</td>
<td>247</td>
<td>&gt;15</td>
<td>Gencay et al. 1998</td>
<td>1993-1994</td>
<td>66 59</td>
<td>64 32</td>
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<td>Canada</td>
<td>107</td>
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<td>Smeja et al. 2001</td>
<td>1991</td>
<td>32 16</td>
<td>32 16</td>
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<td>USA</td>
<td>9035</td>
<td>6mo-80+</td>
<td>Grayston 2000</td>
<td>1963-1989</td>
<td>50-80</td>
<td>45-75</td>
<td>50-80 8</td>
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<td>South America</td>
<td></td>
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</table>
### Summary table 2. Comparative studies on *Chlamydia pneumoniae* MIF and EIA during the 2000’s.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population</th>
<th>Sample size</th>
<th>Antibody types</th>
<th>Companies</th>
<th>Methods</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persson &amp; Boman 2000</td>
<td>ARI</td>
<td>261</td>
<td>G,A,M</td>
<td>La¹, Sa², Me³</td>
<td>CF, MIF, rELISA, EIA</td>
<td>1) Positive finding should be confirmed by MIF.</td>
</tr>
<tr>
<td>Tuuminen <em>et al.</em> 2000a</td>
<td>Mini-review</td>
<td>G,A,M</td>
<td>La¹, Sa², Me³</td>
<td>CF, MIF, rELISA, EIA</td>
<td>2) Standardized reference preparations for serology</td>
<td></td>
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<tr>
<td>Gnarpe <em>et al.</em> 2000</td>
<td>Hypertension</td>
<td>199</td>
<td>G,A</td>
<td>La¹</td>
<td>MIF, EIA</td>
<td>3) EIA cannot be used unless the background antibodies to other chlamydial species have been clarified. MIF must be used as reference.</td>
</tr>
<tr>
<td>Vannen <em>et al.</em> 2001</td>
<td>Diagnosed small AAA</td>
<td>149</td>
<td>G,A</td>
<td>La¹</td>
<td>MIF, EIA</td>
<td>5) EIA has a strong correlation with MIF.</td>
</tr>
<tr>
<td>Schumacher <em>et al.</em> 2001</td>
<td>CHD cases and controls Ø</td>
<td>394</td>
<td>G,A,M</td>
<td>La¹, Me³</td>
<td>MIF, EIA</td>
<td>6) No answer as to which test gives more reliable results.</td>
</tr>
<tr>
<td>Kido <em>et al.</em> 2001</td>
<td>Ischemic heart disease</td>
<td>25</td>
<td>G,A</td>
<td>IH *, Hitazyme</td>
<td>MIF, Elisa, Hitazyme</td>
<td>7) IgA antibodies correlated better than IgG antibodies</td>
</tr>
<tr>
<td>Kido <em>et al.</em> 2001</td>
<td>Healthy adults</td>
<td>18</td>
<td>G,A</td>
<td>IH *, Hitazyme</td>
<td>MIF, Elisa, Hitazyme</td>
<td>Positivity rates for IgG were higher by Hitazyme</td>
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<tr>
<td>Halvorsen <em>et al.</em> 2002</td>
<td>Various</td>
<td>173</td>
<td>G,A</td>
<td>La¹</td>
<td>MIF, EIA</td>
<td>8) Strength of agreement was good.</td>
</tr>
<tr>
<td>Hermann <em>et al.</em> 2002</td>
<td>Healthy volunteers</td>
<td>80</td>
<td>G</td>
<td>11 tests</td>
<td>MIF, rELISA, EIA</td>
<td>9) Sensitivities and specificities varied.</td>
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<tr>
<td>Bennedsen <em>et al.</em> 2002</td>
<td>Various</td>
<td>220</td>
<td>G,A,M</td>
<td>WRF^, MRL, La¹</td>
<td>MIF</td>
<td>10) Three MIF tests acted in the same way.</td>
</tr>
<tr>
<td>Vainas <em>et al.</em> 2003</td>
<td>Patients with atherosclerosis</td>
<td>141</td>
<td>G,A</td>
<td>La¹</td>
<td>MIF, EIA</td>
<td>11) Sufficient agreement.</td>
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<td>Hoymans <em>et al.</em> 2003</td>
<td>Coronary patients vs. healthy</td>
<td>218</td>
<td>G</td>
<td>Fo², La¹, Me³</td>
<td>MIF, rELISA, EIA</td>
<td>12) The result is influenced by the assay applied.</td>
</tr>
<tr>
<td>Hermann <em>et al.</em> 2004</td>
<td>Various</td>
<td>290</td>
<td>G,A</td>
<td>Sa², Me³</td>
<td>MIF, rELISA, EIA</td>
<td>13) ELISAs are a fast and objective way to measure results.</td>
</tr>
</tbody>
</table>

¹ Labsystems, currently AniLabsystems. ² Savyon. ³ Medac. ⁴ Focus, previously MRL. ⁵ Washington Research Foundation. * In house MIF and recombinant MOMP-ELISA.

° = Acute respiratory infection patients. † = Sexual transmitted disease patients. ' = Acute *Chlamydia pneumoniae* infection. × = Abdominal aortic aneurysm. Ø = Coronary heart disease cases and controls.
902. Leskelä, Hannu-Ville (2006) Human bone marrow stem cells—a novel aspect to bone remodelling and mesenchymal diseases


908. Lauronen, Erika (2007) Course of illness, outcome and their predictors in schizophrenia. The Northern Finland 1966 Birth Cohort study


911. Pylkäs, Katri (2007) ATM, ATR and Mre11 complex genes in hereditary susceptibility to breast cancer

Mika Paldanus

SEROLOGICAL STUDIES ON CHLAMYDIA PNEUMONIAE INFECTIONS