Chlamydia trachomatis antibody testing in screening for tubal factor subfertility : clinical application and the pathogenesis paradigm

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**Chlamydia trachomatis antibody testing in
screening for tubal factor subfertility**
Clinical application and the pathogenesis paradigm

De wereld is een groot boek.
Wie het wil lezen,
moet erin gaan.
Wie het wil begrijpen,
moet erin gaan.
Wie het wil voelen,
moet erin gaan.

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Chlamydia trachomatis antibody testing in screening for tubal factor subfertility

Clinical application and the pathogenesis paradigm

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
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Prof. mr. G.P.M.F. Mols,
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Contents

ABBREVIATIONS	7
CHAPTER 1	
General introduction	9
Preface	
Microorganism	
<i>C. trachomatis</i> infection	
Pathogenesis of <i>C. trachomatis</i> infection	
Immune response and immunopathology of <i>C. trachomatis</i> genital tract infection	
Clinical aspects of female <i>C. trachomatis</i> genital tract infection	
Diagnostic tests	
Serologic screening for tubal factor subfertility	
Outline of the thesis and aims of the study	
CHAPTER 2	
Chlamydia antibody testing in screening for tubal factor subfertility: The significance of IgG antibody titre decline over time	43
CHAPTER 3	
<i>Chlamydia pneumoniae</i> and screening for tubal factor subfertility	55
CHAPTER 4	
Performance of five serological Chlamydia antibody tests in subfertile women	67
CHAPTER 5	
<i>Chlamydia trachomatis</i> in subfertile women undergoing uterine instrumentation: Screen or treat?	83

CHAPTER 6

A mouse model for genital tract pathology by <i>Chlamydia trachomatis</i>	91
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CHAPTER 7

Detection of <i>Chlamydia</i> and mouse hsp60 antibodies in murine serum following <i>Chlamydia trachomatis</i> or <i>Chlamydia pneumoniae</i> infection	111
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CHAPTER 8

General Discussion	121
--------------------	-----

SUMMARY	141
---------	-----

SAMENVATTING	145
--------------	-----

DANKWOORD	149
-----------	-----

CURRICULUM VITAE	151
------------------	-----

Abbreviations

C	Chlamydia
CI	confidence interval
CAT	Chlamydia antibody testing
Chsp	Chlamydia heat shock protein
CO value	cut-off value
EB	elementary body
ELISA	enzyme-linked immunosorbent assay
IFU	inclusion forming units
FITC	fluorescein-isothiocyanate-conjugate
Hhsp	human heat shock protein
HSG	hysterosalpingography
Ig	immunoglobulin
κ	kappa
LCR	ligase chain reaction
LGV	lymphogranuloma venereum
LPS	lipopolysaccharide
LR	likelihood ratio
Mhsp	mouse heat shock protein
MIF	micro-immunofluorescence
Min	minutes
MOMP	major outer membrane protein
MoPn	mouse pneumonitis
NPV	negative predictive value
OR	odds ratio
PBS	phosphate-based saline
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
PPV	positive predictive value
RB	reticulate body
SCI	signal to cut-off index
S/CO value	signal to cut-off value
SEM	standard error of mean
STD	sexually transmitted disease
TP	tubal pathology
TWAR	TaiWan Acute Respiratory disease

1 General Introduction

Preface

Chlamydia (C.) trachomatis is the most common sexually transmitted disease worldwide. In up to 80% of female patients *C. trachomatis* infection remains asymptomatic. These asymptomatic infections remain undetected and consequently untreated. The untreated women are at risk of developing late sequelae such as periadnexal adhesions and tubal occlusion. They may present at fertility clinics many years after the infection. For the evaluation of pelvic pathology as a cause of the subfertility, laparoscopy with dye testing is considered the gold standard. Laparoscopy is an invasive and costly procedure. Since a correlation between the level of *C. trachomatis* antibodies and the presence of tubal factor subfertility has been established, *C. trachomatis* antibody testing (CAT) is incorporated in the fertility work-up as a simple and inexpensive screening method for tubal factor subfertility. However, the predictive value of CAT is limited. For clinical practice the availability of a reliable screening method for tubal factor subfertility is important. Adequately estimating a patients' risk for tubal factor subfertility will prevent loss of time by unjustified expectant management, or the institution of ineffective medical treatment. Furthermore, an adequate screening test will minimise the number of unnecessarily performed laparoscopies in patients without tubal pathology.

The thesis will focus on the value of CAT in predicting tubal factor subfertility. At first, the literature on the microorganism *C. trachomatis* and the way infection is induced will be reviewed. Then the pathogenesis, the immune response and its role in the development of tubal pathology will be described. Subsequently the epidemiology, the clinical manifestations of *C. trachomatis* female genital infection and the need for screening programmes will be discussed. Then an overview of the available diagnostic tests will be given and screening for tubal factor subfertility up to now will be reviewed. The chapter ends with an outline of the present thesis and the aims of the study.

Table 1.

Old classification		New classification		Host	Diseases caused
Genus	Species	Genus	Species		
<i>Chlamydia</i>	trachomatis	<i>Chlamydia</i>	trachomatis	humans	genital tract infection, trachoma, LGV, arthritis, neonatal inclusion conjunctivitis, pneumonia
			muridarum suis	mice, hamsters swine	respiratory infections, pneumonia, ileitis conjunctivitis, enteritis, pneumonia
	psittaci	<i>Chlamydophila</i>	abortus psittaci caviae felis	cattle, sheep, goats birds, humans guinea pigs cats	abortion systemic disease, psittacosis conjunctivitis, genital tract infections conjunctivitis, rhinitis
	pecorum		pecorum	cattle, sheep, goats, koalas, swine	abortion, reproductive disease, conjunctivitis, encephalomyelitis, enteritis, pneumonia, polyarthritis
	pneumoniae		pneumoniae	humans, koalas, horses	respiratory tract infections, pneumonia

Microorganism

Chlamydia is a Gram-negative coccoid microorganism that has a unique obligatory intracellular development cycle. Until recently, the classification of Chlamydia was based on phenotypic, morphologic and limited genetic criteria. The family contained a single genus, Chlamydia. The genus Chlamydia was subdivided into four species: *C. pecorum*, *C. psittaci*, *C. pneumoniae* or TWAR (TaiWan Acute Respiratory disease) and *C. trachomatis*. However, phylogenetic analysis has progressed and more obligatory intracellular organisms that have a Chlamydia-like developmental cycle of replication were identified. Based on these developments, Everett *et al.* (1999) proposed a reclassification. The reclassification would replace the genus Chlamydia and its four species, with two genera, Chlamydia and Chlamydophila, and nine species. The genus Chlamydia contains *C. trachomatis*. The mouse and swine strains that were previously grouped with *C. trachomatis* belong to separate species (*C. muridarum* and *C. suis* respectively). Chlamydophila assimilates the current species *C. pecorum*, *C. pneumoniae* and *C. psittaci* to form *Chlamydophila pecorum*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci*. Three new Chlamydophila species are derived from *C. psittaci*: *Chlamydophila felis*, *caviae* and *abortus*. Chlamydophila *pecorum*, *felis*, *caviae* and *abortus* are not pathogenic in humans. However, Schachter and other Chlamydia researchers, have objected to the proposed reclassification. They object because the reclassification is based on minor sequence differences for genus or species differentiation, instead of important biological properties. Furthermore, according to them, there is not sufficient sequence difference that compels a division of the genus into two genera (Schachter *et al.*, 2001).

C. psittaci primarily infects birds and domestic mammals, while in humans infections are rare. *C. pneumoniae* is a respiratory pathogen infecting lung (Kuo *et al.*, 1995; Hammerschlag, 2000; Hahn *et al.*, 2002) and has also been found in vessels, brain and joints (Saikku *et al.*, 1988; Campbell *et al.*, 1998; Grayston, 2000; Kuo and Campbell, 2000; Taylor-Robinson *et al.*, 2000; Dugan *et al.*, 2002; Leinonen and Saikku, 2002; Ngeh *et al.*, 2002). *C. trachomatis* has been isolated from humans and consists of two human biovars (i.e. biologic variants: genetically identical organisms with affinity for different sources) that are transmitted by sexual or other direct contact. The two biovars have been further subdivided in 19 serovars (i.e. serologic variants based on antigenic differences). The biovar trachoma consists of 15 serovars and the remaining 4 serovars are in the biovar lymphogranuloma venereum (LGV). Serovars in both *C. trachomatis* biovars can cause trachoma (Daniell and Taylor, 1997; Mabey and Fraser-Hurt, 2004; Tabbara, 2001), sexually transmitted disease (Sciarro, 1997; Guaschino and De Seta, 2000), some forms of arthritis (Inman *et al.*, 2000; Ishii *et al.*, 2005; Villareal *et al.*, 2002) and neonatal

conjunctivitis (Deschenes *et al.*, 1990; Di Bartolomeo *et al.*, 2001) and pneumonia (Griffin *et al.*, 1990; Hess, 1993; Colarizi *et al.*, 1996; Monno *et al.*, 2002). Table I summarises the old and new classification.

***C. trachomatis* infection**

Chlamydiae are microorganisms, which may exist in two different forms: an infectious form adapted for extra cellular survival and attachment to new host cells (elementary body (EB)), and an intracellular, metabolically active, non-transmissible form (reticulate body (RB)) (Moulder, 1991; Peeling and Brunham, 1996). *C. trachomatis* infection is initiated by the extra cellular EB, which attaches to epithelial cells at mucosal surfaces. The EB is ingested and in the cell an endocytic vesicle will be formed containing the EB. Fusion of different EB containing vesicles takes place. Within 8–12 h after invasion and fusion, EBs develop into the metabolically active RBs. RBs start to divide inside an endosome derived from the host and eventually occupy a large part of the volume of the host cell cytoplasm (termed Chlamydial inclusion). RBs reorganise into EBs 18–30 hours after invasion of host cells and infected cells begin to release infectious EBs by lysis and exocytosis at 48–72 h after infection.

Although little is known about the pathogenicity of the bacteria and the host defense mechanisms against them, it has been established that surface structures of Chlamydiae are involved in the early steps of infection (attachment and ingestion). At the same time, these surface structures are antigens against which antibodies are raised during infection (Holst *et al.*, 1991). The cell wall structure of the EB of Chlamydia contains both an inner and an outer bilayer membrane. The outer membrane, which consists of a lipopolysaccharide (LPS) and outer membrane proteins (OMP), determine the antigenicity of the Chlamydia cell wall. LPS is a genus-specific antigen shared by all Chlamydiae. The OMP, including the major outer membrane protein (MOMP), contain species and subspecies serotype-specific epitopes (Brade *et al.*, 1990). However, regions with high inter-species homology have been identified among the OMP (Yuan *et al.*, 1989). As a result, protective immunity developed during Chlamydia infection is thought to be directed against serovar-specific MOMP epitopes, but non-serovar-specific immunity might develop as well.

During the infective cycle generating new infectious progeny, Chlamydiae express basal levels of two major antigens: MOMP and heat shock protein (hsp) 60. MOMP is the antigen that confers serovar-specific features to Chlamydiae. Protective immunity developed during Chlamydial infection is thought to be

Figure I. The Chlamydia developmental cycle of infection and replication. EB is elementary body and RB is reticulate body.

directed especially towards serovar MOMP determinants (Ward, 1995; Findlay *et al.*, 2005). Hsps are highly conserved proteins present in most prokaryotic and eukaryotic organisms (Kinnunen *et al.*, 2001). These proteins are highly immunogenic, which results in both humoral and cellular immunity. There are four main groups of structurally related hsp's based on their molecular weights: hsp 90, hsp 70, hsp 60 and small hsp's. Hsp expression is enhanced in cells undergoing stress but also in conditions such as heat shock and nutrient deprivation. Figure I summarises the life cycle of *C. trachomatis*.

Besides the above-mentioned acute, productive infection, a chronic infection form has been described (Beatty *et al.*, 1994b and 1995). Although this chronic infection form is not well-defined, *in vitro* studies have shown that under certain circumstances, e.g. by amino acid deprivation (Coles *et al.*, 1987 and 1993), in the presence of penicillin (Beatty *et al.*, 1994a; Dresès Werringloer *et al.*, 2000), or gamma interferon (IFN- γ) (Beatty *et al.*, 1993; Dean *et al.*, 2000), persistence of Chlamydiae may occur. Recently, Dean and coworkers (2001) established a persistent infection when IFN- γ in physiologic concentrations was present. Thus, during the acute phase of infection the production of inflammatory molecules, under which IFN- γ , occurs to eradicate infection. However, IFN- γ may also contribute to initiation of a persistent Chlamydia infection (Dean and Powers, 2001). This persistence is characterised by incomplete Chlamydia replication and the formation of atypical RBs, which are unable to differentiate into infectious progeny (Beatty *et al.*, 1994a; Koehler *et al.*, 1997). The chronic state of Chlamydia infection might therefore be defined as long-term intracellular persis-

tence of non-infectious atypical Chlamydial forms (Beatty *et al.*, 1994a; Dean *et al.*, 2000; Peters *et al.*, 2005). The role of persistence of infection *in vivo* still needs to be studied.

Pathogenesis of *C. trachomatis* infection

C. trachomatis is transmitted by direct sexual contact. The microorganism infects epithelial cells at mucosal surfaces of the genital tract. Infection is characterised by inflammation, but little is known about the mechanisms that initiate and sustain this inflammatory response to Chlamydia. In response to infection, epithelial cells at mucosal surfaces secrete chemoattractant and proinflammatory cytokines. It has been suggested that these cells act as an early warning system for cells in the underlying mucosa. This concept was studied by Rasmussen and coworkers (1997), who showed that infection of cervical epithelial cells with *C. trachomatis* induced the secretion of proinflammatory cytokines that have chemoattractant and proinflammatory functions. Not only invasion of Chlamydia, but especially its intracellular growth is required for this epithelial cytokine response. Thus, the acute inflammatory response induced by Chlamydia infection is mediated by the local production of proinflammatory cytokines from infected epithelial cells. The mechanisms by which *C. trachomatis* induces inflammation that ultimately leads to tissue damage and scarring, resulting in tubal pathology and subfertility, are only partly understood. It has been suggested that the risk of subfertility increases after repeated episodes of salpingitis (Cohen and Brunham, 1999; Weström, 1980 and 1994; Lehtinen and Paavonen, 1994). Subfertility after salpingitis is due to occlusion of the fallopian tubes by fibrous scarring, adhesion formation, mucosal damage and ovarian encapsulation. Tissue damage due to *C. trachomatis* has been assumed to be immunopathologically similar to trachoma, a chronic keratoconjunctivitis due to infection with *C. trachomatis*. In patients with trachoma, it has been demonstrated that tissue destruction and chronic sequelae are only seen in hosts repeatedly exposed to the microorganism (Grayston *et al.*, 1972, 1985). Although the importance of repeated Chlamydia infections in the development of chronic sequelae has been established (Beatty *et al.*, 1995), reactivation of persistent infection might also play a role in the development of these chronic sequelae.

Evidence exists that *C. trachomatis* may persist in the fallopian tubes in a dormant but viable state (chronic infection) for a long period. Pearce and coworkers (1994) showed that *C. trachomatis* DNA or antigens, but not infectious microorganisms, were present in tubal tissue long after the acute phase of infection. The majority of the studied patients had received antibiotic therapy suggesting a persistent infection even after antibiotic therapy. Data of *in vitro*

studies support this suggestion (Beatty *et al.*, 1994). In patients with histologically documented chronic salpingitis and bilateral tubal occlusions *C. trachomatis* antigens in fallopian tube tissue were found in 11 out of 56 (20%) patients (Dieterle *et al.*, 1998). In women presenting with ectopic pregnancy, both *C. trachomatis* DNA and RNA was found in 7/10 fallopian tube biopsy samples (Gérard *et al.*, 1998). The duration of persistence *in vivo* is not known, but recent evidence suggests that organisms may persist for as long as 5 years in the genital tract (Dean *et al.*, 2000).

It remains to be studied how persistent microorganisms contribute to the development of tubal pathology. For this purpose animal models are of great value since they enable us to study several infection modalities such as acute and chronic infections, re-infections and reactivation of infection in well-defined conditions.

Most of the animal studies have been done in mice and focussed on acute infections. Differences in host (mouse strain dependency) and differences in microorganism (human versus mice *C. trachomatis* serovars) were studied. The end-point of most of the animal studies was the development of infertility, defined as a failure to achieve pregnancy after mating with several male mice of proven fertility. De la Maza *et al.* (1994) studied the susceptibility to Chlamydia infection in different mouse strains. They compared C3H, BALB/c and C57BL/6J mice in their ability to develop salpingitis and tubal pathology after intravaginal inoculation with 3×10^7 inclusion forming units (IFU) *C. muridarum* (previously called mouse pneumonitis (MoPn)). Of these three strains of mice, C3H developed the most extensive disease and had the highest rate of infertility, whereas C57BL/6J mice showed the mildest salpingitis. The duration and severity of infection was significantly increased in the C3H strain when compared to that in the C57 strain (Darville *et al.*, 1997). In addition to differences in mouse strain susceptibility to Chlamydia genital infection also the age of the animals was shown to be important. Older animals were more resistant to infection than younger ones (Pal *et al.*, 2001). Mice are considered to be born immunologically mature, but sexual maturation does not occur in most strains of mice until the animals are 6 to 10 weeks of age. In this respect progesterone and estradiol levels can have an effect on the local immune response, which could affect susceptibility to Chlamydia infection (Kaushic *et al.*, 2000). The results suggested that sexual maturation might play a significant role in the decrease in susceptibility to a *C. trachomatis* upper genital tract infection.

Tuffrey *et al.* (1986a, 1986b) compared the *C. muridarum* biovar with human serovars, and showed that intravaginal inoculation of C3H mice with *C. muridarum* led to hydrosalpinx formation and infertility, whereas intravaginal inoculation with human serovars resolved without tubal pathology. Human serovars caused post-infectious sequelae only when high doses were inoculated directly into the uterine horns in mice pre-treated with progesterone. Progesterone was

given before inoculation to synchronise oestrus. It has been shown that during the follicular phase of the cycle mice are less prone to develop an upper genital tract infection than during the luteal phase (Ito *et al.*, 1984; Pal *et al.*, 1998). Why progesterone pre-treatment increases susceptibility to local infection needs to be investigated. One possibility is that progesterone treatment interrupts the normal reproductive cyclicity, which may enhance the attachment of Chlamydiae and establishment of infection in uterine epithelial cells. It is also possible that progesterone causes immunosuppression, decreasing innate immunity to Chlamydia infection (Tuffrey *et al.*, 1981, 1986a, 1986b, 1990; Kaushic *et al.*, 1998; Brunham and Rey-Ladino, 2005).

Pal *et al.* (1998) performed migration studies in C3H mice, which were intravaginally inoculated with *C. muridarum*, and detected infectious particles in the upper genital tract within 24 hours after inoculation. To ascertain the dose needed to induce infertility, groups of C3H mice were inoculated with 10^4 , 10^5 , 10^6 or 10^7 IFU *C. muridarum*, of which 15%, 45%, 70% and 80% respectively became infertile (in contrast to 10% in the control group). From these dose-dependent results Pal *et al.* (1998) concluded that different doses of inocula lead to different degrees of tubal disease.

Only a few animal studies have been performed on *C. trachomatis* re-infection and tubal pathology. *C. trachomatis* re-infection has been described by Patton *et al.* (1989) in macaques, using different serovars of *C. trachomatis*. Their goal was to induce extensive pathology and distal tubal obstruction, in order to develop an animal model that simulated acute pelvic inflammatory disease in women. Patton and coworkers found that re-infection with homologous and heterologous *C. trachomatis* serovars produced a comparable degree of tubal scarring and distal tubal obstruction.

Furthermore, in 1990 Patton and coworkers studied the effects of repeated cervical inoculations followed by a single direct tubal inoculation with *C. trachomatis* in macaques, to test the hypothesis that tubal inoculation after cervical priming causes more extensive disease than primary tubal inoculation alone. In conclusion, they observed the development of peritubal adhesions whether or not the animals had previously been infected in the cervix. More evidence of inflammation was seen in the upper genital tract in macaques that had cervical inoculations prior to tubal inoculation with *C. trachomatis*, as compared to macaques that had tubal inoculations only. This suggests that repeated infections are more likely to cause extensive tissue damage after prior priming of the genital tract with *C. trachomatis*.

The susceptibility to re-infection after a primary genital tract Chlamydia infection was studied by Rank *et al.* (1988) in female guinea pigs. Their goal was to determine the duration of resistance to re-infection after the primary inoculation.

The animals were infected vaginally with *Chlamydophila caviae* (previously called guinea pig inclusion conjunctivitis agent), and re-inoculated at different intervals. Guinea pigs were considered resistant to re-infection when no Chlamydia could be isolated any longer from cervical swabs. Thirty days after primary inoculation the majority of guinea pigs was resistant to re-infection, but after 77 days all animals could become re-infected again. The course of this re-infection was shorter and of lower intensity as compared to the response in animals after primary inoculation. In this study various immune parameters were examined: a decrease in IgA and IgG antibodies was found 30 days after primary inoculation, and cell mediated immunity (the amount of peripheral blood lymphocytes) was at its maximum 30 days after primary inoculation and decreased thereafter.

Ramsey *et al.* (2000) studied the disease outcome subsequent to primary and secondary urogenital infection with *C. muridarum* or *C. trachomatis*. They hypothesised that protection against infection with *C. trachomatis* is serovar-specific, and that re-infections with different serovars are responsible for pathological responses and tissue damage. To confirm this hypothesis, they studied fertility and hydrosalpinx formation in C3H mice after primary vaginal inoculation and re-inoculation with *C. muridarum* and different *C. trachomatis* serovars. First, Ramsey and coworkers confirmed earlier findings by de la Maza *et al.* (1994) that even a single inoculation with *C. muridarum* induced infertility in C3H mice. Furthermore, they observed that mice inoculated primarily with a *C. trachomatis* serovar and re-inoculated with a different serovar remained fertile. On the other hand, mice re-inoculated with the same *C. trachomatis* serovar became infertile. In conclusion, repeated inoculation with the same *C. trachomatis* serovar seems to induce a more profound inflammatory response as compared to repeated inoculation with different *C. trachomatis* serovars. Those results differ from the results of Patton and coworkers (1990) as described above.

Thus, most animal studies concerning *C. trachomatis* inoculation and re-inoculation deal with acute pelvic inflammatory disease with subsequent salpingitis. Although the studies published are heterogenous concerning the animal strains, the *C. trachomatis* and *C. muridarum* administered, the inoculation route and dose of inoculation used, some conclusions can be drawn from these animal studies: different hosts have shown different susceptibility to infection and different inoculation doses induce different degrees of tubal pathology. In mice, some biovars are more virulent than others, *C. muridarum* generally resulting in infertility after one single inoculation only. In guinea pigs a transient resistance to re-infection has been observed. In macaques cervical priming prior to tubal inoculation increased the extent of tubal pathology. In macaques re-inoculation with the same serovar as well as with different serovars induced infertility. In mice however, re-inoculation with the same serovar induced more pronounced tubal pathology whereas mice

re-inoculated with different serovars remained fertile. Extrapolating the results of the animal studies to the human situation remains difficult. These studies all focussed on the course of acute infections resulting in extensive pathology already after primary inoculation. In women however, *C. trachomatis* infections merely induce silent infections eventually leading to chronic sequelae.

Immune response and immunopathology of *C. trachomatis* genital tract infection

Whether *C. trachomatis* infection results in immunity and / or in disease depends on interactions between the host immune system and specific Chlamydia antigens. Up to now, from the very complex structure of *C. trachomatis*, only a limited number of antigens are serologically recognised and studied during infection. Among these, the major serologically defined antigens are the ones most abundantly accessible on the surface of the EB, i.e. MOMP, LPS and hsp antigens. The immune response consists of both a cellular and a humoral response.

The cellular host response to a primary Chlamydia infection occurs at the mucosal surface within one to two days after infection. The cellular response is characterised by inflammation and mucosal infiltration with neutrophilic lymphocytes and small numbers of monocytes (Kuo, 1988). Neutrophilic lymphocytes are recruited and migrate in large numbers to the site of infection, where phagocytosis of Chlamydia elementary bodies is induced (Yong *et al.*, 1986). This host response eliminates or substantially reduces the bacterial load (Rank *et al.*, 1985; Whittum-Hudson *et al.*, 1987; Monnickendam *et al.*, 1980; Brunham *et al.*, 1985).

Besides the cellular immune response a humoral response occurs. The specific interactions of Chlamydia antigens and T cell subsets in the endometrium during an ascending infection are merely unknown, but plasma cell and T helper (Th)-cell infiltration are pathognomonic for endometritis (Paavonen *et al.*, 1985; Lehtinen *et al.*, 1986; Kiviat *et al.*, 1990). Following local antigen stimulation by *C. trachomatis*, specific mucosal T cells (Th1-cells) stimulate the maturation of the plasma cells (Zeitz *et al.*, 1990). Furthermore, plasma cell infiltration is enhanced by inhibition of cytotoxic suppressor cells in response to *C. trachomatis* antigen stimulation (Paavonen and Wolner-Hanssen, 1989; Punnonen *et al.*, 1989). Plasma cells produce immunoglobulin (Ig) A, IgM and IgG antibodies. IgA provides local immunity at mucosal surfaces. IgM is the earliest antibody to appear after *C. trachomatis* antigenic stimulation, but it declines rapidly and is often succeeded by IgG production. IgM is not itself an opsonising antibody, but it activates processes which are able to induce opsonisation. Secondly, IgG antibodies are formed which are the most abundantly present Igs. IgGs are the most signifi-

cant antibody class for neutralizing LPS and MOMP. They act by blocking the sites of the antigenic molecule that determines attachment to cell receptors, and they also enhance phagocytosis of antigens (Sherris and Ray, 1990).

Animal studies have demonstrated a role for antibodies, particularly mucosal IgA, in protection against (or clearance of) vaginal *C. trachomatis* infection and in this way preventing the infection to ascend to the upper genital tract. In vivo, presence of IgA and IgG antibodies against the *C. trachomatis* MOMP reduced vaginal infection rates; reduced the level of shedding of *C. trachomatis* from the vagina and reduced dissemination of infection to the upper genital tract and the associated inflammatory response (Cotter *et al.*, 1995).

The course of *C. trachomatis* infection largely depends on the interaction and balance of cytokines secreted by activated T cells. The role of this T cell response has been reported in animal models (Patton *et al.*, 1994). Th cells can be divided in Th1 and Th2 cells which each produce specific cytokines. IFN- γ , a product of Th1 cells, has been described as the single most important factor in host defence against Chlamydia, while disease susceptibility has been associated with enhanced expression of IL-10, a marker of Th2 cell activation (Beatty *et al.*, 1993). Disturbance of the balance between Th1 and Th2 cell mechanisms, in the favour of Th2, might enable Chlamydia to survive in the host (Morrison *et al.*, 1989; Paavonen and Lehtinen, 1994). The Th1 response induces IFN- γ , and in vitro studies have shown that treatment of infected cells with IFN- γ limits the replication of Chlamydia (Paavonen and Kruse, 1999). Studies in primates have shown that after a single inoculation with *C. trachomatis*, IFN- γ is induced, suggesting that a Th1-like response predominates (Van Voorhis *et al.*, 1996 and 1997). Furthermore, they showed that after re-infection the Th1 response continued to dominate. Since it is known that repeated infections are more likely to cause tubal pathology, one might speculate this Th-1 response to play a role in the induction of *C. trachomatis* induced tubal pathology.

Although the exact role of the cytokines is not fully defined, Th1-like cytokines are important for the killing of intracellular microorganisms, whereas Th2-like cytokines are known to promote persistence of intracellular bacteria. Especially the presence of IFN- γ has been associated with enhanced killing of microorganisms and elimination of Chlamydia infection (Beatty *et al.*, 1993; Starnbach *et al.*, 1994; Rank *et al.*, 1992). Interestingly, as described earlier, IFN- γ also mediates the development of persisting atypical Chlamydia forms in vitro (Beatty *et al.*, 1994). These data suggest that on the one hand Th1-cytokines (especially IFN- γ) may help to recover from a *C. trachomatis* infection but that on the other hand these cytokines induce the development of persistent infection.

Thus, although the host responses to infection have a protective character, i.e. aim to resolve Chlamydia infection, the immune responses might on the other

hand also form the basis of immunopathogenesis. Especially during re-infection or reactivation of persistent infection, the immune system has already been primed and the recurrent inflammatory reaction might ultimately culminate in destructive pathology of the genital tract.

Many authors have associated the development of chronic sequelae after *C. trachomatis* infection with a host response to hsp, secreted by the microorganism (Toye *et al.*, 1993; Witkin *et al.*, 1993; Arno *et al.*, 1995). Hsps are major immune targets for both cellular and humoral immune responses, and their exact role in the development of *C. trachomatis* induced tubal pathology remains an important issue. They function as an important survival mechanism to safeguard cells and microbes from conditions of stress. Although not the only inducers of hsps, infections may activate hsp expression by the release of proinflammatory mediators or by the immune response they induce. Thus the appearance of hsps is an indication of disturbance of the local cellular environment. More specifically, the immune response to Chlamydia hsp (Chsp) 60 may determine the outcome of Chlamydia infection.

In humans hsp60 is found to be associated with subfertility related to tubal occlusion and ectopic pregnancy in women with pelvic inflammatory disease (PID) (Brunham *et al.*, 1985; Sziller *et al.*, 1998; Wager *et al.*, 1990; Ault *et al.*, 1998; Persson *et al.*, 1999). In salpingeal tissue of patients with tubal pathology due to PID, presence of activated Chsp 60 reactive T cells suggests that these cells play a role in the pathogenesis of tissue damage in tubal pathology (Kinnunen *et al.*, 2000).

Chsp 60 antibody levels have been suggested to be predictive of an enhanced risk of PID (Peeling *et al.*, 1997). As shown by Spandorfer and coworkers (1999) not only hsp 60 but also hsp 10, another member of the hsp family, seems to be involved in tubal pathology. In this study it was shown that tubal occlusion was strongly associated with circulating antibodies to both Chlamydia structural antigens and to the Chsp 10.

In animal models of *C. trachomatis* ocular infection (Morrison *et al.*, 1989) or salpingitis (Peeling *et al.*, 1999) purified Chsp 60 elicits an immune response comparable to that in *C. trachomatis* genital infection.

In conclusion, cellular as well as humoral immune responses play a role in the defense against *C. trachomatis* infection but also in the pathogenesis of the disease.

Clinical aspects of female *C. trachomatis* genital tract infection

Over the last decades the incidence of reported *C. trachomatis* infection has increased dramatically. Although this trend probably reflects improvements in screening,

recognition of asymptomatic infection and reporting, rather than a true rise in the incidence of the disease, *C. trachomatis* is the most common sexually transmitted disease (STD) in the Netherlands (van de Laar *et al.*, 1997) and other western countries. In the United States of America an annual incidence of over 4 million new cases of infection is reported by the Centers for Disease Control, 10 million new infections occur in Europe and 89 million cases occur worldwide each year (Guaschino *et al.*, 2000; Low and Cowan, 2002; Marra *et al.*, 1998).

To identify risk factors associated with *C. trachomatis* infection various studies in STD clinics and general practices have been conducted. A number of risk factors are associated with *C. trachomatis* infection, including female sex (Matondo *et al.*, 1996; Simms *et al.*, 1997), young adult age (Oakeshot *et al.*, 1998), particularly teenage girls (Evans *et al.*, 1993; Grun *et al.*, 1997; Simms *et al.*, 1997) and black ethnic origin (Evans *et al.*, 1993; Oakeshot *et al.*, 1998). In addition, young age is associated with increased risk of repeated infection (Hillis *et al.*, 1994 and 1997). Sexual behaviour risk factors include an increased number of sexual partners (Evans *et al.*, 1993; Grun *et al.*, 1997; Oakeshot *et al.*, 1998) and failure to use barrier contraceptives, such as condoms (Evans *et al.*, 1995). Gaydos and coworkers (1998) conducted a large survey among over 13,000 new female army recruits to design a Chlamydia screening program for asymptomatic infection. The new recruits were screened by ligase chain reaction (LCR) for *C. trachomatis* in urine and information on potential risk factors was obtained. They found the following risk factors to be independently associated with Chlamydia infection: having ever had vaginal sex (odds ratio (OR) 5.9), being 25 years of age or less (OR 3.0), being black (OR 3.4), having had more than one sex partner (OR 1.4) or a new partner in the previous 90 days (OR 1.3), having sex without condom use in the previous 90 days (OR 1.4) and having ever had a STD (OR 1.2).

Genital *C. trachomatis* infection is asymptomatic in up to 80% of women (Holmes *et al.*, 1999; Peipert, 2003), which leaves only 20% of infected women presenting with symptoms. The most common clinical manifestation of *C. trachomatis* infection in women is mucopurulent cervicitis. The patient may present with symptoms such as vaginal discharge, post-coital vaginal bleeding, mild abdominal pain and dysuria. On examination cervicitis is characterised by a mucopurulent (green or yellow) endocervical discharge and a cervix that is erythematous, edematous, and friable (Faro, 2001). Chlamydia may ascend into the upper genital tract and cause PID. In 40% of cases of mucopurulent cervicitis, endometritis is also present (Paavonen *et al.*, 1985). In about 10% of patients with endometritis, Chlamydia has been shown to ascend and cause salpingitis (Cates and Wasserheit 1991). Thus, PID can be considered as an infection that started as cervicitis and progressed up to the genital tract to endometritis and eventually salpingitis. In endometritis the patient may present with only mild lower abdom-

inal pain. More intense pain increases the possibility to include salpingitis in the differential diagnosis. Bimanual pelvic examination may show uterine, adnexal or cervical motion tenderness. Furthermore, an elevated erythrocyte sedimentation rate or elevated C-reactive protein and a temperature greater than 38°C are good predictors of laparoscopically confirmed salpingitis (Weström *et al.*, 1984). Laparoscopy remains the gold standard for the diagnosis of acute salpingitis. The tubal surface will be hyperaemic, edematous and covered with a sticky exudate. The severity of clinical disease is unrelated to laparoscopy findings (Livengood *et al.*, 1992).

Infection of the fallopian tubes can result in damage of the ciliated columnar cells that line their inner surface. This damage prohibits the movement of the cilia, which prevents the ovum from migrating down the fallopian tube. In addition, the inflammatory response within the fallopian tube can result in the development of adhesions blocking the passage of the ovum. This might lead to an ectopic pregnancy, since the fertilized ovum is not able to travel throughout the length of the tube to enter into the uterine cavity for implantation.

The inflammatory response due to *C. trachomatis* infection may result in formation of adhesions at the distal end of the tube. The fimbriae become agglutinated, eventually resulting in occlusion of the tube. The fallopian tube fills with purulent exudate forming a pyosalpinx or abscess. The pyosalpinx can either resolve (with treatment) into a hydrosalpinx, or if closely pressed against the ovary, infect the ovary forming a tubo-ovarian abscess (Faro, 2001).

The organism can exit the fallopian tubes and cause a perihepatitis, known as the Fitz-Hugh-Curtis syndrome. The Fitz-Hugh-Curtis syndrome is an extrapelvic manifestation of PID, caused by *C. trachomatis* as well as by *Neisseria (N.) gonorrhoeae* (Lopez-Zeno *et al.*, 1985). It is composed of two phases, acute and chronic. In the acute phase, a perihepatitis and a focal peritonitis result from the transport of inflammatory peritoneal fluid either directly or by lymphatics to the subphrenic and subdiaphragmatic space. Laparoscopically, those patients have a purulent exudate on the liver capsule. The chronic phase is characterised by violin-string adhesions between the anterior surface of the liver and the abdominal wall. Fitz-Hugh-Curtis syndrome tends to be an incidental finding in patients with a clinical diagnosis of PID because their pelvic symptoms are usually more evident (Wølner-Hanssen *et al.*, 1980 and 1982).

Adequate treatment in order to eradicate *C. trachomatis* is very important in preventing the development of long-term sequelae such as ectopic pregnancy or tubal factor subfertility. Tubal factor subfertility has been found to occur in about 11% of women after a single episode of symptomatic PID, and the risk of ectopic pregnancy is increased six- to sevenfold (Weström *et al.*, 1981).

Treatment of *C. trachomatis* genital infection should be instituted prior to the return of confirmatory diagnostic test results to prevent damage of the fallopian tubes. Mucopurulent cervicitis can be effectively treated by azithromycin single dose (1 g orally) (Martin *et al.*, 1992; Thorpe *et al.*, 1996). The clinical severity of the disease will determine whether hospitalisation of the patient with PID is necessary. Besides the appropriate antibiotic regimen, patient education and treatment of sexual partners are part of the treatment. Several antibiotic regimens have been proven effective, and it is not known whether parental regimens are superior to oral regimens. Treatment of *C. trachomatis* PID should also incorporate treatment to *N. gonorrhoeae* and anaerobic microorganisms since co-infection is common. The treatment regimen actually recommended is ofloxacin 2 x 400 mg and metronidazol 2 x 500 mg daily for 10–14 days (Sexually transmitted diseases guidelines 2002; Centers for Disease Control and Prevention).

However, as described before, 80% of *C. trachomatis* infections remain asymptomatic. These infections remain undiscovered (Thejls *et al.*, 1987). Since the infection is asymptomatic no adequate treatment will be instituted. It has been shown that *C. trachomatis* can persist in the genital tract for a long time (Dean *et al.*, 2000). As described earlier, during this persistent phase long-term sequelae of *C. trachomatis* infection may occur. The result of this infection only becomes evident if the patient develops long-term sequelae such as tubal subfertility, ectopic pregnancy or chronic pelvic pain syndrome (Paavonen, 1992; Schachter *et al.*, 1997).

Since part of these women will 10–15 years later consult a specialist because of subfertility, asymptomatic *C. trachomatis* infections are a source of an important public health problem. In order to avoid these long-term complications, the possibilities of implementing cost-effective screening programmes are nowadays being investigated (Kretzschmar *et al.*, 2001; Welte *et al.*, 2000). *C. trachomatis* can be diagnosed by different tests, such as tissue culture, antibody detection methods, and nucleic acid detection methods such as LCR) and polymerase chain reaction (PCR) (Black, 1997). Because LCR- and PCR-based tests have a higher sensitivity than the other tests, and can also be applied in urine samples, in screening population groups they are the tests of first choice.

The cost-effectiveness of screening depends strongly on the prevalence of infections in the target population, and a reliable estimate of the prevalence of asymptomatic infection in the general population is still lacking. To improve our knowledge of especially asymptomatic infection, screening studies in non-clinic population groups using validated and standardised diagnostic tests have been performed. These studies provide accurate estimates of the prevalence of *C. trachomatis* infection and identify risk factors for infection essential for the planning of screening programmes in selected populations (Simms *et al.*, 1997). Paavonen and coworkers (1998a) demonstrated that screening by DNA amplification tests,

and subsequent treatment of women with positive test results is cost-effective in populations with a *C. trachomatis* prevalence of $\geq 4\%$. Health care costs due to short-term and long-term complications resulting from undiagnosed and untreated *C. trachomatis* infections then will exceed the costs of screening an asymptomatic population.

In the Netherlands the prevalence of asymptomatic *C. trachomatis* infections has been estimated by screening general populations derived from general practices, of which urine samples were tested by means of LCR. In 5714 asymptomatic women between 15–40 years of age derived from 16 inner city general practices, van Valkengoed *et al.* (2000) found an overall prevalence of asymptomatic *C. trachomatis* infections of 2.8%. The highest infection rates were found in women who were 21–25 years of age (4.4%). A prevalence study by van den Hoek *et al.* (1997) among heterosexually active women attending a general practice with no STD-related complaints, found an overall prevalence of positive tests for women of 4.9%, and an age-related prevalence of 13.4% (15–19 years), 7.3% (20–24 years), 5.5% (25–29 years), 2.7% (30–34 years) and 2.3% (35–39 years). To investigate the effects of various screening programs on the prevalence and incidence of asymptomatic *C. trachomatis* infections in women, Kretzschmar and coworkers (2001) used a stochastic simulation model for *C. trachomatis* transmission in an age-structured, heterosexual population with a sexually highly active core group. Different screening scenarios were implemented over a time period of 10 years, and prevalence and incidence computed. Through screening of men and women between ages 15 and 24 years, the prevalence of asymptomatic infections in women could be reduced from 4.2% to 1.4% in 10 years. Increasing the age range of screening up to ages 29 or 34 years led to prevalences of 0.4% and 0.06% respectively, after 10 years. Thus, overall prevalences of asymptomatic cervical *C. trachomatis* infections in the Netherlands between 2.8 and 4.9% were found. The prevalence seems to be strongly age related with a peak in late adolescence and early adulthood (15–25 years). Since universal screening may not be appropriate in a low prevalence population, selective screening has been suggested. So far however, no suitable selective screening criteria for the general population could be identified (van Valkengoed *et al.*, 2000).

In conclusion, the incidence of *C. trachomatis* female genital tract infections is increasing. They include a spectrum of clinical manifestations, varying from cervicitis to endometritis or salpingitis. Part of the 80% asymptomatic and untreated cases, cause serious health care problems by the development of late sequelae such as chronic pelvic pain, ectopic pregnancy or tubal factor subfertility. At present screening possibilities to discover asymptomatic *C. trachomatis* infections are evaluated in order to control health care costs due to the long-term sequelae.

Diagnostic tests

For the diagnosis of acute *C. trachomatis* infection, for years, culture of the organism was the only recommended technique. It has been regarded as the "gold standard" method. This method has the benefit of an excellent specificity, but estimates of sensitivity vary from 50–85% (Schachter and Stamm, 1995). Recent advances in molecular biology have allowed for the development of more sensitive diagnostic assays based on the detection of specific nucleic acids in clinical specimens. The PCR and LCR have been shown to be effective alternatives to cell culture for screening for *C. trachomatis* (Schachter *et al.*, 1994; Quinn *et al.*, 1996; Black, 1997; Paavonen, 1998b; Harindra *et al.*, 2003). Both techniques are based on the amplification of plasmid DNA specific for the microorganism, and have a sensitivity of > 90% (Puolakkainen *et al.*, 1998). Therefore these methods should be considered the tests of choice for diagnosing actual *C. trachomatis* infections. *C. trachomatis* can be detected in endocervical specimens obtained by cervical swabs and in first void urine samples by PCR. This latter non-invasive method of specimen collection is valuable when screening high-risk adolescents.

Since the majority of Chlamydia infections are asymptomatic in the acute phase, microorganisms will as a rule not be detected at the acute phase of infection. Patients however may present several years later, with symptoms suspicious of a previous infection. Since at that moment Chlamydia particles may not be present endocervically, the diagnosis of such previous infections is not possible by using the above-mentioned methods. Therefore, for detection of previous *C. trachomatis* infections, serologic tests detecting *C. trachomatis* antibodies are used. The most commonly used method for antibody detection is (micro-)immunofluorescence (MIF). The MIF test was developed in Seattle by Wang and coworkers (1970) to be used in sero-epidemiological studies. The method is based on the attachment of antibodies in a serum sample to Chlamydia antigens fixed onto glass slides. The visualisation of the reaction occurs through the use of a second antibody labelled with fluorescein-isothiocyanate-conjugate (FITC). A positive immunoreaction is microscopically visualised as a "starry sky" appearance: fluorescent green spots on a slightly red background. Initially, antigens from EBs of each of *C. trachomatis* were included in the test, and provided anti-MOMP, serovar-specific antibody testing. The preparation of multiple antigens made the test technically difficult and impractical, and therefore modifications were introduced (Treharne *et al.*, 1977). The number of antigens was reduced by pooling antigens of epidemiologically related serovars (A–C, D–K, L1–L3), or using one broadly reacting serovar (Suchland *et al.*, 1991). Usually serovar L₂ is used, since sera from patients with genital infections tend to cross-react with L₂. Although the MIF technique is generally considered the standard for serodiagnosis of Chlamydia infections, its use

has several limitations. When using a broadly reacting antigen in the MIF test, unintended cross-reactions among *Chlamydia* species (with *C. pneumoniae* in particular) and other *C. trachomatis* serovars (not related to genital infections) cannot be excluded. Furthermore, the MIF test is technically demanding, time consuming, and requires experienced readers for reliable test results. The use of different assay conditions in different laboratories leads to inter-laboratory variability in results (Peeling *et al.*, 1998). In order to decrease the inter-species cross-reactivity in the MIF test, more specific MIF tests have been developed. The *Chlamydia* species share a major genus-specific LPS, which is a component of the OMP of gram-negative bacteria. In the species-specific MIF test, EBs from *C. pneumoniae*, *C. trachomatis* and *C. psittaci* are used, which are spotted separately on a slide. In order to diminish cross-reactivity between *C. trachomatis* and *C. pneumoniae* species, the LPS has been removed in *C. pneumoniae* and *C. trachomatis* antigens. The *C. psittaci* antigen still contains LPS and therefore serves as a control for both LPS and *C. psittaci* positive sera (Chaim *et al.*, 1989).

In the need for rapid, technically less demanding and reliable tests for detection of *Chlamydia* antibodies, enzyme-linked immunosorbent assays (ELISA) have been developed. ELISAs, although developed already in the 1980s, were introduced relatively late in clinical practice, due to problems in preparing genus-specific antigens (Ossewaarde *et al.*, 1994). ELISA utilises the principle of the binding of antibodies, present in a serum sample, to synthetic *C. trachomatis* peptide coated on a plate. The immunoreaction is detected via a second antibody labelled with an enzyme followed by a substrate reaction inducing a colour reaction. The colour intensity is read by a photometer and is directly proportional to the concentration of *C. trachomatis* antibodies in the serum. The antigens are synthetic peptides derived from variable domains of the MOMP of L₂ (Närvänen *et al.*, 1997), or the MOMP of different *C. trachomatis* serovars (Ohana *et al.*, 1996). By using *C. trachomatis* specific peptides (i.e. without homologous sequences with *C. pneumoniae* MOMP), the tests are supposed to exclusively detect *C. trachomatis* antibodies (Tuuminen *et al.*, 2000). ELISA has many advantages over MIF assays, for example, the objective (observer-independent) reading of the results and the possibility for automation. The newly developed ELISAs have been evaluated and compared by MIF by Clad and coworkers (2000). They used five different serological tests in order to detect seroconversion and persistence of *C. trachomatis* antibodies. A species-specific MIF according to Wang and Grayston (1974), a *C. trachomatis* species-specific enzyme immunoassay incorporating LPS-extracted *C. trachomatis* L₂ EBs (ImmunoComb *Chlamydia* bivalent, Organics, Israel), two different synthetic peptide based species-specific tests (*C. trachomatis* ELISA, Labsystems, Finland and SeroCT ELISA, Savyon, Israel) and a recombinant LPS genus-specific ELISA test (rELISA, MEDAC, Germany) were performed. All five

tests were performed on multiple follow-up sera of women with *C. trachomatis* positive cervical swabs with the purpose of verifying the sensitivity of the different tests during *C. trachomatis* seroconversion, re-infection and long-term follow up. They found *C. trachomatis* ELISA to have the highest sensitivity resulting in earlier seroconversion and higher IgG prevalence rates in patients with *C. trachomatis* positive cervical swabs. The increased sensitivity resulted in a higher negative predictive value. The specificity of the test however, still has to be demonstrated.

In order to determine whether ELISAs distinguished patients with tubal factor subfertility or PID from controls, Paukku and coworkers (1998) compared the genus-specific ELISA, based on a recombinant LPS (rELISA, MEDAC, Germany) and the species-specific *C. trachomatis* ELISA (Labsystems, Finland) with a species-specific MIF (a modified version of Wang and Grayston, 1970). The seroprevalence was measured in patients with known tubal factor subfertility, patients with PID and three control groups. Although neither of the tests was as specific as the MIF, both ELISA tests discriminated well between cases and controls. They found that rELISA was used successfully for the screening for Chlamydia antibodies in general and that *C. trachomatis* ELISA detected species-specific antibodies. They suggested that performance of a combination of serologic tests with defined antigens (LPS-based and MOMP-based tests) could contribute to the clinical decision making process among patients undergoing subfertility or PID work-up. Assuming a lower half-life of the LPS antibodies detected by rELISA as compared with the longer persisting anti-MOMP antibodies detected by *C. trachomatis* ELISA, they should be able to discriminate between recent and past infections.

More recently, Morré and coworkers (2002) compared three commercially available peptide-based IgG assays (*C. trachomatis* ELISA, Labsystems, SeroCT ELISA, Savyon and pELISA, MEDAC) to an in-house MIF assay for detection of *C. trachomatis* antibodies in patients screened for asymptomatic *C. trachomatis* infections by PCR. They concluded that the peptide based ELISAs for the serological detection of *C. trachomatis* antibodies performed as well as the MIF assay, and that since the ELISAs are less time consuming, less expensive and easier to perform than the MIF, they might be preferred in the serodiagnosis of acute *C. trachomatis* infection.

Whether these conclusions are also valid for CAT in screening for tubal factor subfertility remains to be established.

Serologic screening for tubal factor subfertility

Laparoscopy with dye testing of the tubes is the best available diagnostic test for evaluation of tubal patency. As an invasive and expensive procedure, laparoscopy is unsuitable for screening on a large scale. Clinicians have searched for inexpensive and non-invasive screening tests to estimate the risk of tubal factor subfertility in a particular patient, in order to subject a high-risk patient to diagnostic testing (i.e. laparoscopy), and to postpone additional testing in a low-risk patient. Since it was noted that the development of PID and its chronic sequelae is associated with Chlamydia IgG antibody formation, the relation between antibody titres and tubal factor subfertility has been demonstrated in many studies (reviewed by Mol *et al.*, 1997). In a meta-analysis of 23 studies with 2729 patients they assessed the discriminative capacity of CAT in the diagnosis of tubal pathology in subfertile patients. Their results showed that the discriminative capacity depended on the type of assay that was used. ELISA or MIF provided a better discrimination than did immunoperoxidase assays (formerly used technique in screening for Chlamydia and based on peroxidase staining of antigen antibody complexes). They furthermore compared the results of CAT to the results of a former meta-analysis by Swart and coworkers (1995) in which the accuracy of hysterosalpingogram in the diagnosis of tubal pathology was studied. They concluded that accuracy of CAT in diagnosing of tubal pathology (defined as occlusion, hydrosalpinx and peritubal adhesions) is comparable to hysterosalpingography in the diagnosis of tubal occlusion. Based on the initial studies on the association of CAT with tubal factor subfertility, CAT has been introduced as a screening test for tubal factor subfertility, and has been incorporated in fertility work-up on a large scale. The test most widely used for CAT is MIF, which has been considered the gold standard in the serological diagnosis of Chlamydia infection. In the Netherlands, CAT is part of the initial work-up of subfertile couples (NVOG, 2004).

A critical reappraisal of the results reported in literature on screening for tubal factor subfertility by CAT however, reveals heterogenous results and indicates that the predictive value for tubal pathology is limited: sensitivity of CAT varies between 30–88% and specificity between 33–100% (Mol *et al.*, 1997). The heterogenous results might be explained by the lack of uniformity in assays and antibodies used, the use of different cut-off levels for titres, and the definitions of tubal pathology applied. A prospective cohort study was performed by Land and coworkers (1998) to evaluate the implications of varying the definitions of tubal pathology and of modifying the cut-off levels on the clinical impact of CAT in predicting tubal factor subfertility. Calculated by statistical means, the optimum cut-off level as the best combination of sensitivity and specificity was 16. This

Table II. Manufacturer's instructions of MIF and ELISA tests mostly used in Dutch laboratories for *C. trachomatis* antibody testing in screening for tubal factor subfertility

Test	Manufacturer	Intended use	Used Antigens	Suggested species specificity	Results expressed as
Chlamydia trachomatis-Spot IF	Biomerieux	Detection of antibodies directed against genus antigen	Serotype L2 group antigens	No	Titres according to dilution steps
<i>C. pneumoniae</i> IgG Micro-IF*	Labsystems	Detection of <i>C. pneumoniae</i> IgG/IgM antibodies	<i>C. pneumoniae</i> EBs, <i>C. trachomatis</i> and <i>C. psittaci</i> EBs are included as controls, LPS has been reduced in <i>C. pneumoniae</i> and <i>C. trachomatis</i> antigens.	Yes, each well contains three spots for <i>C. pneumoniae</i> , <i>C. trachomatis</i> or <i>C. psittaci</i> / LPS	Titres according to dilution steps for each spot on the slide: <i>C. pneumoniae</i> , <i>C. trachomatis</i> and <i>C. psittaci</i> / LPS antigens
<i>C. trachomatis</i> IgG-EIA	Labsystems	Detection of <i>C. trachomatis</i> IgG antibodies in serum or plasma	<i>C. trachomatis</i> peptides	Yes	Negative-borderline-positive-highly positive
<i>C. trachomatis</i> -IgG-pELISA	MEDAC	Detection of <i>C. trachomatis</i> IgG antibodies	Synthetic peptides from the immunodominant region of the MOMP	Yes	Negative-borderline-positive
SeroCT-IgG ELISA	Savoyon	Detection of <i>C. trachomatis</i> IgG antibodies	<i>C. trachomatis</i> specific peptides	Yes	Negative-borderline-positive

* Primarily developed to detect *C. pneumoniae* antibodies, also used for *C. trachomatis* detection.

statistical model assumes false positive results to have the same impact as false negative results. From a clinical point of view, this assumption might not hold true, and another cut-off level may be more suitable. The consequences of a false negative test result might be loss of time for the patient by unjustified management, or the institution of ineffective medical treatment. False positive results increase health care costs by increasing the number of laparoscopies. Therefore, choosing the appropriate cut-off level will depend on patient characteristics, facilities available and on economic factors. Furthermore, it was concluded that CAT was more accurate in predicting severe distal tubal pathology than unspecified tuboperitoneal abnormalities.

Low sensitivity of CAT may be caused by false negative CAT results. Patients who have false negative CAT results have tubal pathology at laparoscopy in spite of negative Chlamydia antibody titres. A number of these patients will have a Chlamydia unrelated cause for adhesions or tubal occlusion (e.g. endometriosis, cornual polyps, salpingitis due to other microorganisms). However, in a number of patients no antibodies will be found despite previous *C. trachomatis* infection. In MIF tests, either pooled antigens or a single broad-reacting antigen (L₂) is used, and not every possible serotype involved in Chlamydia infection may react with the antigens in the MIF test. Although it is assumed that infiltrating Chlamydia infections give rise to persistent antibody formation, there is evidence that IgG titres may decrease over time (Puolakkinen *et al.*, 1986; Chaim *et al.*, 1992; Piura *et al.*, 1993; Henry-Suchet *et al.*, 1994). Furthermore, the possible impact of effective antibiotic treatment on the IgG antibody levels remains to be established.

Decrease of specificity of CAT by MIF is caused by false positive results. In discussing false positive test results for CAT, the possible cross-reactivity in MIF tests with *C. pneumoniae* is a major issue. Since MIF tests have been modified, and serotype-specific antigens are no longer used, cross-reactivity of other Chlamydia species with pooled antigens or with a broad-reacting single antigen has been demonstrated. Chlamydia IgG antibodies have been found in up to 50–70% of the adult population (Grayston, 1992). However, the prevalence of IgG to *C. trachomatis* peaks between 19–29 years, whereas the highest prevalence of IgG to *C. pneumoniae* occurs after the 60 years (Mannion *et al.*, 1991). Although the subfertile population in which CAT is performed consists of females with an average age between 25–35 years, false positive results due to cross-reactivity with *C. pneumoniae* still should be considered.

For CAT, a variety of test methods, among which peptide based ELISA tests, are used. Commonly used tests in the Netherlands have been summarised in table 2. So far, a study comparing CAT by MIF and CAT by species-specific peptide based ELISA in predicting tubal factor subfertility has not been performed.

Outline of the thesis

C. trachomatis infections mostly remain asymptomatic. In fertility work-up a patient's history is therefore not helpful to identify patients at high-risk for tubal pathology as a cause for subfertility. CAT has been found useful in screening for tubal factor subfertility. However, screening for tubal factor subfertility due to *C. trachomatis* infection by CAT has limitations. The studies described in the thesis intend to elucidate factors that have been hypothesised to be a cause for the limited predictive value of CAT. Moreover, factors that have been hypothesised to improve the predictive value of CAT are evaluated. Furthermore, a mouse model has been developed to obtain more insight in the development of tubal pathology after silent *C. trachomatis* infections since this may shed more light on other useful screening parameters.

The **first aim** of the present study is to elucidate the role of IgG antibody decline over time as a possible cause of false negative CAT results (i.e. negative CAT in patients with tubal pathology at laparoscopy). The course of Chlamydia IgG antibody levels in asymptomatic subfertility patients in whom no active *C. trachomatis* infection is assumed to be present will be studied and described in chapter 2. The highest prevalence of Chlamydia infections is in young adults, and, if tubal pathology results, it will take several years before subfertility will become evident. Within this period IgG titres may decline, and at the time of subfertility investigation they may result in false negative CAT results.

Besides false negative CAT results, false positive CAT results occur. The **second aim** was to evaluate the contribution of *C. pneumoniae* antibodies as a cause of these false positive CAT results (i.e. positive CAT in patients without tubal pathology at laparoscopy), due to cross-reactivity in the MIF test. The results regarding this aim will be presented in chapter 3. For this purpose, an ELISA, considered species-specific for *C. trachomatis* and *C. pneumoniae* will be used. Using this technique, the prevalence of antibodies to *C. pneumoniae* in subfertile women will be established. Secondly, cross-reactivity in the MIF test with *C. pneumoniae* antibodies will be evaluated as a possible cause of false positive CAT results. Thirdly, as it is postulated that re-infection or reactivation may be responsible for the induction of tubal pathology, the additive effect of both *C. pneumoniae* and *C. trachomatis* antibodies on the prevalence of tubal pathology will be evaluated.

The **third aim** is to evaluate the impact of different screening tests on the predictive value of CAT as a screening test for tubal factor subfertility. After the introduction of more species-specific *C. trachomatis* serology tests, we hypothesised that the predictive value of CAT may be improved by using one of these new tests. Therefore, in chapter 4 the predictive value of CAT by species-specific MIF or peptide based ELISA in screening for tubal factor subfertility will be evaluated

and dealt with. CAT will be performed by five tests: MIF, a species-specific MIF and three peptide-based species-specific ELISAs. The findings obtained at laparoscopy will be related to the results of the different CAT tests. As described above, CAT by MIF has limited clinical value due to low sensitivity and specificity. It is expected that the use of species-specific tests will decrease inter-species cross-reactivity. Furthermore, the ELISA tests provide automated observer-independent and therefore objective results. The outcomes of the five tests will be compared to the findings at laparoscopy in order to determine their prognostic value of tubal factor subfertility. Sensitivity, specificity, positive predictive value, negative predictive value, likelihood ratios, odds ratios and 95% confidence intervals will be calculated. Furthermore the impact of combining two or more tests on the predictive value will be studied. For all five tests the distribution of *C. pneumoniae* antibodies in CAT positive patients will be compared to the distribution in CAT negative patients in order to evaluate the role of *C. pneumoniae* antibodies in false positive CAT results.

Women who undergo uterine instrumentation are considered at risk for pelvic infections by *C. trachomatis*, which may either derive from ascending endocervical infections or from reactivation of microorganisms persisting in the genital tract from previous Chlamydia infections. Since subfertility patients frequently undergo uterine instrumentation the **fourth aim** is to determine whether they should either undergo endocervical screening for *C. trachomatis* by PCR, or receive prophylactic antibiotic treatment before the procedure, in order to decrease their risk for reactivation of persistent Chlamydia infections. This will be discussed in chapter 5.

The **fifth aim** is to develop a mouse model in which the role of *C. trachomatis* infection on the development of tubal pathology can be studied. Our main interest will be to develop a mouse model that mimics the hypothesised (often asymptomatic) course of Chlamydia infections in women, implying that a primary *C. trachomatis* infection does not result in extensive tubal pathology, and that only subsequent *C. trachomatis* re-infection will induce tuboperitoneal disease. The study in chapter 6 will at first describe a dose finding study in order to find the optimal inoculation dose for inducing infection. IgG antibody formation will be determined. With this optimal dose single inoculation and re-inoculation of C57BL/6J mice with human *C. trachomatis* L₂ will be performed. At three, nine and eighteen weeks after inoculation genital tracts will be evaluated in situ for gross genital tract pathology after which they will be excised for histologic evaluation. Furthermore, the theory of *C. pneumoniae* playing a contributory role in the development of *C. trachomatis* induced genital tract pathology will be explored.

Finally, in chapter 7, we will evaluate whether Chlamydia and mouse hsp60 (Chsp60 and Mhsp60) antibodies can be detected in serum following *C.*

trachomatis or *C. pneumoniae* infection in a mouse model, to investigate whether auto-immunity, based on cross-reactivity between Mhsp60 and Chsp60 antibodies, could be the underlying mechanism of tubal pathology.

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2 Chlamydia antibody testing in screening for tubal factor subfertility:

The significance of IgG antibody decline over time

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Abstract

Background *Chlamydia (C.) trachomatis* antibody testing in screening for tubal factor subfertility is limited by false negative results, i.e. negative Chlamydia antibody tests in patients with tubal pathology at laparoscopy. The present study was performed to determine whether decline in *C. trachomatis* IgG antibodies over time is responsible for those false negative results.

Methods 39 Women with an initial titre of ≥ 64 were serologically restudied after 4–7 years. A new serum sample was collected from each patient. The initial and the second serum sample were tested for *C. trachomatis* IgG antibodies using a micro-immunofluorescence assay (MIF). A species-specific ELISA was used to validate the MIF test results. All patients filled out a questionnaire to determine risk factors for renewed *C. trachomatis* infection between the initial and second serum sample.

Results Seven of the 39 patients (18.0%) showed a significant decline in IgG antibodies by MIF over a period of 4–7 years, but IgG antibodies never became undetectable. In the 7/39 patients who showed a decline by MIF, signal/cut-off values by ELISA did not decrease.

Conclusion A decline in IgG antibody titre is not a significant cause of false negative Chlamydia antibody test results.

Introduction

In women, up to 80% of Chlamydia infections remain asymptomatic (Quinn *et al.* 1996). These untreated women are at risk of developing chronic sequelae such as periadnexal adhesions and tubal occlusion, and of presenting at fertility clinics about 10–15 years after the primary infection.

Chlamydia immunoglobulin (Ig) G antibodies are thought to persist for years and have therefore been used as markers of a previous *C. trachomatis* infection (Ngeow *et al.* 1996). Chlamydia antibody testing (CAT) has been incorporated in fertility work-up on a large scale, and has been shown to be a non-invasive and cost-effective screening method for tubal factor subfertility (Mol *et al.*, 1997).

The clinical significance of CAT however has its limitations, due to false positive and false negative test results (Land *et al.*, 1998). Patients with false negative CAT results (i.e. negative Chlamydia antibody tests in patients with tubal pathology at laparoscopy) may have non-Chlamydia related causes of adhesions or tubal occlusion, or no antibodies may be found despite previous *C. trachomatis* infection. As a cause of false negative CAT, it has been postulated that IgG antibodies may decline over time after *C. trachomatis* infection, in view of the time span between primary infection in adolescence and fertility work-up in adulthood.

So far, little is known about the course of Chlamydia IgG antibodies over time. Four studies have been published on this subject. The post-therapeutic course of serum Chlamydia IgG antibody titres has been studied in 47 women with acute salpingitis (Henry-Suchet *et al.*, 1994), who had positive IgG antibody titres and were treated with doxycycline. Twelve months after treatment, the IgG titre had decreased in 15 of the 47 (31.9%) women. However, only two of the 47 women (4.3%) reverted from IgG sero-positive to sero-negative in the one-year interval studied. Another report on the effect of treatment with doxycycline on serum Chlamydia IgG antibodies is a study performed in 33 women, who had had an episode of acute salpingitis associated with high IgG titres (> 64) (Piura *et al.*, 1993). Mean follow-up time was 34.2 months (range 24–49 months). No significant change in IgG titres was demonstrated in 21 women (63.6%), a significant increase in titre (four-fold or more) was found in eight women (24.2%), and a significant decrease in four women (12.1%). The authors concluded that post-treatment titres remain high in most cases, even when treatment resulted in complete resolution of clinical signs and symptoms of the disease. The third study published deals with the long-term persistence of serum Chlamydia IgG antibodies in patients with high initial IgG antibody titres ($\geq 1:128$) in serum after pelvic inflammatory disease (Puolakkainen *et al.*, 1986). The follow-up time was between 3.1 and 6.3 years. Twenty-six out of 60 patients (43.3%) showed an insignificant change in Chlamydia IgG serum antibody levels, 8 (13.3%) showed a

significant increase and 26 (47.3%) showed a significant decrease. The fourth study published on titre course has been performed in asymptomatic patients during a community oriented screening program among 860 women of reproductive age (Chaim *et al.*, 1992). Twenty-one patients (2.4%) were found to have Chlamydia IgG antibody titres > 64 . These 21 patients were treated with doxycycline, after which IgG titres were followed in 17 of the cases. Over a five-year interval, IgG titres were unchanged in one case (5.9%), had increased twofold in one case (5.9%), decreased significantly (threefold or more) in 11 cases (64.7%) and decreased twofold in four cases (23.5%).

In conclusion, the four studies published on the course of Chlamydia IgG antibody titres describe 153 patients, of which 56 (36.6%) had a significant titre decline during the study period. However, all papers concern the post-therapeutic course of *C. trachomatis* levels in patients with active genital infections, immediately after the institution of therapy. The objective of the present study was to investigate the course of Chlamydia IgG antibody levels in asymptomatic subfertility patients, in whom no active *C. trachomatis* infection is assumed to be present. A MIF test was used, as this test is still considered the best available standard, and a species-specific ELISA was used to validate the MIF test results.

Materials and Methods

In our hospital, CAT has been part of the fertility work-up since 1992. Blood is drawn at the patient's first visit and CAT is performed. Between 1992 and 2000, from 253 patients spare serum (Sample-1) was stored for research purposes. Of those 253 patients, 67 had an antibody titre of ≥ 64 , which we considered positive (Land *et al.*, 1998), and had a follow-up period of at least four years. These women were approached for a second blood sample (Sample-2). Forty-five women could be traced, and 39 women agreed to participate in the study. After a mean follow-up time of 5 years and 7 months (range 4 years and 0 months to 7 years and 2 months), Sample-2 was obtained from these 39 patients. In all 39 patients Sample-1 was re-determined simultaneously with the determination of Sample-2 by MIF. In 33 patients sufficient Sample-1 and Sample-2 serum was available for additional antibody testing by ELISA.

An indirect MIF antibody technique for *C. trachomatis* IgG antibodies was used. In short, 20 μ l of serum was diluted eight times in phosphate buffered saline (PBS) (pH 7.4) and incubated on the *C. trachomatis*-spot immunofluorescence substitute slides (egg grown *C. trachomatis* biovar L₂; Biomerieux, 's Hertogenbosch, the Netherlands) for 30 min at 37°C in a moist chamber. The slides were washed three times for 5 min in PBS and incubated with fluorescein-conjugated rabbit

antihuman IgG (Dako, ITK Diagnostics B.V., Uithoorn, the Netherlands) diluted in PBS for 30 min at 37°C. After three washings in PBS and one in ultrapure water, processed through a milli-Q purifying system (Millipore, Bedford, MA, USA), slides were embedded in Fluoprep mounting medium (Biomérieux, 's Hertogenbosch, the Netherlands) under a cover slip. A positive reaction was characterised by specific fluorescence of the *C. trachomatis* elementary bodies. For a quantitative determination of the antibody titre, two-fold serum dilutions in PBS were made. The reciprocal value of the highest dilution giving a positive signal in the MIF was considered as the antibody titre. All slides were evaluated independently by two readers. In case of disagreement, the independent judgement of a third reader was decisive. Differences in titres were expressed in titre steps. A difference of two titre steps or more between Sample-1 and Sample-2 was considered significant.

For validation of the MIF results, a *C. trachomatis* IgG ELISA (Labsystems, Helsinki, Finland) was performed. Sera were diluted 1:10 with Trisbuffer and tested in microtitre plates coated with synthetic peptides derived from the major outer membrane proteins of *C. trachomatis* (L₂). The plates were incubated 30 min at 37°C. The strips were washed five times in 300 µl washing solution (citrate buffered PBS) and the inverted strips were tapped a few times on a paper towel to remove excess fluid. To each well 200 µl conjugate was added (sheep antihuman IgG/horseradish peroxidase) and the plate incubated again 30 min at 37°C. The washing steps were repeated five times. Citrate-acetate buffered substrate in a volume of 200 µl was pipetted in each well, the plate incubated at room temperature in the dark for 15 min. Finally, 50 µl stopreagens (2M H₂SO₄) was added. The absorbance of the plates was measured in a spectrophotometer at 450 nm. Cut-off indexes and signal/cut-off (S/C) values were calculated and the results interpreted according to the manufacturer's instructions. The results were interpreted to be negative (N) when S/C was < 1.0, equivocal (E) when S/C was ≥ 1.0 and < 1.4, positive (P) when S/C was ≥ 1.4 and < 2.5 and highly positive (HP) when S/C was ≥ 2.5. The washing steps are repeated five times. Citrate-acetate buffered substrate in a volume of 200 µl was pipetted in each well, the plate covered and incubated at room temperature in dark for 15 min. Finally, 50 µl stopreagens (2M H₂SO₄) was added. The strips are cleaned before reading. The absorbance of the plates is measured in a spectrophotometer at 405 nm. Cut-off indexes are calculated according to the manufacturer's instructions. The cut-off index for a positive test is 1.1. Results were considered different when the S/C value of Sample-1 was different from the S/C value of Sample-2.

At the time of Sample-2 all patients filled out a questionnaire in order to determine risk factors for a renewed *C. trachomatis* infection after Sample-1. Questions were asked about abdominal complaints compatible with pelvic inflammatory

disease; fertility treatment in which cervical manipulation is involved, the use of contraceptive methods, and new sexual partners since Sample-1. Furthermore, social habits such as smoking and alcohol use were asked for, since these factors have been associated with the risk of tubal factor subfertility (Bahamondes *et al.*, 1994). For statistical analysis a Fisher's Exact test was used. P-values < 0.05 were considered significant.

Results

Initially, in all 39 women CAT was performed as part of their fertility work-up after they had been trying to achieve pregnancy for at least 12 months. At Sample-1, patients had a mean age of 29 years (range 19-38) and at Sample-2 they had a mean age of 36 years (range 26-45). IgG antibody titres by MIF in Sample-1 and Sample-2 are given in table I. Also in table I, S/C values of Sample-1 and Sample-2 are shown, to allow comparison of MIF and ELISA results. In 32/39 (82.0%) patients, no significant change in Chlamydia IgG antibody titre by MIF was demonstrated between Sample-1 and Sample-2. In 7/39 patients (18.0%) a decline in IgG antibodies of at least two titre steps was found over a period of 4-7 years. However, in no patient IgG antibodies became undetectable.

The ELISA test showed no significant change in 26/33 patients (78.8%), a decline was found in 1/33 patient (3.0%) and an increase in 6/33 patients (18.2%). However, in all 7/39 patients in whom a significant decline in IgG antibody titre was found by MIF, no differences were found by ELISA. In comparing CAT results by MIF and ELISA, a remarkable discrepancy was found. In contrast to MIF, which showed IgG antibodies in all patients for both Sample-1 and Sample-2, the ELISA showed negative values in 7/33 (21.2%) patients in both samples.

For analysis of the questionnaires, patients were divided into two groups, to determine risk factors for renewed *C. trachomatis* infection after Sample-1. Group A consists of patients in whom no significant titre decline by MIF was found, and group B of patients with significant titre decline measured by MIF. Groups A and B were compared for the prevalence of risk factors for renewed infection. The results of the questionnaires are shown in table II. The mean time interval between Sample-1 and Sample-2 did not differ for the two groups: 5 years and 8 months in group A and 5 years and 10 months in group B. Comparison of the group of patients without significant titre decline (group A) with those with significant titre decline (group B) revealed no significant differences in prevalences of the factors associated with renewed infection. As many patients in group A (34.4%) as in group B (28.6%) reported an episode of abdominal complaints after Sample-1. In

Table 1. Chlamydia IgG titres by MIF with differences in titre steps, and signal/cutoff (S/C) values by ELISA with interpretation of S/C values.

patient no	MIF		titre steps	ELISA		Interpretation of S/C values (Sample-1/Sample-2)
	Sample-1 (titre)	Sample-2 (titre)		Sample-1 (S/C value)	Sample-2 (S/C value)	
1	128	256	1	0.03	0.02	N/N
2	128	256	1	1.32	1.89	E/P
3	128	256	1	1.95	1.93	P/P
4	512	1024	1	1.94	1.92	P/P
5	1024	2048	1	1.98	2.00	P/P
6	64	64	0	0.10	0.15	N/N
7	64	64	0	na	na	
8	64	64	0	na	na	
9	64	64	0	2.03	2.74	P/HP
10	64	64	0	1.94	2.62	P/HP
11	256	256	0	2.02	2.78	P/HP
12	512	512	0	1.97	1.95	P/P
13	512	512	0	1.95	1.78	P/P
14	512	512	0	1.97	1.98	P/P
15	1024	1024	0	1.21	1.11	E/E
16	1024	1024	0	na	na	
17	2048	2048	0	1.99	1.98	P/P
18	4096	4096	0	2.01	1.99	P/P
19	64	32	-1	na	na	
20	128	64	-1	na	na	
21	128	64	-1	na	na	
22	128	64	-1	1.29	1.34	E/E
23	128	64	-1	1.16	1.22	E/E
24	256	128	-1	0.97	0.92	N/N
25	256	128	-1	0.11	0.04	N/N
26	256	128	-1	2.25	2.15	P/P
27	512	256	-1	2.25	2.32	P/P
28	512	256	-1	0.03	0.05	N/N
29	512	256	-1	2.22	2.51	P/HP
30	512	256	-1	1.50	1.40	P/P
31	512	256	-1	2.15	2.64	P/HP
32	1024	512	-1	1.77	1.25	P/E
33	64	16	-2	2.11	2.03	P/P
34	512	128	-2	2.09	2.08	P/P
35	1024	256	-2	0.05	0.06	N/N
36	1024	256	-2	1.67	1.66	P/P
37	2048	512	-2	1.61	1.74	P/P
38	2048	512	-2	0.05	0.04	N/N
39	1024	128	-3	2.25	2.28	P/P

S/C values are interpreted negative (N) when <1.0 , equivocal (E) when ≥ 1.0 and <1.4 , positive (P) when ≥ 1.4 and <2.5 , highly positive (HP) when ≥ 2.5 na is not available.

Table II. Anamnestic prevalence of variables presuming Chlamydia reactivation or re-infection between Sample-1 and Sample-2.

Patient groups	A	B	p-value
Total no. of patients	32	7	
Pelvic / abdominal complaints	11 (34.4%)	2 (28.6%)	0.57
surgical intervention	2 (6.3%)	1 (14.3%)	0.46
antibiotic treatment	0	0	
Cervical manipulation in subfertility treatment	14 (43.8%)	4 (57.1%)	0.41
Contraceptive methods			
no	21 (65.6%)	5 (71.4%)	0.57
intra uterine device	0	0	
condom	1 (3.1%)	0	0.83
oral	7 (21.9%)	2 (28.6%)	0.52
other	3 (9.4%)	0	0.54
No. of sexual partners between Sample-1 and Sample-2			
same partner, no other partners	28 (87.5%)	5 (71.4%)	0.29
1 other sexual partner	4 (12.5%)	0	0.44
different sexual partners	0	2 (28.6%)	0.28
Smoking habits			
no	14 (43.8%)	3 (42.9%)	0.65
1-15 cigarettes / day	6 (18.8%)	2 (28.6%)	0.45
>15 cigarettes / day	12 (37.5%)	2 (28.6%)	0.51
Alcohol consumption			
no	17 (53.1%)	4 (57.1%)	0.59
1-7 units / week	14 (43.8%)	3 (42.9%)	0.65
>7 units / week	1 (3.1%)	0	0.82

Sample-1 is initial serum sample; Sample-2 is second serum sample.

Group A shows patients with no titre decline between Sample-1 and Sample-2.

Group B shows patients with a significant titre decline between Sample-1 and Sample-2.

P-values were calculated to determine significance of differences between factors in groups A and B.

group A, 2/32 patients (6.3%) had had pelvic or abdominal surgery as compared to 1/7 patients (14.3%) in group B. No patient had received antibiotic treatment for abdominal complaints in the period between Sample-1 and Sample-2. In group A, 14/32 patients (43.8%) had undergone fertility investigations and / or treatment that involved cervical manipulation, as compared to 4/7 patients (57.1%) in group B. No significant difference in use of contraceptives was found between the groups, and especially no IUCD use was reported between Sample-1 and Sample-2. Twenty-eight out of 32 patients (87.5%) in group A and 5/7 patients (71.4%) in group B reported to have had the same sexual partner between Sample-1 and Sample-2. Furthermore, 18/32 patients (56.3%) in group A reported cigarette smoking versus 4/7 patients (57.1%) in group B. Alcohol consumption was reported by 15/32 patients (46.9%) in group A and 3/7 patients

(42.9%) in group B. At statistical analysis the above mentioned factors showed no significant differences between groups A and B.

Discussion

CAT has been introduced as a simple and inexpensive screening test for tubal factor subfertility. However, little attention has been paid to its limitations. Although it is assumed that Chlamydia infections give rise to persistent IgG antibody formation, the exact course of titres over time is still poorly understood. The four studies published to date on the course of Chlamydia IgG antibody titres together describe a significant decline of titres of 36.6% in 153 patients tested, but describe seroconversion from positive to negative as a rare occurrence. However, these studies have all concerned patients recently treated for acute pelvic inflammatory disease only. To our knowledge, the present study is the first to investigate the course of Chlamydia IgG antibody levels in asymptomatic subfertility patients.

In our study, by using MIF a decline of IgG antibody titres of at least two titre steps was found in 7/39 patients (18.0%) over a period of 4–7 years. However, in no patient IgG antibodies became undetectable. The persistently high Chlamydia IgG antibody titres in 82.0% of patients might theoretically be explained by re-infections by Chlamydia. Firstly, Chlamydia re-infection might occur by endogenous reactivation of the microorganism that has become latent after a previous infection (Forsey *et al.*, 1990). Subfertility patients are considered to be at risk for endogenous reactivation by cervical manipulation during fertility investigations (e.g. hysterosalpingography) or treatment (e.g. intra-uterine insemination, IVF). Secondly, exogenous re-infection after a solved primary infection (Stamm *et al.*, 1986) may occur. A questionnaire was composed to estimate the risks of *C. trachomatis* re-infection. We are aware of the fact that a questionnaire is a weak instrument, but there was no better method available to determine the risk of re-infection between the two time points studied. The questionnaire showed that there were as many patients who had undergone cervical manipulation in the group with titre decline as in the group without titre decline. Furthermore, the prevalence of active cervical *C. trachomatis* infections in subfertility patients has been reported to be < 2% (Eggert-Kruse *et al.*, 1997; Macmillan *et al.*, 1999). This also suggests the chance for endogenous reactivation in subfertility patients to be small. Our estimation of the risk of exogenous re-infection had to rely on patients' answers in the questionnaire, as patients were not screened for active *C. trachomatis* infections. Questions concerning risk factors associated with infection or re-infection were not answered differently by patients with no titre decline (group A) as compared to patients with titre decline (group B). Nevertheless, although the

questionnaire indicated exogenous re-infection between the time points studied to be unlikely, re-infection can not be excluded completely. Summarising the results of the questionnaire, it can be concluded that in our study *C. trachomatis* re-infection by either endogenous reactivation or exogenous re-infection appears to be no major cause of persistence of Chlamydia IgG antibody titres in the subfertile women studied.

MIF is the most widely used test method for CAT in subfertility patients for screening for tubal pathology due to earlier *C. trachomatis* infections. CAT by MIF has been shown to have clinical value in predicting tubal pathology (Land *et al.*, 1998). During *C. pneumoniae* infection however, antibodies are also formed against the common antigenic epitopes present in all members of the genus Chlamydia. As the MIF test is not species-specific, cross-reaction between *C. trachomatis* and *C. pneumoniae* might occur (Mannion *et al.*, 1991, Moss *et al.*, 1993, Gijzen *et al.*, 2001). Furthermore, interpretation of the MIF results is subjective, and inter-observer differences are inevitable. Recently, ELISAs have become available which are more species-specific, and the results can be objectively analysed by computer. In the present study, an ELISA was used to validate the results on titre decline found by MIF. Overall, in 78.8% of cases, the ELISA showed no change in the interpretation of S/C values between the two samples, and in particular no decline in S/C value was found in the 7/39 patients that showed a significant titre decline by MIF. Remarkably however, in 7/33 patients (21.2%) who had Chlamydia IgG antibodies by MIF, ELISA showed negative S/C values. The discrepancy found between MIF and ELISA results might be due to cross-reaction in the MIF test with other Chlamydia species than *C. trachomatis*. On the other hand, since in ELISAs synthetic peptides derived from different domains of the major outer membrane are used as antigens, these peptides will affect the specificity of the test. Therefore, patients without antibodies against the particular peptide used in the assay, will have negative ELISA results. Although the ELISA has been shown to be highly sensitive in detecting Chlamydia IgG antibodies in patients with proven acute *C. trachomatis* infections (Närvänen *et al.*, 1997), it has not yet been evaluated in screening for tubal factor subfertility in patients with previous subclinical infections.

In conclusion, this is the first paper to report on the course of *C. trachomatis* IgG antibodies over time in asymptomatic and untreated female subfertility patients. In 39 patients with initial *C. trachomatis* IgG antibody titres ≥ 64.7 (18.0%) showed a decline of at least two titre steps over a 4–7 year interval. However, in spite of this decline, all patients continued to test positive for IgG antibodies. Responses to a questionnaire showed that reactivation or re-infection does not seem to be responsible for the persistence of Chlamydia IgG antibodies, since patients without titre decline did not differ from those with titre decline as regards the risk factors associ-

ated with renewed *C. trachomatis* infection. We therefore conclude from this study that in subfertility patients, decline in IgG antibody titres over time is not a significant cause of false negative Chlamydia antibody test results (i.e. patients testing negative for Chlamydia antibodies, but with tubal factor subfertility found at laparoscopy).

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3 *Chlamydia pneumoniae* and screening for tubal factor subfertility

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Abstract

Chlamydia (C.) antibody testing (CAT) by microimmunofluorescence (MIF) tests, has been introduced into the fertility work-up as a screening test for tubal factor subfertility. In this study the role of *C. pneumoniae* antibodies, as a cause for false positive CAT results due to cross-reactivity with *C. trachomatis* antibodies in the MIF test, has been evaluated. In 240 subfertile women serologic data were compared to laparoscopy findings.

The prevalence of *C. pneumoniae* antibodies using ELISA was 75% and did not differ between patients with and without tubal pathology. *C. pneumoniae* antibodies were found in 87% of women with a positive MIF test (≥ 32), and in 66% with a negative MIF test ($p < 0.0005$). Using ELISA instead of MIF for the detection of *C. trachomatis* antibodies, *C. pneumoniae* antibodies were found in 87% of *C. trachomatis* positive women, and in 69% of *C. trachomatis* negative women ($p < 0.0005$).

Patients without tubal factor subfertility but a positive MIF test showed *C. pneumoniae* antibodies more frequently than patients without tubal factor subfertility and a negative MIF test. Therefore it was suggested that *C. pneumoniae* antibodies may be the cause of false positive CAT results. Remarkably, tubal pathology was more common in patients who had antibodies to both *C. trachomatis* and *C. pneumoniae*.

Introduction

Nowadays, *C. trachomatis* is the most common sexually transmitted disease in industrialised countries. In the majority of female patients *Chlamydia* infections remain asymptomatic, but they may eventually compromise tubal function and increase the risk of tubal factor subfertility. Clinicians have searched for simple and inexpensive screening tests to estimate the risk for tubal factor subfertility in a particular patient, in order to subject a high-risk patient to diagnostic testing (i.e. laparoscopy), and to postpone additional testing in a low-risk patient. Since it was noted that the development of pelvic inflammatory disease and its chronic sequelae are associated with *C. trachomatis* IgG antibody formation, the relation between antibody titres and tubal factor subfertility has been confirmed in many studies (summarised by Mol *et al.*, 1997).

Chlamydia antibody testing (CAT), e.g. by micro-immunofluorescence (MIF) tests, has been introduced into the fertility work-up as a simple and inexpensive screening test for tubal factor subfertility. The clinical significance of CAT is limited by patients with Chlamydia antibodies but without tubal pathology at laparoscopy (defined as false positive CAT results), and by patients without Chlamydia antibodies but tubal pathology at laparoscopy (defined as false negative CAT results). False negative CAT results may be explained by non-Chlamydia causes of tubal pathology. False positive CAT results increase health care costs by increasing the number of laparoscopies. Therefore, if CAT is applied for selecting patients for laparoscopy, the number of false positive CAT results should be minimised.

In discussing false positive CAT results (i.e. patients with positive antibody titres, without tubal pathology at laparoscopy), the possible cross-reactivity in MIF tests between *C. trachomatis* and *C. pneumoniae* antibodies is a major issue. Initially, in the MIF test, antigens from elementary bodies of each of the serotypes of *C. trachomatis* were included in the test, and provided serotype-specific antibody testing (Wang *et al.*, 1970). The preparation of multiple immunotype antigens made the test technically difficult to perform and impractical, and therefore modifications were introduced (Treharne *et al.*, 1977). By pooling antigens of epidemiologically related serotypes, or using one broadly reacting serotype (usually L₂), the number of antigens was reduced. Since MIF tests have been modified, and serotype-specific antigens are no longer used, cross-reactivity with other *Chlamydia* species (*C. pneumoniae* in particular) has been suggested (Mannion *et al.*, 1991). The aim of this study was to evaluate the significance of *C. pneumoniae* antibodies in false positive CAT results in female subfertility patients. Recently, new test methods have been developed, which are considered species-specific for *C. trachomatis* and *C. pneumoniae*. These tests are commercially

available enzyme-linked immunosorbent assays (ELISA), which have not yet been evaluated in a clinical setting for CAT in subfertility patients. We used these tests to establish the prevalence of antibodies to *C. pneumoniae* in subfertile women. Secondly, we evaluated cross-reactivity in the MIF test with *C. pneumoniae* antibodies as a possible cause of false positive CAT results.

Materials and Methods

A prospective cohort study was performed in consecutive patients who sought treatment for subfertility in our clinic. Since 1992, CAT using MIF is a routine procedure in our fertility work-up. In all female patients blood is drawn at their initial visit and an indirect micro-immunofluorescent antibody technique for *C. trachomatis* antibodies is used. All spare serum samples are cryopreserved. Between January 1992 and April 1999, 240 patients underwent laparoscopy and tubal testing with methylene blue dye as part of their fertility work-up and took part in the study. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or caesarean section) were excluded. For the sake of the study, tubal pathology at laparoscopy was defined as extensive periadnexal adhesions and/or distal occlusion of one or both tubes (Land *et al.*, 1998). A true positive CAT result was defined as a positive antibody titre using MIF, in a patient with tubal pathology at laparoscopy. A false positive CAT result was defined as a positive antibody titre using MIF, in a patient without tubal pathology at laparoscopy.

After thawing the spare sera of the participating patients, an ELISA for *C. trachomatis* antibodies (Labsystems, Helsinki, Finland) and an ELISA for *C. pneumoniae* antibodies (Bioclone Elegance, Marrickville, Australia) were performed. These ELISAs are considered specific for *C. trachomatis* antibodies (Närvänen *et al.*, 1997) and for *C. pneumoniae* antibodies respectively.

Serological methods

For the indirect micro-immunofluorescent IgG antibody test, 20 µl of the serum is diluted eight times in phosphate buffered saline (PBS) and incubated on the *C. trachomatis*-spot immunofluorescence substitute slides (egg grown *C. trachomatis* biovar L₂; Biomerieux, 's Hertogenbosch, The Netherlands) for 30 min at 37°C in a moist chamber. The slides are washed three times for five min in PBS and incubated with fluorescein-conjugated rabbit antihuman IgG (Dako, ITK Diagnostics BV, Uithoorn, The Netherlands) diluted in PBS for 30 min at 37°C. After three washings in PBS and one in ultra-pure water processed through a Milli-Q puri-

fying system (Millipore, Bedford, MA, USA), slides are embedded in Fluoprep mounting medium (Biomérieux, 's Hertogenbosch, The Netherlands). A positive reaction is characterised by specific fluorescence of the *C. trachomatis* elementary bodies. For quantitative determination serial dilutions in PBS are performed. All slides are evaluated independently by two readers. In case of disagreement, the judgement of a third reader is decisive. According to receiver operating characteristic curves constructed, an antibody titre of 32 appeared to be the optimum cut-off level in predicting tubal pathology (Land *et al.*, 1998). Therefore, a positive MIF result was defined as a titre ≥ 32 .

For the *C. trachomatis* IgG ELISA (Labsystems, Helsinki, Finland), sera are diluted 1:10 with Trisbuffer and tested in microtitre plates coated with synthetic peptides derived from the major outer membrane proteins of *C. trachomatis* (L₂). The plates are incubated 30 min at 37°C. The strips are washed five times in 300 µl washing solution (citrate buffered PBS) and dried. To each well 200 µl conjugate is added (sheep antihuman IgG/horseradish peroxidase). The plate is incubated again 30 min at 37°C. The washing steps are repeated five times. Citrate-acetate buffered substrate in a volume of 200 µl was pipetted in each well, the plate incubated at room temperature in dark for 15 min. Finally, 50 µl stopreagens (2M H₂SO₄) was added. The absorbance of the plates is measured in a spectrophotometer at 405 nm. Cut-off indexes are calculated according to the manufacturer's instructions. The cut-off index for a positive test is 1.1. The washing steps are repeated five times. Citrate-acetate buffered substrate in a volume of 200 µl was pipetted in each well, the plate covered and incubated at room temperature in dark for 15 min. Finally, 50 µl stopreagens (2M H₂SO₄) was added. The strips are cleaned before reading. The absorbance of the plates is measured in a spectrophotometer at 405 nm. Cut-off indexes are calculated according to the manufacturer's instructions. The cut-off index for a positive test is 1.1.

For the *C. pneumoniae* IgG ELISA (Elegance, Marrickville, Australia), human sera are diluted 1:210 with washing solution and tested in strips coated with highly purified *C. pneumoniae*-specific outer membrane complexes. The plate is covered and incubated for 30 min at 37°C. The strips are washed three times with 300 µl washing solution. To each well, 100 µl *C. pneumoniae* IgG antibody reagent (labeled anti-human IgG polyclonal antibodies) is added, the plate is incubated again for 60 min. The plate is washed as described before. To each well 100 µl substrate solution (diethanolamine) is pipetted. The plate is incubated at room temperature for 10 min. Finally, 25 µl Chlamydia stop solution is added and the fluid on the plate gently mixed. The Optical Density of the plates is measured in a spectrophotometer at 405 nm. The cut-off index for a positive test, as recommended by the manufacturer, is 1.1.

Statistical methods

Data obtained at laparoscopy were correlated to serological test results, using the Chi-square test. P-values < 0.05 were considered significant. Differences between proportions were calculated by using proportion tests for two independent proportions. 95%-Confidence intervals for proportion differences and p-values were calculated. P-values < 0.05 were considered significant.

Results

In 240 patients serologic data as well as laparoscopy results were available for analysis. The mean age of the patients was 30.3 years (range 19 to 40 years).

C. pneumoniae antibodies were detected by ELISA in 75% of patients. In 40% of patients a positive MIF test for *C. trachomatis* antibodies was found. *C. pneumoniae* antibodies were found in 83/95 (87%) of patients who had a positive MIF test for *C. trachomatis*, and in 96/145 (66%) of patients with a negative MIF test ($p < 0.0005$). *C. pneumoniae* antibodies were found in 66/76 (86%) of patients who had a positive ELISA for *C. trachomatis* antibodies, and in 51/164 (69%) of patients who had a negative ELISA for *C. trachomatis* antibodies ($p < 0.0005$). Table I summarises positive serologic test results in patients with and without tubal pathology at laparoscopy respectively.

Cross-reactivity in the MIF test between *C. pneumoniae* and *C. trachomatis* antibodies is a possible cause for false positive CAT results. In order to study cross-reactivity, *C. trachomatis* and *C. pneumoniae* ELISAs were performed. The prevalence of *C. pneumoniae* antibodies has been studied in two different subgroups of patients in whom no tubal pathology was found at laparoscopy (table II).

Table I. Positive antibody tests to different *C. trachomatis* and *C. pneumoniae* antigens in patients with and without tubal factor subfertility at laparoscopy.

Antibody test	Total group of patients (n=240) ³	Patients with tubal pathology (n=67) ³	Patients without tubal pathology (n=173) ³
<i>C. trachomatis</i> MIF (Biomerieux) ¹	95 (40%) ^a	39 (58%) ^b	56 (32%) ^a
<i>C. trachomatis</i> ELISA (Labsystems) ²	76 (32%) ^a	35 (52%) ^b	41 (24%) ^a
<i>C. pneumoniae</i> ELISA (Bioclone Elegance) ²	179 (75%)	51 (76%)	128 (74%)

¹ Cut-off titre for positive test 32.

² Cut-off titre for positive test 1:1.

³ Different superscripts depict significant differences; $p < 0.01$.

Table II. Prevalence of *C. pneumoniae* antibodies in 173 patients without tubal pathology at laparoscopy and positive vs. negative MIF test results.

Antibody test results ¹	Number of patients	Prevalence of <i>C. pneumoniae</i> antibodies
<i>C. trachomatis</i> MIF positive	56	84% ²
<i>C. trachomatis</i> MIF positive and <i>C. pneumoniae</i> ELISA positive	47	
<i>C. trachomatis</i> MIF negative	117	
<i>C. trachomatis</i> MIF negative and <i>C. pneumoniae</i> ELISA positive	81	69% ²

¹ Cut-off titre for positive MIF test 32, Cut-off for positive *C. pneumoniae* ELISA 1:1.² 95% Confidence interval for the difference between proportions is 1-30; $p < 0.05$.**Table III.** Prevalence of *C. pneumoniae* antibodies in 173 patients without tubal pathology at laparoscopy and positive vs. negative ELISA *C. trachomatis* results.

Antibody test results ¹	Number of patients	Prevalence of <i>C. pneumoniae</i> antibodies
<i>C. trachomatis</i> ELISA positive	41	83% ²
<i>C. trachomatis</i> ELISA positive and <i>C. pneumoniae</i> ELISA positive	34	
<i>C. trachomatis</i> ELISA negative	132	
<i>C. trachomatis</i> ELISA negative and <i>C. pneumoniae</i> ELISA positive	94	71% ²

¹ Cut-off for positive *C. trachomatis* ELISA 1:1, cut-off for positive *C. pneumoniae* ELISA 1:1.² 95% Confidence interval for the difference between proportions is -4-20; $p = 0.11$.

C. pneumoniae antibodies were found in 47/56 (84%) of patients who had a positive MIF test and no tubal pathology at laparoscopy (false positive CAT). In a control group of patients who had a negative MIF test and no tubal pathology (true negative CAT), 81/117 (69%) had *C. pneumoniae* antibodies ($p < 0.05$). In table III, in the same subgroups, ELISA for *C. trachomatis* was performed, which confirmed the results found by MIF. *C. pneumoniae* antibodies were found in 34/41 (83%) of patients who had a positive ELISA for *C. trachomatis* and no tubal pathology at laparoscopy. In a control group of patients who had a negative ELISA for *C. trachomatis* and no tubal pathology, 94/132 (71%) had *C. pneumoniae* antibodies ($p = 0.11$).

Table IV. Relation between *C. trachomatis* antibodies (MIF), and *C. pneumoniae* antibodies (ELISA), and tubal pathology at laparoscopy in 240 subfertility patients.

<i>C. trachomatis</i> MIF ¹	<i>C. pneumoniae</i> ELISA ²	Number of patients n=240	Patients with tubal pathology ³ n=67 (%)
Negative	Negative	49	13 (27) ^a
Negative	Positive	96	15 (16) ^b
Positive	Negative	12	3 (25)
Positive	Positive	83	36 (43) ^c

¹ Cut-off titre for positive test 32² Cut-off titre for positive test 1.1³ Defined as extensive periadnexal adhesions and/or distal occlusion of both tubes^{a-c} reflects significant difference with $p < 0.05$.^{b-c} reflects significant difference with $p < 0.0001$.**Table V.** Relation between *C. trachomatis* antibodies (ELISA), and *C. pneumoniae* antibodies (ELISA) and tubal pathology at laparoscopy in 240 subfertility patients.

<i>C. trachomatis</i> ELISA ¹	<i>C. pneumoniae</i> ELISA ¹	Number of patients n=240	Patients with tubal pathology ² n=67 (%)
Negative	Negative	51	13 (26) ^a
Negative	Positive	113	19 (17) ^b
Positive	Negative	10	3 (30)
Positive	Positive	66	32 (49) ^c

¹ Cut-off for positive test 1.1² Defined as extensive periadnexal adhesions and/or distal occlusion of both tubes^{a-c} reflects significant difference with $p < 0.05$.^{b-c} reflects significant difference with $p < 0.0001$.

In table IV the correlation between the presence of antibodies to *C. trachomatis* by MIF and/or to *C. pneumoniae* using ELISA, and tubal pathology found at laparoscopy is given. The presence of both antibodies to *C. trachomatis* and to *C. pneumoniae* was associated with a higher rate of tubal factor subfertility (43%), as compared to no antibodies (27%; $p < 0.05$), antibodies to *C. trachomatis* only (25%; $p = 0.23$) or *C. pneumoniae* only (16%; $p < 0.0001$).

In table V the correlation between the presence of antibodies to *C. trachomatis* using ELISA and/or to *C. pneumoniae* using ELISA, and tubal pathology found at laparoscopy is given. The presence of both antibodies to *C. trachomatis* using ELISA and to *C. pneumoniae* was associated with a higher rate of tubal factor subfertility (49%), as compared to no antibodies (26%; $p < 0.05$), antibodies to *C. trachomatis* only (30%; $p = 0.2$) or *C. pneumoniae* only (17%; $p < 0.0001$).

Discussion

The aim of performing CAT as a standard procedure in subfertility patients is to identify patients with previous *C. trachomatis* infections. Using MIF, a positive test result is assumed to be associated with *C. trachomatis* antibodies, and the patient is considered to be at high risk for tubal pathology. However, the results reported in literature on screening for tubal factor subfertility by CAT using MIF are heterogeneous. Correlating CAT results with laparoscopy results, both false positive and false negative results are found. In the present study we evaluated the role of *C. pneumoniae* antibodies, as a cause of false positive CAT results, due to cross-reactivity of *C. pneumoniae* antibodies with *C. trachomatis* antibodies. Furthermore the prevalence of *C. pneumoniae* antibodies in subfertile women was calculated.

From table I it can be concluded that the prevalence of *C. trachomatis* antibodies in the group of subfertile women studied here was comparable using MIF and ELISA, 40% and 32% respectively. Using ELISA the prevalence of *C. pneumoniae* IgG antibodies was 75%. In patients with tubal pathology at laparoscopy the prevalence was 76%. It was 74% in women without tubal factor subfertility (table I). Several reports on the prevalence of *C. pneumoniae* antibodies in West European countries have been published. In 21% of over 4500 women attending a genito-urinary clinic *C. pneumoniae* antibodies were found using MIF (Moss *et al.*, 1993). In adolescents the prevalence of *C. pneumoniae* antibodies using MIF was 69% in men, and 57% in women (Freidank and Brauer, 1992). Another study confirmed the prevalence of *C. pneumoniae* antibodies to be higher in men (72%) than in women (56%), and showed the prevalence to increase with increasing age (Karvonen *et al.*, 1994). In female subfertility patients the prevalence of *C. pneumoniae* antibodies was significantly higher in patients with bilaterally occluded tubes at laparoscopy (75%), as compared to patients with normal tubes (56%) (Freidank *et al.*, 1995). The prevalence of *C. pneumoniae* antibodies found in our study (75%) is comparable with the prevalences reported in literature in asymptomatic women and subfertility patients.

In discussing false positive test results for CAT, cross-reactivity in MIF tests with *C. pneumoniae* is a major issue. Since MIF tests have been modified and serotype-specific antigens are no longer used, cross-reactivity with Chlamydia species other than *C. trachomatis* has been demonstrated (Mannion *et al.*, 1991).

Comparison of subfertility patients with positive and negative MIF test results respectively, revealed a significant difference in the prevalence of *C. pneumoniae* antibodies (87% and 66% respectively). A possible explanation for the difference found is cross-reactivity between *C. pneumoniae* and *C. trachomatis* antibodies in the MIF test. To explore the likelihood of cross-reactivity further, the prevalence of *C. pneumoniae* antibodies has been analysed in two subgroups of patients (tables

II and III). In patients without tubal factor subfertility but a positive MIF test result (table II), *C. pneumoniae* antibodies were found more frequently than in patients without tubal factor subfertility and negative MIF test results. These data suggest that *C. pneumoniae* antibodies can be a cause of false positive CAT results. Analysis of the same groups using *C. trachomatis* ELISA instead of MIF (table III) showed the same.

Reports in the literature are rare and controversial concerning cross-reactivity between *C. pneumoniae* and *C. trachomatis* antibodies in MIF tests. In female patients with tubal factor subfertility and pregnant controls, the presence of *C. trachomatis* and *C. pneumoniae* antibodies has been studied (Persson *et al.*, 1999). *C. trachomatis* antibodies were detected more often in patients (88%) than in controls (48%). In contrast, no difference was found for *C. pneumoniae* antibodies between patients (52%) and controls (58%). The authors concluded that cross-reactivity did not occur, as *C. trachomatis* antibodies were equally common in patients with and without *C. pneumoniae* antibodies (89% and 87% respectively). A study (Moss *et al.*, 1993) in over 4500 serum samples of women attending a genitourinary clinic, using a modified MIF test to detect type-specific antibodies to Chlamydia species, has been performed. The IgG responses to Chlamydia species were detected in three distinct forms: species-specific (where IgG was directed against one species only), cross-reactive (where the IgG level against one species was twofold or higher than the IgG levels against other species), or group-specific (where IgG levels were similar against all species tested). In 67% of women Chlamydia IgG antibodies were found. Species-specific or cross-reactive IgG against *C. trachomatis*, *C. pneumoniae* and *C. psittaci* was present in 38%, 21% and 0.1% of the samples respectively. In 8% of samples, the IgG level was similar against all three species (group-specific). The authors conclude that antibodies to *C. pneumoniae* may account for up to half of all Chlamydia positive test results using MIF. From the latter study (Moss *et al.*, 1993) and our study it can be concluded that, due to cross-reactivity, *C. pneumoniae* antibodies are probably major contributors to the low specificity of those MIF tests in which pooled antigens are used. Species-specific antibody tests for *C. trachomatis* are prerequisites for improving the predictive value of CAT.

In our study, tubal pathology was more frequent in patients in whom both antibodies to *C. trachomatis* and to *C. pneumoniae* were found (tables IV and V). The precise mechanism by which Chlamydia causes tissue damage remains to be elucidated, but immune-mediated inflammatory responses seem to be involved in the process (Brunham and Peeling, 1994). It has been suggested that primary Chlamydia infections do not necessarily cause tubal tissue damage, but that tissue damage will develop only after re-infection or after reactivation of a primary infection (Patton and Kuo, 1989). Re-infection or reactivation of primary infections is

considered to induce a booster immune response and subsequently tubal pathology. It has been hypothesised that this immune response may not be specific for *C. trachomatis*, and may be induced by *C. pneumoniae* antibodies as well (Freidank *et al.*, 1995). In their study Freidank *et al.* (1995) found the presence of both antibodies to *C. trachomatis* and *C. pneumoniae* in the MIF test to be associated with a significantly higher rate of tubal occlusion. Our findings are in agreement with the findings of Freidank *et al.* (1995), i.e. more tubal pathology was found in patients with both *C. trachomatis* and *C. pneumoniae* antibodies. This supports the contention that *C. trachomatis* infection and (highly prevalent) *C. pneumoniae* infections might have a synergistic effect on the development of tubal pathology. However, the exact role of *C. pneumoniae* in this process needs further exploration.

In conclusion, in 240 subfertility patients a prevalence of *C. pneumoniae* antibodies of 75%, using a specific ELISA for *C. pneumoniae* was found. Comparison of laparoscopic and serologic data suggests cross-reactivity between antibodies to *C. trachomatis* and antibodies to *C. pneumoniae* in the MIF test, as a cause for low specificity of CAT. The simultaneous presence of antibodies to *C. trachomatis* and to *C. pneumoniae* was associated with a significantly increased rate of tubal factor subfertility. *C. pneumoniae* antibodies, which are widespread, may be involved in Chlamydia upper genital tract infections and/or subsequent tubal damage.

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Performance of five serological Chlamydia antibody tests in subfertile women

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Abstract

Background Micro-immunofluorescence (MIF) is widely used for Chlamydia antibody testing (CAT). Recently a species-specific MIF and three enzyme-linked immunosorbent assay (ELISA) tests have been introduced. We compared five commercially available CAT tests, using laparoscopy as a reference, and evaluated whether combinations of tests could improve the predictive value of CAT.

Methods In a consecutive cohort of 315 subfertile women, results of the five CAT tests were correlated to findings at laparoscopy. Likelihood (LR) and odds ratios (OR) were calculated for single tests and for combinations of tests.

Results Of the tests evaluated, MIF Labsystems had the best diagnostic performance (OR 15.7), while pELISA Medac (OR 8.2) was the best of the three ELISA tests. Stepwise logistic regression analysis showed that performance of MIF Labsystems could not be improved by adding a second test. Significant cross-reactivity with *C. pneumoniae* antibodies was found in all tests evaluated, except in pELISA Medac.

Conclusions In screening for tubal factor subfertility, MIF Labsystems was superior to the ELISA tests evaluated, and combining two CAT tests did not improve its predictive value. Economic analysis will show whether serial testing by pELISA Medac, and retesting positive samples by MIF Labsystems, is most cost-effective. In CAT, cross-reactivity with *C. pneumoniae* antibodies is still a major concern.

Introduction

Since the association between *C. trachomatis* antibodies in serum and tubal pathology was noticed (Punnonen *et al.*, 1979), Chlamydia antibody testing (CAT) has been used in the fertility work-up as a screening test for tubal factor subfertility. A widely used test for CAT is the micro-immunofluorescence test (MIF), which has been considered the gold standard in the serological diagnosis of Chlamydia infection (Dowell *et al.*, 2001). Initially, antigens from elementary bodies of each of the serotypes of *C. trachomatis* were included in the MIF test, which provided serotype-specific antibody testing (Wang and Grayston, 1970). Later, for practical reasons, the number of antigens has been reduced by pooling antigens of epidemiologically related serotypes, or by using one broadly reacting serotype (usually L₂) (Treharne *et al.*, 1977). However, in modified MIF tests, cross-reactivity between *C. trachomatis* and *C. pneumoniae* occurs (Mannion *et al.*, 1991; Gijzen *et al.*, 2001). To overcome this, species-specific MIF tests have been developed. In these tests, the cross-reactivity between the different Chlamydia species has been reduced by subtracting lipopolysaccharide (LPS), a common component of the outer membrane of all Chlamydiaceae, from *C. pneumoniae* and *C. trachomatis* antigens. Other disadvantages of the MIF tests are that they are labour intensive, their reading is observer dependent, and inter-laboratory variation is significant (Peeling *et al.*, 2000). To overcome these drawbacks, enzyme-linked immunosorbent assay (ELISA) tests with high specificity have been developed, using LPS-stripped elementary bodies as antigens (Ossewaarde *et al.*, 1994). Recently assays have become commercially available in which specific synthetic peptides are used. These peptides are based on the major outer membrane protein (MOMP) of *C. trachomatis*, which contains species-specific and serotype-specific epitopes (Närvänen *et al.*, 1997). These ELISA tests are easy to perform and are well standardized (Tuuminen *et al.*, 2000). But the value of CAT using ELISA and species-specific MIF in predicting tubal factor subfertility has not been evaluated yet.

The first aim of this study was to compare five commercially available Chlamydia IgG antibody tests in their accuracy to predict tubal factor subfertility, using laparoscopy as a reference. The performances of a species-specific MIF test and three ELISA tests were compared with findings obtained with a MIF test that had been used in our hospital for 10 years. Cross-reactivity with *C. pneumoniae* antibodies was evaluated in the five CAT tests as a possible cause of false positive CAT results. The second aim was to evaluate whether combinations of two tests could improve the predictive value of CAT.

Materials and methods

The study was performed in female patients who sought treatment for subfertility in our clinic, a tertiary care center. Since 1992, CAT using MIF has been a routine procedure in our fertility work-up. In all female patients blood is drawn at their initial visit and a MIF test for *C. trachomatis* IgG antibodies (Biomerieux, The Netherlands) is performed, and all spare serum samples are cryopreserved at -20°C . CAT is used for selecting patients at high risk for tubal factor subfertility. Patients who have antibody titres < 64 are considered low risk and tubal factor is evaluated initially by hysterosalpingography (HSG). In cases of an abnormal HSG or failure of pregnancy to occur in the 6 months following HSG, the fertility work-up is concluded by laparoscopy and tubal testing with methylene blue dye. In patients who have antibody titres ≥ 64 , no HSG is performed, but tubal factor is evaluated primarily by laparoscopy.

Between June 1992 and September 2001, 315 successive patients underwent laparoscopy as part of their fertility work-up. All took part in this study. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded.

All laparoscopy reports were scored by two gynaecologists, who were blinded for the CAT results. For the sake of the study, tubal pathology at laparoscopy was defined as extensive periadnexal adhesions and/or distal occlusion of both tubes, since this has been shown to reflect Chlamydia associated tubal disease most accurately (Land *et al.*, 1998).

After thawing the cryopreserved sera of the participating patients, the five different CAT tests were performed. A number of patients had participated in a previous study (Land *et al.*, 1998), but for the present study all serum samples were retested. Finally, data obtained at laparoscopy were correlated to the serological test results.

Serological methods

All assays and calculations were performed according to the manufacturers' instructions. For each test all sera were tested simultaneously using the same batch of test kits, to avoid inter-test variability.

MIF Biomerieux: *Chlamydia trachomatis*-spot IF test (Biomerieux). In this indirect fluorescent IgG antibody test, *C. trachomatis* L₂ is used as a group antigen. A positive reaction is characterized by specific fluorescence of the *C. trachomatis* elementary bodies. For quantitative determination serial dilutions are made. In this study all slides were evaluated independently by two readers. In case of disagree-

ment, the judgement of a third reader was decisive. In this study a third reader was needed in four cases.

MIF Labsystems: *Chlamydia pneumoniae* IgG/IgM micro-IF test (Labsystems, Finland). In this species-specific MIF test *C. pneumoniae*, *C. trachomatis* and *C. psittaci* elementary bodies are used as antigens. In order to diminish cross-reactivity the immunological activity of *Chlamydia* LPS in *C. pneumoniae* and *C. trachomatis* antigens has been reduced. In this study the test was used to detect anti-*C. trachomatis* and anti-*C. pneumoniae* IgG antibodies. A positive reaction is characterized by specific fluorescence of the respective elementary bodies. For quantitative determination serial dilutions are made. In this study all slides were evaluated independently by two readers. In case of disagreement, the judgement of a third reader was decisive. In this study a third reader was needed in three cases.

ELISA Labsystems: *Chlamydia trachomatis* IgG EIA (Labsystems, Finland). In this indirect enzyme immunoassay synthetic peptides are used, which are derived from MOMP of *C. trachomatis* (Närvänen *et al.*, 1997). The optical density of the test plates is read in a spectrophotometer, and the signal to cut-off indices (SCI) are categorized as negative, equivocal, positive or highly positive.

pELISA Medac: *Chlamydia trachomatis*-IgG-pELISA (Medac, Germany). Medac has a LPS based, genus-specific test (rELISA) and a species-specific ELISA (pELISA) on the market. The pELISA has been used in this study, and, according to the manufacturers information it is based on "a synthetic peptide" from MOMP of *C. trachomatis*. The optical density of the test plates is read in a spectrophotometer, and the SCI are categorized as negative, equivocal, or positive.

ELISA Savyon: Sero-CT-IgG (Savyon, Israel). In this ELISA *C. trachomatis* species-specific peptides, derived from "different serotypes" are used. The optical density of the test plates is read in a spectrophotometer, and the SCI are categorized as negative, equivocal, or positive.

Statistical methods

The outcomes of the five tests were compared to the findings at laparoscopy in order to determine their prognostic value of tubal factor subfertility. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio (LR), odds ratio (OR) and 95% confidence interval (CI) were calculated.

For comparison of LR of the results of the CAT tests, the Chi-square test was used. To test the difference between OR of the different tests, the bootstrap technique was used (Efron and Tibshiram, 1993). Two sided $P < 0.05$ were considered significant. A stepwise logistic regression analysis was used to select the best combination of tests. For evaluation of agreement between tests, κ was calculated,

and $\kappa > 0.7$ were considered to indicate good agreement. For comparison of the distributions of *C. pneumoniae* titres in patients with a positive and negative Chlamydia antibody test respectively, the Mann-Whitney U test was used. $P < 0.05$ were considered significant.

Results

In 315 subfertile women, results of five different CAT tests and laparoscopy were available for analysis. In 51 patients (16%) tubal pathology was found at laparoscopy. In table I results are given for each test, reflecting its accuracy in predicting tubal factor subfertility. The effect of using different cut-off levels for a positive test is shown. Within each test, increase of the cut-off titre or SCI increased specificity and PPV at the expense of sensitivity. When using 32 or 64 as the cut-off level for a positive test, the OR of MIF Labsystems differed significantly from all OR of the other four CAT tests. For further analyses not all different cut-off levels and SCI were used. MIF Biomerieux and MIF Labsystems were considered positive if the IgG titre was ≥ 32 , ELISA Labsystems was considered positive if SCI was positive or highly positive, and pELISA Medac and ELISA Savyon if SCI was positive.

When MIF Biomerieux was used 132 women had a positive CAT test, 52 women had a positive test by MIF Labsystems, 53 by ELISA Labsystems, 62 by pELISA Medac and 87 women had a positive test when ELISA Savyon was used (table I). The OR of MIF Labsystems (15.7) was significantly better than the OR of all other tests evaluated. pELISA Medac had the highest OR (8.2) of the three ELISA tests.

In 158 patients all five CAT tests were negative, 67 patients had one positive test, 25 had two positive tests, 18 had three positive tests, 20 had four positive tests and in 27 patients all five tests were positive. Table II gives the number of positive CAT tests in 315 patients. The LR of tubal factor subfertility was correlated to the number of positive tests. A statistically significant difference was found between the LR of three, four and five simultaneously positive tests as compared with the LR of one positive test only.

The impact of combining two different tests on the predictive value for tubal factor subfertility was studied. Three different strategies were evaluated. First, a stepwise logistic regression analysis was performed to investigate whether combinations of tests would perform better than the best single CAT test (i.e. MIF Labsystems). From the logistic regression analysis it could be concluded that the diagnostic performance of MIF Labsystems could not be improved significantly by adding another CAT test.

Table I. Chlamydia antibody testing and tubal pathology at laparoscopy in 315 patients, using five different serological tests and different cut-off levels.

Test	Cut-off*	No patients with pos test	No patients with pos test and TP	Sens (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-	OR	95% CI
MIF Biomeneux	8	231	45	88	30	19	93	1.3	0.4	3.1	1.2-9.9
	16	149	39	76	58	26	93	1.8	0.4	4.6	2.1-10.3
	32	132	37	73	64	28	92	2.0	0.4	4.7 ^a	2.3-10.2
	64	104	36	71	74	35	93	2.7	0.4	6.9	3.3-14.9
MIF Labystems	128	60	29	57	88	48	91	4.8	0.5	9.9	4.7-21.1
	8	91	32	61	77	34	91	2.7	0.5	5.3	2.6-10.8
	16	75	32	61	83	41	92	3.6	0.5	7.8	3.7-16.2
	32	52	30	59	92	58	92	7.1	0.4	15.7 ^b	7.1-35.1
ELISA Labystems	64	37	24	47	95	65	90	9.6	0.6	17.2	7.1-42.4
	128	19	13	25	98	68	87	11.2	0.8	14.7	4.6-52.4
	Equivocal	84	23	45	77	27	88	2.0	0.7	2.7	1.3-5.4
	Positive High pos	53	19	37	87	36	88	2.9	0.7	4.0 ^c	1.9-8.4
pELISA Medac	Equivocal	26	12	24	95	46	87	4.4	0.8	5.5	2.0-14.4
	Positive	74	28	55	83	38	90	3.2	0.5	5.8	2.8-11.8
ELISA Savyon	Equivocal	62	28	55	87	45	91	4.3	0.5	8.2 ^d	3.9-17.3
	Positive	99	26	51	72	26	88	1.8	0.7	2.7	1.4-5.4
		87	25	49	77	29	89	2.1	0.7	3.1 ^e	1.6-6.3

Tubal pathology (TP) was found in 51 patients.

* IgG titre in MIF tests, and signal to cut-off index in ELISA tests.

Sens = sensitivity; spec = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = likelihood ratio of positive test; LR- = likelihood ratio of negative test; OR = odds ratio; CI = confidence interval.

b-a, b-c, b-d b-e, d-c, d-e Differences statistically significant.

Table II. Number of positive Chlamydia antibody tests in 315 patients, and the likelihood of tubal pathology at laparoscopy.

No. of positive tests	MIF Bio-merieux ¹	MIF Lab-systems ¹	ELISA Lab-systems ²	pELISA Medac ³	ELISA Savyon ³	LR	95% CI
0	183	263	262	253	228	0.5	0.3-0.7
1	46	-	4	3	14	0.4 ^a	0.2-0.9
2	22	5	4	5	14	1.0	0.4-2.6
3	17	6	10	9	12	2.0 ^b	0.8-5.0
4	20	14	8	18	20	4.2 ^b	1.9-9.4
5	27	27	27	27	27	6.5 ^b	3.2-12.7

¹ IgG titre ≥ 32 .

² Signal to cut-off index positive or highly positive.

³ Signal to cut-off index positive.

LR = likelihood ratio; CI = confidence interval.

^{a-b} Difference statistically significant.

As a second strategy the CAT tests were divided into two groups: the ELISA tests, as they can be automated, and the more laborious MIF tests. Based on stepwise logistic regression analysis with the three ELISA tests in the first block and the two MIF tests in the second block, pELISA Medac was chosen as the first test, and MIF Labsystems as the second test (table III). In retesting of initially positive sera the serial set was considered positive if the second test was also positive, and in retesting of initially negative sera the serial set was considered negative if the second test was also negative. Using pELISA Medac on all serum samples, 62 patients (20%) had a positive test. After retesting these 62 sera by MIF Labsystems, 44 sera (14%) remained positive. An initial negative test was found in 253 patients (80%), and after retesting these 253 sera, 245 were negative (78%). The results of serial testing with the chosen set of CAT tests (i.e. pELISA Medac and MIF Labsystems) are given in table III. The OR of both serial sets (14.2 and 9.2 respectively) were lower than the OR of MIF Labsystems as a single test (15.7; table I).

In the third strategy it was hypothesised that the two CAT tests which differed most from each other may cover a wider spectrum of serotypes, and act complementary in identifying women with tubal pathology. In order to select the two tests to be used in the model, the agreement between the five tests was established (table IV). For evaluation of the third strategy the set with the lowest κ , i.e. ELISA Labsystems and MIF Biomerieux ($\kappa = 0.35$), was performed on all serum samples. The result of the set was defined as positive if both tests were positive, whereas all other combinations of test results were considered negative. The result of the set was considered negative if both tests had a negative test result, and all other combinations of test results were considered positive. The results of serial testing with the

Table III. Chlamydia antibody testing and tubal pathology at laparoscopy in 315 patients. Results for serial testing with two different sets of tests.

Sets of tests	No patients with pos test	Sens (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-	OR	95% CI
Serial set^a									
- retest positive results ^c	44	51	93	59	91	7.5	0.5	14.2	6.2-32.6
- retest negative results ^c	70	61	86	45	92	4.2	0.5	9.2	4.4-19.5
Low κ set^b									
- retest positive results ^c	47	35	89	38	88	3.2	0.7	4.4	2.0-9.6
- retest negative results ^c	138	75	62	28	93	2.0	0.4	4.8	2.3-10.6

^a pELISA Medac (signal to cut-off index positive) and MIF Labsystems (IgG titre ≥ 32).

^b ELISA Labsystems (signal to cut-off index positive or highly positive) and MIF Biomerieux (IgG titre ≥ 32).

^c Positive, retesting of all positive test results; negative, retesting of all negative test results.

Sens = sensitivity; spec = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = likelihood ratio of positive test; LR- = likelihood ratio of negative test; OR = odds ratio; CI = confidence interval.

Table IV. κ -values for agreement in predicting tubal pathology at laparoscopy between five Chlamydia antibody tests.

	MIF Biomerieux ^a	MIF Labsystems ^a	ELISA Labsystems ^b	pELISA Medac ^c	ELISA Savyon ^c
MIF Biomerieux ^a	—	0.43	0.35	0.42	0.45
MIF Labsystems ^a	—	—	0.49	0.72	0.48
ELISA Labsystems ^b	—	—	—	0.50	0.55
pELISA Medac ^c	—	—	—	—	0.59
ELISA Savyon ^c	—	—	—	—	—

^a IgG titre ≥ 32 .

^b Signal to cut-off index positive or highly positive.

^c Signal to cut-off index positive.

low κ set are given in table III. The OR of the low κ set (4.4 and 4.8 respectively) did not differ significantly from the OR of the single tests of which the sets were composed (4.0 for ELISA Labsystems and 4.7 for MIF Biomerieux respectively; table I). This indicates that serial testing with the two CAT tests which differed most from each other could not improve the predictive value of CAT.

In order to study cross-reactivity with *C. pneumoniae* antibodies in the five CAT tests, in all 315 patients *C. pneumoniae* IgG antibody titres were determined by

MIF Labsystems. In 41 patients the IgG titre was < 8 , in five patients it was 8, in 33 patients it was 16, in 61 patients it was 32, in 83 patients it was 64, in 56 patients it was 128, in 20 patients it was 256 and in 16 patients it was > 256 . Based on the results of the CAT test, patients were divided into two groups, i.e. those with a positive test result (ELISA positive or highly positive, or MIF titre ≥ 32) and those with a negative test result. The distribution of the *C. pneumoniae* antibody titres in the CAT positive patients was compared with the distribution in the CAT negative patients. This comparison was made for all five CAT tests separately. Significant differences were found between the distributions of *C. pneumoniae* antibodies in the CAT positive and CAT negative patients when CAT was performed by MIF Biomerieux ($p < 0.00001$), MIF Labsystems ($p = 0.001$), ELISA Labsystems ($p = 0.02$) or ELISA Savyon ($p = 0.02$), whereas no significant difference was found by pELISA Medac ($p = 0.06$). This suggests significant cross-reactivity with *C. pneumoniae* antibodies in all CAT tests evaluated, except in pELISA Medac.

Discussion

Active genital tract infections with *C. trachomatis* can be diagnosed by direct detection of the microorganism from the infected site. After the acute episode however, the microorganism may not be detectable any longer, and Chlamydia antibodies in serum may be the only indication of previous Chlamydia involvement. The aim of screening subfertile women by CAT is to identify patients with previous *C. trachomatis* infections, who are at increased risk for tubal pathology. It has become evident, however, that not all women develop *C. trachomatis* antibodies after Chlamydia infection (Schachter *et al.*, 1979), and that not all women with antibodies have tubal pathology. Although the immunopathology underlying Chlamydia infection is still poorly understood, antibody tests have been developed for clinical application. The information provided by the manufacturers about the antigenic epitopes used in their tests is very limited, and is restricted to statements that the test contains "a" or "some" specific peptides. Furthermore, manufacturers modify the epitopes used in their tests, which may remain unnoticed by the customers who therefore cannot anticipate changes in test performance. The present study is a clinical comparison of five commercially available Chlamydia antibody tests in their ability to predict tubal pathology in subfertile women. Four of these tests had not been evaluated as screening tests for tubal factor subfertility before. We are aware that the results of the study show the diagnostic performances of the tests at a given moment in time only.

In the present study CAT results were compared to the findings at laparoscopy, and therefore only women who had undergone a laparoscopy were included.

Patients with low Chlamydia antibody titres are less likely to have tubal pathology and were less likely to be included in the study, since many will have conceived before laparoscopy can be done. This verification bias (Mol *et al.*, 1999) will influence predictive values of CAT, which are dependent on the prevalence of disease. Verification and selection bias is hard to prevent in clinical studies, however, unless one is prepared to perform the complete fertility investigation of a patient on a single day. Although the bias will result in overestimation of the LR, the overestimation will be similar for all tests investigated.

In comparing the five CAT tests considerable variation was found in the number of patients with a positive test (table I). An IgG titre ≥ 32 was found in 132 women using MIF Biomerieux and in 52 using MIF Labsystems, 53 had a positive test by ELISA Labsystems, 62 by pELISA Medac and 87 by ELISA Savyon. The tests with the highest number of positive test results had the lowest PPV, indicating the highest rates of false positive test results. False positive CAT results (i.e. positive CAT tests in patients without tubal pathology at laparoscopy) may be explained by cross-reactivity with *C. pneumoniae* antibodies, which can be found in about 70% of subfertile women (Gijsen *et al.*, 2001). In this study a significant correlation was found between *C. pneumoniae* antibody titres and CAT results in four tests, in particular in MIF Biomerieux ($p < 0.00001$). This confirms the findings of our previous study using a *C. pneumoniae* IgG ELISA, in which we found cross-reactivity to occur between *C. pneumoniae* and *C. trachomatis* antibodies in MIF Biomerieux. Since the only CAT test (pELISA Medac) in which no significant correlation with the distribution of *C. pneumoniae* titres was found did not have the highest PPV, cross-reactivity with *C. pneumoniae* antibodies does not seem to be the only explanation for the high rates of false positive test results obtained in the CAT tests. Since not all women with Chlamydia antibodies have tubal pathology at laparoscopy, it has been suggested that genetic factors in the host may also play a role, by modulating immune defense mechanisms and the development of late sequelae (Kinnunen *et al.*, 2002).

Comparing MIF and ELISA, the results (table I) suggest that ELISA tests tend to have lower sensitivity and NPV, i.e. more false negative test results. In ELISA tests chosen for this study, specific synthetic peptides are used which are considered analogous to the serotype-specific antigenic determinants of MOMP of *C. trachomatis* (Närvänen *et al.*, 1997). These serotype-specific determinants differ between tests, and may explain the differences found in test performances between ELISA tests of different manufacturers. Furthermore, tests based on highly specific peptides may be so specific that they are not able to detect all relevant antigens (Bas *et al.*, 2001). Variants of serotypes have been identified in urogenital isolates (Morré *et al.*, 1998) and mutations have been shown to occur in positions within MOMP (Dean *et al.*, 2000). Consequently, highly specific tests may not be able to

identify all serotypes involved in Chlamydia infection, and cause false negative CAT results (i.e. negative CAT tests in patients with tubal pathology at laparoscopy).

There are a few reports in the literature on the diagnostic accuracies of different antibody tests for *C. trachomatis*, in which the tests evaluated in our study have been included. In these studies either tubal pathology, or the direct demonstration of the microorganism in the genital tract, have been used as reference standards for the serologic tests. Each reference standard has its limitations, however. Since tubal pathology can be caused by other microorganisms in addition to *C. trachomatis*, it is obvious that tests based on Chlamydia antibodies will be imperfect in predicting all tubal pathology. Studies in which the direct detection of the microorganism is used as a reference have limited diagnostic accuracy as well (Chernesky *et al.*, 1998; Bas *et al.*, 2001), since superficial infections may provide a poor stimulus for antibody formation. Paukku *et al.* (1998) did not find a significant difference between the presence of IgG antibodies in 78 patients with tubal factor subfertility, using a modified MIF test and ELISA Labsystems. In a serological follow-up study of 16 women with *C. trachomatis* positive cervical swabs, ELISA Labsystems, ELISA Savyon and a MIF test have been used (Clad *et al.*, 2000). ELISA Labsystems was found to be the most sensitive test, and it was concluded that ELISA Savyon did not cover all Chlamydia serotypes. Morré *et al.* 2002 studied the IgG prevalences in 43 women with PCR positive cervical swabs and 106 PCR negative women. Results obtained by two in-house MIF tests were compared to results by ELISA Labsystems, pELISA Medac and ELISA Savyon. The authors concluded that the ELISA tests performed equally well as the MIF tests used.

In the present study the likelihood of tubal factor subfertility improved as the number of positive CAT tests in a patient increased (table II). Compared with one positive CAT test, the LR improved significantly in patients in whom three, four or five positive tests were found. But from a clinical point of view, performing more than two CAT tests in patients is impractical and expensive. Therefore, we evaluated whether two serially performed tests could be of any benefit in predicting tubal factor subfertility. Firstly, from a stepwise logistic regression analysis it was concluded that the diagnostic performance of the best single CAT test (i.e. MIF Labsystems) could not be improved by adding a second CAT test. Secondly, we evaluated the performance of a serial set of tests from a laboratory perspective, by constructing a model in which pELISA Medac was performed as the first test and MIF Labsystems as the second test. Although pELISA Medac was found to have a significantly lower OR (8.2) as compared with MIF Labsystems (15.7), ELISA tests have the advantage over MIF tests of being less laborious. From our results it can be concluded that if pELISA Medac is performed as the first test on all samples, and all samples with positive test results (i.e. 20% of all samples) are

retested with MIF Labsystems, the predictive value of the set (OR 14.2) is comparable to the predictive value of MIF Labsystems as a single test (OR 15.7). Cost-effectiveness analysis has to demonstrate which strategy is to be preferred: MIF Labsystems as a single test on all samples, or pELISA Medac on all samples and retesting of the positive ones with MIF Labsystems. Thirdly, we hypothesised that two tests with poor agreement (low κ) might have different antigenic properties, and might react with a greater number of serotypes and therefore complement each other. From the results presented in table III it can be concluded that the low κ set of tests (ELISA Labsystems and MIF Biomerieux) did not improve the predictive value of CAT significantly, compared with the predictive values of the single tests of which the set was composed. Therefore the hypothesis of complementary testing with the set of tests with low κ had to be rejected.

The results of the present study show that there still is no excellent screening test for tubal pathology in subfertile women. In order to develop more accurate tests for the prediction of Chlamydia-associated tubal pathology, future research should focus on the immunopathology of Chlamydia infections. Evidence exists that patients with chronic, persisting Chlamydia infections are particularly at risk for developing late sequelae. Although IgG antibodies are markers of a previous infection, they do not reflect an ongoing chronic inflammation properly. Candidates to be introduced into screening for tubal factor subfertility, in addition to specific *C. trachomatis* IgG antibodies, are anti-HSP60 and anti-LPS antibodies. HSP60 has been shown to play a prominent role in chronic inflammation and scarring (Claman *et al.*, 1997), and anti-LPS antibodies might be indicators of ongoing Chlamydia infection (Tuuminen *et al.*, 2000).

In conclusion, although ELISA tests have been claimed to be highly sensitive and specific, in the present study they were not superior in predicting tubal factor subfertility. Of the five CAT tests evaluated, MIF Labsystems had the best diagnostic performance, and among the three ELISA tests, pELISA Medac performed best. MIF Biomerieux had the largest number of false positive test results, probably due to cross-reactivity with *C. pneumoniae* antibodies. Combining two different CAT tests did not improve the predictive value for tubal factor subfertility. Health care evaluation from an economic perspective has to prove whether serial testing with the automated pELISA Medac as a first test, and retesting of all positive serum samples with the more laborious MIF Labsystems, is to be preferred to testing of all samples with MIF Labsystems only.

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Chlamydia trachomatis in subfertile women undergoing uterine instrumentation: screen or treat?

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Abstract

Women who undergo uterine instrumentation are considered at risk for pelvic infections by *Chlamydia (C.) trachomatis*, which may derive either from ascending endocervical infections or from reactivation of microorganisms persisting in the genital tract after previous Chlamydia infections. Women presenting at fertility clinics frequently undergo uterine instrumentation (e.g. hysterosalpingography and laparoscopy with hydrotubation).

Studies in subfertile women using DNA amplification techniques have shown that the prevalence of endocervical Chlamydia infections is low (1.8%). In contrast, in 30–60% of subfertile women Chlamydia IgG antibodies can be found in serum, indicating previous Chlamydia infections. It has been demonstrated that, several years after Chlamydia infections, viable microorganisms may still be present in the upper genital tract. Therefore, subfertile women with Chlamydia antibodies should be considered at risk for reactivation of persistent Chlamydia infections after uterine instrumentation, even after exclusion of endocervical Chlamydia infections. Moreover, in subfertile women without Chlamydia antibodies, the presence of viable microorganisms in the genital tract cannot be excluded. As a consequence, prophylactic antibiotics before uterine instrumentation should be considered in all subfertile women, instead of endocervical screening for *C. trachomatis* and treatment of positive cases only.

Introduction

C. trachomatis is the most common sexually transmitted disease in industrialised countries. Chlamydia infections remain often undiagnosed, as they are asymptomatic in the majority of patients. Undiagnosed and untreated Chlamydia infections, however, may have considerable consequences in women, by increasing the risk for pelvic inflammatory disease, tubal factor subfertility and ectopic pregnancy. In women with asymptomatic endocervical Chlamydia infections, ascending spread of microorganisms from the cervix can cause upper genital tract infections. Women with endocervical Chlamydia infections are considered particularly at risk for ascending infections during uterine instrumentation.

Hysterosalpingography (HSG) and laparoscopy with hydrotubation are common procedures of uterine instrumentation in subfertile women. Clinical pelvic infection following HSG has been reported in up to 4% of cases, and in 10% of patients with tubal disease (Forsey *et al.*, 1990). In a number of patients with post-HSG infections due to *C. trachomatis*, the microorganism could be isolated from the cervix prior to HSG, indicating spread of the microorganism from the cervix into the upper genital tract. However, in some patients with post-HSG infections due to *C. trachomatis*, the microorganism could not be recovered from the cervix prior to HSG, suggesting post-HSG infection to be caused by reactivation of viable microorganisms which had persisted in the genital tract (Forsey *et al.*, 1990).

Although pelvic Chlamydia infections may derive from ascending endocervical infections as well as from reactivation of microorganisms persisting in the upper genital tract, different intervention strategies have been proposed for the prevention of ascending infections only. In 1996, the Royal College of Obstetricians and Gynaecologists recommended that all women undergoing uterine instrumentation should be screened for Chlamydia, or should receive prophylactic antibiotics (Royal College of Obstetricians and Gynaecologists, 1996). In 2000, in its more recent guidelines, the Royal College is ambiguous in its recommendations: screening for Chlamydia prior to uterine instrumentation should be considered only in patients at risk, i.e. women < 25 years, but protection against upper genital tract infections should be considered in all women presenting for subfertility investigation (Royal College of Obstetricians and Gynaecologists, 2000). Therefore, the controversy between the administration of prophylactic antibiotics and a screen-and-treat policy remains still unresolved.

Endocervical Chlamydia infections in subfertile women

Studies published on endocervical Chlamydia colonization in subfertility patients have reported the prevalence to range from 0–10.4% (Macmillan and Templeton, 1999; for summary). Most studies used cell culture, which has a sensitivity of < 70%. In DNA amplification tests, polymerase chain reaction (PCR) and ligase chain reaction (LCR), sensitivity is > 90% (Puolakkainen *et al.*, 1998) and therefore these must be considered the tests of choice for diagnosing *C. trachomatis* infections. Studies in subfertility patients using LCR on endocervical specimens have reported prevalences of 1.3% (95% CI 0.2–4.7%) (Eggert-Kruse *et al.*, 1997) and 1.9% (95% CI 0.5–4.8%) (Macmillan and Templeton, 1999). We performed a study in 255 female subfertility patients by PCR, and found endocervical Chlamydia infections in 2.0% (95% CI 0.6–4.5%) (unpublished data). From the combined data of the three studies using DNA amplification tests, it can be concluded that endocervical Chlamydia infections are infrequent in subfertility patients (prevalence 1.8%, 95% CI 0.9–3.2%). Screening women for asymptomatic endocervical Chlamydia infections, in order to reduce the rate of pelvic inflammatory disease, has been debated. It has been demonstrated that screening by DNA amplification tests, and subsequent treatment of women with positive test results, is only cost-effective in populations with a prevalence of *C. trachomatis* of $\geq 4\%$ (Paavonen *et al.*, 1998).

In order to identify high prevalence populations for Chlamydia infections, criteria have been developed for high-risk patients. Age < 25 years, two or more sexual partners in the past year, and single status have independently been associated with an increased risk for endocervical Chlamydia infection (Stergachis *et al.*, 1993). The majority of female subfertility patients are > 25 years of age (in our study 4.0% of women were < 25 years old), and couples attending fertility clinics usually have had monogamous relationships for several years. Therefore, factors identifying high-risk patients for Chlamydia infections in a general population are not applicable in subfertility patients.

Chlamydia IgG antibodies have been found in 30–60% of subfertile women (Mol *et al.*, 1997; for review), and are considered markers for past pelvic infections. Cervical infection causes only rarely a rise in Chlamydia antibody titre (Tayler-Robinson, 1997). Therefore, Chlamydia IgG antibody testing is not helpful in identifying patients with active endocervical infections. This is in agreement with findings by others (Eggert-Kruse *et al.*, 1997), who found no relation between LCR results and Chlamydia IgG antibody titres in 150 patients, of whom two were LCR positive. In fact, more data on LCR / PCR positive subfertile women are needed to confirm these findings, but the low prevalence of endo-

cervical infections in subfertility patients makes studies with adequate numbers difficult to perform.

Persisting Chlamydia microorganisms in the upper genital tract: the role of screening

PCR testing of endocervical swabs is based on DNA amplification of Chlamydia antigen obtained from the lower genital tract. Evidence exists however that *C. trachomatis* may persist in the upper genital tract despite negative PCR test results from the cervix. In patients with bilateral tubal occlusion due to Chlamydia infection, tubal biopsies have been evaluated by a fluorescent antibody test for the presence of the major outer membrane protein (MOMP) of *C. trachomatis* (Dieterle *et al.*, 1998). MOMP was found in tubal specimens from 11/56 women, in whose endocervical specimens the presence of *C. trachomatis* could not be demonstrated. PCR testing of Fallopian tube tissue from 10 women with ectopic pregnancies (Gérard *et al.*, 1998) showed that 7/10 tubes were positive for Chlamydia DNA. In each PCR-positive sample, transcripts from Chlamydia genes were present, indicating that the Chlamydia cells identified were viable and metabolically active at the time of biopsy. Evaluation by in-situ hybridisation or immunoperoxidase staining technique of tubal specimens of patients with tubal pathology showed DNA or antigens in 19/24 women (Patton *et al.*, 1994). There was no difference in detection rate of *C. trachomatis* between patients who had been treated by antibiotics (tetracycline, ofloxacin or roxythromycin) and untreated patients. These studies indicate that *C. trachomatis* may persist in a viable state in Fallopian tubes for a long period, even after antibiotic treatment. Reactivation of Chlamydia infection may occur, but it is still speculative which stimuli may induce reactivation of upper genital tract infection. Uterine instrumentation might be such a stimulus. Therefore, reactivation of Chlamydia upper genital infections may occur in subfertility patients with Chlamydia antibodies (indicating a previous infection), despite the fact that no Chlamydia DNA can be detected in the cervix. This questions screening by cervical swabs in subfertile women before uterine instrumentation, and also raises questions considering PCR-negative patients not at risk for subsequent upper genital tract Chlamydia infections.

Prophylactic antibiotics

Since the presence of viable Chlamydia microorganisms in the upper genital tract can be excluded by invasive methods only, there is no feasible screening method available in daily practice. Therefore, prophylactic antibiotics may be considered in subfertility patients before uterine instrumentation. Guidelines for the treatment of patients with genital Chlamydia infections (Centers for Disease Control and

Prevention, 1998) have recommended azithromycin as a single oral 1 g dose as equivalent to the 7 day regimen of doxycycline (100 mg twice daily) for treating uncomplicated genital Chlamydia infections. Pharmacokinetic studies in man have shown the time to adequate tissue concentrations after a single oral dose to be 12-14 h, tissue concentrations being 10-100 times higher than in serum and to remain high for several days (Foulds *et al.*, 1990). These pharmacokinetic characteristics of azithromycin encourage its use as prophylaxis in subfertility patients before procedures which might reactivate Chlamydia infections.

Conclusions

Upper genital tract Chlamydia infections may derive from ascending endocervical infections, but reactivation of dormant microorganisms in the upper genital tract is another possibility. Endocervical infections can be excluded by LCR or PCR, but the prevalence of active endocervical Chlamydia infections in subfertile women is low (1.8%). Concerning reactivation, it has been shown that several years after Chlamydia infections viable *C. trachomatis* microorganisms may still be present in the upper genital tract. Therefore, subfertile women with Chlamydia antibodies (indicating a previous Chlamydia infection) should be considered at risk for reactivation of persistent Chlamydia microorganisms after uterine instrumentation, even after exclusion of Chlamydia in the cervix. From this point of view, prophylactic antibiotics should be considered in subfertile women who have Chlamydia antibodies in serum and have to undergo uterine instrumentation, as opposed to endocervical screening for *C. trachomatis* and treatment of positive patients only. In subfertile women who have no Chlamydia antibodies in serum, the presence of Chlamydia in the genital tract cannot be excluded however. Since iatrogenic spread of Chlamydia in subfertile women has severe consequences for the preservation of fertility, prophylactic antibiotics before uterine instrumentation should also be considered in subfertile women without Chlamydia antibodies. Clinical studies which allow the assessment of the effectiveness of antibiotic therapy in preventing pelvic infections after uterine instrumentation are impracticable, since the majority of Chlamydia infections will remain subclinical and therefore will go undiagnosed. Thus, clinical guidelines concerning precautions in subfertile women undergoing uterine instrumentation can be based on theoretical arguments only. Prophylactic antibiotics (e.g. azithromycin in a single oral 1 g dose, 12 h before the planned procedure) should replace endocervical screening for *C. trachomatis* and treatment of positive cases only.

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6

A mouse model for genital tract pathology by *Chlamydia trachomatis*

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Introduction

C. trachomatis is the most common sexually transmitted disease in industrialised countries. In the majority of female patients, Chlamydia infections remain asymptomatic (silent infections) (Thejls *et al.*, 1987). In a number of these asymptomatic patients involvement of the upper genital tract occurs, leading to pelvic inflammatory disease (PID) and finally resulting in tubal factor subfertility or ectopic pregnancy (Paavonen and Wølner-Hanssen, 1989). The largely unknown pathogenesis of tubal factor subfertility after silent *C. trachomatis* infections, and the unknown role of immunologic or genetic factors in this process hinder the search for a clinically significant screening test for tubal factor subfertility.

Animal studies have been performed in order to shed more light on the pathogenesis of *C. trachomatis* genital tract infections and re-infections. These papers report on acute ascending *C. trachomatis* genital infections and subsequent infertility, using macaques (Patton *et al.*, 1987, 1989, 1990), guinea pigs (White *et al.*, 1979) and mice. In mice most experiments have been done using the *C. trachomatis* mouse pneumonitis (MoPn) strain (Barron *et al.*, 1981; Tuffrey *et al.*, 1981, 1986a, 1986b, 1990; Swenson *et al.*, 1983 and 1984; de la Maza *et al.*, 1994; Perry *et al.*, 1999; Pal *et al.*, 1998; Ramsey *et al.*, 2000). However, this MoPn strain is much more virulent than human serovars and causes infections throughout the genital tract in mice (Williams *et al.*, 1981). Furthermore, since genome analysis has shown that human isolates are genetically clearly different from the MoPn strain, MoPn has recently been reclassified as a new species within the genus Chlamydia and is now named *C. muridarum* (Everett *et al.*, 1999; Read *et al.*, 2000). Because of the genomic differences between *C. muridarum* and human strains, use of *C. muridarum* as a model for human pathology has become a matter of debate (Morré *et al.*, 2000; Morrison *et al.*, 2000).

Knowledge about the pathogenesis of long-term sequelae of human *C. trachomatis* serovars in mice is rather limited. Human biovars have been demonstrated to cause post-infectious sequelae only if high doses were inoculated directly into the uterine horns of mice pre-treated with progesterone. A reason for the effect of progesterone pre-treatment could be a local action of progesterone in the genital tract, e.g. by increasing the susceptibility of the epithelium to an infection with *C. trachomatis*, or by a generalized immunosuppressive effect of this hormone (Tuffrey *et al.*, 1981, 1986a, 1986b, 1990).

Re-infection has been studied in macaques and in mice. In macaques cervical priming with *C. trachomatis* prior to tubal inoculation increased the extent of tubal pathology (Patton *et al.*, 1990). In mice, re-inoculation with the same serovar induced pronounced tubal pathology whereas mice re-inoculated with different serovars remained fertile (Ramsey *et al.*, 2000).

Tubal pathology due to *C. trachomatis* infection is assumed to be immunopathologically mediated (Morris *et al.*, 1985; Brunham and Peeling, 1994). Although early studies on tubal pathology did not concentrate on *C. trachomatis* specifically, in patients with acute PID it was shown that the infertility rates after one, two and three episodes of symptomatic PID were 13%, 20% and 75% respectively (Weström and Mårdh, 1975). Similarities exist between the *C. trachomatis* induced immune response occurring in trachoma, i.e. keratoconjunctivitis due to *C. trachomatis*, and the response in genital tract infection (Brunham *et al.*, 1985). Animal studies on the pathogenesis of trachoma have shown that primary Chlamydia infection does not lead to permanent tissue damage, but that re-infection induces an inflammatory reaction that may ultimately culminate in fibrosis and scarring and lead to ocular damage (Grayston *et al.*, 1972, 1985). The specific immunologic pathways that underlie this tissue damaging response to infection and re-infection are unknown (Patton and Kuo, 1989; Monnickendam *et al.*, 1980).

Moreover, in assuming that the immune response not only consists of a species-specific response, but also contains genus-specific features, the development of tubal pathology might not only be induced by *C. trachomatis* infection alone, but 'antigenic related' other Chlamydia infections might also play a role. Chlamydia species which, besides *C. trachomatis*, can cause infections in humans are *C. pneumoniae* and *C. psittaci*. The presence of *C. psittaci* in the general population is, with a prevalence of 0.1% *C. psittaci* antibodies, very low (Moss *et al.*, 1993; Wong *et al.*, 1999). *C. pneumoniae* however is a widespread pathogen in humans and (re-)infections commonly occur (Thom *et al.*, 1991). A prevalence of *C. pneumoniae* antibodies of 63–74% in healthy males and females has been found (Karvonen *et al.*, 1994; Wong *et al.*, 1999). The microorganism causes acute respiratory infections (Kalayoglu *et al.*, 1998; Monno *et al.*, 2002), but has also been associated with several chronic diseases, like asthma, reactive arthritis and atherosclerosis (Campbell *et al.*, 1998; Grayston, 2000).

As expected, the prevalence of *C. pneumoniae* IgG antibodies in subfertile women was comparable to the prevalence in healthy males and females, ranging from 56 to 76%, regardless of the cause of subfertility (Freidank *et al.*, 1995; Gijzen *et al.*, 2001). Because of the high antibody prevalence in subfertile women, we postulated *C. pneumoniae* to be a possible contributor to the development of *C. trachomatis* induced tubal pathology. Previous data in our laboratory are in agreement with this hypothesis by suggesting a correlation between the presence of both antibodies to *C. trachomatis* and *C. pneumoniae*, and tubal pathology at laparoscopy. The presence of both antibodies to *C. trachomatis* and to *C. pneumoniae* was associated with a higher rate of tubal factor subfertility (49%), as compared to

no antibodies (26%, $p < 0.05$), antibodies to *C. trachomatis* only (30%, $p > 0.05$) or *C. pneumoniae* only (17%, $p < 0.0001$) (Gijzen *et al.*, 2001).

Animal studies on *C. trachomatis* thus far have focussed on the development of acute PID and not on silent infections and their chronic sequelae. We hypothesised that in silent infections a primary *C. trachomatis* infection does not result in extensive genital tract pathology, and that only subsequent *C. trachomatis* re-infection induces tubal disease. Our main goal was to develop a small-animal model that mimics this hypothesised course of Chlamydia infections in women. The main part of the current study evaluates infection of a low susceptible mouse strain, i.e. C57BL/6J mice (De la Maza *et al.*, 1994), using a human *C. trachomatis* serovar (experimental design A). For this purpose mice were inoculated intravaginally and the development of histopathologic changes and tissue damage in the genital tract at different time points after infection were studied. Of these mice, one group was submitted to re-infection while the others were not re-infected. Furthermore, to explore whether other Chlamydia species, i.e. *C. pneumoniae*, contribute to the development of *C. trachomatis* induced tubal pathology, a small pilot study with *C. pneumoniae* inoculation was performed (experimental design B). The results of experimental design B are, due to technical problems, incomplete, and therefore only shortly described. For this reason, the discussion and conclusions of the present chapter deal with experimental design A only.

Materials and methods

Microorganisms

C. trachomatis (human serovar L₂) and *C. pneumoniae* (TWAR2043) were inoculated in flasks (75 cm²) containing Hep-2C (ATCC CCL-23) monolayers. The flasks were centrifuged for 1 hour at 3000g. Chlamydia growth medium was added, containing Eagles minimal essential medium (Bio Whittaker, Verviers, Belgium) (pH 7.5), 0.15% NaHCO₃, 10% fetal calf serum, 1% non essential amino acids, 1% glutamine, gentamicin (25 µg/ml) and cycloheximide (1 µg/ml). After 72 hours the monolayer was harvested by scraping the cells, and sonicated twice for 15 sec using a Branson sonifier 250 (Brouwer Scientific BV, Soest, The Netherlands). Cell debris was pelleted by centrifugation for 10 min at 100g, after which the supernatant was used for pelleting the Chlamydia elementary bodies at 30.000g for 45 minutes at 4°C. The pellet was resuspended in Sucrose Phosphate Glutamate medium (SPG) and stored at -80°C. The concentration of inclusion forming units (IFU) was determined by culturing a serial tenfold dilution range of the isolated Chlamydia in a 96-well culture dish containing Hep-2C monolayers.

After fixation of the cell monolayer, the concentration of *C. trachomatis* IFU was determined by immunofluorescence staining with monoclonal antibodies CF2 (Washington Research Foundation, Seattle, Washington, USA) and FITC-labeled rabbit anti-mouse conjugate (DAKO F313, Glostrup, Denmark). The concentration of *C. pneumoniae* IFU was determined by immunofluorescence staining with monoclonal antibodies RR402 (DAKO 6600, Glostrup, Denmark) and FITC-labeled rabbit anti-mouse conjugate (DAKO F313, Glostrup, Denmark).

Animals

Specific-pathogen-free eight week old female C57BL/6J mice were obtained from Charles River Laboratories (Somerens, The Netherlands). They were kept in an isolator-type cage under standard environmental conditions. Mice were given subcutaneous injections containing 2.5 mg of medroxyprogesteronacetate (Anovulin®, Produlab Pharma, Raamsdonkveer, The Netherlands) in 100 µl of phosphate buffered saline (PBS) ten and three days prior to inoculation with *C. trachomatis*, *C. pneumoniae* or PBS (control group), to synchronise oestrus (Tuffrey and Taylor-Robinson, 1981). For vaginal inoculation the mice were manually fixated and the urine bladder emptied by light rubbing of the abdomen. This was performed to prevent the mice from expelling the inoculum by urinating immediately in response to the inoculation. For *C. pneumoniae* inoculation the mice were fixated manually, and *C. pneumoniae* was injected intraperitoneally, as described by Ezzahiri and coworkers (2002 and 2003).

Experimental design A: C. trachomatis infection experiments

A dose finding study was performed to establish the optimal dose for infection. In this study three groups of twelve mice were inoculated intravaginally with 10^3 , 10^5 and 10^7 IFU *C. trachomatis* L₂ respectively in 10 µl PBS medium, and control mice were given 10 µl PBS only. Infection was assessed by determining *C. trachomatis* IgG antibodies in serum three and nine weeks after inoculation using the micro-immunofluorescence (MIF) assay, as described below.

Five groups of mice were entered in the *C. trachomatis* pathogenesis study. Three groups of six mice each were inoculated once with 10^7 IFU, the dose of *C. trachomatis* selected from the dose finding study. Mice were sacrificed at regular intervals, three weeks (single-3), nine weeks (single-9) and eighteen weeks after inoculation (single-18). Six mice were inoculated with 10^7 IFU *C. trachomatis* and re-inoculated after nine weeks and sacrificed eighteen weeks after the primary inoculation (re-inoc group). The control group was sacrificed nine weeks after inoculation (PBS-control group).

Before sacrifice, mice were anaesthetised with a weight-adjusted dose of pentobarbital (Nembutal, Sanofi Sante BV, Maassluis, the Netherlands) and sacrificed by performing a heart puncture. Gross genital tract pathology was examined and scored *in vivo*, and genital tracts were excised for histologic evaluation.

Experimental design B: C. pneumoniae infection experiments

A dose finding study was performed to study whether *C. pneumoniae* is able to induce pathologic changes in the genital tract without the interference of *C. trachomatis*. To establish the optimal dose for infection, three groups of six mice were inoculated with 10^3 , 10^5 and 10^7 IFU *C. pneumoniae* TWAR respectively in 200 μ l PBS medium according to a standardised procedure as described by Ezzahiri *et al.* (2002 and 2003). Infection was assessed by determining *C. pneumoniae* IgG antibodies in serum three weeks after inoculation using MIF. Gross genital tract pathology was examined and scored *in vivo*, and genital tracts were excised for histologic evaluation.

Subsequently, experiments using successive inoculations with *C. pneumoniae* and *C. trachomatis* were done. Three groups of six mice were inoculated with 10^7 IFU *C. pneumoniae* per mouse and / or 10^7 IFU *C. trachomatis* as described before. One group was inoculated with *C. pneumoniae* and re-inoculated with PBS (single-Cp group). A second group was inoculated primarily with *C. pneumoniae* and re-inoculated with *C. trachomatis* (Cp-Ct group). The third group was inoculated primarily with *C. trachomatis* and re-inoculated with *C. pneumoniae* (Ct-Cp group).

For all three groups re-inoculation was planned at nine weeks, and sacrifice at 18 weeks after primary inoculation. However, due to miscalculation Cp-Ct mice were re-inoculated after 18 weeks and subsequently sacrificed after 27 weeks.

Antibody detection

Mice were tested for the presence of *C. trachomatis* IgG antibodies using a commercially available indirect micro-immunofluorescent (MIF) antibody technique (*C. pneumoniae* IgG Micro-IF, Labsystems, Helsinki, Finland). This test was originally developed to detect *C. pneumoniae* antibodies, but each well also contains a spot appropriate for *C. trachomatis* detection. Moreover, in screening for human tubal factor subfertility, this test was superior to four other Chlamydia antibody tests evaluated (Land *et al.*, 2003). In short, 10 μ l serum was diluted 10 times in PBS (pH 7.4) and incubated on the spots containing Chlamydia antigens, for 30 min at 37°C in a moist chamber. The slides were washed two times for five min in PBS and once in ultrapure water, processed through a Milli-Q purifying system

(Millipore, Bedford, MA, USA) and placed into 37°C until dry. The spots were incubated with 10 µl rabbit anti-mouse IgG-FITC-labeled conjugate (DAKO F313, Glostrup, Denmark) and placed in a moist chamber for 30 min at 37°C. The slides were washed after serum incubation, and placed into 37°C until dry. Slides were embedded in mounting fluid under a cover slip. A positive reaction was characterised by specific fluorescence of the respective elementary bodies. All slides were evaluated independently by two readers. In the case of disagreement, the judgement of a third reader was decisive. A positive MIF result was defined as a titre ≥ 10 .

Gross genital tract pathology

After sacrifice of the mice, the genital tracts were exposed by an abdominal midline incision and examined for gross pathology in situ, using a light-microscope. In the present study, colour changes of the uterine horns and tubes were scored (as normal, red or pale) as parameters of inflammation. Since genital tract infections in mice can cause fluid retention and enlargement of the uterine horns and tubes (hydrosalpinx), dilatation of the uterine horns and tubes was scored (as absent or present) (Cotter *et al.*, 1997). Thereafter, the genital tracts were excised completely for histologic examination.

Histology

Paraffin embedded genital tracts were sectioned in 4 µm slides and evaluated after hematoxylin and eosin staining. Blinded sections were scored by an experimental pathologist (M. Gijbels), and histopathologic evaluation of the genital tracts focussed on several infection parameters. In stroma, values were assigned to the amount of plasma cells, lymphocytes and neutrophilic granulocytes. The endometrium was scored for atypia, presence of mitosis, apoptosis and T cells, and the endometrial crypts for presence of mitosis and apoptosis. The severity of the inflammatory response, as indicated by the infiltration of different inflammatory cells, mitosis and apoptosis, was scored as absent (0), very mild (1), mild (2), moderate (3) or severe (4). Atypia of the endometrium was scored as absent (0), focal (1), multifocal (2) or extensive (3). The sum of the values per parameter was calculated and the average $\pm$ standard error of mean (SEM) was given for each group of mice. For statistical analysis a Mann-Whitney test was used. P-values of < 0.05 were considered to reflect statistical significance.

Results of experimental series A

Determination of optimal dose for C. trachomatis intravaginal infection

Since it is known that different mouse strains show different susceptibilities to infection with *C. trachomatis*, a dose finding study with *C. trachomatis* L₂ was performed in order to determine the optimal inoculation dose to induce infection in C57BL/6J mice. Two mice died during the experiments: one mouse inoculated with 10³ IFU died due to dental problems and one scheduled for inoculation with 10⁷ IFU died between progesterone pre-treatment and inoculation.

In order to confirm whether the animals were infected with *C. trachomatis*, IgG antibody detection was performed. Table I summarises the results of the *C. trachomatis* antibody detection at three and nine weeks after inoculation. Mice inoculated with the lowest dose (10³ IFU) did not develop IgG antibodies three weeks after inoculation, and only 1 out of 6 mice showed anti-*C. trachomatis* antibodies nine weeks after inoculation. The number of sero-positive animals increased with higher doses of *C. trachomatis*, resulting in 100% sero-positivity in mice nine weeks after inoculation with 10⁷ IFU. Based on these data, it was decided to use 10⁷ IFU *C. trachomatis* as inoculation dose for further experiments.

C. trachomatis pathogenesis study

Mice were sacrificed at several time points after inoculation in order to evaluate the development of gross (Table II) and microscopic morphological changes (Table III) of the genital tracts.

One mouse from the single-9 group died before inoculation due to an unknown cause. In the PBS-control group no gross abnormalities were noticed. As shown in table II, in the *C. trachomatis* inoculated animals, in 3 mice redness was observed in the single-3 group. The single-3 group showed no other abnormalities, while the single-9 group showed paleness, dilatation of uterine horns and tubes in about one third of the animals. These aspects had disappeared nine weeks later, i.e. the single-18 group showed redness in 3 mice and dilatation of uterine horns in only one. However, 4/6 mice from the re-inoc group, did show dilatation of uterine horns and tubes. In this re-inoc group dilatation was demonstrated more often than in the single-18 group. In summary, these results show that in a number of mice gross genital tract pathology developed in nine weeks after *C. trachomatis* inoculation. The effects had a mild and temporary character, since eighteen weeks after inoculation gross genital tract pathology had resolved. After re-inoculation gross genital tract pathology was noted again. Since the end-point of the study was nine weeks after re-inoculation, we could not evaluate whether the effects seen after re-inoculation were permanent.

Table I. IgG antibody detection by MIF in mice after intravaginal inoculation with different doses of *C. trachomatis*.

IFU/mouse	No. of mice with <i>C. trachomatis</i> IgG antibodies / total no. of mice inoculated	
	3 weeks after inoculation	9 weeks after inoculation
10 ⁷	5/5	5/5
10 ⁵	2/6	2/6
10 ³	0/5	1/5
0 ^b	ND ^a	0/6

^a ND: not determined; ^b Mice inoculated with PBS.

Table II. Presence of gross genital tract pathology in *C. trachomatis* inoculated mice sacrificed at different time points after inoculation.

Gross genital tract pathology	Inoculation groups ^a				
	Single-3 ^b	Single-9 ^b	Single-18 ^b	Re-inoc ^b	PBS-control ^b
redness	3/6	0/5	3/6	0/6	0/6
palleness	0/6	2/5	0/6	1/6	0/6
ardilatation uterine horns	0/6	2/5	1/6	4/6	0/6
dilatation tubes	0/6	2/5	0/6	2/6	0/6

^a Number of mice with gross genital tract pathology / total number of mice

^b Single-3, single-9 and single-18: mice sacrificed respectively three, nine and eighteen weeks after inoculation; re-inoc: mice re-inoculated nine weeks after the primary inoculation and studied nine weeks after re-inoculation; PBS-control: control mice inoculated with PBS.

Table III summarises histologic parameters for the different inoculation groups. In the stroma of the PBS-control group very mild infiltration of inflammatory cells was observed and the endometrium of two out of six animals showed focal atypia. After single inoculation with *C. trachomatis* the infiltration of inflammatory cells (lymphocytes, macrophages and granulocytes) increased with time ($p < 0.05$ in the single-18 group as compared to the single-3 group), while in the endometrium of these animals no abnormalities were observed in comparison to the PBS-control group. Mitosis and apoptosis in the endometrium and crypts were present more often in the single-9 and PBS-groups compared to the other inoculation groups. Atypia of the endometrium was found more often (multifocally) in the re-inoc group as compared to the single inoculation groups in which atypia was scored as focal. In the re-inoc group infiltration of inflammatory cells was significantly increased as compared to the PBS-control group. Figure I shows hematoxylin and eosin stained sections of uterine horns from mice of the PBS-control group and the re-inoc group.

Table III. Histologic scores in *C. trachomatis* inoculated mice sacrificed at different time points after inoculation.

Histologic parameters ^a	Inoculation groups ^b				
	Single-3 (n = 6)	Single-9 (n = 5)	Single-18 (n = 6)	Re-inoc (n = 6)	PBS-control (n = 6)
Inflammatory cells					
plasma cells	0.3 ± 0.2	0.8 ± 0.2	0.3 ± 0.2	1 ± 0.4	0.2 ± 0.2
lymphocytes/macrophages	0.8 ± 0.2 ^c	1.6 ± 0.6	2 ± 0.4 ^d	2.5 ± 0.3 ^d	0.6 ± 0.3 ^c
granulocytes	0 ^c	2 ± 0.6	2.3 ± 0.5 ^d	2.5 ± 0.4 ^d	0.6 ± 0.3 ^c
Endometrium					
atypia	0 ^c	0.6 ± 0.4	0.3 ± 0.3	1.8 ± 0.4 ^d	0.8 ± 0.5
mitosis	0.2 ± 0.2 ^c	2 ± 0.7 ^d	0.8 ± 0.5	0.7 ± 0.5	2 ± 0.9 ^d
apoptosis	0.2 ± 0.2	2 ± 0.6 ^d	0 ^c	1.7 ± 0.5	1.2 ± 0.5
T cells	0	0.4 ± 0.4	0.3 ± 0.33	0.3 ± 0.2	1 ± 0.6
Crypts					
mitosis	0.2 ± 0.2 ^c	2 ± 0.5 ^d	0.7 ± 0.5	1 ± 0.5	0.8 ± 0.9
apoptosis	0.3 ± 0.3 ^c	0.4 ± 0.4 ^c	0 ^c	2.3 ± 0.3 ^d	1.6 ± 0.6

^a Mean values ± S.E.M for histologic parameters in each group. The infiltration of different inflammatory cells, mitosis and apoptosis, was scored as absent (0), very mild (1), mild (2), moderate (3) or severe (4). Atypia of the endometrium was scored as absent (0), focal (1), multifocal (2) or extensive (3).

^b Single-3, single-9 and single-18: mice sacrificed respectively three, nine and eighteen weeks after inoculation; re-inoc: mice re-inoculated nine weeks after the primary inoculation and studied nine weeks after re-inoculation; PBS-control: control mice inoculated with PBS.

^{c-d} Reflect significant differences with $p < 0.05$.

Figure 1. Hematoxylin and eosin stained sections, 100X.

A: Uterus of PBS-control group: mitosis is observed (asterisk) and some inflammatory cells (arrow head). B: Uterus of re-inoculation group: many apoptotic cells in the glandular epithelium (small arrow) and in the stroma macrophages (thick arrow) and neutrophilic granulocytes (arrow head).

Table IV. Presence of gross genital tract pathology in *C. pneumoniae* inoculated mice sacrificed three weeks after inoculation.

Gross genital tract pathology ^a	Inoculation dose (IFU)		
	10 ³	10 ⁵	10 ⁷
redness	0/6	0/6	2/6
paleness	0/6	0/6	1/6
dilatation uterine horns	0/6	0/6	1/6
dilatation tubes	0/6	0/6	0/6

^a Number of mice / total number of mice inoculated

Results of experimental series B

C. pneumoniae dose finding experiment

Since the susceptibility of mice genital tracts to *C. pneumoniae* has not been described before, a dose finding study with *C. pneumoniae* was performed. Sero-logic response, gross and microscopic genital tract pathology were studied three weeks after inoculation.

In the group of mice inoculated with the lowest dose (10³ IFU) no *C. pneumoniae* IgG antibodies were detected. The number of sero-positive animals increased with higher doses of *C. pneumoniae*. In the group of mice inoculated with 10⁵ IFU *C. pneumoniae*, three out of six mice sero-converted while in six mice inoculated with 10⁷ IFU *C. pneumoniae* 100% sero-positivity was found, indicating that inoculation with high doses of *C. pneumoniae* (10⁷ IFU) leads to infection of all mice.

As shown in table IV, no gross abnormalities were observed in both lower doses (10³ and 10⁵ IFU), while inoculation with 10⁷ IFU *C. pneumoniae* resulted in genital tract redness in two out of six mice, with dilatation of uterine horns in one of these and paleness in one other mouse. After *C. pneumoniae* inoculation the infiltration of inflammatory cells increased with higher doses, but in the endometrium of these animals no abnormalities were observed (table V). Based on these data, it was decided to use 10⁷ IFU *C. pneumoniae* as inoculation dose for the *C. pneumoniae* study.

C. pneumoniae pathogenesis study

In order to study the role of *C. pneumoniae* as a contributor to the development of *C. trachomatis* induced tubal pathology, mice were successively inoculated with *C. pneumoniae* and *C. trachomatis* or vice versa. As described earlier, miscalculation of

Table V. Histologic scores in *C. pneumoniae* inoculated mice sacrificed three weeks after inoculation.

Histologic parameters ^a	Inoculation dose (IFU)		
	10 ³ (n = 6)	10 ⁵ (n = 6)	10 ⁷ (n = 6)
Inflammatory cells			
plasma cells	0.8 ± 0.2	1.5 ± 0.2	3 ± 0
lymphocytes/macrophages	0	1.5 ± 0.2	3 ± 0
granulocytes	0	0.2 ± 0.2	0.5 ± 0.2
Endometrium			
atypia	0	0.5 ± 0.2	0
mitosis	0.2 ± 0.2	1.4 ± 0.5	0.2 ± 0.2
apoptosis	0	0	0
T cells	0.3 ± 0.4	0	0
Crypts			
mitosis	0.7 ± 0.4	0.8 ± 0.3	1 ± 0.5
apoptosis	0	0	0.2 ± 0.2

^a Mean values ± S.E.M for histologic parameters in each group. The infiltration of different inflammatory cells, mitosis and apoptosis, was scored as absent (0), very mild (1), mild (2), moderate (3) or severe (4). Atypia of the endometrium was scored as absent (0), focal (1), multifocal (2) or extensive (3).

the time of re-inoculating of the Cp-Ct mice has precluded from comparing them with single-Cp or Ct-Cp inoculated mice. For the sake of completeness the results of the Cp-Ct group are presented in the tables separately.

As shown in table VI, the presence of redness and paleness as parameters for gross genital tract parameters was not different between the single-Cp and Ct-Cp groups. The single-Cp group showed no other abnormalities, while dilatation of uterine horns and tubes was observed in the Ct-Cp group in 3 mice.

Due to technical problems in the preservation process, histologic material of all six mice in the Ct-Cp group became unusable. After single inoculation with *C. pneumoniae* mild to moderate infiltration of inflammatory cells in the stroma was observed, and the endometrium showed multifocal atypia. These results indicate that inoculation with *C. pneumoniae* may induce genital tract inflammatory processes without the interference of *C. trachomatis*. Tables VI and VII summarise macroscopic and microscopic changes of the genital tract for the different study groups.

Table VI. Gross genital tract pathology in mice after single *C. pneumoniae* inoculation and combined *C. pneumoniae* / *C. trachomatis* inoculation or vice versa.

Gross genital tract pathology	Inoculation groups ^a		
	Single-Cp ^b	Ct-Cp ^b	Cp-Ct ^b
redness	2/5	1/6	2/6
palleness	1/5	1/6	1/6
dilatation uterine horns	0/5	0/6	1/6
dilatation tubes	0/5	3/6	2/6

^a Number of mice with gross genital tract pathology / total number of mice.

^b Single-Cp, Ct-Cp: mice re-inoculated after nine weeks and sacrificed eighteen weeks after inoculation; Cp-Ct: due to miscalculation mice were re-inoculated after 18 weeks and sacrificed 27 weeks after inoculation.

Table VII. Histologic scores in mice after single *C. pneumoniae* inoculation and successive *C. pneumoniae* / *C. trachomatis* inoculation or vice versa.

Histologic parameters ^a	Inoculation groups ^b		
	Single-Cp (n = 5)	Ct-Cp (n = 6)	Cp-Ct (n = 6)
Inflammatory cells			
plasma cells	1.6 ± 0.3	n.a	0.8 ± 0.4
lymphocytes/macrophages	3.2 ± 0.2	n.a	2.4 ± 0.4
granulocytes	3.2 ± 0.5	n.a	2.6 ± 0.4
Endometrium			
atypia	1.8 ± 0.5	n.a	0.8 ± 0.5
mitosis	1.2 ± 0.7	n.a	1 ± 0.5
apoptosis	2 ± 0.9	n.a	1.4 ± 0.9
T cells	0	n.a	0.2 ± 0.2
Crypts			
mitosis	0.8 ± 0.6	n.a	0.4 ± 0.6
apoptosis	1 ± 0.6	n.a	0.6 ± 0.3

^a The infiltration of different inflammatory cells, mitosis and apoptosis, was scored as absent (0), very mild (1), mild (2), moderate (3) or severe (4). Atypia of the endometrium was scored as absent (0), focal (1), multifocal (2) or extensive (3). Mean values ± S.E.M for histologic parameters in each group.

^b Single-Cp, Ct-Cp: mice re-inoculated after nine weeks and sacrificed eighteen weeks after inoculation; Cp-Ct: due to miscalculation mice were re-inoculated after 18 weeks and sacrificed 27 weeks after inoculation.

n.a = not available.

Discussion

In contrast to earlier animal studies which addressed acute PID and the induction of infertility, the current study presents a mouse model that mimics the hypothesised course of silent *C. trachomatis* infection. It was hypothesised that a primary *C. trachomatis* infection results in an immune response without the development of tubal pathology, and that a subsequent *C. trachomatis* re-infection induces genital tract pathology. In developing the model, knowledge was derived from earlier animal studies for *C. trachomatis* infections, in which acute PID with short-term infertility had been studied.

For our study, the choice for the C57BL/6J mouse was based on the study by De la Maza *et al.* (1994), who compared C3H, Balb/c and C57BL/6J mice in their ability to develop salpingitis and tubal pathology after intravaginal inoculation with *C. muridarum*, and found C57BL/6J mice to develop the mildest disease. Since our main interest was to induce silent infections without extensive tubal pathology after the first inoculation, the C57BL/6J mouse strain was chosen for our studies.

For the same reason in our studies L₂, a human serovar of *C. trachomatis* was used. As compared to *C. muridarum*, human serovars have been shown to be less virulent in mice (Williams *et al.*, 1981). After vaginal inoculation *C. trachomatis* human biovars induce a mild infection of the mouse genital tract (Perry *et al.*, 1999). From our results we conclude that using C57BL/6J mice and intravaginal inoculation of a human *C. trachomatis* strain, high inoculation doses (10⁷ IFU) are required to induce an immune response with IgG antibody formation (Table I). This finding is in agreement with other studies using human serovars of *C. trachomatis* (Johansson *et al.*, 1997). However, studies using the *C. muridarum* agent have shown that much lower concentrations of the microorganism can be used for infection experiments (Perry *et al.*, 1999; Pal *et al.*, 1998).

No data are available in the literature on specific parameters that adequately reflect the changes associated with silent *C. trachomatis* infection of the genital tract in mice. Again, knowledge was derived from studies on acute *C. trachomatis* genital tract infection. It has been shown that genital tract infection stimulates the immune response in the endometrium, inducing increased influx of inflammatory cells. Based on this, the number of inflammatory cells, and the presence of mitotic and apoptotic cells were scored as parameters for infection. Eventually, the stromal infiltration of inflammatory cells and endometrial atypia appeared to be the parameters specifically related to infection, since those are the parameters increasing with time after inoculation and after re-inoculation. However, the presence of mitosis and apoptosis was not clearly related to infection or re-infection.

We assumed the endometrium in silent Chlamydia infections to respond with increased cell activity, reflected in an increase in mitotic cells. In case of re-infec-

tion, increased cell activity may result in cell death by apoptosis. Subsequently, apoptosis may induce atypia in the endometrium. Results in mice sacrificed nine weeks after single inoculation showed gross genital tract pathology (i.e. uterine and tubal dilatation) whereas those sacrificed eighteen weeks after inoculation seemed to have recovered. However, stromal inflammatory cells remained present, without the development of atypia in the endometrium. This may suggest a persisting chronic inflammatory process. After re-inoculation, the inflammatory response may be reactivated and finally the endometrium may be unable to recover, resulting in multifocal atypia. To evaluate whether gross genital tract pathology after re-inoculation would persist, a longer follow up period would have been necessary. The results are according to those found in trachoma, in which a primary infection primes the immune system and re-infection induces tissue damage (Grayston *et al.*, 1985). The explanation for this chronicity might be that endometrium does not fully recover after a single inoculation, possibly due to the persistence of dormant, but still viable, Chlamydia (Barlow *et al.*, 2001). It may be hypothesised that it is not re-infection with *C. trachomatis* per se, but reactivation of persistent Chlamydia that is able to chronically stimulate the immune responses which are responsible for the development of tubal pathology (Beatty *et al.*, 1994).

From our *C. pneumoniae* pilot study (part B), due to technical problems no major conclusions can be drawn. However, some observations will be discussed. It was postulated that *C. pneumoniae* is able to induce tubal pathology as well. The role of *C. pneumoniae* in the development of tubal pathology may be explained, by its high inter-species antigen homology with *C. trachomatis*. In case of an infection with a particular Chlamydia species, Chlamydia antigens will result in activation of the immune system, leading to the production of genus-specific and species-specific antibodies. It may be hypothesised that the genus-specific antibody response to *C. pneumoniae* may contribute to the development of tubal pathology.

Although a direct effect of *C. pneumoniae* on the genital tract might seem unlikely and in any case unexpected, it has been suggested that *C. pneumoniae* dissemination can occur by circulating infected monocytes and macrophages spreading the microorganism via the blood and / or lymphatic system to almost all organs (Ezzahiri *et al.*, 2002; Gieffers *et al.*, 2001; Maass *et al.*, 2000; Rupp *et al.*, 2005). In mice, the microorganism has been detected by polymerase chain reaction (PCR) in the liver, spleen and lymph nodes following intranasal or intraperitoneal inoculation (Moazed *et al.*, 1997). In accordance with this, *C. pneumoniae* may be postulated to directly affect the genital tract as well.

Summarising our results, the inflammatory response in mice after *C. pneumoniae* intraperitoneal inoculation on the genital tract is obvious. Although no

major gross or microscopic genital tract pathology was observed in mice three weeks after single *C. pneumoniae* inoculation, more inflammatory cells were present (table V) than after *C. trachomatis* inoculation (table III, single-3). Furthermore, eighteen weeks after inoculation, inflammatory response and atypia of the endometrium was more evident in *C. pneumoniae*- (table VII, single-Cp) compared to *C. trachomatis* inoculated mice (table III, single-18). Considering re-inoculation (Ct-Cp and Cp-Ct), data on histologic parameters are incomplete. However, whereas only redness was found in mice after single *C. pneumoniae* inoculation, dilatation of uterine horns and tubes was found in *C. pneumoniae* / *C. trachomatis* or vice versa re-inoculated mice (table VI). In *C. trachomatis* re-inoculated mice however (table II, re-inoc), more hydrosalpinges were described as compared to Cp-Ct or Ct-Cp. Since a direct effect of *C. pneumoniae* on the genital tract is rather unexpected, the remarkable inflammatory response might be due to the intraperitoneal route of inoculation. Therefore, in future experiments, intranasal inoculation should be considered, as this way of inoculation may better mimic the infection course in humans.

In conclusion, part A of the present study describes a mouse model that mimics the hypothesised course of mild Chlamydia infections in women, i.e. that primary *C. trachomatis* infections do not result in the development of extensive genital tract pathology. Our results indicate that single intravaginal inoculation of C57BL/6J mice with the human *C. trachomatis* L₂ strain induces an infection without extensive pathology, but with a chronic inflammatory cell response. Re-inoculation stimulates this process, finally resulting in the induction of atypia in the endometrium. Changes at the tissue and cellular level were all more evident after re-inoculation, but were also mild. Despite our assumption, that extensive pathology would be found after re-inoculation, only mild genital tract pathology and histologic changes were found. This might be due to various factors in the model. We combined the use of C57BL/6J mice, which have demonstrated only mild disease in earlier studies (De la Maza *et al.*, 1994), with the inoculation of a human *C. trachomatis* serovar, which has been shown to be less virulent in mice (Tuffrey *et al.*, 1986a, 1986b, 1990). This combination might have led to only mild genital tract pathology after re-inoculation. When using a different strain of mice (e.g. C3H or BALB/c), which has been shown to be more susceptible to *C. muridarum* (de la Maza *et al.*, 1994), re-inoculation might have led to the induction of more extensive genital tract pathology. Furthermore, increase of the number of inoculations might reinforce the inflammatory response and tissue reaction in the genital tract. Rank and coworkers (1988) demonstrated a short-term immunity to re-infection in guinea pigs after primary inoculation, suggesting that lengthening the time interval between the initial and the re-inoculation might overcome the risk of mice still being immune following the primary inoculation.

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7

Detection of Chlamydia and mouse hsp60 antibodies in murine serum following *C. trachomatis* or *C. pneumoniae* infection

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Introduction

In the search for markers to be used in screening for tubal factor subfertility, it is important to elucidate its etiology. In this respect, it has been suggested that persistence of *C. trachomatis* infection in the host may be important (Debattista *et al.*, 2003; Stephens, 2003). However, mechanisms by which *C. trachomatis* induces tubal damage have not been determined exactly yet. It has been postulated that the immune response following repeated or chronic exposure to *C. trachomatis* antigens may form the basis for pathology resulting in tissue destruction (Brunham *et al.*, 1985; Beatty *et al.*, 1994a; Mabey *et al.*, 2001). This immune response may either be protective or pathological, depending on the respective contributions of the humoral and cellular immunity. Stimulated by infection, T helper (Th) lymphocytes will respond by following cellular or humoral immune responses, i.e. Th1 cells are involved in cellular immunity and Th2 cells are responsible for humoral immunity. Th1 and Th2 cells each produce distinct cytokines. It is the ability or inability to generate specific cytokines or cytokine patterns in response to a particular infection that will determine the clinical course of the infection, and whether this will result in successful clearance or persistence of the microorganism (Stephens, 2003).

In case of persistence, an autoimmune response has been supposed to underlie the process from persistent *C. trachomatis* infection to tubal pathology, with a central role for antibodies directed against heat shock protein 60 (hsp60) (Domeika *et al.*, 1998). In clinical studies, Chsp60 antibodies, considered as markers of chronic infection, have already been associated with the development of tubal pathology (Toye *et al.*, 1993; Arno *et al.*, 1995; Claman *et al.*, 1997).

Hsp60 is a member of the heat shock protein family, a group of highly conserved cellular stress proteins, expressed by prokaryotic and eukaryotic cells under physiological conditions or in response to stress (e.g. heat or infection). They participate primarily in mediating intracellular functions, but they are also involved in protecting the cell from adverse effects on cellular metabolism due to stress. Since their amino-acid composition is highly conserved during evolution, hsps of highly divergent organisms are very similar to one another (Neuer *et al.*, 2000). With respect to Chlamydia, Chlamydia hsp60 (Chsp60) shares approximately 50% homology with the human hsp60 (Hhsp60).

Based on this high degree of homology, it has been suggested that antibodies, initially directed against Chsp60 may cross-react with Hhsp60 (Wick *et al.*, 2001). This cross-reactivity may ultimately result in tissue damage when Hhsp60 is translocated to the cell membrane in cases of cellular stress. Such auto-immune mechanism has previously been suggested to be responsible for the adverse effects of chronic *C. pneumoniae* infections in diseases such as atherosclerosis or arthritis

(van Eden, 1999; Hannu *et al.*, 1999; Kalayoglu *et al.*, 2002; Schumacher *et al.*, 1999; Wick *et al.*, 2001; Xu *et al.*, 1992 and 1996).

When extrapolating this auto-immune hypothesis to the development of tubal pathology, we postulate that a persistent *C. trachomatis* genital infection may not be directly responsible for the destruction of susceptible epithelial cells (with subsequent tubal damage), but that it induces the expression of Hhsp60 on the surface of these cells instead. Assuming that repeated or chronic *C. trachomatis* infection additionally results in the production of Chsp60 antibodies (Beatty *et al.*, 1994a and b), cross-reaction of Chsp60 antibodies with Hhsp60 expressed on tubal epithelial cells may consequently lead to tubal damage. Moreover, the development of tubal pathology might not only be induced by *C. trachomatis* hsp60 antibodies, but infections with 'antigenic related' microorganisms might also play a role. Because of its high antibody prevalence in subfertile women (Freidank *et al.*, 1995; Gijzen *et al.*, 2001), we postulated *C. pneumoniae* to be a possible contributor as well.

In the present study we evaluated whether Chlamydia- and mouse hsp60 (Mhsp60) antibodies could be detected in serum following *C. trachomatis* or *C. pneumoniae* infection in a mouse model, to evaluate whether auto-immunity, based on cross-reactivity could be the underlying mechanism of tubal pathology.

Materials and methods

Microorganisms

C. trachomatis (human serovar L₂) and *C. pneumoniae* (TWAR2043) were cultured in Hep-2C (ATCC CCL-23) monolayers as previously described (Roblin *et al.*, 1992). The concentration of *C. trachomatis* inclusion forming units (IFU) was determined by titration in Hep-2 cells and immunofluorescence staining with monoclonal antibodies CF2 (Washington Research Foundation, Seattle, Washington, USA) and fluorescein-isothiocyanate-conjugate (FITC)-labeled rabbit anti-mouse conjugate (DAKO F313, Glostrup, Denmark). The concentration of *C. pneumoniae* IFU was determined by immunofluorescence staining with monoclonal antibodies RR402 (DAKO 6600, Glostrup, Denmark) and FITC-labeled rabbit anti-mouse conjugate (DAKO F313, Glostrup, Denmark).

Animals

Specific-pathogen-free (SPF) eight week old female C57BL/6J mice were obtained from Charles River Laboratories (Somerer, The Netherlands). They were kept in an isolator-type cage under standard environmental conditions. Mice

were given subcutaneous injections containing 2.5 mg of medroxyprogesteronacetate (Anovulin®, Produlab Pharma, Raamsdonkveer, The Netherlands) in 100 µl of phosphate buffered saline (PBS) ten and three days prior to inoculation with *C. trachomatis*, *C. pneumoniae* or PBS (control group), to synchronise oestrus (Tuffrey and Taylor-Robinson, 1981). For vaginal inoculation the mice were manually restrained and the urine bladder emptied by light rubbing of the abdomen. This was performed to prevent the mice from expelling the inoculum by urinating immediately in response to the inoculation. For *C. pneumoniae* inoculation mice were fixated manually, and *C. pneumoniae* was injected intraperitoneally as described by Ezzahiri and coworkers (2002 and 2003).

Additionally, one group of sera, from mice participating in an atherosclerosis study, was added to the study (kindly provided by R. Ezzahiri, Department of Microbiology, Maastricht, the Netherlands). Consequently, the latter animals' characteristics were different. Seventeen week old SPF C57BL/6J mice were fed an atherogenic diet and injected with 10 monthly intraperitoneal inoculations of 5×10^7 IFU *C. pneumoniae*.

Experimental design

First, the presence of Chsp60 and Mhsp60 antibodies was determined in mice inoculated once at an age of 8 weeks with 10^7 IFU *C. trachomatis* or *C. pneumoniae*. Mice were sacrificed at three (single-3), nine (single-9) and eighteen (single-18) weeks after inoculation. One mock infected group was included (PBS-control).

Further, sera from mice after intravaginal *C. trachomatis* and / or intraperitoneal *C. pneumoniae* re-inoculation were used. Sera from three different groups were used: mice inoculated twice with *C. trachomatis* (Ct-Ct group), mice inoculated with *C. trachomatis* and re-inoculated with *C. pneumoniae* (Ct-Cp group) and mice inoculated with *C. pneumoniae* and re-inoculated with *C. trachomatis* (Cp-Ct group). For details, see legend of table II.

Finally, sera originating from mice that underwent 10 monthly repeated inoculations of 5×10^7 IFU *C. pneumoniae* were included (10-Cp). Sera were collected at sacrifice, twenty-five days after the last inoculation. In order to assure that we used sera from mice that were properly infected, only sera from mice that seroconverted for *C. trachomatis* and / or *C. pneumoniae* IgG, as determined by an indirect micro-immunofluorescent antibody technique (*C. pneumoniae* IgG Micro-IF, Labsystems, Helsinki, Finland), were included.

Chsp60 and Mhsp60 IgG antibody detection

Antibodies to Chlamydia and mouse hsp60 were determined in serum by an 'in-house' ELISA. This experimental assay has been developed in the Department of Immunology and performed according to the following procedure. Recombinant Chlamydia hsp60 or recombinant mouse hsp60 (Stressgen Bioreagents, Victoria, BC, Canada) were diluted to 5 µg/ml in 100 mM carbonate buffer pH 9.6, and Maxisorp microtitre plates (NUNC, Roskilde, Denmark) were coated with 50 µl per well; wells incubated with 50 µl carbonate buffer only served as blank controls.

After coating overnight at 4°C, plates were filled with 100 µl/well of 1% BSA in carbonate buffer and incubated overnight at 4°C to block remaining binding places. Plates were then washed three times with 0.05% Tween 20 in PBS pH 7.4 (PBS-T) and incubated for one hour at room temperature with 100 µl/well of 0.05% Tween 20 + 1% bovine serum albumin (BSA; Merck, Darmstadt, Germany) in PBS (PBS-T-BSA). Sera were diluted 1/50 in PBS-T-BSA and 50 µl of each serum was added in duplicate to both hsp60-coated wells and uncoated control wells; plates were then incubated overnight at 4°C. After a five-fold wash with 10 mM TRIS pH 8.0 + 150 mM NaCl + 0.05% Tween 20, plates were incubated with a 1/2000 dilution of an alkaline phosphatase conjugated goat F(ab)₂ anti-mouse IgG Fc (Jackson Laboratories, Pennsylvania, USA) in 100 mM TRIS + 300 mM NaCl + 0.05% Tween 20 pH 8.0 for 1 hour at 37°C. After a final five-fold wash with TRIS/NaCl/Tween, 100 µl of NPP/DEA substrate was added. Optical density (OD) was read after 60 minutes at 405 nm. For each serum sample, mean OD value of uncoated wells was subtracted from mean OD value in hsp-coated wells; samples were considered positive if the resulting net OD exceeded 0.1. Mouse serum samples, strongly seropositive or negative during earlier pilot studies to both Chsp60- and Mhsp60 IgG, served as positive and negative control samples for the test. These samples were added to each microtitre plate. In all cases, the positive control was seropositive indeed and the negative control remained seronegative.

Results

Table I summarises the results of serum Chsp60 and Mhsp60 IgG antibodies in mice three, nine and eighteen weeks after a single 10⁷ IFU *C. trachomatis* or *C. pneumoniae* inoculation. All mock infected mice (PBS-control group) remained seronegative. No antibodies were found in mice three and eighteen weeks after single *C. trachomatis* inoculation. In only 1 out of 16 mice, Chsp60 IgG antibodies

Table I. Presence of serum Chlamydia- and Mouse hsp60 IgG antibodies in mice at different time points after single intravaginal *C. trachomatis* or intraperitoneal *C. pneumoniae* inoculation.

Inoculation groups ^a	Chsp60 IgG ^b	Mhsp60 IgG ^b
PBS-control	0/6	0/6
<i>C. trachomatis</i>		
Single-3	0/5	0/5
Single-9	1/16	0/16
Single-18	0/3	0/3
<i>C. pneumoniae</i>		
Single-3	0/5	0/5
Single-9	0/5	0/5
Single-18	0/5	0/5

^a PBS-control: control mice inoculated intravaginally with PBS; Single-3, single-9 and single-18: mice sacrificed respectively three, nine and eighteen weeks after inoculation.

^b Number of mice with Chlamydia and /or Mouse hsp60 IgG antibodies / total number of mice.

Table II. Presence of serum Chlamydia and Mouse hsp60 antibodies in mice after intravaginal *C. trachomatis* and / or intraperitoneal *C. pneumoniae* inoculation and re-inoculation.

Inoculation groups ^a	Chsp60 IgG ^b	Mhsp60 IgG ^b
Ct-Ct	0/5	0/5
Ct-Cp	2/6	0/6
Cp-Ct	0/6	1/6
10-Cp	5/9	0/9

For the Ct-Ct, Ct-Cp and Ct-Cp groups, first inoculations were all given at 9 weeks of age.

^a Ct-Ct and Ct-Cp: mice re-inoculated after nine weeks and sacrificed eighteen weeks after inoculation; Cp-Ct: mice re-inoculated after 18 weeks and sacrificed 27 weeks after inoculation; 10-Cp: mice sacrificed following 10 monthly *C. pneumoniae* inoculations.

^b Number of mice with Chsp60 and /or Mhsp60 IgG antibodies per total number of mice.

were found nine weeks after *C. trachomatis* inoculation. In none of the *C. trachomatis* inoculated mice serum Mhsp60 antibodies were found.

C. pneumoniae inoculated mice did not show Chsp60- or Mhsp60 IgG antibodies after a single inoculation.

As shown in table II, serum Chsp60 and Mhsp60 IgG antibody formation in mice after re-inoculation was low: only 2 out of 17 mice showed Chsp60 IgG antibodies and in one mouse Mhsp60 IgG antibodies were found. In the sera from mice adopted from the atherosclerosis study, 5 out of 10 animals showed Chsp60 IgG antibodies, but no Mhsp60 IgG seroconversion was found.

Discussion

In this mouse model we evaluated whether single or repeated *C. trachomatis* and / or *C. pneumoniae* infections resulted in the formation of antibodies directed at Chsp60. Additionally we determined whether this was accompanied by the formation of auto-reactive antibodies, cross-reacting with Mhsp60, which may support the hypothesis that auto-immune processes contribute to the pathogenesis of Chlamydia induced tubal pathology. Single inoculation of C57BL/6J mice with either *C. trachomatis* or *C. pneumoniae* resulted in Chsp60 antibody formation in only 3% of the animals, and no cross-reactive Mhsp60 antibodies could be detected. Re-inoculated mice showed Chsp60 and Mhsp60 antibodies in 12 and 6% of cases respectively. Frequent re-inoculation, i.e. 10 monthly intraperitoneal *C. pneumoniae* doses, increased the Chsp60 IgG antibody level to 56%, but also in these mice no Mhsp60 antibodies were detected. Since all mice were *C. trachomatis* and / or *C. pneumoniae* IgG seropositive, it can be excluded that these negative results are due to unsuccessful inoculations of the mice. However, we cannot eliminate the possibility that infections had been successfully cleared, and that it was not possible to induce a persistent or chronic infection with the subsequent production of Chsp60 and /or Mhsp60 antibodies in these mice (Brunham and Rey-Ladino, 2005). This may eventually be related to the inherent predisposition of the C57BL/6J mouse strain to develop a specific immune response, which results in the early eradication of infection (Darville *et al.*, 1997) before chronicity develops and hsp antibodies are formed. Furthermore, it has been demonstrated that this specific mouse strain develops poor immunity against Chlamydia, which may explain the absence of (auto-reactive) antibodies against hsp60 (Huang *et al.*, 2002). This is in contrast to findings by Erkkilä and coworkers (2004), who did show Mhsp60 antibody formation in C57BL/6J mice after repeated intranasal *C. pneumoniae* infection combined with a cholesterol-enriched diet. Therefore, instead of Mhsp60 antibodies not being produced, it may as well be that the antibodies, although present, were not detected by our assay. Until now, no commercial assay is available for Chsp60 and Mhsp60 antibody detection in mice. The assay we used is still experimental. It has been developed for human serum. And although this assay has been evaluated thoroughly in experimental settings, it is possible that Mhsp60 antibodies can not be properly detected with the assay. The fact that no Mhsp60 antibodies could be detected may theoretically be due to the biochemical conformation of the recombinant Msp60 used. It is possible that even small structural differences between the recombinant Mhsp60 and mouse hsp60 are crucial for recognition, subsequently leading to failure of Mhsp60 IgG antibody attachment. Finally, as postulated by Stephens (2003), pathogenesis might primarily be based on cellular responses instead of auto-immune reactivity. In this

hypothesis, Chsp60, produced during (persistent) infection might play a role in the induction of cellular responses rather than auto-immunity (Stephens, 2003).

In conclusion, the results of the pilot studies in our mouse model do not support the contention of auto-immunity, based on cross-reactivity between Mhsp60 and Chsp60 IgG antibodies, since Chsp60- and Mhsp60 IgG antibody levels, even in frequently re-infected mice, were low or absent. Further studies are needed to assess the relationship, if any, between *C. trachomatis* and *C. pneumoniae* induced immune-mediated pathogenic mechanisms and the development of tubal pathology.

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General Discussion

***C. trachomatis* course of infection**

C. trachomatis is a major cause of sexually transmitted urogenital tract infection in men and women. Manifestations and consequences affect woman's health more than man's. Infection in women starts with invasion of the endocervical epithelium with *C. trachomatis* elementary bodies. From this point onwards the infection may run a symptomatic or an asymptomatic course. In the majority of cases infections remain asymptomatic (Peipert, 2003), and are therefore left untreated, which may enable the organism to ascend into the upper genital tract causing pelvic inflammatory disease (PID). PID may either resolve spontaneously, progress to symptomatic PID, or lead to asymptomatic (silent) PID with a risk of developing late sequelae. In this latter case patients suffering from late sequelae may eventually present with chronic pelvic pain, ectopic pregnancy or subfertility (Wiesenfeld *et al.*, 2002). Figure I schematically present the various symptomatic and asymptomatic courses of *C. trachomatis* genital tract infection and possible clinical manifestations.

In this general discussion, topics that have contributed to the current understanding and approach of asymptomatic *C. trachomatis* infections and its late sequelae will be highlighted, using figure I for guidance. More specifically, attention will be paid to screening for tubal factor subfertility by serum Chlamydia Antibody Testing (CAT), as this is the main subject of the present thesis. Finally, the most important findings of the present investigations will be summarised.

Spontaneous clearance

Although data on the natural course of *C. trachomatis* infections are rather poor, it has been shown that cervical infections may resolve spontaneously (Golden *et al.*, 2000; Brunham and Rey-Ladino, 2005). Previous studies showed spontaneous clearance rates between 16–50%, with 7–18% development of symptomatic PID

Figure 1. Symptomatic and asymptomatic courses of *C. trachomatis* genital tract infection and its possible clinical manifestations.

(McCormack *et al.*, 1979; Rahm *et al.*, 1986). However, those studies concerned only small patient groups and used conventional and rather insensitive *C. trachomatis* detection systems. Morré and coworkers (2002) studied thirty women with a *C. trachomatis* positive urine sample in a cohort based nested case-control study ($n = 744$ women). Urine specimens at one, six and twelve months were analysed for the presence of *C. trachomatis*-DNA, and questionnaires, concerning demographic and sexual behaviour variables and questions on self-reported urogenital complaints, were completed. The findings of this study showed that, without using antibiotics, almost half of the asymptomatic *C. trachomatis* infections in women cleared during one year of follow-up, and that none of the untreated *C. trachomatis* positive women developed symptomatic PID. Molano and coworkers (2005) performed a 5-year follow-up analysis of asymptomatic *C. trachomatis*-positive women. They retrospectively used clinical specimens (i.e. cervical smears) for polymerase chain reaction analyses (PCR) and questionnaire data of women originally participating in a study on the natural course of human papillomavirus infection in relation to cervical intraepithelial neoplasia. In accordance with Morré and coworkers, approximately 46% of the infections were persistent at 1 year. In 18% infections persisted at 2 years of follow-up, and in 94% of the women, *C. trachomatis* infections had cleared spontaneously without causing complaints at 4 years of follow-up.

Microorganism related factors associated with infection outcome

Whether *C. trachomatis* leads to symptomatic or asymptomatic infection, and whether infection leads to sequelae might be due to various factors. Among those, serovar specific properties have been suggested, based on variation in the major outer membrane protein. First, in the earlier described study of Molano on spontaneous clearance, differences in rate of clearance were found between serovars (Molano *et al.*, 2005). Serovars were grouped on the basis of amino acid similarity: group B (serovars B, D and E), group C (serovars H, I, J and K) and an intermediate group (serovars F and G). Of the serovars identified in single-serovar infections, 35% belonged to group B, 29% to group C and 36% to the intermediate group. The most common serovars identified were D, E, F and G. The rate of clearance was lower for serovars of groups B and C than for serovars of the intermediate group. The authors suggested this higher rate of persistence of groups B and C serovars to be due to an ability to escape the host immune response (Molano *et al.*, 2005).

The association between serovars and clinical manifestation of cervical infection has been studied by several groups (Persson *et al.*, 1993; Workowski *et al.*,

1994; Dean *et al.*, 1995; van de Laar *et al.*, 1996). None of them found an association between specific clinical manifestations and certain serovars or genotypes of serovars, suggesting no serovar specific differences in virulence. Van Duynhoven and coworkers (1998) however, were the first to suggest an association between *C. trachomatis* genotypes and patients' self reported symptoms. In women, genotypes of the F and G groups seemed to cause more complaints of lower abdominal pain. Signs upon examination (such as easily induced bleeding of the cervix, cervical ectopy and mucopurulent discharge) were not associated with any *C. trachomatis* genotype. However, the study concerned a small number of symptomatic women, and before these *C. trachomatis* genotypes can serve as prognostic indicators for complicated infections of the upper genital tract, the association needs to be confirmed in larger studies. To some extent, the association is supported by a study in 33 women with cervical or endometrial infections. Variants of genotype F were associated with PID and presence of plasma cells and neutrophils in the endometrium (Dean *et al.*, 1995).

To determine whether certain *C. trachomatis* serovars are preferentially associated with a symptomatic or an asymptomatic course of infection, *C. trachomatis* serovar distributions were analysed in symptomatic and asymptomatic males and females (Morré *et al.*, 2000). The most prevalent serovars (D, E and F), comprising 73% of the *C. trachomatis* serovars, showed no association with either a symptomatic or asymptomatic course of infection. However, differences were found for the less frequent *C. trachomatis* serovars: serovar Ga was associated with dysuria in men, serovar Ia was detected only in asymptotically infected persons. Furthermore, serovar K was associated with the specific clinical symptom of abnormal vaginal discharge in women (Morré *et al.*, 2000).

Taken together, some associations were detected, although the observed inconsistencies in reported results warrant further investigation to establish whether a causal relationship exists between serovar and virulence.

Host related factors associated with infection outcome

Host variation might also play an important role in the susceptibility for *C. trachomatis* infection and in the outcome of an infection. The use of human leucocyte antigen (HLA) typing revealed that PID was related to specific HLA types (Cohen *et al.*, 2000 and 2003; Ness *et al.*, 2004). Differences in HLA types may be responsible for the fact that not all women with previous *C. trachomatis* infections develop late sequelae such as tubal pathology. For example, the balance between the T helper 1 (Th)1 and Th2 immune response, characterized by interferon (IFN)- γ or interleukin (IL)-10 secretion respectively, has been suggested to be

controlled by epitopes selected by the HLA class II molecules. An association of the cellular immune response to *Chlamydia* hsp 60 (Chsp60), HLA class II alleles and IL-10 promoter genotypes was revealed in patients with *C. trachomatis* tubal factor subfertility (Kinnunen *et al.*, 2002). Cohen and coworkers (2003) studied the presence of certain HLA class II alleles and polymorphisms in selected cytokine genes and compared the results among tubal factor subfertility patients with and without *C. trachomatis* antibodies. They found that two class II alleles were detected less commonly in sero-positive than in sero-negative women, and suggested that these alleles may induce an immunologically mediated mechanism of protection against *C. trachomatis* infection, or alternatively increase the risk for tubal scarring due to another cause.

Morré and Lyons and coworkers (2003 and 2004a) investigated polymorphisms in a gene encoding for Toll like receptor 4 function, a receptor supposed to play an important role in *C. trachomatis* infection outcome. Their study did not confirm any over- or under-expression of polymorphisms of the gene in women with or without tubal pathology. Furthermore they investigated the role of IL-1B and IL-1 receptor antagonist gene polymorphisms in tubal pathology, and the presence of these gene polymorphisms in *C. trachomatis* IgG positive women with and without tubal pathology. Although the IL-1 gene cluster has been linked to inflammatory and infectious diseases, their data suggested that specific IL-1 gene polymorphisms are not associated with tubal pathology or with the development of *C. trachomatis*-based post-infectious sequelae (Murillo *et al.*, 2003).

The host genetic background may still be an additional variable influencing the susceptibility to and the course of *C. trachomatis* infection, but so far there is little evidence to support this.

Screening for asymptomatic *C. trachomatis* cervicitis

Since the majority of *C. trachomatis* cervicitis remains asymptomatic, these infections are left unrecognised and hence untreated, unless screening is being performed. Community based screening programs for asymptomatic *C. trachomatis* infections have been the subject of interest of several large-scale studies. The aforementioned study by Morré and coworkers (2002) on spontaneous clearance of microorganisms, suggested that the eventual clinical complication rate of asymptomatic *C. trachomatis* infections might be much lower than the previously published 2-20% (Paavonen *et al.*, 1998; Weström *et al.*, 1992). As a consequence, their study questions the usefulness of screening programmes, since the *C. trachomatis* prevalence above which screening may be cost effective, might be much higher than the 3.9% calculated by Paavonen and coworkers (1998).

Recently, three studies on cost-effectiveness of screening women for asymptomatic *C. trachomatis* infections were reviewed (van Valkengoed *et al.*, 2004). In all three studies, the decision models are based on assumptions about the risk of complications (i.e. expected complication rates). The expected rate for developing PID varied from 15 to 80% (symptomatic and asymptomatic PID). For tubal infertility, ectopic pregnancy, and chronic pelvic pain after an asymptomatic Chlamydia infection the expected rates were 10-20%, 5-25% and 18-30% respectively. In addition, the probability of complications of *C. trachomatis* was calculated based on available registration data and data from several studies (i.e. observed complication rates). The observed complication rates were 0.43% clinical PID, 0.02% tubal infertility and 0.07% ectopic pregnancy. For all complications of *C. trachomatis*, the expected complication rates are higher than the observed complication rates. Therefore, our current estimate of the complication rates after asymptomatic *C. trachomatis* infection is likely to be too high. The effect of overestimation is potentially the largest in populations with a low prevalence, and the currently assumed cost savings associated with screening may disappear when using more realistic estimates for complications.

In accordance with the Dutch Institute for Health Care Improvement revised guideline (Bleker *et al.*, 2003), community based screening for asymptomatic *C. trachomatis* infections has been designated for Amsterdam, a high-risk area, since it was calculated to be cost effective there. For other parts of the country, prevalence studies should be performed first. Until these studies will be available, according to the same guideline, risk groups eligible for screening on *C. trachomatis* infection in the Netherlands include: partners of *C. trachomatis*-positive persons, visitors of clinics for sexually transmitted diseases, women who will undergo an induced abortion, mothers of newborns with conjunctivitis or pneumonitis, young persons of Surinam or Antillean descent, young women with new sexual relationships and individuals whose history indicates risky sexual behaviour.

Persistence of *C. trachomatis* infection and clinical implications for subfertility patients

The ability of *C. trachomatis* to persist in the genital tract is becoming increasingly evident (Beatty *et al.*, 1994b and 1995). This persistence is characterised by incomplete Chlamydia replication and the formation of atypical reticulate bodies (RBs), which are unable to differentiate into infectious progeny (Beatty *et al.*, 1994a; Koehler *et al.*, 1997). The duration of persistence in vivo is not known, but recent evidence suggests that organisms may persist for as long as 5 years in the genital tract (Dean *et al.*, 2000).

Figure II. Revised Chlamydia developmental cycle of infection and replication. EB is elementary body and RB is reticulate body. Persistent Chlamydia infection is characterised by incomplete Chlamydia replication and the formation of atypical RBs.

The ability of *C. trachomatis* to become “reactivated” from a persistent state has been demonstrated in both mice (Cotter *et al.*, 1997), humans (Forsey *et al.*, 1990) and in vitro (Beatty *et al.*, 1993). The aberrant forms again recover the shape of normal RBs and replication may resume. The mechanism leading to persistence and the capacity to enter into a reversible latent form have not been fully defined in vivo. Figure II schematically depicts a revised *C. trachomatis* life cycle in which persistence of *C. trachomatis* is incorporated.

The ability of *C. trachomatis* to persist may have clinical relevance for subfertility patients. Since most of these patients will undergo uterine instrumentation as part of their fertility workup, they are, in case of an active or persistent *C. trachomatis* endocervical infection, at risk for induction of an ascending infection. Different intervention strategies have been proposed for the prevention of ascending infections by uterine instrumentation. In chapter 5 of the present thesis we discuss screening for endocervical *C. trachomatis* infections in subfertility patients before uterine instrumentation. We argue that endocervical PCR screening is inappropriate since not all persisting Chlamydia infections can be identified in cervical samples. Because of these “false negative” PCR results, selective treatment of only positive cases will not suffice. Furthermore, screening by CAT is unsuitable as well, since it will only determine previous exposure to the organism, without providing information as to whether viable Chlamydia will be actually present (Thomas *et al.*, 2000). Furthermore, negative CAT does not exclude *C. trachomatis* cervical infection, since cervical infections only rarely cause

a rise in Chlamydia serum antibody titre (Taylor-Robinson, 1997). Thus, up to now, no adequate non-invasive screening test is available for identifying all patients with Chlamydia in any part of their genital tracts. Since iatrogenic spread of Chlamydia in subfertile women has severe consequences for the preservation of their fertility, clinical guidelines concerning precautions in subfertile women undergoing uterine instrumentation are essential. Based on the theoretical arguments of inadequate screening possibilities, we proposed that prophylactic antibiotics (i.e. azithromycin in a single oral dose, 12 hours before the planned procedure) should replace endocervical screening for *C. trachomatis* and treatment of positive cases only (chapter 5). This policy has been criticized by others (Witkin *et al.*, 2002; Ng *et al.*, 2002), postulating that the universal prophylaxis approach might contribute to the development of antibiotic resistance (Somani *et al.*, 2000). Reports from the literature indicate that antibiotics may not eliminate Chlamydia from all host cells in vitro (Drees-Werringloer *et al.*, 2001), in macaques (Patton *et al.*, 1997) and in humans (Bragina *et al.*, 2001). In patients with treated Chlamydia infections, atypical small intracellular inclusions have been found, suggesting that antibiotics may induce persistence by modulating the microorganism, rendering it less susceptible to antibiotics. These observations deserve further exploration, since they question the principles and efficacy of treatment of acute as well as chronic Chlamydia infections.

Screening for tubal factor subfertility

In case spontaneous clearance of infection has not occurred and *C. trachomatis* infection has been able to ascend from the cervix into the upper genital tract, asymptomatic (silent) PID may occur, with the risk of inducing tubal pathology. The best available standard for diagnosing tubal pathology as a late sequel of *C. trachomatis* infection is direct visualization of the pelvic organs at laparoscopy and dye testing, a technique that is invasive, costly, and that worldwide has limited availability. Because it was noted that the development of tubal pathology was associated with *C. trachomatis* IgG antibody formation, CAT was developed as an inexpensive and non-invasive technique for distinguishing patients at high risk for tubal factor subfertility.

The micro-immunofluorescence (MIF) test, widely used for CAT, has several limitations. Since in the MIF test the number of antigens has been reduced, or only one broadly reacting antigen is used, there is lack of species-specificity, and unintended cross-reactivity with *C. pneumoniae* antibodies in the MIF may occur, resulting in false positive CAT results. Moreover, the test is time consuming and inter-observer and inter-laboratory variabilities exist. In order to overcome the

clinical limitations of MIF, enzyme-linked immuno sorbent assays (ELISA) have been developed as a standardized and less labour-intensive alternative.

In chapter 4 a comparison is presented of five commercially available tests (MIF Biomerieux, MIF Labsystems, ELISA Labsystems, pELISA Medac, ELISA Savyon) in respect of their accuracy to predict tubal pathology. The results showed that there still is no single outstanding screening test for tubal pathology in subfertile women. Although ELISAs have been claimed to be highly sensitive and specific, they are not superior to MIF in predicting tubal factor subfertility. In all but one test (pELISA Medac), a cross-reaction was found with *C. pneumoniae* antibodies. Significant differences were found between the distributions of *C. pneumoniae* antibodies in the CAT positive and CAT negative patients when CAT was performed by MIF Biomerieux, MIF Labsystems, ELISA Labsystems or ELISA Savyon. These results suggest cross-reactivity with *C. pneumoniae* as a cause for false positive CAT results and confirm our previous findings (chapter 3), suggesting an additive role of *C. pneumoniae* in the development of tubal pathology. However, after enlargement of the cohort and using a logistic regression model, additive determination of IgG antibodies to *C. pneumoniae* did not improve the predictive value of *C. trachomatis* IgG antibody testing in screening for tubal factor subfertility. The odds ratio (OR) of screening by *C. trachomatis* IgG only was 13.9 and an OR of 15.4 was found for the combination of *C. trachomatis* IgG and *C. pneumoniae* IgG (den Hartog *et al.*, 2004).

Besides false positive CAT results, also false negative CAT were found, in which ELISAs tended to have more false negative results than MIF (chapter 4). The more general hypothesis that false negative CAT results may be due to disappearance of IgG antibodies between infection and fertility investigation 10–15 years later could be refuted (chapter 2). In patients with an initial CAT > 32 by MIF, a specific decline in IgG antibody titres (defined as a decline of at least two titre steps) was found in only 18%, and in no patient did IgG titres become undetectable in 5 years. With ELISA, in chapter 2 used as a validation of MIF, signal / cut-off values did not decrease.

Also, the specific synthetic peptides used in the species-specific ELISA could be responsible for false negative test results. These peptides are derived from the variable domain region of the major outer membrane of *C. trachomatis*. These highly specific epitopes may not be able to be recognized by all serotypes involved in *C. trachomatis* infection, and therefore cause false negative results (Bas *et al.*, 2001).

Finally, besides assay-related factors as a cause for false positive and false negative CAT results, the aforementioned serotype-related factors and genetic factors in the host may play a role as well. Host genetic factors may be responsible for the fact that not all patients with *C. trachomatis* antibodies develop tubal pathology (causing false positive CAT) (Kinnunen *et al.*, 2002). Variants of serotypes (Morré

et al., 1998) as well as mutations in the position within MOMP (Dean *et al.*, 2000) have been described, through which a test may not be able to detect the specific antibodies (causing false negative CAT).

In conclusion, there is still no outstanding screening test for tubal pathology in subfertile women, for which several reasons are held responsible. For the improvement of screening for *C. trachomatis* induced tubal pathology, IgG testing as an indicator of previous *C. trachomatis* infection should be combined with, or even replaced by, markers more specifically related to the immunopathology.

Immunopathogenesis of *C. trachomatis* induced tubal pathology

In the present thesis we studied some of the clinical limitations of CAT. In order to develop more accurate tests for the prediction of tubal pathology, future research should focus on the immunopathology of silent *C. trachomatis* infections.

The flow chart in figure III attempts to provide a simple model of *C. trachomatis* invasion, the interplay between cellular and humoral immunity, infection and pathology. Progression along a particular pathway is determined by the immune response, which in turn is determined by a range of both host and pathogen factors as described earlier in this chapter. The exact mechanism by which changing cytokine patterns influence a particular immune pathway, i.e. shift away from balance in favour of either Th1 or Th2, is yet to be resolved (Debattista *et al.*, 2003). Because of the asymptomatic course of infection these interactions are difficult to study in humans. Therefore, a mouse model has been developed (chapter 6).

Since it has become increasingly evident that for development of tubal pathology chronic infection, by repeated or persisting *C. trachomatis* infection, is necessary, we focussed on single and repeated *C. trachomatis* vaginal inoculation of female C57BL/6J mice. Despite our hypothesis that extensive tubal pathology will be found after re-inoculation, only mild genital tract pathology and histological changes were encountered. Nevertheless, some findings are worth mentioning. Single intravaginal inoculation induced an infection without extensive pathology, but with an inflammatory cell response in the endometrium. Stromal inflammatory cells remained present, suggesting a persisting chronic inflammatory process. Re-inoculation stimulated the process, finally resulting in the induction of atypia of the endometrium. Based on these data, we suggested that re-inoculation might (re)activate the inflammatory response. Eventually, in the mouse, the endometrium may be unable to recover, resulting in pathology of the upper genital tract.

Figure III. Postulated immunopathogenesis of *C. trachomatis* infection (adapted from DeBattista et al. 2003)

An auto-immune response based on hsp60 antibody cross-reactivity has been hypothesised to underlie the evolvement from *C. trachomatis* infection to tubal pathology (Domeika *et al.*, 1998). In chapter 7 we evaluated whether single or repetitive *C. trachomatis* and / or *C. pneumoniae* infections resulted in the formation of Chsp60 antibodies, and additionally whether this was accompanied by the formation of auto-reactive antibodies (mouse hsp60 (Mhsp60) antibodies). In our study we found that Chsp60 and Mhsp60 antibody levels were low, even in frequently infected mice. The results therefore did not support the hypothesis of auto-immunity, based on cross reactivity of Chsp60 and Mhsp60 IgG antibodies, being the underlying mechanism of tubal pathology induction, at least in this mouse model. In summary, in the present model no extensive tubal pathology was found, and neither could auto-immunity due to hsp60 IgG antibody cross-reactivity be demonstrated.

In reference to figure III, host related immune factors might play a role in *C. trachomatis* infection outcome, i.e. the inherent predisposition of a mouse strain to develop a certain immune response. Since C57BL/6J mice are known to predominantly conduct a Th1 response (Darville *et al.*, 1997), resolution of infection may

have occurred before chronicity and the formation of hsp antibodies and auto-immunity could develop. Lyons and coworkers (2004b) compared two immunologically different strains of mice, Th1 inherent C57BL/6J mice and BALB/c mice with inherent Th2 immune response polarity. The role of inherent Th1/Th2 polarity bias on the course of infection and on the nature of the immune response to female genital tract infection was studied. Their results showed significant differences among the mouse strains. Once established, the duration of infection was longer in C57BL/6J mice. However, their level of bacterial shedding during infection was less, and assessments of quantitative and qualitative cellular and humoral responses to infection showed stronger Th1 type immune response in C57BL/6J compared to BALB/c mice. However, regardless of inherent Th1/Th2 polarity, a significant level of acquired immunity to re-infection was induced in both mouse strains. Combining these results with our findings suggests that, in order to study tubal pathology due to persistent infection, the use of a predominantly Th2 responding mouse strain may have to be preferred. Furthermore, as reviewed by Stephens (2003), as an alternative to the immunological paradigm for Chlamydia pathogenesis, it might rather be that pathogenesis is primarily based on the cellular responses elicited by mucosal epithelial cells, their secretion of chemokines and growth factors, and secondarily their interaction with the adaptive immune system.

In conclusion, attention should be paid to improvement of the mouse model focussing on the influence of the various factors. As is the case with human *C. trachomatis* genital infection, both host and microorganism related factors play a role (i.e. differences in mouse strains and serovars), and complicate the development of a useful model. However, a suitable model would ultimately provide opportunities to study the individual factors involved in the inflammatory response to infection and re-infection and the role of different serovars and host immunogenetic factors in the etiology of tubal factor subfertility.

Recent insights in serological screening for tubal factor subfertility

Elucidating the etiology of *C. trachomatis* induced tubal pathology may provide insights that could lead to the development of new markers that could subsequently be introduced in screening for tubal factor subfertility. Persistent exposure to *C. trachomatis* may result in a chronic inflammatory response and increase the risk of tubal damage. Although IgG antibodies are markers of a previous infection, they may not reflect a persistent chronic inflammation properly. Recent studies have focussed on anti-lipopolysaccharide (LPS) antibodies, Chsp60 antibodies, *C.*

trachomatis IgA antibodies, and C-reactive protein (CRP), assuming these factors to more specifically reflect persistent infection.

There is a high interspecies homology in the outer membrane protein LPS between *Chlamydia* species. Although it is known that genus-specific anti-LPS-antibodies increase rapidly in the early phase of infections, in case of infection with *C. trachomatis* and re-infection with a particular *Chlamydia* species or endogenous "reactivation", a chronic inflammatory response may be induced leading to the continuous production of anti-LPS-antibodies. In this perspective, anti-LPS-antibodies were hypothesised to serve as a marker for chronic inflammation. Anti-LPS antibodies were found significantly more often in women with distal tubal pathology as compared to women without distal tubal pathology. However, the presence of anti-LPS antibodies was no independent predictor for tubal disease (den Hartog *et al.*, 2004).

Chsp60 has been postulated to play an important role in chronic inflammatory processes, immune responses and in the development of tubal pathology (Domeika *et al.*, 1998; Witkin *et al.*, 1998), and antibody responses to Chsp60 may signal chronically persistent or repeated infection. In small series, Chsp60 antibodies, considered as markers for chronic infection, have already been associated with the development of tubal pathology (Toye *et al.*, 1993; Arno *et al.*, 1995; Claman *et al.*, 1997). Although the studies were performed in small patient groups, a Chsp60 antibody test showed sensitivities of 44–76% and specificities of 81–92% for detecting tubal factor subfertility. When the tests were used in combination with *C. trachomatis* IgG antibodies by MIF, sensitivity and specificity of Chsp60 antibody testing showed even more promising results (70–92% and 70–93% respectively) (Freidank *et al.*, 1995; Claman *et al.*, 1997). When both the MIF for *C. trachomatis* IgG and Chsp60 test were used, Freidank and coworkers (1995) reported a positive likelihood ratio of 10 and a negative likelihood ratio of 0.32, suggesting the combination of *C. trachomatis* IgG and Chsp60 to be a promising marker for predicting *C. trachomatis* induced tubal factor subfertility. In a cohort of 313 women, den Hartog and coworkers (2005) studied whether Chsp60 antibodies, *C. trachomatis* IgA antibodies or elevated CRP, alone or in combinations, could identify tubal pathology in subfertile women better than currently used *C. trachomatis* IgG antibodies. *C. trachomatis* IgG antibody testing remained the best single predictor for tubal pathology. For Chsp60 antibodies, an OR of 5.9 was found, which is significantly lower than the OR of 13.9 for *C. trachomatis* IgG antibodies. This lower OR may be explained by cross-reactivity with *C. pneumoniae*, being highly prevalent in subfertile women. The same held true for *C. trachomatis* IgA antibodies. Although associated with chronic inflammation and infection in studies considering *C. pneumoniae* and cardiovascular diseases (Falck *et al.*, 2002), IgA antibodies did not prove to be adequate predictive markers for

tubal factor subfertility (OR 6.1). A forward stepwise logistic regression analysis revealed that only adding the CRP-test significantly improved the diagnostic performance of the *C. trachomatis* IgG test (OR 39.7) (den Hartog *et al.*, 2005).

In considering asymptomatic *C. trachomatis* infection as a chronic disease, similarities may be drawn with the pathogenesis of other chronic diseases in which inflammatory processes play a role. Over the past few years, the association of chronic *C. pneumoniae* infection with vascular diseases, i.e. atherosclerosis and its complications, has become apparent (Leinonen *et al.*, 2002; Libby *et al.*, 2002a en b). An observation that has generated interest is that slightly elevated CRP levels represent a strong predictor of myocardial infarction, stroke and vascular death (Ridker *et al.*, 2001a en b; 2002). Further studies suggest that CRP may not only be an inflammatory marker of atherosclerosis but also a mediator of the disease, because it contributes to lesion formation, plaque rupture, and coronary thrombosis by interacting with and altering the endothelial cell phenotype (Wang *et al.*, 2003; Bisoendial *et al.*, 2005). Extrapolating these findings to the development of tubal pathology, it was found that measuring *C. trachomatis* IgG antibodies (markers of a previous infection) in combination with CRP (a marker of the course of the infection) seems to identify a subset of subfertile women with the highest risk of persistent infections and distal tubal pathology (den Hartog *et al.*, 2005).

In summary, the most important findings of the present study are:

- A decline in IgG antibody titre over time is not a significant cause of false negative Chlamydia antibody test (CAT) results (chapter 2).
- The prevalence of *C. pneumoniae* antibodies in subfertility patients is 75% (chapter 3).
- The prevalence of *C. pneumoniae* antibodies did not differ between patients with and without tubal pathology (chapter 3).
- *C. pneumoniae* antibodies, due to cross-reactivity, may be the cause of false positive CAT results (chapter 3).
- Tubal pathology is more common in patients who have both antibodies to *C. trachomatis* and *C. pneumoniae* (chapter 3).
- In screening for tubal factor subfertility, significant differences in predictive value exist between different commercially available MIF and ELISA tests (chapter 4).
- Combining two CAT tests does not improve the predictive value of CAT (chapter 4).
- In subfertile women undergoing uterine instrumentation, prophylactic antibiotics should replace endocervical screening for *C. trachomatis* and treatment of positive cases only (chapter 5).

- Single intravaginal inoculation of C57BL/6J mice with the human *C. trachomatis* L₂ strain induces an infection without extensive pathology, but with a chronic inflammatory cell response in the endometrium (chapter 6).
- Re-inoculation of C57BL/6J mice stimulates the inflammatory process, finally resulting in the induction of atypia of the endometrium (chapter 6).
- No extensive genital tract pathology and histologic changes are found in the C57BL/6J mouse, possibly due to various factors in the model (chapter 6).
- The results in our mouse model do not support the contention of auto-immunity, based on cross-reactivity between Mhsp60 and Chsp60 IgG antibodies, since Chsp60- and Mhsp60 IgG antibody levels, even in frequently re-infected mice, were low or absent (chapter 7).

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S

Summary

The present thesis focussed on the role of *Chlamydia (C.) trachomatis* antibody testing (CAT) in screening for tubal factor subfertility. CAT was developed as an inexpensive and non-invasive technique for distinguishing high-risk patients from those with a low risk for tubal factor subfertility due to silent *C. trachomatis* infection. Ideally, based on the results of CAT, high-risk patients would be subjected to diagnostic testing (i.e. laparoscopy) while additional testing of tubal patency could be postponed in low-risk patients. The clinical significance of CAT, however, has its limitations due to false negative and false positive results.

The aim of the current study was to elucidate factors that have been hypothesised as a cause of limiting the predictive value of CAT. Moreover, also factors that have been suggested to improve the predictive value of CAT were studied. To obtain more insight in *C. trachomatis* induced tubal pathology a mouse model was developed.

In **chapter 1**, the literature on the microorganism *C. trachomatis* and the way it induces infection has been reviewed. Furthermore, the pathogenesis, the immune response, and its role in the development of tubal pathology were described. Subsequently, the epidemiology, the clinical manifestations of female *C. trachomatis* genital tract infection and the need for screening programmes were discussed. An overview of the available diagnostic tests was given and screening for tubal factor subfertility was reviewed.

In **chapter 2**, it was postulated that IgG antibodies might decline over time after *C. trachomatis* infection. In view of the time span between primary *C. trachomatis* infection in adolescence and fertility work-up in adulthood, decline of *C. trachomatis* IgG antibodies over time as a potential cause of false negative CAT results was studied. A number of 39 women with an initial titre of ≥ 64 were restudied serologically after 4-7 years. The initial and a new serum sample were tested for *C. trachomatis* IgG antibodies using a micro-immunofluorescence assay (MIF). A species-specific enzyme-linked immunosorbent assay (ELISA) was used to validate the MIF test results. All patients filled out a questionnaire to determine risk factors for renewed *C. trachomatis* infection between the initial and second serum sample. Although seven of the 39 patients (18.0%) showed a significant decline in IgG antibodies by MIF over a period of 4-7 years, IgG antibodies never

became undetectable. In the 7/39 patients who showed a decline by MIF, signal/cut-off values by ELISA did not decrease. From the results of the questionnaire, it was concluded that *C. trachomatis* re-infection appeared to be no major cause of persistence of Chlamydia IgG antibody titres in the subfertile women studied. We concluded that a decline of IgG antibodies did occur, but could not be considered as a significant cause of false negative results.

In **chapter 3**, the role of *C. pneumoniae* antibodies, as a potential cause of false positive CAT results due to cross-reactivity with *C. trachomatis* antibodies in the MIF test, has been evaluated.

In 240 subfertile women serologic data were compared to laparoscopy findings. The prevalence of *C. pneumoniae* antibodies using ELISA was 75% and did not differ between patients with and without tubal pathology.

C. pneumoniae antibodies were found in 87% of women with a positive MIF test (≥ 32), and in 66% with a negative MIF test ($p < 0.0005$). Similar percentages were found when using ELISA instead of MIF for the detection of *C. trachomatis* antibodies, i.e. 87% of *C. trachomatis* positive women versus 69% of *C. trachomatis* negative women had *C. pneumoniae* antibodies ($p < 0.0005$).

Patients without tubal factor subfertility but a positive MIF test (i.e. false positive CAT result) showed *C. pneumoniae* antibodies more frequently than patients without tubal factor subfertility and a negative MIF test (true negative CAT result), indicating that *C. pneumoniae* is a major contributor of false positive CAT results by MIF. Remarkably, tubal pathology was more common in patients who had antibodies to both *C. trachomatis* and *C. pneumoniae*, supporting the contention that *C. pneumoniae* may be involved in Chlamydia upper genital tract infections and/or subsequent tubal damage.

Chapter 4 describes a comparison of five commercially available CAT tests (MIF Biomerieux, MIF Labsystems, ELISA Labsystems, pELISA Medac, ELISA Savyon) in respect of their accuracy in predicting tubal pathology was performed. In a prospectively collected cohort of 315 subfertile women results of the five CAT tests were correlated to findings at laparoscopy. Furthermore it was evaluated whether combinations of tests could improve the predictive value of CAT. Of all tests evaluated, MIF Labsystems had the single best diagnostic performance (OR 15.7), and among the three ELISA tests, pELISA Medac performed best (OR 8.2). Stepwise logistic regression analysis showed that the performance of MIF Labsystems could not be improved by adding a second test. Furthermore, except for pELISA Medac, significant cross-reactivity with *C. pneumoniae* antibodies was found in all tests.

Chapter 5 deals with screening of endocervical *C. trachomatis* infections in subfertility patients. Most of these patients will undergo uterine instrumentation as part of their fertility work-up. They are, in case of an active or dormant *C.*

trachomatis endocervical infection or colonization, considered at risk for ascending infection. Since endocervical PCR screening may not identify all dormant *C. trachomatis*, and CAT will only determine previous exposure to the organism, without providing information as to whether Chlamydia is actually present, no adequate non-invasive screening test for genital tract infection is available. Based on these theoretical arguments, we argue that prophylactic antibiotics (i.e. azithromycin in a single oral dose, 12 hours before the planned procedure) should replace endocervical screening for *C. trachomatis* and selective treatment of positive cases only.

In **chapter 6**, the development of a mouse model was described, to study the largely unknown pathogenesis of tubal factor subfertility after silent *C. trachomatis* infections. It was hypothesised that primary *C. trachomatis* infection would not result in extensive genital tract pathology, and that only subsequent *C. trachomatis* re-infection would induce tubal disease. Therefore, female C57BL/6J mice were inoculated intravaginally with human *C. trachomatis* serovar L₂. Mice were sacrificed at regular intervals, three, nine and eighteen weeks after inoculation, and the development of histopathologic changes and tissue damage in the genital tract was studied. One group of mice was submitted to re-inoculation to investigate the effect of re-infection on the development of genital tract pathology. Furthermore, to explore whether *C. pneumoniae* would contribute to the development of *C. trachomatis* induced tubal pathology, a pilot study with *C. pneumoniae* was performed. The results indicated that single intravaginal inoculation of C57BL/6J mice with the human *C. trachomatis* L₂ strain induces an infection without extensive pathology, but with a chronic inflammatory cell response. Re-inoculation stimulated the process, finally resulting in the induction of atypia of the endometrium. Changes at the tissue and cellular level were all more evident after re-inoculation. Despite our assumption that extensive pathology would be found after re-inoculation, only mild genital tract pathology and histologic changes were found. Various factors in the model that might be held responsible for the findings, e.g. the use of C57BL/6J mice and inoculation with a human *C. trachomatis* strain, were discussed.

In **chapter 7**, it was postulated that auto-immunity, based on cross-reaction of Chlamydia hsp60 (Chsp60) antibodies with human hsp60 expressed on tubal epithelial cells during persistence of infection, might be the underlying mechanism of tubal pathology. We evaluated in a mouse model whether Chsp60 and mouse hsp60 antibodies could be detected in serum following *C. trachomatis* and *C. pneumoniae* infection. The results of our pilot studies did not support the hypothesis of auto-immunity, since Chsp60 and mouse hsp60 antibody levels, even in frequently inoculated mice, were low. We recognize however the limitations of our model.

In chapter 8 topics that have contributed to our current understanding and approach of asymptomatic *C. trachomatis* infections and its late sequelae were highlighted. In this context, the results of the present studies were discussed more specifically and the most important findings were summarised.

S

Samenvatting

De best beschikbare standaard voor het diagnostiseren van tubapathologie ten gevolge van *Chlamydia* (*C.*) *trachomatis* infectie is de laparoscopie met tubatesten. Daar dit een invasief en duur onderzoek is, is het niet geschikt voor het screenen van groepen patiënten. Als screeningstest voor tubapathologie wordt binnen het oriënterend fertiliteitsonderzoek de Chlamydia IgG antilichaam titer (CAT) bepaling gebruikt, met als doel patiënten met een hoog risico op tubapathologie ten gevolge van *C. trachomatis* infectie te onderscheiden van die met een laag risico. Idealiter zouden patiënten at risk voor tubapathologie vroeg in het fertiliteitsonderzoek invasieve diagnostiek kunnen ondergaan (laparoscopie met tubatesten), terwijl dit in patiënten met een laag risico kan worden uitgesteld en het fertiliteitsonderzoek zich meer kan richten op andere oorzaken en behandeling van de subfertiliteit. Helaas zijn de klinische resultaten met betrekking tot het voorspellen van tubapathologie teleurstellend door het voorkomen van zowel fout-positieve als fout-negatieve CAT uitslagen.

Het huidige onderzoek richtte zich op factoren waarvan gesuggereerd wordt dat ze mogelijk verantwoordelijk zouden kunnen zijn voor de beperkte voorspellende waarde van CAT. Vervolgens werden mogelijkheden voor verbetering van de voorspellende waarde van CAT onderzocht. In het laatste deel van het proefschrift wordt aandacht besteed aan de ontwikkeling van een muismodel, daar een beter begrip van de pathogenese noodzakelijk is voor het ontwikkelen van een accurate screeningstest voor tubapathologie ten gevolge van asymptomatische *C. trachomatis* infecties.

Hoofdstuk 1 betreft een literatuuroverzicht over het micro-organisme *C. trachomatis* en het infectieverloop. Aan bod komen de pathogenese, de immuunrespons en de rol hiervan in de ontwikkeling van tubapathologie. Verder wordt aandacht besteed aan de epidemiologie, het klinische beeld, en de noodzaak van screeningsprogramma's. Er wordt een overzicht gegeven van de beschikbare diagnostische tests en screeningstests voor tubapathologie. De doelstellingen van het proefschrift worden uiteengezet.

Een mogelijke verklaring voor het voorkomen van fout-negatieve CAT is het dalen van titers in de tijdsperiode tussen de oorspronkelijke infectie en het fertili-

teitsonderzoek. In **hoofdstuk 2** bestudeerden we 39 patiënten met een CAT ≥ 64 ten tijde van hun fertiliteitsonderzoek. Bij hen werd 4-7 jaar later opnieuw serum afgenomen, en werd een vragenlijst afgenomen om het risico van een her-infectie in de tussenliggende periode in te schatten. CAT met behulp van een micro-immunofluorescence test (MIF) werd uitgevoerd op het initiële en het nieuwe serummonster. Ter validatie van de MIF test werd tevens een species-specifieke ELISA verricht. Zeven van de 39 (18%) patiënten toonden een significante afname van IgG antilichamen (minimaal twee titerstappen). Seroconversie van positief naar negatief werd slechts bij één patiënt aangetoond. De ELISA resultaten voor deze groep toonden geen daling van IgG antistoffen. Uit de resultaten van de vragenlijst kon worden geconcludeerd dat het vóórkomen van risicofactoren voor her-infectie gelijk was voor patiënten met en patiënten zonder titerdaling. Afname van de IgG antilichaam titer lijkt derhalve geen belangrijke oorzaak voor fout-negatieve CAT uitslagen te zijn.

In **hoofdstuk 3** onderzochten we de rol van *C. pneumoniae* als potentiële oorzaak voor het voorkomen van fout-positieve CAT uitslagen. Fout-positieve CAT kunnen het gevolg zijn van kruisreactie in de MIF test tussen *C. trachomatis* en andere species, bijv. *C. pneumoniae*. In een groep van 240 patiënten werden serologische data vergeleken met de laparoscopische bevindingen. De prevalentie van *C. pneumoniae* IgG antistoffen bedroeg 75%, en was vergelijkbaar voor patiënten met en zonder tubapathologie.

Van de patiënten met een positieve MIF test (≥ 32) was 87% *C. pneumoniae* IgG positief, versus 66% van de patiënten met een negatieve MIF test ($p < 0.0005$). Wanneer de ELISA werd gebruikt voor het aantonen van *C. trachomatis* IgG waren de cijfers respectievelijk 87% van de *C. trachomatis* positieve patiënten versus 69% van de *C. trachomatis* negatieve patiënten ($p < 0.0005$). In de groep patiënten zonder tubapathologie maar een positieve MIF test ("fout-positieve" CAT) werden relatief vaker *C. pneumoniae* IgG antistoffen aangetroffen dan in de groep patiënten zonder tubapathologie en een negatieve MIF test ("terecht negatieve" CAT). Kruisreactie in de MIF tussen *C. trachomatis* en *C. pneumoniae* speelt dus een rol bij het vóórkomen van fout-positieve CAT uitslagen. Opvallend was dat tubapathologie vaker voorkwam bij patiënten met zowel *C. trachomatis* IgG en *C. pneumoniae* IgG antistoffen, implicerend dat *C. pneumoniae* een rol zou kunnen spelen in het ontstaan van *C. trachomatis* gerelateerde tubapathologie.

Hoofdstuk 4 beschrijft de voorspellende waarde van CAT voor vijf commercieel beschikbare tests (MIF Biomerieux, MIF Labsystems, ELISA Labsystems, pELISA Medac, ELISA Savyon). In een prospectief verzameld cohort van 315 patiënten werden de resultaten van de vijf tests gecorreleerd aan de bevindingen bij laparoscopie. Verder werd onderzocht of een verbetering van de voorspellende waarde mogelijk was door het combineren van een of meer tests. MIF labsystems

bleek superieur aan de andere 4 tests (OR 15.7), en van de drie ELISA's, kwam pELISA van MEDAC als beste test naar voren. Logistische regressie analyse toonde geen verbetering van predictieve waarde door het combineren van twee tests. In alle tests, behalve in pELISA van MEDAC, werd een significante kruis-reactiviteit met *C. pneumoniae* antistoffen gevonden.

Hoofdstuk 5 beschrijft screening van endocervicale *C. trachomatis* infecties bij subfertiliteitspatiënten. Als onderdeel van het fertiliteitsonderzoek of -behandeling vindt frequent intra-uteriene instrumentatie plaats. De aanwezigheid van actieve danwel persisterende endocervicale *C. trachomatis* infecties vormen derhalve een risicofactor voor het ontstaan van opstijgende infecties. Endocervicale PCR screening detecteert met name actieve infectie, waardoor een persisterende infectie mogelijk niet wordt onderkend en CAT toont eerdere expositie aan *C. trachomatis*, zonder informatie over de actuele aanwezigheid van *C. trachomatis* in de endocervix. Er is geen adequate non-invasieve screeningstest voor de aanwezigheid van persisterende infectie in de tractus genitalis. Gebaseerd op deze theoretische argumenten, adviseren we profylactische antibiotica (eenmalig azythromycine, 12 uur voor de geplande ingreep) voor elke subfertiële patiënte voorafgaand aan intra-uteriene instrumentatie, in plaats van endocervicale *C. trachomatis* screening en behandeling van de patiënten met een positieve test uitslag.

In **hoofdstuk 6** beschrijven we de ontwikkeling van een muismodel, ter bestudering van het nog grotendeels onbekende ontstaan van tubapathologie ten gevolge van *C. trachomatis*. Er werd verondersteld dat uitgebreide tubapathologie niet ontstaat in aansluiting aan een primaire (subklinische) *C. trachomatis* infectie, maar dat met name herhaalde infecties kunnen leiden tot uitgesproken afwijkingen. Vrouwelijke C57BL/6J muizen werden intravaginaal geïnoculeerd met de humane *C. trachomatis* L2 stam. Intervalsgewijs werden dieren opgeofferd en de histopathologische veranderingen en weefselschade in de tractus genitalis bestudeerd. Een groep muizen onderging een tweede inoculatie om het effect van her-infectie op het ontstaan van tubapathologie te evalueren. Tevens werd een pilot-studie verricht waarin de eventuele bijdrage van *C. pneumoniae* aan *C. trachomatis*-geïnduceerde pathologie werd bestudeerd. Enkelvoudige intravaginale inoculatie van C57BL/6J muizen met humane *C. trachomatis* L2 induceerde een infectie zonder uitgebreide tubapathologie, maar met een chronische ontstekingsreactie. Re-inoculatie stimuleerde dat proces, uiteindelijk leidend tot atypie van het endometrium. Afwijkingen op weefsel- alsmede cellulair niveau waren meer uitgesproken na her-inoculatie, alhoewel uitgebreide tractus genitalis pathologie niet werd waargenomen. Diverse factoren, mogelijk verantwoordelijk voor de milde bevindingen werden bediscussieerd.

Hoofdstuk 7 vooronderstelt dat auto-immuniteit, gebaseerd op kruis-reactiviteit van Chlamydia hsp60 (Chsp60) antistoffen met humaan hsp60, ten grondslag

zou kunnen liggen aan het ontstaan van tubapathologie. In een muismodel werden in aansluiting aan infectie met *C. trachomatis* en / of *C. pneumoniae* Chsp60 en mHsp60 (mHsp60) antistoffen bepaald. De resultaten ondersteunden de hypothese niet, daar slechts sporadisch Chsp60 en mHsp60 antistoffen konden worden aangetoond.

In **hoofdstuk 8** worden onderwerpen beschreven die bijdragen aan ons huidige begrip en de benadering van asymptomatische *C. trachomatis* infecties en de late gevolgen ervan. In deze context bediscussieerden we de resultaten van de gepresenteerde studies. De belangrijkste bevindingen van het onderzoek werden samengevat.

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Het is af.

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S Curriculum vitae

Tanja Gijsen werd op 8 november 1971 te Nederweert geboren. In 1990 voltooide zij het VWO aan de Philips van Horne Scholengemeenschap te Weert, waarna zij startte met de studie Geneeskunde aan de Universiteit Maastricht. Na het artsexamen in juni 1997 werkte zij als Assistent Geneeskundige Niet in Opleiding op de afdeling Gynaecologie van het Elkerliek ziekenhuis te Helmond. Vervolgens werkte zij van mei 1998 tot april 2002 als IVF-arts in het Academisch Ziekenhuis in Maastricht. Naast het klinisch werk op de IVF-afdeling, werd gestart met het onderzoek beschreven in dit proefschrift. Het onderzoek werd uitgevoerd binnen het onderzoeksinstituut Groei en Ontwikkeling (GROW), afdeling Obstetrie en Gynaecologie (begeleider: prof. dr. J.L.H. Evers) en de afdeling Medische Microbiologie (begeleider: prof. dr. C.A. Bruggeman). Vanaf april 2002 is zij in opleiding tot gynaecoloog. De opleiding werd gestart in het Atrium Medisch Centrum te Heerlen (opleider dr. F.J.M.E. Roumen) en vanaf april 2005 vervolgd in het Academisch ziekenhuis te Maastricht (opleider prof. dr. J.L.H. Evers).