A multidisciplinary approach to the study of chlamydia trachomatis infections
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A multidisciplinary approach to the study of

*Chlamydia trachomatis* infections

Female urogenital and male anorectal infections
The work described in this thesis was performed at the Municipal Health Service, Amsterdam, the Netherlands (Public Health Laboratory and outpatient STD clinic) (Former head: Prof. R. A. Coutinho, MD, PhD), and the VU University Medical Center, Laboratory of Immunogenetics (Head: Prof. A. S. Peña, MD, PhD, FRCP), Amsterdam, the Netherlands. This thesis was enrolled in the research line “Immunogenetics of Infectious Diseases” (Project-leader: Dr. S. A. Morré).
A multidisciplinary approach to the study of *Chlamydia trachomatis* infections

Female urogenital and male anorectal infections
PROMOTIECOMMISSIE

Promotores
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Prof. dr. C.M.J.E. Vandenbroucke-Grauls

Faculteit der Geneeskunde
This thesis is dedicated to the three most important men in my life – my grandfather Jan Spaargaren, who was far ahead of his own life time – my father Jan Spaargaren, who understood the nature of things by inborn experience – my beloved Kees, for his supportive and loving presence in my life.
- Concernant la couverture de cette these -

La seduction de l’orchidée

Si les plantes fleurissent, c’est pur se reproduire!
Le pollen fécondant est transporté d’une fleur à l’autre par le vent ou par des insects…

Certaines orchideées utilisent les services d’une abeille mâle sauvage.
Pour l’attirer, l’orchidée est “déguisée” en femelle-abeille:
l’un de ses petals mime la forme, les couleurs et le pelage de cet insecte.
Le male-abeille, dupe, tente de s’accoupler avec la fleur…
et l’orchidée depose alors du pollen sur sa tête!
Frustré de cette rencontre, l’insecte vole visiter une autre fleur:
il y abandonne le précieux pollen…
assurant ainsi la reproduction de l’orchidée.

(Pris de: Centre de Vulgarisation de la Connaissance
www.cvc.u-psud.fr/cvc)
CONTENTS

General Introduction

- *Chlamydia trachomatis* infections: a multidisciplinary approach - 9
- Lymphogranuloma venereum infections - 29

Aim and outline of the thesis

PART 1 UROGENITAL *CHLAMYDIA TRACHOMATIS* INFECTIONS

1A. BACTERIAL FACTORS

Aims and Outline of part 1A - 41

Chapter 1 Analysis of *Chlamydia trachomatis* serovar distribution changes in the Netherlands (1986 – 2002) - 45
Adapted from: Sexually Transmitted Infections 2004 Apr;80(2):151-2

Chapter 2 Interrelationship between polymorphisms of *incA*, fusogenic properties of *Chlamydia trachomatis* strains and clinical manifestations in patients in The Netherlands - 57
Adapted from: Journal of Clinical Microbiology May 2005;43:2441-2443

1B. ENVIRONMENTAL FACTORS AND EPIDEMIOLOGICAL STUDIES

Aims and Outline of part 1B - 67

Chapter 3 Population prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in the Netherlands. Should asymptomatic persons be tested during population-based Chlamydia screening also for gonorrhoea or only if chlamydial infection is found? - 69
BMC Infectious Diseases 2006 Mar 7;6(1):e42

Chapter 4 Association of antibody response to *Chlamydia pneumoniae* with lower abdominal pain in Dutch Caucasian women with urogenital *Chlamydia trachomatis* infection - 77
Submitted

1C. HOST SEROLOGICAL RESPONSES AND HOST IMMUNOGENETIC FACTORS

Aims and Outline of part 1C - 87

Chapter 5 *Chlamydia trachomatis* heat-shock-protein 60 (cHSP60) antibodies in women without and with tubal pathology using a new commercially available assay. - 89
Sexually Transmitted Infections 2004 Oct;80:415-6

Chapter 6 The first strong genetic susceptibility marker for *Chlamydia trachomatis* infections: the interleukin 1 receptor antagonist IL-1RN +2018 C>T gene polymorphisms - 95
Submitted
Chapter 7  
The CD14 functional gene polymorphism -260 C>T is not involved in either the susceptibility to *Chlamydia trachomatis* infection or the development of tubal pathology.  
BMC Infectious Diseases 2005 Dec 20;5:e114.

**PART 2  LYMPHOGRANULOMA VENEREUM**

Aims and Outline of part 2 - 119

Chapter 8  
New lymphogranuloma venereum *Chlamydia trachomatis* variant, Amsterdam - 121  
Emerging Infectious Diseases 2005;11(7):1090-1092

Chapter 9  
Real-time PCR to diagnose lymphogranuloma venereum - 129  
Emerging Infectious Diseases 2005;11(8):1311-1312

Chapter 10  
Diagnostic and clinical implications of anorectal lymphogranuloma venereum in men who have sex with men: a retrospective case-control study -135  
Clinical Infectious Diseases 2006;42:(2):186-194

Chapter 11  
Slow epidemic of lymphogranuloma venereum L2b strain - 151  
Emerging Infectious Diseases 2005;11(11):1787-1788

General discussion - 157

Summary - 171

Samenvatting - 181

**ADDENDUM**

Dankwoord - 193

Curriculum Vitae - 195

Publications - 196
1. **SUMMARY**

*Chlamydia trachomatis* is the most prevalent sexually transmitted disease worldwide. The majority of infections run an asymptomatic course and therefore will not be treated. Those patients, most of them are women, may play an important role in the transmission of infections with an increased risk for long-term sequelae. The course of an urogenital *C. trachomatis* infection can not be predicted exactly at present. Clear differences in the progress of the infection with late complications are due to the interaction between bacterial features, (e.g. virulence factors among different serovars), the local environmental situation in the urogenital tract, (e.g. whether the local flora can keep the pH in balance, whether other microorganisms like *C. albicans* are present) and the local as well as the systemic host responses upon a urogenital *C. trachomatis* infection. We describe an integrated approach on urogenital *C. trachomatis* infections in order to define (a) risk profile(s) for women for the development of late complications like PID, tubal scarring and infertility. This translational approach will help to gain further and valuable insight into the immunopathogenesis of this sexually transmitted infection (STI) and to define the development of new intervention strategies, including the necessary vaccine and screening programs to effectively diagnose and treat *C. trachomatis* infection and prevent long term serious complications.

2. **INTRODUCTION**

The *Chlamydiales* are bacteria that are nonmotile, gram-negative, obligate intracellular parasites of eukaryotic cells with a distinctive developmental cycle for their replication. Descriptions of a “chlamydia-like” disease of human eyes resembling the disease now known as trachoma (meaning: ‘rough eye’) have been found in ancient Chinese and Egyptian manuscripts. In 1907, Halberstaedter and von Prowazek, working in Java, described the transmission of trachoma from man to orang-utans by inoculating their eyes with conjunctival scrapings. In Giemsa-stained conjunctival epithelial cells, they found intracytoplasmic vacuoles (chlamydial inclusions) containing numerous minute particles.
General Introduction

(small chlamydial elementary bodies (EBs) and larger chlamydial reticulate bodies (RBs)) which they correctly inferred represented the causal agent of trachoma.

Figure 1: Halberstaedter and von Prowazek's drawings of a normal conjunctival epithelial cell (left), an infected cell (centre) and free chlamydial particles (right). 1907.

Figure 2 (left): Field emission scanning electron micrograph (by M. E. Ward and C. Inman, Southampton Biomedical Imaging Unit) of a HeLa 229 cell infected for 40 hrs with *C. trachomatis*.

Figure 3 (right): A freeze-fractured inclusion of *C. abortus* at 30 hours post infection showing how chlamydiae pack around the edge of the inclusion on the inclusion membrane. Such chlamydiae are well placed to interact via their projections or their tts system with the host cell cytoplasm. Unpublished electron micrograph of M. Ward and C. Inman, Southampton.

The newly discovered organisms were called the *Chlamydozoa* (from the Greek khlamus, a mantle/cloak) because of the blue-staining matrix in which the particles were apparently embedded *Chlamydia* (1).
Similar inclusions were subsequently described in the conjunctival cells of babies with non-gonococcal *ophthalmium neonatorum*, in the uterine cervix from some of their mothers and in the urethral epithelium from male patients with non-gonococcal urethritis. Thus trachoma, inclusion conjunctivitis of the newborn and infection of the adult genital tract were caused by similar infective agents (now *Chlamydia trachomatis*). The term ‘*Chlamydia*’ appeared in the literature in 1945. That Chlamydiae were not viruses became evident in 1965 with the advent of tissue culture techniques. In the 1990s, with the introduction of new diagnostic methods like DNA-DNA reassociation studies and subsequently gene sequencing lead to two new species, *Chlamydia pneumoniae* and *Chlamydia pecorum* (now both placed into the genus *Chlamydophila*). Nucleic amplification methods led to chlamydiae being discovered in tissues and cells never before reported (joints, atherosclerotic plaques, brains) and associated with diseases of previously unknown aetiology (arthritis, Alzheimer disease, coronary artery disease, etc.) (2-6). The new molecular knowledge led to a new taxonomy of the order *Chlamydophila* (means ‘like chlamydia’), which split the former family *Chlamydiaceae* into two genera, *Chlamydia* and *Chlamydophila*, encompassing nine species, and added three new non *Chlamydiaceae* families, the *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae* (7).

**Developmental cycle**

Sporelike forms of chlamydiae known as chlamydial elementary bodies (EBs) are small, round or occasionally pear shaped, electron-dense structures approximately 0.3 microns in diameter. Chlamydial EBs are unusual in that little or no peptidoglycan is present in the cell wall. Structural rigidity is thought to be due to the highly cross-linked nature of the outer-membrane complex among which cysteine rich proteins are important like OmpA. Electron microscopic examination demonstrated the presence of surface projections, which extend app. 30 nm from the surface. These structures are thought to correspond to Type III secretion system (TTSS) “needle” structures like those seen in *Salmonella enterica* (8). The initial reversible attachment of EBs with host cells occurs through electrostatic interactions most probably through binding to the surface exposed OmpA protein. A second, irreversible binding stage may be associated with protein disulfide isomerase, a component...
of the estrogen receptor complex, immediately followed by phosphorylation of the translocated actin-recruiting phosphoprotein, CT456 (TARP). EBs are the only infectious stage of the chlamydial developmental cycle and function. Enclosed within a pinched-off piece of the cell’s outer membrane (known as an entry vacuole), EBs begin differentiating into noninfectious reticulate bodies (RB) whose purpose is to permit chlamydial survival and replication in the non-supportive environment outside the host cell. RBs are larger than EBs (ca. 1 µm) and the cytoplasm appears granular with diffuse, fibrillar nucleic acids, in contrast with the highly condensed nucleic acid content of the EB. RBs are bounded by an inner and outer-membrane, resembling other, Gram-negative, eubacteria. The surface of RBs is covered with projections and rosettes that extend from the bacterial surface through the inclusion membrane. RBs undergo binary fission throughout the middle part of the developmental cycle (9).

Figure 5: A schematic representation of the Chlamydial developmental cycle (9). The host cell cytoplasmic membrane (red line) is shown to depict the interactions of Chlamydial EBs and the origin of the inclusion membrane. The major events in the developmental cycle are categorized as described. TARP (Translocated Actin-Recruiting Phosphoprotein, CT456), TTSS (Type III Secretion System), MEP (non-MEvalonate Pathway), CPAF (Chlamydial Protease/proteasomelike Activity Factor).
The bacteria thrive by extracting nutrients from the host cells’ cytoplasm. The developmental cycle from entry to release from the host cell takes between 48-72 hours. The most prominent component of the cell membrane is the major outer membrane protein (MOMP), which comprises 60% of the dry weight of the outer membrane complex in EBs and nearly 100% in RBs (10). This major structural protein (40 kDa) has a unique function in maintaining the structural integrity of the cell wall and forms with two other cysteine rich proteins, Omp2 (60 kDa) and Omp3 (12 kDa), a disulfide cross-linked complex in the cell wall (11). The MOMP is a transmembrane protein with surface antigenic components which can be used to identify the different C. trachomatis serovars.

Sofar, 19 different serovars have been identified by serotyping. In addition to the known serovars numerous variants have been characterized by serotyping, genotyping, and sequencing of the ompA gene. The ompA gene contains four variable sequence regions (VS1-VS4) and is interspaced and enclosed by five "constant" sequence regions (CS1-CS5). The variable MOMP protein domains (VD1-VD4) protrude from the chlamydial membrane with VD3 as the least protruding. VD1 and VD2 contain serovar-specific epitopes (12), while subspecies-, serogroup-, and species-specific determinants are found in VD4 (13-18). In addition, evidence suggest either that a serovar-specific epitope is present in VD4, and that VD4 influences antibody binding to VD1 and VD2.

**Chlamydia genes and proteins**

In 1998 the *Chlamydia trachomatis* genome project (19) showed that the chlamydial genome consists of a single circular 1.042.519 base pair chromosome (58.7% A+T) and a 7493 base pair plasmid (nucleotide sequence at http://chlamydia-www.violet.edu:4231 and GenBank under accession number AE001273). Analysis of the genome resulted in the identification of 894 putative protein encoding genes. Similarity searching identified functional assignment of 604 (68%) encoded proteins of which 35 (4%) were similar to putative proteins from other bacteria. The remaining 255 (28%) predicted proteins were not similar to other sequences deposited in GenBank to date. Many genes, operons and pathways were identified and two intriguing dogmas were unravelled. Firstly, *Chlamydiae* are thought to be "energy parasites" because they import ATP from their host cell. The presence of two genes encoding ATP translocases supports this hypothesis. However, the genome sequence analysis supports some, perhaps limited, capacity for substrate-level phosphorylation by phosphoglycerate kinase, pyruvate kinase, and succinate thiokinase. Furthermore, a vacuolar-type *Archaeae*-like ATPase (possibly to energize the chlamydial membrane, or to serve as a Na⁺ pump) and two flagellar-type ATPases (likely to be involved in proton-coupled transport) were found. Although the function of these genes, potentially involved in ATP synthesis, remains to be experimentally determined, *Chlamydiae* seem to be not strictly ATP auxotroph.

Secondly, it has been proposed that the *Chlamydiae* cell wall lacks peptidoglycan because muramic acid has not been biochemically detected or only in relatively small amounts (20). However, genes encoding proteins for the entire pathway for peptidoglycan synthesis, and membrane assembly and
recycling are present in the chlamydial genome as shown in the *C.trachomatis* genome project (19). This directly explains why *C. trachomatis* is sensitive to penicillin, a β-lactam antibiotic that exerts its activity due to inhibition of bacterial wall synthesis.

3. **CLINICAL COURSE of INFECTION**

**In women**

Almost 70% of the *C. trachomatis* infections in women run an asymptomatic course and therefore will not be treated with antibiotics. Those women may play an important role in the transmission of infections with the risks of long-term sequelae. Clinical manifestations of (primary) urogenital infections in women include cervicitis, urethritis, endometritis and pelvic inflammatory disease. Worth noting is the presence of oropharyngeal symptoms due to the nature of sexual activities practiced. Symptoms most often reported and related to *C. trachomatis* infections are abnormal vaginal discharge, dysuria, postcoital bleeding and in case of pelvic inflammatory disease (PID) sometimes subtle pelvic, uterine, and/or adnexal pain not gastro-intestinal and menses related. PID is the result of an ascending *C.trachomatis* infection and is responsible for most of the morbidity and costs due to tubal scarring and ectopic pregnancy eventually after repeated episodes resulting in tubal infertility.

**In men**

*C. trachomatis* infections are usually urethral in men. Recent figures show that up to 50% of the *C. trachomatis* infections in men are asymptomatic (21,22). Chlamydia urethritis is usually diagnosed by dysuria and urethral discharge, symptoms which occur 1 to 3 weeks following exposure to *C. trachomatis*. Untreated infections may lead to Reiter's syndrome. Reiter's syndrome consists of arthritis, urethritis and conjunctivitis, and is diagnosed 20 times more frequently in men than women and is significantly more often found in HLA B27 antigen-positive individuals (23,24). Epididymitis i.e. infection of the sperm ducts of the testicles, is most often due to *C.trachomatis* or *Neisseria gonorrhoeae* in young, sexually active men younger than 35 years of age (25). Epididymitis is diagnosed by unilateral scrotal pain, scrotum swelling, tenderness, and fever (26). Another disease caused by *C. trachomatis* is prostatitis. *C. trachomatis* could be cultured from urethral swabs of men with non-bacterial prostatitis after prostatic massage (27). *C. trachomatis* has also been implied in male infertility but there is a great variability in the reported prevalence of *C. trachomatis* in infertile men, partially related to the methods used.

**In neonates and infants**

*C. trachomatis* is the most common cause of neonatal conjunctivitis and one of the most common causes of pneumonia in early infancy (28-29). Neonates who are exposed at birth to a (asymptomatic) *C. trachomatis* infected mother, usually develop symptoms of conjunctivitis within 2 weeks after
delivery, and pneumoniae at 4 to 17 weeks after birth (28). Infants with chlamydial pneumonia are at increased risk for later pulmonary dysfunction and possibly for chronic respiratory disease (30). Besides maternally transmission during delivery, transmission of C. trachomatis to neonates can also occur after birth (31).

Relation between urogenital C. trachomatis infections and sequelae
The most important organisms involved in upper genital tract infections, specifically PID, are C. trachomatis and NG. The etiologic role of C. trachomatis and NG infections reflect the prevalence of the organisms in a particular population but in general 50% of the PIDs are caused by these two sexually transmitted microorganisms (32-33). In the Netherlands around 60,000 symptomatic C. trachomatis infections are estimated to occur. The estimated number of secondary complications are shown in Table 1.

Table 1: Number of symptomatic C. trachomatis infections in the Netherlands in men and women and the number of sequelae.

<table>
<thead>
<tr>
<th>Chlamydial infections and sequelae</th>
<th>Number per year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women: Chlamydial infections</td>
<td>33,000</td>
</tr>
<tr>
<td>Endometritis</td>
<td>14,000</td>
</tr>
<tr>
<td>Salpingitis/PID</td>
<td>7,000</td>
</tr>
<tr>
<td>Tubal infertility</td>
<td>1,000</td>
</tr>
<tr>
<td>Ectopic pregnancy</td>
<td>300</td>
</tr>
<tr>
<td>Men: Chlamydial infections</td>
<td>27,000</td>
</tr>
<tr>
<td>Epididymitis/proctitis</td>
<td>1,000</td>
</tr>
</tbody>
</table>


The role of C. trachomatis in PID, ectopic pregnancy and tubal infertility has been established by many epidemiological studies based on C. trachomatis serology (34-37). C. trachomatis antibody responses are strongest in the more severe complication: 72% in tubal infertility, 56% in ectopic pregnancy and 22% in controls (38). C. trachomatis is also detected directly in upper genital tract specimens derived from women with late complications (ectopic pregnancy, tubal infertility) using cell culture, EIA and PCR (39-41). These studies showed that the percentage of C. trachomatis infections that results in PID is between 8-20% and are based mainly on symptomatic C. trachomatis infections. This is an important issue since screening for C. trachomatis is mainly focused of the 70% asymptptomatically infected women, and which percentage of these asymptomatic C. trachomatis
infections leads to complications is difficult to investigate. Most studies show an indirect relation between *C. trachomatis* and late complications and between PID and the subsequent development of ectopic pregnancy and tubal infertility. In short, there are striking interindividual differences in the clinical course of *C. trachomatis* infection.

4. **AN INTEGRATED APPROACH TO THE STUDY *C. TRACHOMATIS* INFECTIONS**

Although progress has been made in the past years, it is still unclear why some people are susceptible to infection and develop symptoms and why others do not. The *C. trachomatis* infections reported are mainly symptomatic, since patients consult a physician due to clinical symptoms and complaints. However, while it is known that *C. trachomatis* can also run an asymptomatic course, exact percentages concerning asymptomatic infections are lacking and data range from 60–80% of infections in women and 30–50% in men (50-51). In addition, it is also mostly unclear why in some women upper genital tract progression occurs with in some cases detrimental results, and why in other the infection seems to clear rapidly, apparently without any clinical consequences. *C. trachomatis* infections can ascend to the upper genital tract resulting in pelvic inflammatory disease, ectopic pregnancy and tubal infertility. Uncontrolled immune reactions in the fallopian tubes are believed to contribute to the disease pathogenesis. Also repeated infections are associated with the development of these late complications. Some patients clear the infection spontaneously, while in others the infection persists for years. Some of the treated infections seem to reappear despite treatment of partners (52-55). However, the infections result in secondary complications in only some women (54 and 56). Furthermore not all partners of a *C. trachomatis*-positive index patient are *C. trachomatis* positive (confounding factors such as condom use were excluded). Transmission of the infection from the index patient to the partner is observed in 45–70% of cases (57-59) with the lower frequencies in those people having an asymptomatic course of infection.

For *C. trachomatis* infections, as well as many other infectious diseases, it is clear that differences in the susceptibility to and severity of infections are due to a complex interaction between bacterial, environmental (such as co-infections) and host factors (Figure 6).
Chlamydia trachomatis infections: a multidisciplinary approach

Figure 6: Factors influencing the course of *C. trachomatis* infection.

Below the three main factors, bacterial, environmental and host, influencing the clinical course of *C. trachomatis* infection will be discussed briefly.

### 4.1 Bacterial factors

With these factors mainly potential so-called virulence factors are the topic of interest. Among these are for instance the omp, inc and pmp genes all potentially influencing the clinical presentation and course of infection.

Serovar determination

**Serotyping**

Serotyping is performed after *C. trachomatis* culture using polyclonal and later monoclonal antibodies raised against the major outer membrane protein (MOMP) of *C. trachomatis*. Identification of a *C. trachomatis* serovar is based on the reactivity pattern of the isolate to a panel of monoclonal antibodies generated from all individual reference strains. Different formats can be used to identify all 19 different serovars and its variant that are known till now. The major drawback of serotyping is its laborious performance with the requirements of cell culture and a large panel of monoclonal antibodies (60-65)
Genotyping

To overcome the shortcomings and drawbacks of serotyping, genotyping is a good alternative. In 1991, Frost et al. (66), Sayada et al. (67), and Rodriguez et al. (68) showed that amplification of the \textit{omp1} gene by PCR followed by restriction analysis and gel electrophoresis of the obtained fragments could identify all serovars (PCR-based RFLP). Using the \textit{AluI} restriction analysis, the serovars can be divided into three geno-groups: the B-group (B, E, D, Da, L1, L2, L2a), the C group (C, A, H, I, Ia, J, K), and the intermediate group (F, G, Ga). These three groups also reflect the immunological relationship between the serovars as determined by serotyping. One of the major advantages is that culture is no longer needed, since amplification can be performed directly on the cervical and urethral swabs collected in an appropriate buffer as shown by Lan et al. (69). Beside this, the sensitivity of the PCR-based RFLP could be improved using a nested PCR format.

\textit{OmpA} sequencing

The most detailed way of typing \textit{C. trachomatis} serovars is nucleotide sequencing of the complete \textit{omp1} gene. From most of the 19 serovars, the complete \textit{ompA} sequence has become available (70), except for Ga and L2a from which only partial \textit{omp1} sequences have been described (71-73). When the nucleotide sequences are used for phylogenetic comparisons, the described three genogroups are identified (the B group, C group, and intermediate group, see serotyping). Sequencing identified numerous new CT variants like D\textsuperscript{+}, G\textsuperscript{I} (Identical to G/IOL-238/R which was in 1994 typed Ga (*4) (prototype G: G/UW-57/CX)), G\textsuperscript{I}, Jv, D\textsuperscript{+}, and I\textsuperscript{I} (73-75).

The identified nucleotide mutations as compared to the prototype strain can have consequences at the amino acids level. Most nucleotide changes in the VSs result in amino acid changes. The nucleotide changes in the CSs are more often silent mutations most likely since the amino acid sequence of the CDs is important for the transmembrane structure of the MOMP protein (76-77).

Serovar human

In humans, Morrè et al. (78) demonstrated that serovar Ga in men was associated with symptoms specifically dysuria and in women serovar K was associated with vaginal discharge. The finding that less prevalent serovars were associated with clinical symptoms was supported by Antilla et al. (79).

They showed that serovar G was most strongly associated with subsequent development of cervical squamous cell carcinoma. Other serotypes associated with cervical squamous cell carcinoma were I and D. In contrast, others (80) showed that, after controlling for age and race, women who reported abdominal pain and/or dyspareunia were more often infected with serovar F, a high prevalent serovar. On the other hand, a study typing the infecting chlamydiae among female sex workers in Senegal found that serovar E, also a high prevalent serovar, was less associated with visible signs of cervical inflammation than other serovars (81). Also, upper versus lower genital tract infections have been
investigated for a relation with particular serovars. While one study suggested an association between serovar F variants and symptomatic, severe endometrial disease, whereas E genotypes were associated with asymptomatic, milder infections (82) another study did not find any apparent association of any specific serovar with PID (83). The possible relationship of recurrent chlamydial cervicitis to the infecting serovar in women was examined by Dean et al. (84). The usual assumption is that recurrence of infection with a new chlamydial serovar indicates reinfection, whereas same-serovar recurrences may be due to persisting infection. A study of 552 women with more than three recurrent infections over 2 years found that 24% had same-serovar recurrences of which 45% were the less common subgroup C serovars; this was significant [statisticians: OR 2.4; 95% CI 1.7-3.5; P<.0001]. Further study indicated that cervical infections with C subgroup serovars particularly, may be persistent for years, perhaps because these organisms are able to adapt especially flexibly to immune pressure from the host.

Serovar Murine model
In mice Lyons et al. (85) have demonstrated that in an animal model of lower genital tract infection there are differences among serovars both in the duration of infection and in the ability to establish upper tract (uterine horn) infections. In that, the longest animal infections were in the B complex and the shortest were in the C complex, with intermediate serovars (F and G) in between. Upper genital tract progressions occurred more often in animals infected with serovar D than the serovar H group. Lyons et al. (86) also demonstrated variation in the course of infection in the murine model between serovar D and H. Serovar D was both more virulent (longer duration of infection) and immunogenic (higher level of circulating and vaginal IgG and higher incidence of IgA in vaginal secretions) in the mouse genital tract. In addition, prior infection with serovar D resulted in significant reduction in the median duration of infection against both homotypic and heterotypic reinfection as compared to prior infection with serovar H. Extension of this study assessed the in vitro characteristics and EB associated cytotoxicity of these 2 serovars in order to identify phenotypic difference(s) that might explain the previously reported variation in virulence in the mouse as a model to throw light on the variation in clinical presentation of a human urogenital infection (87). When compared to serovar D, an infection with serovar H resulted in the production of lesser numbers of progeny per unit input with less cytotoxicity providing.

IncA
Chlamydiae occupy a non-acidified vacuole (the inclusion) during their entire intracellular developmental cycle. They produce a set of proteins (Inc proteins including IncA) that localize to the surface of the inclusion within infected cells which in turn modify this surface through insertion of chlamydial proteins. Mutations in incA are thought to be associated with aberrant fusing patterns of the inclusions and probable differences in clinical course of infection.
Geisler et al (88) showed that female patients infected with nonfusing mutant *C. trachomatis* strains had fewer symptoms and were infected with lower inclusion-forming unit counts as compared to women infected with wild-type fusing strains. They suggest that these data underscore the importance of screening programs to detect and treat inapparent *C. trachomatis* infections.

**Pmp genes**
Furthermore a family of polymorphic membrane protein genes of *C. trachomatis*, resembling autotransporter proteins, has recently been discovered in *C. trachomatis*. Two studies have suggested (89-91) that the evolution of at least one of them, PmpH, showed three groups that reflect disease groups, suggesting this protein might play a role in pathogenesis. For the rest, it is known that polymorphisms in tryptophan synthase reflect functional changes in the organism’s sensitivity to IFN-γ. Future studies focused on defining whether these specific mutations correlate with different clinical manifestations of chlamydial genital infection are to be done.

**Plasticity zone**
A region of the chlamydiae chromosome termed the “plasticity zone” has undergone genetic reorganization to a much higher degree than the rest of the chromosome, is suspected to be involved in pathogenesis and contains the tryptophan biosynthesis genes. Mutations in this region representing differences in synthesizing tryptophan from indole might also be important for the persistence of *C. trachomatis* within the genital tract epithelium, with important consequences for disease transmission as well as for the inflammatory sequelae associated with chronic infection has been proposed by Fehlner-Gardiner et al (91-92).

**4.2 Environmental factors**
The susceptibility to a *C. trachomatis* infection is influenced by many factors including the presence and composition of the vaginal microflora. *C. trachomatis* infections give a broad range of clinical symptoms such as fluor or abdominal pain and can be caused by more than one etiologic agent. For instance, *Neisseria gonorrhoeae* and *Candida albicans* can result in similar and dissimilar symptomatology as compared to a *C. trachomatis* infection. Knowledge on the co-infection status on clinical presentation and susceptibility to infection is an essential step forward in unravelling the clear differences in the clinical course of a *C. trachomatis* infection between women.
The most significant complication of sexually transmitted diseases in women is pelvic inflammatory disease (PID), which is responsible for considerable medical, social, and economic problems. *C. trachomatis, N. gonorrhoeae* or both cause PID in at least 50% of cases.
Other microorganisms that are part of the abnormal vaginal flora also cause PID. One study has shown the results of interaction and persistence of human papillomavirus as most significant risk factor when
previous *C. trachomatis* infection was present (93). On the other hand associations between *Mycoplasma genitalium*, *C. trachomatis* and PID have not confirmed yet (94).

### 4.3 Host serological responses and host immunogenetic factors

#### Human serological responses

Chlamydiae contain common immunodominant antigens like the genus-specific lipopolysaccharide (LPS). To perform species specific serology *C. trachomatis*-specific immunogenic structures are needed. These structure are located in the variable domains of the major outer membrane protein (MOMP). Due to the fact that most *C. trachomatis* infections run an asymptomatic course, most infections will go undetected and may develop, in a suitable host in the right environment, a chronic disease sustained by the ascended, persisting agent. It is thought that the host immunity plays an important role in controlling *C. trachomatis* infections.

Associations between antibodies to chlamydial heat shock protein 60 (cHSP60) and the chronic sequelae of a chlamydia infection have been shown. In one study of 313 subfertile women, as graded by laparoscopy, cHSP60 IgG was significantly more prevalent in women with tubal pathology (95). This finding was supported by Karinen et al. (96) who demonstrated also that antibodies to cHSP60 was associated with female subfertility defined as time to pregnancy $\geq$ 12 months in a population-based sample. However this does not prove directly that autoimmune responses necessarily play a significant role in the immunopathogenesis of subfertility, but the results suggest that the autoimmune response to human hsp60 can develop following *C. trachomatis* upper genital tract infection in women, probably as a consequence of an immune response to an epitope of chlamydial hsp60 cross-reactive with the human hsp60 (97).

#### Serological responses in mice

Lyons *et al.* (86) also demonstrated variation in the course of infection in the murine model between serovar D and H. Serovar D was immunogenic (higher level of circulating and vaginal IgG and higher incidence of IgA in vaginal secretions) in the mouse genital tract. Although both serovars induced cross-reacting antibodies during the course of primary infection, prior infection with serovar H resulted in only a slight reduction in the median duration of infection against homotypic reinfection, while prior infection with serovar D resulted in significant reduction in the median duration of infection against both homotypic and heterotypic reinfection when compared to primary infection in age and conditions matched controls.

#### Immunogenetic factors

Gene association studies (whether case-control or cohort) can be used either in an “indirect” manner as a tool for mapping genes using linkage disequilibrium or in a “direct” manner for evaluating associations with postulated causal (“candidate”) genes. The latter approach involves selection of the
gene encoding the protein which is thought to function abnormally, identification of one (or, preferably, several) informative polymorphisms in, or very close to, the gene in question and the application of both association and linkage studies to determine whether there is any relationship between those variants and disease risk within populations of families.

Fundamental aspects of *C. trachomatis* infection have been investigated using murine and pig-based experimental models. For example, knockout mice have been used to assess the relevance of specific genes such as TLR4 or IFN-γ on the course of *C. trachomatis* infection. The findings for primary infection have been extrapolated to a human cohort with uncomplicated infections in a population with STIs, while for the translation of the murine findings after reinfection, a human cohort of women with subfertility has been used. In these human cohorts candidate gene approaches have been used to investigate whether the murine findings can be extrapolated to humans to identify important genes which regulate the susceptibility to and severity of infection, and thus potentially identify women at risk of either infection in general or the development of late complications.

Kinnunen et al (98) studied the relationship between *C. trachomatis* tubal factor infertility (TFI) and the host's immunoregulatory genes. DQA1*0102 and DQB1*0602 alleles together with IL-10 -1082AA genotype were found significantly more frequently in the tubal factor infertility patients than in the controls (0.18 and 0.02 respectively; P = 0.005). Furthermore the relationship between interleukin-10 (IL-10) promoter -1082 polymorphism and cell-mediated immune response during *C. trachomatis* infection in vitro, lymphocyte proliferation and cytokine (IL-10, IFN-gamma, TNF-alpha, IL-2, IL-4 and IL-5) secretion was analysed in subjects with different IL-10 genotypes. Enhanced IL-10 secretion and reduced antigen-specific lymphocyte proliferative and IFN-gamma responses were found in subjects with IL-10 -1082 GG genotype when compared to those with -1082 AA genotype indicating that impaired cell-mediated response to *C. trachomatis* might be associated with IL-10 genotype in subjects with high IL-10 producing capacity. A comparison of immune markers between subjects with a history of noncomplicated and complicated infection is needed to further understand the confounding factors associated with the development of *C. trachomatis* associated sequelae (99).

### 4.4 The integrated approach

The critical evaluation of these host, bacterial, environmental, clinical and epidemiological data and the results of experimental studies conducted both *in vitro* and *in vivo* using both animal models and human cohorts will give valuable insight into the immunopathogenesis and lead to an understanding of the disease process, including both susceptibility to and severity of disease. This will contribute to the development of new intervention strategies, including screening programs, that are necessary to effectively diagnose, treat and prevent *C. trachomatis* infection. This multidisciplinary approach to study *C. trachomatis* infections is referred to as the integrated approach to *C. trachomatis* infections (Figure 7).
Integrated Approach to *Chlamydia trachomatis* Infections

Promotes a synergism between epidemiology, immunogenetics, functional biology, and clinical studies

![Integrated Approach to *Chlamydia trachomatis* Infections](image)

Figure 7: The integrated approach of *C. trachomatis* infections in which bacterial studies, animal and human models participate.

5. REFERENCES

8. Ho,T.D., Starnbach,M.N. The *Salmonella enterica* serovar typhimurium-encoded type II secretion systems can translocate *Chlamydia trachomatis* proteins into cytosol of host cells. Infection and Immunity 2005;73(2), 905-911


Lymphogranuloma venereum infections
(adapted from Morbidity and Mortality Weekly Report 2004;53(42):985-988. (1))

1. Abstract
2. Clinical spectrum of disease
3. Diagnosis
4. Treatment
5. The current outbreak or continuous spread
6. References
1. ABSTRACT

This chapter describes lymphogranuloma venereum (LGV), a systemic, sexually transmitted disease (STD) which is caused by *Chlamydia trachomatis* serovars L1 to L3. The prevalence of LGV is greatest in Africa, Southeast Asia, Central and South America, and Caribbean countries (2) and this disease rarely occurs in the United States and other industrialized countries. However, in December 2003 local health authorities in Rotterdam reported 13 cases of LGV. We describe the clinical spectrum, the diagnosis and the initial phase of the current outbreak in industrialized countries, focussing on The Netherlands (3).

![Way of entry of LGV into the human body through the lymphatic system to reside in the lymph nodes.](www.indepthlearning.org)

2. CLINICAL SPECTRUM of DISEASE

Although LGV is a relatively rare sexually transmitted disease in industrialized countries, its aggressive nature and serious late sequelae make early recognition and treatment imperative. The way of entry into the human body is shown in Figure 1. The course of the disease is divided into three stages: primary genital lesions, secondary inguinal bubo development, and late fistula and stricture formation.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time after infection</th>
<th>Clinical signs and symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>3-30 days</td>
<td>Urogenital lesion: painless, transient</td>
</tr>
<tr>
<td>Secondary</td>
<td>1-several weeks</td>
<td>Lymphadenopathy: inguinal, retro-peritoneal (bubo)</td>
</tr>
<tr>
<td>Tertiary</td>
<td>Years</td>
<td>Proctitis, recto-vaginal fistulae. D.d.: M. Crohn</td>
</tr>
</tbody>
</table>
Primary stage
After an incubation period ranging as wide as 3 to 30 days, a lesion reportedly appears on the glans of the penis or, in women, on the vaginal wall, labia or, occasionally, the cervix. Rarely lesions may occur at extra-genital sites such as the anus, fingers or tongue. The initial lesion is usually transient, barely perceptible and painless; occurring as a papule, a shallow ulcer / erosion, a herpes (cold-sore) - like lesion or as a non specific urethritis reflecting an intra-urethral lesion. This so-called primary lesion is seen in around 4% of LGV patients, either because being painless the patient doesn't seek medical attention, or because the lesions described are not characteristic for LGV (4). This would explain the apparent unique ability of this biovar to replicate in dead, keratinized epithelium. Syphilis, chancroid, Donovan's disease, *Herpes simplex*, trauma and abrasions are all potential causes of such indeterminate lesions. There can, however, be no doubting in the next stage.

Secondary stage
Occurs from one to several weeks after infection. Most patients attend clinics because of the marked lymphadenopathy associated with this stage. The lymphadenopathy is usually unilateral, involving the retro-peritoneal lymph nodes in women or the inguinal lymph nodes in men. In the so-called bubonic form, both the inguinal and femoral lymph nodes may be involved giving rise, because of the separating Pupart's ligament, to the "groove sign" considered a characteristic of this disease, but one which is only seen in a small proportion of patients (4). Other lymph nodes may become involved by lymphatic drainage of infection from the infected node, giving rise in some cases to a whole chain of swollen nodes. The infected nodes become abscesses which eventually suppurate and may give rise to draining fistulae. In a small proportion of patients chronic lymphadenopathy may persist for years. Swollen buboe may need aspiration to avoid rupture.
Tertiary stage.
If left untreated, progressive spread of the infection leads to increasing and devastating tissue destruction (5). LGV proctitis initially occurs, followed by rectal damage, strictures and, in women, the formation of recto-vaginal fistulae. Two separate reports of LGV rectovaginal fistula have occurred recently in the world literature (5,6) after a gap of almost thirty years (5). There may be substantial urethral destruction also. Epithelial tissue is destroyed and replaced with granulation tissue and infiltrating plasma cells which histologically and on endoscopic examination may mimic Crohn's disease (6, 20, 22, 23). Rectal infection with the LGV biovar of C. trachomatis is relatively common in homosexual men and is accompanied by signs of more systemic cachexic illness than is usually seen with chlamydial proctitis due to the usual oculo-genital serovars D thru K.

3. DIAGNOSIS
LGV is a lymphotropic infection and it induces a major cell mediated immune response. The Frei test, the classic delayed hypersensitivity skin test for LGV, is obsolete (4) and non-specific. In LGV there is usually, but not always (6) a high titre antibody response to the infecting organism which may be demonstrated by the micro-immunofluorescence test or with a C. trachomatis peptide based IgG ELISA. However none of these tests are well defined for the diagnosis LGV. There have been few studies of modern molecular methods of diagnosing chlamydial infection in suspected LGV. However the studies of Htun (7) and others suggest that nowadays the best strategy in genital ulcer disease or lymphadenopathy is to test with the (commercial) nucleic acid based approaches including PCR based RFLP analysis to separate LGV strains from non LGV strains and the use of real-time PCR approaches (21) to assess the presence of the LGV agent in the genital tract or in material aspirated from affected lymph nodes.

4. TREATMENT
The recommended treatment is administration of 100 mg of doxycycline, twice a day for 21 days, with erythromycin listed as alternative regimen. In addition, fluctuant buboes should be aspirated to prevent rupture and sinus tract formation. Sex partners who had contact with the patient within 30 days of the patient’s onset of symptoms should be evaluated; in absence of of symptoms, they should be treated with either 1 g azithromycin in a single dose, or 100 mg of doxycycline, twice a day for 7 days.

5. THE CURRENT OUTBREAK
In December 2003 local health authorities in Rotterdam reported 13 cases of LGV. After alerting other local health departments and STD clinics an additional 92 confirmed cases were found in The
Netherlands. At that time preliminary evaluation of the unconstrained epidemiological data showed that all the patients were white men having sex with men (MSM) and that, among the 30 MSM whose HIV status was known, 23 (77%) were HIV positive. Other preliminary findings suggested that concurrent sexually transmitted infections were prevalent and that the majority had participated in casual sex gatherings (e.g., "leather scene" parties) and unprotected anal intercourse or other unprotected anal penetration (e.g., fisting) during the 12 months before onset of symptoms. These LGV infections were diagnosed by conducting polymerase chain reaction (PCR) tests on rectal swab specimens and performing subsequent restriction endonuclease pattern analysis of the amplified outer membrane protein A gene to determine the genotype or by performing a real-time PCR test on the rectal samples.

For the current outbreak in the Netherlands diagnostic case definitions were developed (see Table 1):

**Confirmed LGV cases** were those in patients with proctitis or contact with a patient with a confirmed LGV diagnosis, a positive PCR test for *C. trachomatis* on a urine or rectal specimen and a L1, L2 or L3 genotype as determined by RFLP.

**Probable LGV cases** were those in patients whose illness was consistent with the first two criteria and who also had a positive serologic test for *C. trachomatis* as determined by peptide based enzyme immunoassay, but did not meet the third criterion because specimens were not available for genotyping.

**Possible LGV cases** were in patients who met only the first criterion and had a positive serologic test.

Table 1: diagnostic criteria for LGV

<table>
<thead>
<tr>
<th>NAAT on rectal swab or on aspirated pus from bubo</th>
<th>Genotype</th>
<th><em>C. trachomatis</em> specific serology</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>L1, L2, L3 or their variants</td>
<td>Relative high titer</td>
<td>Confirmed case</td>
</tr>
<tr>
<td>Positive</td>
<td>Not known or not L1-L3</td>
<td>Relative high titer</td>
<td>Probable case</td>
</tr>
<tr>
<td>Negative or not available</td>
<td>Not known</td>
<td>Positive</td>
<td>Possible case</td>
</tr>
</tbody>
</table>
Recent reports from Belgium, France, and Sweden confirm that LGV is occurring elsewhere in Europe (8-10). Additional reports have followed and have increased awareness of the outbreak. In July 2004, CDC identified an L2 LGV strain on a rectal swab specimen from a patient in the United States who had signs and symptoms similar to those of the patients in the Netherlands. In this case, no known exposure to European MSM was reported; U.S. contacts of the patient were evaluated and treated. Although some of the patients in this LGV outbreak reported having multiple sex partners in cities in Europe and the United States (11), limited information has been reported regarding detailed epidemiological information regarding the LGV occurrences outside the Netherlands.

In 2004 an intensified surveillance was started on LGV in The Netherlands and in Europe. In January 2006 179 confirmed cases were reported in The Netherlands: in 2002/2003 65 cases were retrospectively confirmed, in 2004 77 and in 2005 37 confirmed cases were reported (Figure 5). Although the factors for a new outbreak of LGV or another STI are probably still present in the high risk groups it appears that the LGV epidemic is declining in Amsterdam (Figure 4) and overall in The Netherlands too (Figure 5). However, in other European countries still new confirmed cases are diagnosed (Figure 6). Nevertheless, continuous observation is recommended.

![Figure 4: Lymphogranuloma venereum in Amsterdam, The Netherlands after 2003. (personal communication dr H.J. de Vries, STD clinic Amsterdam.)](image-url)
Figure 5: number of confirmed cases of LGV in The Netherlands. 

Figure 6: number of confirmed cases of LGV reported in the literature from 2002 until December 2005. 
*: the total number of confirmed cases in France and the UK is times 10.
6. REFERENCES


Aims and outline of the thesis

*Chlamydia trachomatis* is the most prevalent bacterial sexually transmitted agent worldwide. Most infections run an asymptomatic course, in women around 70%, and subsequently will be transmitted unknowingly and left untreated. In women this might result in severe complications like pelvic inflammatory disease (PID), ectopic pregnancy and tubal infertility. When symptoms do occur, abnormal vaginal discharge, dysuria, and postcoital bleeding are mostly reported. Symptoms of chlamydial PID, which may be subtle, include pelvic, uterine, and/or adnexal pain. It is still mostly unclear why some women develop symptoms and why others do not, why in some women upper genital tract progression occurs with in some cases detrimental results and why in others the infection seems to clear rapidly, apparently without any clinical consequences. However, it is likely, that, as for other infectious agents, differences in the susceptibility to and severity of infections are due to a complex interaction between bacterial, environmental and host factors.

The aim of this thesis was twofold:

In **Part 1** we address the role of bacterial factors, amongst others *C. trachomatis* serovars (part 1A), environmental factors and epidemiological variables (part 1B), and host factors, among others serological responses and immunogenetic responses (candidate gene approach based; part 1C), to assess their role, individual or combined, in the susceptibility to and severity of *C. trachomatis* infections.

The Municipal Health Service in Amsterdam has as one of its main goals to provide clinically based general health care in the sexual transmitted disease (STD) field. From this point of view in **Part 2** we describe a recently identified, still ongoing, Lymphogranuloma Venereum (LGV) outbreak among men having sex with men in Amsterdam. To characterize and describe this current clinically relevant topic we used retrospective and prospective epidemiological studies, we addressed different approaches to diagnose these LGV infections, we characterized the LGV strain identified in these men and looked at algorithms to identify LGV positive patients.
Aims and outline
PART 1

UROGENITAL CHLAMYDIA TRACHOMATIS INFECTIONS

1A. BACTERIAL FACTORS
Aims and outline
Aims and outline of Part 1A

The course of *C. trachomatis* infections in influenced by bacterial factors, environmental factors and host factors. This section A of Part 1 will focus on *C. trachomatis* bacterial (virulence) factors. Virulence factors might explain in part the observed differences in the clinical course of *C. trachomatis* infection. These factors could influence the efficiency to attach to and infect human cells. On the other hand they could be involved in alteration of the host intracellular environment in such a way that persistent infection occurs and they could be involved in the level of innate and/or adaptive immune responses following infection. The different scenarios could result in either a more symptomatic or more asymptomatic course of infection and depending on other factors result in an enhanced risk for late complications.

The aim of the following studies was to investigate the role of two potential bacterial factors encoded by either *ompA* gene encoding a Chlamydial membrane protein, or the *IncA* gene, encoding a Chlamydial inclusion membrane protein.

**Chapter 1** Does the *C. trachomatis* serovar distribution change in time in The Netherlands?

To answer this question we determined the serovar distribution at the Municipal Health Services in Amsterdam around 2002 and compared the results to already known and published distributions in The Netherlands ever since 1986 to reveal possible information on changes in the epidemiology of *C. trachomatis* infection and clinical courses.

**Chapter 2** What is the *incA* sequence variation in *C. trachomatis* serovar isolates from Dutch Caucasian cohort and is there a relationship between *incA* sequencetype(s) and the presentation of infection as symptomatic or asymptomatic?

A recent report in the USA showed that among 11,440 isolates, only 1.5% had the so-called non-fusogenic phenotype while in an other publication it was shown that non-fusogenic strains were found more often among patients with less signs of infection as compared to fusogenic strains. We determined the percentage of non-fusogenic strains *C. trachomatis* isolates obtained from Dutch Caucasian women and the IncA sequence types in relation to the symptom presentation.
Chapter 1

Analysis of *Chlamydia trachomatis* serovar distribution changes in the Netherlands (1986-2002)

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Abstract

**Introduction:** Determination of *Chlamydia trachomatis* serovars is used to get insight in the epidemiology and transmission of *C. trachomatis* infections. In addition, associations between different *C. trachomatis* serovars and clinical presentations have been studied.

**Aim:** To investigate whether significant shifts in serovar distribution could be demonstrated in The Netherlands from 1986 through 2002.

**Material and methods:** First, we determined the most recent *C. trachomatis* serovar distribution in a large STD population in Amsterdam during 2000 – 2002. Second, we compared all published serovar distributions (n=9, 2185 isolates) in the Netherlands to one another. Serovars were determined by PCR-based RFLP analysis of the *ompA* gene.

**Results:** This largest and most recent study on the serovar distribution of *C. trachomatis* in the Netherlands showed that 85 (20.8%) isolates accounted for serogroup C, 132 (32.3%) belonged to the Intermediate group and 192 (46.9%) to the B group. In both men and women, the most prevalent serovar was F followed by E, D, and G. The serovar distribution was: B=0.2%; D=12%; Da=0.2%; D-=1%; E=33%; F=23%; G=4%; Ga=5%; H=8%; I=6%; Ia=1%; J=3%; K=2%. No significant shifts in serovar distribution could be demonstrated in The Netherlands from 1986 through 2002. However, when the serovar distributions were compared to each other based upon the two geographically distinct locations from which these serovar distributions were obtained it appeared that serogroup C was found more frequently in Rotterdam as compared to Amsterdam: 30 vs 19% (p<0.0001; OR 1.8 (95% CI: 1.4-2.3)), of which the most prominent serovar difference was serovar K (10.6 vs 3.2%, p<0.0001; OR 3.6 (95% CI 2.4-5.3)). The Intermediate-serogroup was found less frequently in Rotterdam: 21 vs 31% (p=0.0002; OR 1.6 (95% CI: 1.2-2.0)), of which the most prominent serovar difference was serovar F (15 vs 22%, p=0.0018; OR 1.6 (95% CI: 1.2-2.1)). Serogroup B was stable between the two cities (49% vs 50%).

**Conclusions:** No major differences in serovar distributions in time were observed in The Netherlands, but different stable geographical-based serovar distributions could be demonstrated.
Introduction

*Chlamydia trachomatis* infection is the leading cause of sexually transmitted diseases worldwide. In women 70% of the infections runs an asymptomatic course. Due to this asymptomatic state, those women will most probably not be treated, may serve as an important source of transmission and are at risk for late complications like pelvic inflammatory disease (PID), ectopic pregnancy and tubal infertility. The incidence and prevalence of *C. trachomatis* are influenced by a complex interaction of environmental, demographic and behavioural factors, including gender, age at first sexual intercourse, number of lifetime sexual partners, frequency of partner change, unsafe sex and socio-economic status.

*Chlamydia trachomatis* is an obligate intracellular bacterium with a unique bimorphic lifecycle. Up till now 19 different serovars have been identified. The serovars A, B, Ba, and C infect mainly the conjunctivae, the serovars D, Da, E, F, G, Ga, H, I, Ia, J, and K are predominantly isolated from the urogenital tract and the serovars L1, L2, L2a, and L3 can be found in the inguinal lymph nodes. In addition to these 19 serovars numerous variants have been characterized (1,2). Serovar determination can be based upon both the protein level (major outer membrane protein (MOMP)) as well as on the DNA level using the *omp A* gene which encodes for MOMP. An increasing number of isolates is typed worldwide and these studies have provided a wealth of information on the epidemiology of *Chlamydia trachomatis* infections. In most studies the serovars D, E and F are the most prevalent and comprise around 70% of the serovars typed. Recently, studies have suggested an association between the serovar distribution and the clinical course of infection. E.g. the results of the longitudinal study by Anttila and colleagues (6) suggests that specific serotypes (G, I and D) were more likely to be associated with squamous cell carcinoma than other types. In addition, although contradictory results have been obtained, associations between serovar and upper genital tract progression or symptomatology have been reported. In general, determination of *Chlamydia trachomatis* serovars is used to get insight in the epidemiology and changes in the epidemiology and transmission of *Chlamydia trachomatis* infections (3-5).

The aim of this study was to determine whether significant shifts in serovar distribution could be demonstrated in The Netherlands from 1986 through 2002. First, we determined the most recent *Chlamydia trachomatis* serovar distribution in a large sexual transmitted diseases outpatient clinic (STD) population in Amsterdam during 2000-2002. Second, we compared all published serovar distributions in The Netherlands, starting from 1986, to one another to identify potential shift(s).
Chapter 1

Material and Methods

Patients and clinical specimens of the most recent serovar distribution

Of people attending the STD outpatient clinic in Amsterdam from 2000-2002, clinical specimens found *C. trachomatis* positive (n=409) by LCx (Abbott Laboratories, Chicago, Ill) were used for serovar determination (1). First-void urine specimens and urogenital scrapes were obtained from randomly selected females and males. All participants gave informed consent.

*C. trachomatis* detection and typing

*C. trachomatis* DNA was detected by LCx (Abbott Laboratories, Chicago, Ill.) according to the instructions of the manufacturers. For *C. trachomatis* typing total DNA, including *C. trachomatis* DNA, from urines was isolated by dissolving the pellet (from 1,5 ml urine) in 100 µl Phosphate Buffered Saline (pH 7,4) and lyse the sample in 500 µl in the chaotropic agent guanidinium thiocyanate L6 during 30 minutes at 65 °C. Released DNA was precipitated with 96% pure ethanol in the presence of a coprecipitant. The pellet was dissolved in 50 µl 10 mM Tris-HCl, pH 8.0, and stored at −20 °C until amplification. The same procedure was followed for the urogenital swabs except that 100 µl swab elutionbuffer was added directly to the lysisbuffer L6.

*C. trachomatis* typing was performed by amplification of the *ompA* gene (1.1 Kb) in a nested PCR using the primers sero1A (sense) (5’- ATGAAAAAATCTTGTGAAATCGG – 3’) and sero2A-AT (antisense) (5’- TTTCTAGATTTCATTTTGTT – 3’) / sero2A-AC (antisense) (5’- TTTCTAGATTTCATTTTGTT – 3’) as initial primers and the primers CTOMPN1 (sense) (5’- CTTTGAGTTCTGCTTCCT – 3’) and CTOMPN2 (antisense) (5’- AGGAACGATTGCTATTG – 3’) as nested primers. The PCR mixture (final volume 25 µl) contained 50 mM NaCl, 2 mM MgCl₂, 10 mM Tris/HCl (pH8.0), 200 µM (each) deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP), 160 pg of each primer, and 0.5 U Taq polymerase (Supertaq, Eurogentec).The reaction mixture was overlaid with a few drops of liquid paraffin to prevent evaporation. The PCR amplification was carried out in a thermocycler (PTC-200 Peltier Thermal Cycler, MJ Research Inc., Mass., USA) starting with 3 min of denaturation at 94 °C and continuing for 30 cycles of amplification. Each cycle consisted of denaturation at 93 °C for 30 sec., annealing at 45 °C for 30 sec. and chain elongation at 72 °C for 1 min. The PCR product was checked on agarose gel for its length. Subsequently, 10µl PCR product was digested using different restriction enzymes as described previously. Serovars and variants were identified by their RFLP patterns after polyacrylamide gel electrophoresis.
Published serovar distributions in The Netherlands

Literature reviews were performed to identify articles containing data on the serovar determination of *C. trachomatis* in The Netherlands.

**Statistical analysis**

Chlamydia serovars were classified in three groups (referred to as serogroups) based on their nucleotide relatedness and serological reactivity in previous studies: the B-complex comprising the serovars B, Ba, D, Da, D-, E, L1, and L2; the intermediate serogroup or F/G-group comprising the serovars F, G, and Ga; and the C-complex comprising the serovars C, H, I, I', Ia, J, K, and L3. Trends in infection rates for the three different serogroups were examined from 1986 – 2002. If trends were found, analyses on the serovar level were performed.

The results from the present serovar distribution were compared together with all published serovar distributions in The Netherlands from 1986 through 2002 for potential serovar distribution shifts in time. PubMed, EMBASE and information from the Dutch Chlamydia Society were used to identified previous published Dutch *C. trachomatis* serovar distribution studies.

The following presumptions were made: 1) no distinguishing between male and female participants were made (most available studies did not provide this information); 2) no distinguishing between asymptomatic and symptomatic infection episodes were made (most available studies did not provide this information); 3) serovar determination with different typing techniques were analyzed together (serovars are identically typed used MOMP or ompA serovar determination techniques); 4) serovar B/Ba were excluded in the analysis due to the low numbers; 5) Double infections were excluded due to low numbers; and finally 6) for serovar level analyses D/D-/Da, serovars G/Ga and serovars I/I-/Ia were taken together and designated serovar D, G and I respectively, since most studies did not distinguish between these serovars.

Comparison of the serogroups and the different serovar(s) were performed using the Chi-square test. If numbers were not sufficient (n<5) the Fisher’s Exact test was used. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. We calculated the proportions of each of the three serogroup for all published serovar distributions. 95% Confidence intervals were calculated using CIA, version 1 (MJ Gardner; British Medical Journal).

**Results**

The distribution assessed in the present study of the different serogroups in Amsterdam from 2000-2002 is shown in Table 1. Eighty-five (20.8%) were typed as serogroup C, 132 (32.3%) belonged to the intermediate group and 192 (46.9%) to the B group. In both men and women, the most prevalent serovar was F followed by E, D, and G. The following serovar distribution was found: B=1.0%; D=12%; Da=0.2%; D-=1%; E=33%; F=23%; G=4%; Ga=5%; H=8%; I=6%; Ia=1%; J=3%; K=2%.
Table 1: Publications containing data on the serogroup determination of *C. trachomatis* from 1986 – 2002 in the Netherlands; proportion of the different serogroups with their 95% confidence intervals (CI) are calculated. STD: sexual transmitted diseases cohort; Gyn.: gynaecology cohort; Asymp.: asymptomatic cohort; Mixed: asymptomatic and STD cohort.

<table>
<thead>
<tr>
<th>Study</th>
<th>First author</th>
<th>Year of publication</th>
<th>Cohort collection</th>
<th>Year of collection</th>
<th>Study Location</th>
<th>n</th>
<th>C (strains)</th>
<th>95% CI</th>
<th>Int. Group: 95% CI</th>
<th>B Group: 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wagenvoort</td>
<td>1988</td>
<td>STD</td>
<td>1986</td>
<td>Rotterdam</td>
<td>190</td>
<td>67</td>
<td>43</td>
<td>35% 29-42</td>
<td>23% 17-29</td>
</tr>
<tr>
<td>3</td>
<td>Morré</td>
<td>1998</td>
<td>STD</td>
<td>1985-1990</td>
<td>Amsterdam</td>
<td>90</td>
<td>30</td>
<td>23</td>
<td>33% 24-44</td>
<td>26% 17-36</td>
</tr>
<tr>
<td>4</td>
<td>Ossewaarde</td>
<td>1994</td>
<td>STD</td>
<td>1992</td>
<td>Amsterdam</td>
<td>289</td>
<td>45</td>
<td>90</td>
<td>16% 11-20</td>
<td>31% 26-37</td>
</tr>
<tr>
<td>5</td>
<td>Lan</td>
<td>1995</td>
<td>screening</td>
<td>1993</td>
<td>Amsterdam</td>
<td>51</td>
<td>14</td>
<td>17</td>
<td>27% 16-42</td>
<td>33% 21-48</td>
</tr>
<tr>
<td>7</td>
<td>Morré</td>
<td>2000</td>
<td>Mixed</td>
<td>1996</td>
<td>Amsterdam</td>
<td>426</td>
<td>69</td>
<td>129</td>
<td>16% 13-20</td>
<td>30% 26-35</td>
</tr>
<tr>
<td>8</td>
<td>Morré</td>
<td>1998</td>
<td>Asymp.</td>
<td>1997</td>
<td>Amsterdam</td>
<td>74</td>
<td>12</td>
<td>19</td>
<td>16% 9-27</td>
<td>26% 16-37</td>
</tr>
<tr>
<td>9</td>
<td>This study</td>
<td>2004</td>
<td>STD</td>
<td>2000-2002</td>
<td>Amsterdam</td>
<td>409</td>
<td>85</td>
<td>132</td>
<td>21% 17-25</td>
<td>32% 28-37</td>
</tr>
</tbody>
</table>
C. trachomatis serovar distribution in the Netherlands from 1986-2002

Using PubMed, EMBASE and information from the Dutch Chlamydia Society we identified 8 previous published Dutch C. trachomatis serovar distribution studies. Table 1, applying the presumptions as described earlier, summarizes all typing results by first author, by year of publication, by year of cohort collection, by known characteristics of the studied population, by total number of patient samples studied and by serogroup and serovar respectively. Figure 1 shows a graphical presentation of the results including 95% confidence intervals. In general, no statistical significant serovar distribution trends in time were observed between 1986 and 2002 when all studies were taken together. Of the 9 studies, study 1 and 6 represent serovar distributions from STD populations in Rotterdam and showed no significant changes in general or over time (mean: C-group: 30%; Int-group: 21%; B-group: 49%). Studies 2, 3, 4 and 9 represent serovar distributions from STD populations in Amsterdam and showed no significant changes either (mean: C-group: 20%; Int-group:
31%; B-group: 49%). Studies 5, 7 and 8 represent serovar distributions from mixed symptomatic and asymptomatic infected persons (5 and 7) and asymptotically infected populations in Amsterdam. They showed no significant changes in general, over time, or as compared to the Amsterdam STD based serovar distribution (C-group: 17%; Int-group: 30%; B-group: 53%). However, when the two geographically derived serovar distributions were compared to each other serogroup C was found more frequently in Rotterdam: 30 vs 19% (p<0.0001; OR 1.8 (95% CI: 1.4-2.3)), of which the most prominent serovar difference was serovar K (10.6 vs 3.2%, p<0.0001; OR 3.6 (95% CI 2.4-5.3)). The Intermediate-serogroup was found less frequently in Rotterdam: 21 vs 31% (p=0.0002; OR 1.6 (95% CI: 1.2-2.0)), of which the most prominent serovar difference was serovar F (15 vs 22%, p=0.0018; OR 1.6 (95% CI: 1.2-2.1)). Serogroup B was stable between the two cities (49% vs 50%) (Figure 2).

Figure 2: Serovar distribution studies in the Netherlands from 1986 to 2002. The time of cohort collections is shown since the year of publication can be different from the year of cohort collection. Differences in serovar distributions in time were analysed. Each study is indicated by first author, year of publication, and number of isolates included: 1, Wagenvoort1, 1998, n = 190; 2, vd Laar2, 1996, n = 372; 3, Morré3, 1998, n = 90; 4, Ossewaarde4, 1994, n = 289; 5, Lan5, 1995, n = 51; 6, v Duynhoven6, 1998, n = 305; 7, Morré7, 1998, n = 74; 8, Spaargaren, this study, n = 407. C = serogroup C (serovars H, I, Ia/I', J, Jv, K); Int = intermediate serogroup (serovars F, G, Ga); B = serogroup B (serovars D, Da, D-, E).
Discussion

In this study we could not demonstrate significant shifts in the *C. trachomatis* serovar distribution in The Netherlands from 1986 through 2002. However, we found clear stable differences in the serovar distributions between the two geographical locations from which all serovar distributions were obtained. In the Rotterdam serovar distributions as compared to the Amsterdam studies, serogroup C was significantly more prevalent with serovar K as most prominent while the intermediate group was less significant with serovar F as most prominent. Serogroup B was stable between the two cities.

To analyze serovar distribution shifts in time we first determined the most recent serovar distribution in The Netherlands in a STD clinic in Amsterdam. In this newly determined serovar distribution we showed that the serovars D, E and F comprised about 69% of all serovars typed and that members of the Intermediate serogroup and of serogroup B account for most of all the serovars diagnosed between 2000-2002 which is in agreement with other studies worldwide and in The Netherlands. Subsequently we analysed all published serovar distribution studies published in The Netherlands including this new one for shifts in time. Although the composition of the 9 studies differed considerably, i.e. asymptomatic screening population vs. gynaecological outpatient and STD population, no significant serovar distribution changes in general or over time were found (Figure 1). However, when the two geographically derived serovar distributions were compared to each significant changes were identified. The Rotterdam population differed significantly from the Amsterdam population in having a lower incidence of the Intermediate group serovars and a higher incidence of C-group serovars, albeit an identical B-group serovar distribution.

This can be explained in part by differences in study size and population composition like different ethnic compositions of the studied cohorts or other yet unknown confounding factors between Rotterdam and Amsterdam. Martin *et al.* (15) investigated the distribution of *C. trachomatis* *ompA* genotypes from the early 1980s through the mid of 1990s in New Orleans. Sequence analysis, a form of genomic comparison, of the *ompA* gene was used to study the possible emergence of new gene polymorphisms within different serovars. Also their results, shown at the ISSTDTR meeting in Berlin 2001, gave evidence that there was no or at most a slight indication for a shift of *C. trachomatis ompA* genotypes over a period of nearly 20 years in New Orleans, LA.

Since linkage of specific serovars to clinical syndromes would be promising, these results are important from an epidemiological as well as from a microbiological point of view. Serotype G has been associated with symptomatic infections and upper genital tract infections and was also associated with the development of cervical carcinoma. In addition others have shown differences between serovars, the presence or absence of clinical symptoms, specific clinical symptoms, and gender. Differences were most striking among the less frequent *C. trachomatis* serovars Ga, Ia and K: serovar Ga was associated with dysuria in men and serovar Ia was detected only in asymptomatically infected men and women. Furthermore, serovar K was associated with the
specific clinical symptom of abnormal vaginal discharge in women (16). From a microbiological standpoint it might be speculated that specific C. trachomatis serotype(s) might be more virulent than others and perhaps less sensitive to appropriate antimicrobial therapy, and could thus play a part in carcinogenesis, indicating a possible role of genotyping of the bacterium in epidemiology and public health. Experimental work in a lower genital tract animal model by Ito and Lyons (17) has shown that there are differences among serovars both in duration of infection and in the ability to establish upper tract infection. Indicating that there might be virulence factors or antigens that correlate with typing antigens in vivo. Interesting, the duration on infection in this murine model mimics exactly the prevalence of the different serovars and serogroups. Future research is needed to elucidate these differences in humans.

In conclusion, no changes in serovar distribution differences were found over time in the Netherlands in general or within the two different geographic areas. However, the Rotterdam population differed significantly from the Amsterdam populations. These findings could be due to different ethnic compositions of the studied cohorts or other confounding factors between Rotterdam and Amsterdam and further study is necessary to elucidate these differences.

References


Chapter 2
Chapter 2

Interrelationship between polymorphisms of $incA$, fusogenic properties of $Chlamydia trachomatis$ strains, and clinical manifestations in patients in The Netherlands.

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Abstract

Of 98 *Chlamydia trachomatis* isolates, two carried an *incA* with a premature stop-codon, lack IncA and were non-fusogenic, while 96 contained an intact *incA*, expressed IncA and were fusogenic. Nine IncA sequence types were found, which were randomly distributed among symptomatic and asymptomatic patients. In conclusion, IncA variation and clinical manifestations are not related.
Introduction

Chlamydia trachomatis is an obligate intracellular pathogen, responsible for sexually transmitted disease (STD). The bacteria replicate intracellularly in a membrane bound vacuole (inclusion) that is modified through the insertion of chlamydial proteins, collectively classified as Inc proteins, among them IncA (5, 11). Inc proteins are generally characterized by a typical bi-lobed hydrophobic domain of approximately 60 amino acids, but lack overall amino acid homology (1). Strains with mutations in incA, resulting into a premature stop codon, reside in multiple small homotypic inclusions, that, in contrast to inclusions containing wild type incA strains, do not fuse with each other (12). This strongly suggests involvement of IncA in the homotypic fusion of the inclusions. It has been reported, that among 11,440 isolates, only 176 (1.5%) had the non-fusogenic phenotype (16). Involvement of incA sequence polymorphisms in the clinical outcome of infection has been suggested by the observation that non-fusogenic strains were found more often among patients with less signs of infection then fusogenic strains. (3).

We have previously reported the incA sequence polymorphisms and fusogenic properties of a set of 25 C. trachomatis laboratory reference strains comprising most serovars (9). All off these laboratory strains contained an incA gene encoding for full length protein and a total of 12 amino acid sequence types (STs) were identified. The strains studied were all fusogenic and expressed IncA in the inclusion membrane (IM), irrespective of the IncA ST found (2, 9, 13).

Here, we present the incA sequence polymorphisms and fusogenic properties of 98 C. trachomatis clinical isolates from Dutch female patients. In addition, we investigated IncA expression and the relationship between IncA ST and the clinical outcome of infection.

Material and Methods

Patients and clinical specimens

Females (age range 14 to 33 years; median 22 years), visiting our Public Health Laboratory, participated after signing an informed consent. A cervical swab was taken for the detection of C. trachomatis DNA as described previously (14). A second cervical swab was placed in 0.4 M sucrose phosphate buffer (4SP) medium and used for C. trachomatis culture and isolation of DNA. Females with co-infections with other STD causing microorganisms, cultured or detected by procedures described elsewhere (2, 13, 17) were excluded. Co-infection negative females were classified symptomatic based upon at least one positive answer on a questionnaire, regarding their complaints, varying from increased discharge, having bloody discharge during and/or after coitus, abdominal pain and/or dysuria. The first 98 consecutive enrolled C. trachomatis positive females without co-infection, 45 with symptoms (46%) and 53 without symptoms (54%), were included in this study.
Laboratory methods

*C. trachomatis* was cultured in HeLa 229 cells (ATCC CCL2), harvested and stored as described elsewhere (7, 8). DNA was extracted from the 4SP collected clinical sample (7) and incA was amplified by thermocycling and sequenced as described previously (9).

Results

Sequence analysis (Staden Package version 4.7 (www.mrclmb.cam.ac.uk/pubseq/)) revealed that 96 females were infected with a *C. trachomatis* strain harbouring an incA gene encoding a putative full length protein. Among the 96 IncA sequences, nine amino acid sequence types (STs) were observed. Four STs represented 94% of the strains, indicating that incA variation is limited. These four predominant types are ST1 (identical to the prototypic sequence) (27%), ST2 (I47T) (36%), ST3 (I47T and E116K) (26%) and ST4 (I218T) (5%), respectively. ST1 to ST3, found in a frequency of 88% in this study, were also the most common STs (64%) found among the collection of reference laboratory strains, previously indicated as STa, b and c (9). ST4 was also described previously (12). In contrast, ST5 to ST9 represent novel incA alleles (Table 1).

Table 1: *C. trachomatis* IncA amino acid sequence types and the distribution among symptomatic and asymptomatic females

<table>
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<tr>
<th></th>
<th>Accession no.</th>
<th>Polymorphic amino acid sites</th>
<th>No. of females:</th>
<th>p-value^d</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Symptomatic (n=43) (%)</td>
<td>Asymptomatic (n=53) (%)</td>
</tr>
<tr>
<td>1</td>
<td>AY683465</td>
<td>M K I P Q E N T</td>
<td>11 (26)</td>
<td>15 (28)</td>
</tr>
<tr>
<td>2</td>
<td>AF683466</td>
<td>. . T . . . .</td>
<td>12 (28)</td>
<td>23 (43)</td>
</tr>
<tr>
<td>3</td>
<td>AF683467</td>
<td>. . T . . K .</td>
<td>14 (33)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>4</td>
<td>AF683468</td>
<td>. . . . . . . I</td>
<td>2 (5)</td>
<td>3 (6)</td>
</tr>
<tr>
<td>5</td>
<td>AF683469</td>
<td>L . T . . . .</td>
<td>1 (2)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>6</td>
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<td>. . T . . . S</td>
<td>1 (2)</td>
<td></td>
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<tr>
<td>7</td>
<td>AF683471</td>
<td>. . . L . . .</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AF683472</td>
<td>. . . E . . .</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AF683473</td>
<td>. R . . . . . I</td>
<td>1 (2)</td>
<td></td>
</tr>
</tbody>
</table>
The prototype amino acid sequence IncA deduced from the DNA sequence of strain D/UW-3/Cx is indicated as ST1 (15). ST1, ST2, and ST3 were previously annotated as STa, STb, and STc, respectively (9).

The GenBank accession number of the incA sequence, representative for a ST. The sequences of two strains with insertions in incA, resulting in premature stop codons, are not presented in the table but are available at GenBank through accession no. AY683474 (strain 190-163-02) and AY683475 (strain 190-059-03).

Polymorphic amino acid sites of IncA at positions 29 (M), 33 (K), 47 (I), 63 (P), 64 (Q), 116 (E), 161 (N), and 218 (T), respectively. Numbering according to the amino acid numbering of the prototype IncA sequence (15). A dot indicates amino acid identity with ST1.

P values (two-tailed) are based on chi-square analysis. NS, not significant; ND, not determined

Taken together these data, and additional incA sequences already deposited in GenBank, 19 IncA STs are now reported. Silent mutations were only observed in incA of two strains with an ST3 IncA (not shown). Two females (2%) were infected with C. trachomatis harbouring an incA gene with a mutation leading to a premature stopcodon. The incA gene, amplified of the cotton swab specimen of subject 46, had a single-nucleotide insertion at position 175, leading to a frame shift and a premature stopcodon at position 265. In addition, the incA, amplified of the cotton swab specimen of subject 64 had a 20 bp nucleotide duplication at position 54, leading to a frame shift and a premature stopcodon at position 142. We next assessed inclusion morphology and IncA expression by fluorescence microscopy of infected cells using anti-MOMP (Chlamydia direct IF identification kit, bioMérieux, sa Marcy l’Etoile, France) and anti-IncA (10). HeLa 229 cells cultured on coverslips were infected with each of the isolates at a multiplicity of infection (MOI) of 5-10. Inclusion morphology and IncA expression was independently judged by two persons, blinded for the code of the isolates. Repropagation was successful with 88 of the 98 isolates. One large single inclusion containing the bacteria was observed in infected HeLa cells with 86/88 isolates using anti-MOMP (Figure 1A). Of these 86 isolates, expression of IncA and its localization to the IM of the inclusion was demonstrated by bright fluorescent labeling of the IM of the infected cells, using anti-IncA (not shown). HeLa cell infection with the isolates of two females resulted in a completely different inclusion morphology demonstrated by fluorescence microscopy using anti-MOMP (Figure 1B and C).
Figure 1: Fluorescence microscopic analyses of HeLa cells infected with clinical isolates (MOI 5-10) containing an \textit{incA} gene encoding for full length IncA (A), or an \textit{incA} gene with insertions, leading to premature stopcodons (B,C). Immunostaining with anti-MOMP revealed a single inclusion in HeLa cells infected with \textit{C. trachomatis} isolates (n = 86), containing full length IncA, irrespective of its ST. In contrast, multiple small inclusions were observed in HeLa cells infected with strain 190-163-02 (B) or strain 190-059-03 (C) carrying an \textit{incA} with a premature stopcodon. Bar indicates 10 $\mu$m.

These isolates were phenotypically characterized by the formation and persistence of multiple inclusions in one single infected cell, indicating non-fusogenicity. IncA labelling of the IM or any other parts of the HeLa cells infected with these two isolates could not be detected (not shown).

The stability of the non-fusogenic phenotype and the lack of IncA expression by both isolates was confirmed by serial passage in HeLa cells. Decoding of the identity of the isolates revealed that the non-fusogenic isolates, lacking IncA expression, correspond to the two isolates carrying \textit{incA} genes with an insertion leading to a premature stopcodon.

Conclusions

In our study, 2% (2/88) of the isolates were non-fusogenic, consistent with the results of Suchland and co-workers in the USA (16), indicating that in the Netherlands, as well as in the USA, nonfusogenicity of \textit{C. trachomatis} isolates is a rare phenomenon. The two non-fusogenic strains described in our study were isolated from symptomatic female patients. Geisler and colleagues found that females infected with non-fusogenic strains were to have fewer signs and symptoms of infections then females infected with fusogenic strains (3, 16). The low frequency in which non-fusogenic strains occur makes it difficult to predict whether the presence or absence of IncA may lead to differences in the course of infection in our population. However, the fact that these strains are found in only 2% of the cases, while in case of chlamydial infection 50-80% of the infections run an asymptomatic course (4, 6), makes a direct relationship between these two parameters unlikely. In addition, IncA STs were randomly distributed among symptomatically and asymptotically patients (Table 1). In conclusion, a correlation between clinical manifestations in \textit{C. trachomatis} infected females and sequence polymorphisms of IncA is absent.
Sequence accession numbers

The nucleotide sequences of incA, representative for ST1 to 9, and the sequences of incA with a premature stop codon, are deposited in the GenBank under the accession numbers indicated in Table 1.

Acknowledgements

We gratefully acknowledge Daniel D. Rockey for providing us with anti-IncA serum. Wim van Est is thanked for the artwork.

References


PART 1

UROGENITAL CHLAMYDIA TRACHOMATIS INFECTIONS

1B. ENVIRONMENTAL FACTORS AND EPIDEMIOLOGICAL STUDIES
Aims and outline
ENVIRONMENTAL FACTORS AND EPIDEMIOLOGICAL STUDIES

Aims and outline of Part 1B

The course of *C. trachomatis* infections is influenced by bacterial factors, environmental factors and host factors. This section B of Part 1 will focus on environmental factors using epidemiological studies. The susceptibility to a *Chlamydia trachomatis* infection is influenced by many factors including the presence and composition of the vaginal microflora. *C. trachomatis* infections give a broad range of clinical symptoms such as flor or abdominal pain and can be caused by more than one etiologic agent. For instance, *Neisseria gonorrhoeae* and *Candida albicans* can result in similar and dissimilar symptomatology as compared to a *C. trachomatis* infection. Knowledge on the co-infection status on clinical presentation and susceptibility to infection is an essential step forward in unravelling the clear differences in the clinical course of a *C. trachomatis* infection between women.

The aim of the following studies was to study the effect of environmental factors in and on *Chlamydia trachomatis* infections using epidemiological studies on women with uncomplicated *C. trachomatis* infections (in part STD clinic based).

**Chapter 3** *Is it worthwhile, in terms of population prevalence, to screen asymptomatic persons for gonorrhoea if found Chlamydia trachomatis positive during population based screening?*

To answer this question we determined the community prevalence of gonococcal infections in *C. trachomatis* DNA positive samples obtained from a population-based screening.

**Chapter 4** *Is there an association between C. trachomatis positive women, the presence of lower abdominal pain, and the presence of serological responses to C. pneumoniae?*

Clinical manifestations of an upper genital tract infection with *C. trachomatis* is thought to occur not only due to local tissue injury but also as an outcome of (auto-) immune reactions. Murine model studies support this e.g. pulmonary *C. pneumoniae* can moderate the inflammatory response of a *C. trachomatis* infection in the upper urogenital tract. Therefore, we determined the role of *C. pneumoniae* responses in women with lower abdominal pain (not gastrointestinal or menstrual related) and a urogenital *C. trachomatis* infection.
Chapter 3
Chapter 3

Population prevalence of Chlamydia trachomatis and Neisseria gonorrhoeae in the Netherlands.

Should asymptomatic persons be tested during population-based chlamydia screening also for gonorrhoea or only if chlamydial infection is found?

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Abstract

Background
Screening and active case finding for Chlamydia trachomatis (CT) is recommended to prevent reproductive morbidity. However insight in community prevalence of gonococcal infections and co-infections with Neisseria gonorrhoea (NG) is lacking.

Methods
Nested study within a large population-based Chlamydia Screening Pilot among 21,000 persons 15-29 year. All CT-positive (166) and a random sample of 605 CT-negative specimens were as well tested for gonococcal infection.

Results
Overall Chlamydia prevalence in the Pilot was 2.0% (95% CI: 1.7-2.3), highest in very urban settings (3.2%; 95% CI: 2.4-4.0) and dependent of several risk factors. Four gonococcal infections were found among 166 participants with CT infection (4/166=2.4%; 95% CI: 0.1%-4.7%). All four had several risk factors and reported symptoms. Among 605 CT-negative persons, no infection with NG could be confirmed.

Conclusions
A low rate of co-infections and a very low community prevalence of gonococcal infections were found in this population based screening programme among young adults in the Netherlands. Population screening for asymptomatic gonococcal infections is not indicated in the Netherlands. Although co-infection with gonorrhoea among CT-positives is dependent on symptoms and wellknown algorithms for elevated risks, we advise to test all CT-positives also for NG, whether symptomatic or asymptomatic.
Background

Chlamydial and gonococcal infections are important causes of reproductive morbidity (1-3). Nucleic Acid Amplification tests (NAATs) on self-obtained specimens (urine, vaginal swabs) make it feasible to detect these infections in a very effective manner, inside as well as outside conventional clinic settings (4-6). In fact these new technologies prelude a potential revolution in our ability to control Sexually Transmitted Infections (STI). The vast majority of STI is asymptomatic or sub-clinical and these “hidden infections” are the key to persistence and ongoing transmission on a population level. Merely treatment of symptomatic cases will not be able to influence transmission dynamics significantly.

Therefore, in many countries screening or active case finding for Chlamydia trachomatis (CT) is recommended. Although information on population prevalence of CT becomes more widespread, unfortunately little information on population prevalence of gonococcal infections in the general young adult population is available. As the feasibility of combined testing increases, this lack of information hampers insight in the question whether or not to integrate testing for Neisseria gonorrhoeae (NG) in Chlamydia screening programmes. Insight in the rate of NG (co-)infections in asymptomatic persons could fuel cost-effectivity analysis and offer evidence-based information about the need for persons found positive in Chlamydia screening to be tested for NG co-infection as well. Although patients at STI clinics get a full STI screen even if asymptomatic, this is not a routine procedure in primary care. In the Netherlands health care seeking behaviour for STI is geared towards primary care, the General Practitioner (GP) addressing the majority of the STI-related problems (7).

We wanted to estimate community prevalence of NG infections and the number of dual infections in CT infected participants in a population based screening programme in the Netherlands.

Methods

A large population based Chlamydia screening was performed (2003) by inviting 21,000 persons in urban and rural areas for home-based urine testing. Design and results of this study has been described in detail elsewhere (8). In summary, this representative cross-sectional study was a stratified national probability survey according to ‘area address density’. Twenty-one thousand random-selected women and men in 4 regions, aged 15-29 years, received a home-sampling kit and a questionnaire. Urine-samples were returned by mail, pooled by 5 and tested by polymerase chain reaction (PCR Roche Diagnostic Corp., Indianapolis, IN, USA). Positive pools were individually retested. Treatment was possible via the GP, STI- or MHS-clinic. 82% of patients that were tested positive in our home-based CT screening program went to the GP for treatment. For the current research question all Chlamydia positive (n=166) and a random sample of 605 Chlamydia negative
urine specimens (out of a total 8217 negatives) were as well tested for NG infection according the manufacturer’s instructions (Roche Diagnostic Corp., Indianapolis, IN, USA). Confirmation of NG positive results was performed by detecting the cppB gene and the multicopy opa genes with a real-time PCR method using the Rotorgene instrument (9,10).

**Results**

In the initial Chlamydia Screening Study 10.610 persons responded: 11% sent in a refusal card and 41% (n=8383) participated by sending in urine and questionnaire. Non-response analysis showed a balance of high and low risk categories among participants. Details have been reported elsewhere (8,11). Overall Chlamydia prevalence was 2.0% (95% CI: 1.7-2.3); 2.5% (2.0-3.0%) in women and 1.5% (1.1-1.9) in men. Chlamydia prevalence was significantly higher in very high urbanised areas 3.2% (95% CI: 2.4-4.0) compared to rural areas 0.6% (0.1-1.1). Infection was also associated with self-reported ethnicity (especially Surinamese/Antillean 8.2% [95% CI: 3.9-12.5]), number of sex partners and symptoms. Among 166 samples of persons who tested positive for *Chlamydia trachomatis* infection, 4 gonococcal infections were diagnosed (4/166=2.4%. 95% CI: 0.1%-4.7%). Initially 9/166 were reactive, but only 4 out of 9 were positive in confirmatory PCR NG testing. All 4 persons coinfected with NG were either 17 or 18 year, 3 reported 6-10 lifetime partners (and 2 had 2-5 partners in the past 6 month). All 4 reported symptoms (lower abdominal pain, intermenstrual bleeding, dysuria) and no condom-use during last sex contact. Two reported a Surinamese-Antillean background. Main characteristics and riskfactors of the 4 dually infected persons are listed in Table 1. Among 605 Chlamydia-negative persons, no gonococcal infections were diagnosed. Initially 16/605 were reactive for NG, but none could be confirmed with the additional confirmatory test.

**Table 1**: Main characteristics and risk factors of the 4 dually infected CT-positive and NG-positive cases

<table>
<thead>
<tr>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>Female</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>17 yr</td>
<td>17 yr</td>
<td>17 yr</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td>Dutch</td>
<td>Suriname</td>
<td>Dutch</td>
</tr>
<tr>
<td><strong>Level of urbanisation</strong></td>
<td>High urban</td>
<td>Very high urban</td>
<td>Very high urban</td>
</tr>
<tr>
<td><strong>Level of education</strong></td>
<td>Unknown</td>
<td>Intermediate</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Symptoms:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermenstrual and/or postcoital bleeding</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Painfull/frequent mixturation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lower abdominal pain</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Lifetime partners</strong></td>
<td>2-5</td>
<td>6-10</td>
<td>6-10</td>
</tr>
<tr>
<td><strong>Partners previous 6 months</strong></td>
<td>1</td>
<td>1</td>
<td>2-5</td>
</tr>
<tr>
<td><strong>New partner previous 2 months</strong></td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Condom use last sexual contact</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>History of previous STI</strong></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* According to Area Address Density: very high urban (>2500 addresses/km²); high urban (1500-2500 addresses/km²); moderate urban (1000-1500 addresses/km²); low urban (500-1000 addresses/km²); rural (<500 addresses/km²). www.cbs.nl.
Conclusion

In this large national representative population based Chlamydia Screening in the Netherlands among 21,000 persons we have reported an overall CT prevalence of 2.0%. In order to gain insight in NG dual infections we retested all positive CT specimens and found a low rate of NG co-infections (2.4%; 4/166) among CT positives. We found no NG infections at all among a random subset of 605 CT negative samples, suggesting a very low community prevalence of gonococcal infections in the young adult population. Given this very low NG prevalence, general population screening for asymptomatic NG infections is not indicated in the Netherlands and targeted screening is a better and more cost-effective option. For instance, at the Amsterdam STI clinic 10% of the (heterosexual) visitors had CT infection and 2.5% GC infection; with much higher rates in MSM (CT:13% NG:14%) and in Surinamese-Antillean population (CT: 16% NG:7.6%)(12). The inequalities in rates of CT and NG in black ethnic groups are well known (13,14). We also found considerable higher CT prevalence (8.2%) among Surinamese Antillean persons in our initial CT screening, and ethnicity remained an independent variable in our prediction rule for selective CT-screening[11]. Also 2 out of the 4 positive NG participants had a Surinamese/Antillean background, which is remarkable because only 1.6% of all participants in our screening belonged to this ethnicity. NG infections are even more than CT infections concentrated within particular risk groups, within specific risk networks and entangled in specific risk environments (15). The number of reported dual infections in the literature varies considerably, from less than 1% up to more than 40% (16-18). Most studies have been performed in clinical settings among selected patient groups and often relate to the proportion concurrent CT infections in NG infected persons. This relation has been reported consistently high and justifies the policy of giving antibiotic treatment for Chlamydia at the time of Gonorrhoea diagnoses, when CT results are not available. However, the opposite – concurrent infection with NG if CT is diagnosed – is less often the case, and even less in CT cases found in home- or community - based screening programmes. In a Chlamydia screening programme in the UK, prevalence of gonorrhoea among CT-positives was 4.6% for women and 6.3% for men in STI clinics but only 0.2% for women and 1.2% for men in the CT-positives found via community screening (19). In the US, a nationally representative prevalence study, found a CT prevalence of 4.2% and a low infection rate for NG (0.43%) and prevalence of co-infection was only 0.3% (20). Substantial racial/ethnic disparities in prevalence of both infections were reported. Some regional home surveys in the US reported substantially higher NG prevalence (5.3% from the Baltimore’s household survey, and 3.9% in San Francisco) (5).

We could not confirm the majority of our initially positive NG results as true positives. Certain strains of Neisseriaceae, considered as commensal organisms and Lactobaccillus species are known to produce false-positive results. This underlines once more the necessity of confirmatory testing in a screening programme, with a test that is more specific and at least as
sensitive (9,10). The few persons tested positive for NG in our study were all young women (17, 18 year) with a high risk profile ( > 6 lifetime partners, no condom use during last sex and two had Surinamese/Antillean ethnicity). All reported in the questionnaire subjective complaints. This means that these patients, who came to the doctor for their treatment for the CT infection detected by home-based screening, are in fact entitled for a STI screen according to current guidelines and algorithms (symptomatic patients with a risk profile should be tested both for CT and NG). This suggests that even in participants who turn out CT-positive in a population screening programme in a low prevalence area, a routine NG screen would not be required if proper risk-assessment is made by the physician to tailor further need for a full STI screen. However, risk assessment in primary care is not always optimal and discussing sexual health in GP is not always easy, not for the doctor, nor for the patient (21). We would argue therefore that pursuing in primary care the old paradigm: “always look for another STI if one STD is found” would be most practical. However, cost-effectiveness of such a strategy would depend very much on regional STI epidemiology. As integrated combo-tests for diagnosing CT, NG, but also for Trichomonas, Mycoplasma, and even HIV might become within reach in the near future, and incremental costs for testing for these additional STI will become more favourable from a cost-effective point of view, special consideration should be paid to the potential negative side-effects and the enhanced likelihood of false-positive results if screening takes place in very low prevalence settings (22).

**In conclusion:** Based on our results, population screening for gonococcal infections is not indicated in the Netherlands. NG co-infection in persons who tested Chlamydia positive in population screening programmes in young adults is dependent on symptoms and well known algorithms for elevated risks. Routine screening for dual infections in CT-positive participants is still recommended, whether symptomatic or asymptomatic. Compared to other countries, the Netherlands still has a low burden of STI.

**Acknowledgements**

Prof. PJE Bindels, AJP Boeke MD PhD, Prof. JDF Habbema, JAR van den Hoek MD PhD, L Jacobi MSc, SA Morré PhD were scientific advisors (scientific advisory board). This study was financed by a grant of ZonMw (the Netherlands Organisation for Health Research and Development).
References


12. **Fennema, J.S.A.** Annual report 2002 STI-clinic Municipal Public Health Service Amsterdam, 2003, Amsterdam, The Netherlands


14. **Aral, S.O.** Understanding racial-ethnic and societal differentials in STI. Sex Transm Infect. 2002 Feb;78(1):2-4


18. **Centers for Disease Control and Prevention.** Sexually Transmitted Infections guidelines 2002.


Chapter 4

Association of antibody response to *Chlamydia pneumoniae* with lower abdominal pain in Dutch Caucasian women with urogenital *Chlamydia trachomatis* infection

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Submitted
Abstract

Clinical manifestations of an upper genital tract infection with *Chlamydia trachomatis* is thought to occur not only due to local tissue injury but also as an outcome of (auto-) immune reactions. Murine model studies support this notion; e.g. pulmonary *C. pneumoniae* can moderate the inflammatory response of a *C. trachomatis* infection in the upper urogenital tract.

The aim of this study was to investigate the potential association between *C. trachomatis* positive women, the presence of lower abdominal pain and the presence of serological responses to *C. pneumoniae*.

As part of a prospective study of Dutch Caucasian women (n=500) visiting the STD outpatient clinic in Amsterdam, the presence of *C. trachomatis* in the urogenital tract was assessed by PCR (Roche) and sera were collected for analysis of IgG antibodies against *C. pneumoniae* and *C. trachomatis* at the time of consultation. Questionnaires were filled regarding their self reported complaints including recent lower abdominal pain (not gastrointestinal or menses related).

From the 170 *C. trachomatis* DNA positive women 152 had IgG antibodies against *C. pneumoniae* and from those 22% reported lower abdominal pain while from the 18 women without antibodies to *C. pneumoniae* none reported LAP (p=0.025, OR 10.4).

In conclusion, *C. pneumoniae* infection seems to be associated with the presentation of lower abdominal pain in women with a PCR confirmed urogenital *C. trachomatis* infection. These data provide new insight in the immunopathological mechanisms of *C. trachomatis* associated symptomatology.
**Introduction**

Upper genital tract infection with *Chlamydia trachomatis* could eventually result in fallopian tube damage which is thought to occur not only due to direct tissue damage from infection, but also due to immunologic chronic inflammation and auto-immune reactions (1,2). Why only part of the women with a urogenital *C. trachomatis* infection actually develops late complications is still largely unknown but risk factors including previous or repeated infections and the presentation with symptoms, especially lower abdominal pain, have been described (3,4). Nakabe and colleagues showed, in a murine model, that it is possible that different species within the genus Chlamydiae, specifically respiratory acquired *C. pneumoniae* (intranasal administration), can be detected in remote organs, contributing to an enhanced inflammatory process in the fallopian tubes of female mice with a *C. trachomatis* infection (5).

Interestingly, a recent publication by Price and colleagues showed that intranasal administration of recombinant *Neisseria gonorrhoeae* transferring binding proteins A and B conjugated to a Cholera Toxin B subunit, induced systemic and vaginal antibodies in mice (6). Intranasal immunization resulted in both IgA and IgG in the genital tract. However, subcutaneous immunization mainly generated IgG. Also without the Cholera Toxin B subunit the *N. gonorrhoeae* transferring binding proteins were immunogenic and induced serum and mucosal responses in the urogenital tract. The data from both Nakabe (5) and Price (6), though in different aspects dissimilar, show the potential relevance of systemic and remote local immune responses after nasally acquired proteins for microorganisms in the urogenital tract and the course of their infection and the subsequent inflammation.

Combining the work from Nakabe and colleagues showing the presence of *C. pneumoniae* proteins in the upper genital tract of mice, the fact that respiratory *C. pneumoniae* infections can disseminate to remote tissues and organs and the recent findings from Gijsen et al. (7) who reported that tubal pathology was more common in patients who had antibodies to both *C. trachomatis* and *C. pneumoniae*, it seems that *C. pneumonia* could potentially contribute to the course and symptomatology of urogenital *C. trachomatis* infections in women.

To elucidate the role of *C. pneumoniae* in women with lower abdominal pain (not gastrointestinal or menstrual related) and a urogenital *C. trachomatis* infection we collected sera from female attendants of our STD outpatient clinic in Amsterdam with a proven urogenital *C. trachomatis* infection (PCR based), to assess if serological IgG responses against *C. pneumoniae* were linked to the presentation with lower abdominal pain.
Patients and Methods

Patients

Hundred and seventy C. trachomatis positive women of Dutch Caucasian origin, under the age of 33 (range 14 to 33 years; median 22 years and visiting the STD outpatient clinic in Amsterdam, The Netherlands, were included in this study (collection period: July 2001 – April 2003). These 170 women were part of a cohort of 500 women whom were consecutively included as the first part of a large prospective study. For every C. trachomatis-DNA positive woman (see section C. trachomatis DNA detection) 2 consecutive C. trachomatis-DNA negative controls were included in the prospective study on the same day. The women were asked to sign an informed consent and to fill out a questionnaire, regarding their urogenital complaints, varying from increased discharge, having bloody discharge during and/or after coitus, recent lower abdominal pain (not gastrointestinal or mensis related) and/or dysuria.

Chlamydial detection by PCR and serology

A cervical swab was taken for the detection of C. trachomatis DNA (CT-DNA) by PCR (COBAS AMPLICOR; Hoffman – La Roche, Basel, Switzerland) (8). In addition, from all C. trachomatis positive samples, serovars were assessed by PCR-based restriction fragment length polymorphism (RFLP) analysis targeting the outer membrane protein gene (ompA) as described previously to confirm true C. trachomatis positivity (9). Peripheral venous blood was collected for the analysis of IgG antibodies against C. pneumoniae and C. trachomatis (Medac Diagnostika mbH, Hamburg, Germany). A titer of both ELISA’s of ≥ 1:50 was considered positive. Samples with greyzone values, e.g. cut off ± 10%, were repeated and considered positive when the result was positive or again within the greyzone. N. gonorrhoeae was detected according to the methods described previously to correct for this bacterial infection which can also cause lower abdominal pain (10).

Statistical analysis

The incidence of lower abdominal pain in women with C. trachomatis DNA was compared between women with and without a C. pneumoniae serological IgG response. Women with a N. gonorrhoeae infection were excluded in the analysis since N. gonorrhoeae is directly related to the presentation of lower abdominal pain and thus could be a potential confounding factor in the study. For specificity purposes the C. pneumoniae IgG responses were compared between the C. trachomatis DNA and C. trachomatis IgG serological positive and negative women. Two-by-two tables (Chi-square test or Fisher’s exact test when appropriate) were used to compare groups. Differences were considered statistically significant if p<0.05 and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Statistical analysis were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA).
Results

*C. trachomatis* DNA detection
None of the 170 women who were *C. trachomatis* DNA positive were also positive for *N. gonorrhoeae*. All 170 *C. trachomatis* DNA positive women in the Roche PCR could be typed for the *C. trachomatis* serovar (confirming true *C. trachomatis* positivity) using a PCR-based RFLP analysis of the *ompA* gene: C-group 17% (serovars H, I, Ia, J, and K), Intermediate group 30% (serovars F, G, and Ga) and the B-group 53% (serovars B, E, D, Da, and D').

Chlamydial serology
The seroprevalence of *C. trachomatis* was 68.2% among the *C. trachomatis* DNA positive women, while 152 (89.4%) women had IgG antibodies against *C. pneumoniae*. The prevalence of *C. pneumoniae* antibodies did not differ between women with or without *C. trachomatis* IgG responses.

Chlamydial infection and lower abdominal pain
Data regarding the presence or absence of lower abdominal pain and IgG antibodies to *C. pneumoniae* in relation with *C. trachomatis* DNA positivity are presented in Table 1. Lower abdominal pain was only reported in women with a serological response to *C. pneumoniae*: 22% (33/152) versus 0% (0/18), p=0.0255; OR 10.4. The median age and range distribution in the *C. pneumoniae* positive and negative women was similar (23 versus 22 years) as was the case for the women with serological IgG responses to *C. pneumoniae* and a positive PCR for the presence of *C. trachomatis* when comparing those with or without lower abdominal pain (23 versus 23 years) (Table 1).

Table 1: Relationship between the presence of lower abdominal pain (LAP) in *C. trachomatis* DNA positive women in relation to *C. pneumoniae* IgG serological responses

<table>
<thead>
<tr>
<th>Group</th>
<th>Total (n)</th>
<th>Age (years) Median (range)</th>
<th>LAP n (%)</th>
<th>Age (years) Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT(+) CP(+)</td>
<td>152</td>
<td>23 (16-32)</td>
<td>33 (22)</td>
<td>23 (17-30)</td>
</tr>
<tr>
<td>CT(+) CP(-)</td>
<td>18</td>
<td>22 (16-31)</td>
<td>0 (0)</td>
<td>--</td>
</tr>
</tbody>
</table>
Chapter 4

1 CT(+): presence of *C. trachomatis* DNA as assessed by commercially available nucleic acid amplification assays
2 CP(+) CP(-): presence (+) or absence (-) of *C. pneumoniae* IgG responses
3 LAP in the CT(+)/CP(+) versus the CT(+)/CP(-) group: p=0.025, OR 10.4

Discussion

We showed for the first time that the presence of IgG antibodies against *Chlamydia pneumoniae* may be associated with the presentation of lower abdominal pain in women with a proven urogenital *Chlamydia trachomatis* infection as assessed by the presence of *C. trachomatis* DNA.

Different potential confounding variables for our finding are discussed below. Our results were not confounded by potential serologically cross reactivity between IgG responses against either *C. pneumoniae* or *C. trachomatis* as shown by the identical responses in *C. trachomatis* DNA or IgG positive and negative women (11). In our study the IgG responses against *C. pneumoniae* in *C. trachomatis* positive women was 89.4% (see results) which is identical to the IgG responses against *C. pneumoniae* in *C. trachomatis* negative women: 85.2% (data not shown). We checked *C. trachomatis* DNA positive women for a potential coinfection with *N. gonorrhoeae* since *N. gonorrhoeae* can also cause symptoms of lower abdominal pain, we did so to strengthening the relation between lower abdominal pain and *C. trachomatis* as the causative agent. We could genotype all *C. trachomatis* positive samples for epidemiological purposes (not shown in detail in this manuscript) by restriction fragment length polymorphism indicating that all *C. trachomatis* by PCR were true *C. trachomatis* positive samples. The age distribution was assessed in the *C. pneumoniae* positive and negative women to address the fact that older age is associated with a higher *C. pneumoniae* IgG prevalence due to the cumulative exposure risk (12) and may thus be potentially be linked to lower abdominal pain, however, this was not the case. Since we used short specific questionnaires, the self-reported symptom score lower abdominal pain, has been assessed reliable, however, due to medical ethical restrains we were not allowed to used physician based clinical assessment of lower abdominal pain. Finally, we should realize that the prominent effect of the lack of *C. pneumoniae* IgG antibodies and the subsequent absence of lower abdominal pain is an effect that is seen in 18 out of 170 *C. trachomatis* DNA positive women, indicating that larger studies are needed to confirm the results obtained.

Although all women were consistently evaluated and they underwent a systematic detailed history, which was recorded on standardized forms, we did not include questions on previous *C. trachomatis* infections and can therefore not address the issue whether proceeding or repeated infections and/or probable longer duration of urogenital chlamydial infections and/or reduced clearance of the bacterium may have influenced the results. However, as mentioned above the presence of serological IgG responses to *C. trachomatis* was no confounder. In our study we focused specifically on women from Dutch Caucasian ethnicity. This was done since ethnicity can be related to the susceptibility to
infection, although for chlamydial infection this topic has not been addressed in detail (13-15), but in our study we can exclude ethnicity issues as a confounder for our findings.

If we review literature no relation between *C. pneumoniae* and *C. trachomatis* responses is described in a STD population. However, Gijssen and co-workers (7), have shown that, although the prevalence *C. pneumoniae* antibodies was equally distributed among patients with and without laparoscopically proven tubal pathology, tubal pathology was significant more frequent in patients in whom both antibodies to *C. pneumoniae* and *C. trachomatis* were present. So their findings also suggest a role for *C. pneumoniae* in the development of late upper genital tract disease and pathology. Previously, Nakabe *et al.* (5) showed that *C. pneumoniae* may trigger autoimmune reactions in the urogenital tract by first inoculating BALB/C mice directly in the fallopian tubes with *C. trachomatis* and subsequently nasal inoculations with *C. pneumoniae*. Immunostaining of the fallopian tubes showed that salpingitis was associated with nasal *C. pneumoniae* inoculation in the *C. trachomatis* inoculation group.

Although the studied groups were small it indicates that once a urogenital infection is established, a protein similar to *C. trachomatis* remains in the tissue and that immunologic inflammatory tissue damage may occur when other organs are infected by microorganisms of the same genus.

The immunopathogenic role of *C. pneumoniae* in lower genital tract disease remains to be elucidated. In recent years fallopian tube damage in *C. trachomatis* infection is thought to occur not only due to direct tissue damage from infections, but also due to immunologic chronic inflammation, repeated infections and autoimmune reactions (4). Priming and/or molecular mimicry are thought to play an important role in the development of late complications (2). Therefore transgenic mouse models have to be used to address this issue, including both combinations of active and previous *C. pneumoniae* and/or *C. trachomatis* infections and the effect of IgA and IgM responses. In addition, since the results are obtained in a specific high-risk group of Dutch Caucasian woman attending a STD clinic other settings and women from other ethnicity should be studied.

In conclusion, *C. pneumoniae* infection seems to be associated with women presenting with lower abdominal pain and a urogenital *C. trachomatis* infection. This interesting phenomenon sheds new light on the immunopathogenesis of urogenital *C. trachomatis* infections.

References


PART 1

UROGENITAL *CHLAMYDIA TRACHOMATIS* INFECTIONS

1C. HOST SEROLOGICAL RESPONSES AND HOST IMMUNOGENETIC FACTORS
Aims and outline
HOST SEROLOGICAL RESPONSES AND HOST IMMUNOGENETIC FACTORS

Aims and outline of Part 1C

The course of *C. trachomatis* infections is influenced by bacterial factors, environmental factors and host factors. This section C of Part 1 will focus on the influence of host factors in the susceptibility to and severity of *C. trachomatis* infections. Two types of host responses are studied in this Part 1C, serological responses to infection with *C. trachomatis* and the influence of functional genetic variation in genes potentially modulation infection. Serological responses are studied in general by looking at IgG, however since heat shock proteins (HSP), especially hsp60, between humans and Chlamydia are alike resulting in autoimmune reaction, hsp60 serology has been developed recently and could be of clinical relevance. Genetic variation could influence the inflammatory response modulated through NF-kB activation. Interleukin-1 receptor antagonist as well as the Toll-like receptors (TLRs) which recognize components of the infectious agents, can trigger an inflammatory cascade that results in the expression of the array of cytokines, chemokines and other molecules.

The aim of the following studies was to investigate host immune system in order to get insight in the clear difference in the clinical course of infection between infected women. Both women with uncomplicated *C. trachomatis* infection (those attending a STD clinic) and women with late complication (those women attending a gynaecology or subfertility clinic) were included in our studies.

**Chapter 5** Do *C. trachomatis* heat-shock-protein 60 antibodies have a link with the severity of late complications caused by this bacterium?

To answer this question we determined the cHSP60 and IgG antibody responses in women with and without confirmed tubal pathology and a group of pregnant women.

**Chapter 6** Does functional genetic variation in the Interleukin-1 receptor antagonist play a role in the course and outcome of *C. trachomatis* female genital tract infection?

We used immunogenetic analyses (candidate gene approach) in women with a recent urogenital tract infection with *C. trachomatis*, to determine if the IL1RN gene was associated with the susceptibility to infection.
Chapter 7 Does genetic variation in the LPS-sensing TLR4 co-receptor CD 14 play a role in the course and outcome of C. trachomatis female genital tract infection?

The Toll-like receptors enables the innate immune system to detect the presence and the nature of the pathogen and provides the first elements to control the subsequent immune responses. The aim of this study was to determine if the CD14-260 C>T SNP influenced the susceptibility to infection (STD cohort) and/or the severity of a C. trachomatis infection (subfertility cohort).
Chapter 5

*Chlamydia trachomatis* heat shock protein 60 (cHSP60) antibodies in women without and with tubal pathology using a new commercially available assay

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Chapter 5

Abstract

A commercially available cHSP60 serological assay was used to determine the anti-cHSP60 responses in three gynaecologically well defined groups of women. The results indicate, although obtained in small groups of patients, that this assay clearly makes distinctions between the severity of the tubal pathology and can be used in practice.
Introduction

Besides commercially available serological assays that detect antibodies to major outer membrane protein (MOMP) and lipopolysaccharide (LPS) "in-house" chlamydial heat shock protein 60 (cHSP60) assays are extensively used in assessing serological responses to urogenital Chlamydia trachomatis infection. Although comparison of the different "in-house" assays is difficult owing to a lack of standardisation, there is a consensus among the users of these assays that the anti-cHSP60 responses in women increase with the severity of C trachomatis associated disease, leading to the suggestion that the high amino acid sequence homology between chlamydial and human HSP60 results in autoimmune mediated fallopian tube damage. Owing to the significance of the possible association of the response to cHSP60 and progressive disease, a commercially produced assay that employs defined cHSP60 epitopes should allow for the comparison of results obtained in different laboratories, as well as forward the use of cHSP60 as a diagnostic tool if the assay proves to be relevant in predicting pathology or clinical outcome of a urogenital chlamydial infection.

Materials and Methods

This study evaluated a recently introduced commercially available cHSP60 serological assay and determined the anti-cHSP60 responses in three gynaecologically well defined groups of women. Group 1 consisted of women without tubal pathology as assessed by either hysterosalpingography or laparoscopy (n = 21), group 2 consisted of pregnant women (unknown tubal status, proved fertility; n = 86), and group 3 consisted of women with confirmed (based on hysterosalpingography or laparoscopy) tubal pathology (n = 11). C trachomatis positivity was assessed previously using one of the following serological assays: microimmunofluorescence (MIF) (BioMérieux’s Hertogenbosch, Netherlands), BAG Chlamydia EIA (Biologische Analysensystem GmbH, Lich, Germany) and the CT-pELISA (Medac, Wedel, Germany). The study groups and techniques were described previously. The cHSP60 assay (Medac, Wedel, Germany) was performed according to the manufacturer’s instructions.

Results

Results are shown in Figure 1. C trachomatis IgG positivity was previously determined to be 19% for group 1, 40% for group 2, and 64% for group 3, showing the expected clear difference in IgG seroprevalence between women with and without procedure confirmed tubal pathology, while an intermediate prevalence observed in pregnant women. The same pattern but with lesser incidence was observed in the anti-cHSP60 responses being 4.8%, 16%, and 27%, for groups 1–3, respectively.
(χ² test for trend: χ² = 3.1, p = 0.079, group 1 v group 3: p = 0.096, OR 10.6). The incidences of anti-cHSP60 were increased in the CT IgG positive subgroups to 25%, 35%, and 43%, for groups 1–3, respectively, while only 3.8% anti-cHSP60 titres were observed in the C trachomatis IgG negative subgroups, all in subgroup 2 (unknown tubal status, proved fertility). This indicates that the concordance between CT IgG and cHSP60 positivity is high, almost 90%; however, clearly a different subgroup of women is identified by the cHSP60 assay since only 40% of the C trachomatis IgG positive women has a cHSP60 response (measurement of agreement: kappa 0.371). Finally, the median cHSP60 titres increased from groups 1–3: 50, 100, and 200, respectively, suggesting an association between the level of cHSP60 response and tubal pathology.

Figure 1: Chlamydia trachomatis IgG and cHSP60 antibody responses in Dutch white women with different degrees of tubal pathology.

Conclusion

As far as we know this is the first study evaluating the commercially available cHSP60 assay in women with different degrees of tubal pathology. Two abstracts were published in the ISSTDR meeting Vienna, Austria in 2002 on cHSP60 antibodies in women with pelvic inflammatory disease (85% in patients with C trachomatis positive swabs and patients with occluded tubes, 20% in blood donors) and in women with open or occluded fallopian tubes (31% and 70% respectively).

The standardisation provided through this new commercially available assay will potentially enhance the comparability of cHSP60 results between laboratories. The results presented here, although obtained in small but well defined groups, look suggestively promising. Indeed, power calculations (alpha = 0.5, beta = 0.1) show that doubling (1.7 times) the size of the (sub)groups would results in significant p values instead of clear trends. However, further studies are needed in larger groups with different degrees of pathology because of C trachomatis infections to further determine the diagnostic, prognostic, and clinical relevance of this new assay.
References


Chapter 5
Chapter 6

The first strong genetic susceptibility marker for *Chlamydia trachomatis* infections: the interleukin 1 receptor antagonist IL-1RN +2018 T>C gene polymorphisms

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\(^\S\)On behalf of the ICTI consortium

Submitted
Abstract

*Chlamydia trachomatis* is the most prevalent sexually transmitted disease with clearly different courses of infection in different infected patients. No clear relations have been identified between bacterial factors and susceptibility to disease and evidence for the influence of host genetic factors on *C. trachomatis* pathogenesis is just emerging. The *IL-1RN*+2018 T>C polymorphisms is strongly reduced (p: 0.0005, OR: 1.5) in women with urogenital *C. trachomatis* infections and may indicate a potential therapeutic target.
Introduction

About half of all deaths and diseases are associated with preventable behaviours and avoidable exposures, which would seem to leave the remainder attributable to genetic influences. Confirming this almost certainly oversimplified subtraction and filling in the details that will be of practical clinical value is the task that genetic epidemiology is addressing (1). For reasons mainly of statistical power recruitment of large samples, genetic epidemiology is moving away from linkage studies based on families to allelic association studies based on unrelated individuals. The biology underpinning genetic epidemiology offers a potential useful way to study environmental determinants including infectious agents in disease without residual confounding (2).

*Chlamydia trachomatis* is the most common sexually transmitted bacterial infection strongly associated with tubal infertility. The course of *C. trachomatis* infection is quite different in different individuals: only part of the people gets infected and only part of those infected develops more severe disease. Although both environmental, bacterial and host factors are known to be involved in disease course, no clear bacterial virulence factors have been identified in relation to disease susceptibility and the first relevance of genetic variation in host factors have been published recently (3,4). For instance, the *CD14* -260 C>T polymorphism is not associated with *C. trachomatis* infection or it’s late complications (3), while specific *HLA* and *IL-10* genotypes have been associated with *C. trachomatis* infection and late sequelae (4) and twin studies showed a 40% genetic predisposition (7). The IL-1 receptor antagonist (IL-1RN) is the natural inhibitor of the proinflammatory cytokine IL-1 and plays an important role in the regulation of inflammation, infection, and immune responses. We studied the *IL-1RN* +2018 T>C polymorphism in relation to the susceptibility to *C. trachomatis* infections.

Participants and methods

Twelve hundred and thirteen Dutch Caucasian women visiting the STD outpatient clinic in Amsterdam, The Netherlands were included in this study. The cohort (as described previously (3)) consisted of 748 *C. trachomatis* DNA positive women and 465 *C. trachomatis* DNA negative women. Analyses for the presence of *Candida albicans*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, and Herpes simplex virus type 1/2 were included in our analyses since infections with these microorganisms may also result in symptoms similar to a *C. trachomatis* infection. The functional *IL-1RN* +2018 T>C polymorphism was determined using TaqMan analysis. Fisher’s exact and $\chi^2$ tests were used when appropriate. A p-value <0.05 was considered significant.
Chapter 6

Results

The carriage of the IL-1RN2 allele was significantly decreased in CT DNA positive women (39%) compared to CT DNA negative women (50%) ($p: 0.0005$, OR: 1.5, 95% Confidence interval: 1.2 – 1.9). When CT serology was introduced, similar results were found. The IL-1RN2 allele was significantly reduced in CT DNA positive / CT IgG positive women (41%) when compared to CT DNA negative / CT IgG negative women (52%) ($p: 0.0144$, OR: 1.6, 95% confidence interval: 1.1 – 2.3). The results are summarized in Figure 1. Introduction of coinfection status or symptomatology did not alter the results.

![IL1RN +2018 T>C](image)

**Figure 1: IL-1RN genotype distribution.**
Depicted on the left is the distribution in the cohort, divided in CT DNA positive and CT DNA negative.
Depicted on the right is the IL-1RN genotype distribution for the samples with CT serology. The bars represent the IL-1RN distribution between CT DNA positive and CT DNA negative women. The differences in colours represent the relative distribution of IgG positivity and negativity, with each bar representing 100%. The figures next to the bars represent the relative percentage of IgG positive women in that group.

Comment

We have shown that the carriage of the IL-1RN2 allele is significantly reduced in women with *C. trachomatis* infection.
This allele has previously been associated with increased expression of IL-1ra and an anti-inflammatory immune response. Previous studies have shown that the carriage of the IL-1RN2 allele is associated with protection against infection related pre-term birth (5). A previous study of our group did not show an association between IL-1RN and the late complication of C. trachomatis infection, tubal pathology (6). This indicates that genetic variation in IL-1RN might protect against primary C. trachomatis infections but may not influence strongly the development of late complications, reflecting complex underlying pathogenic mechanisms, and indicating the potential existence of other factors influence to risk tubal pathology. From these data it can be hypothesized that stimulation of IL-1ra might be used as a potential treatment in patients infected with C. trachomatis and inhibition of IL-1ra may reduce infection related pre-term births (5).

Although further studies are required, the identified defects in different molecular signalling pathways will provide extensive insight in the individual differences in the human immunopathogenesis of C. trachomatis disease, knowledge which will be translated to improve infectious diseases and health care issues.

References


Chapter 7

The CD14 functional gene polymorphism –260 C>T is not involved in either the susceptibility to *Chlamydia trachomatis* infection or the development of tubal pathology

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Chapter 7

Abstract

Objectives: The functional polymorphism -260 C>T in the LPS sensing TLR4 co-receptor CD14 gene enhances the transcriptional activity and results in a higher CD14 receptor density. Individuals carrying the T/T genotype also have significantly higher serum levels of soluble CD14. The T allele of this polymorphism has recently been linked to *Chlamydia pneumoniae* infection.

Aim: To investigate the role of the CD14 –260 C>T polymorphism in the susceptibility to and severity (defined as subfertility and/or tubal pathology) of *C. trachomatis* infection in Dutch Caucasian women.

Methods: The different CD14 –260 C>T genotypes were assessed by PCR-based RFLP analysis in three cohorts: 1) a cohort (n=576) of women attending a STD clinic, 2) a cohort (n=253) of women with subfertility, and 3) an ethnically matched control cohort (n=170). The following variables were used in the analysis: In cohort 1 the CT-DNA status, CT IgG serology status, self-reported symptoms and in cohort 2, the CT IgG serology status and the tubal status at laparoscopy.

Results: In the control cohort the CC, CT and TT genotype distribution was: 28.2%, 48.2%, and 23.5% respectively. No differences were found in the overall prevalence of CD14 –260 genotypes (28.1%, 50.7%, and 21.2%) in cohort 1 when compared to the control cohort. Also no differences were observed in women with or without CT-DNA, with or without serological CT responses, with or without symptoms, or in combinations of these three variables. In subfertile women with tubal pathology (cohort 2, n=50) the genotype distribution was 28.0%, 48.0%, and 24.0% and in subfertile women without tubal pathology (n=203), 27.6%, 49.3% and 23.2%. The genotype distribution was unchanged when CT IgG status was introduced in the analyses.

Conclusions: The CD14 –260 C>T genotype distributions were identical in all three cohorts, showing that this polymorphism is not involved in the susceptibility to or severity of sequelae of *C. trachomatis* infection.
Introduction

Chlamydia species are related to a broad clinical spectrum of human disease including *Chlamydia pneumoniae* in lung and cardiovascular disease, *C. psittaci* in pulmonary emphysema and psittacosis, and *C. trachomatis* in ocular and urogenital infections (1-3).

*C. trachomatis* is the most prevalent sexually transmitted disease in Europe and the USA. Due to the mostly asymptomatic course of infection, these women will most likely not be treated resulting in an enhanced risk for the development of late complications, which include pelvic inflammatory disease (PID), ectopic pregnancy and tubal infertility.

The female reproductive tract is a very complex system where many factors, including hormones, vaginal flora and immune mediators, combine to provide protection on the one hand, while on the other hand maintaining an environment suitable for conception (4). Clear differences in the clinical course of infection have been described and are due to an interaction between environmental (e.g. co-infection), bacterial (e.g. virulence factors) and host factors (genetic differences between individuals).

In previous studies no clear associations have been demonstrated between *C. trachomatis* serotype, *C. trachomatis* genotype, and the course of *C. trachomatis* infection (5,6), although differences in cytotoxicity for different serovars have been described (7) and an association between *C. trachomatis* serovar G and cervical squamous cell carcinoma has been suggested (8). In addition, virulence gene expression studies, and genomic comparisons of strains, isolated from clearly symptomatic or asymptomatic infected persons, revealed no strong role for the *C. trachomatis* bacterium in relation to the course of infection (9,10).

A limited number of studies have recently demonstrated the influence of host genetic factors on the susceptibility to and the severity of *C. trachomatis* infection. Host factors including HLA-DQ and interleukin 10 (IL-10) have been associated with Chlamydia infection (11). The Toll Like Receptor (TLR) family is a group of pattern recognition receptors, which recognise several microbial products, including bacterial cell wall components and DNA (12). Poltorak et al. associated TLR4 with lipopolysaccharide (LPS) recognition in mice (13). Further studies in mice corroborated these data (14,15), while studies in humans demonstrated associations between TLR4 mutations and LPS hyporesponsiveness (16). We did not observe an association between the TLR4 Asp299Gly polymorphism in patients with tubal pathology although the study population was relatively small (17). The lack of association can be explained by recent publications showing that heterozygous carriage of the TLR4 Asp299Gly mutation does not affect LPS responsiveness and that only the rare homozygous carriers are less responsive to LPS (18). CD14 acts as a co-receptor for TLR4 and confers responsiveness to LPS, a component of the cell wall of most Gram-negative bacteria. CD14 forms a complex with LPS and the LPS-binding protein (LBP) (Figure 1) (19).
Figure 1: CD14 localisation.
Panel A: Membrane-bound CD14 (mCD14) complexed with TLR4 and the LBP – LPS complex. Panel B: Soluble CD14 (sCD14). Abbreviations: TLR: Toll-Like Receptor; LBP: LPS Binding Protein; LPS: Lipopolysaccharide; NF-κB: Nuclear Factor κ B.

Combined with TLR4 this complex induces NF-κB associated immune responses including the release of a broad spectrum of cytokines that include tumour necrosis factor alpha (TNF-α), IL-1, IL-6, and IL-8 to initiate immune response (20). The promoter region of the CD14 gene contains a single nucleotide polymorphism (SNP) at position -260. The -260 C>T genetic variation affects the binding of transcription factors (21) and has been associated with levels of sCD14 and inversely associated with serum IgE levels (20). This SNP has been associated myocardial infarcton (22), Crohn’s disease (23) and an increased susceptibility to develop chronic spondyloarthropathy in women (24). Eng et al. demonstrated that carriers of the T allele of this promoter polymorphism have a higher expression of both mCD14 and sCD14 and that TNFα production is increased in the homozygous CD14 -260 T carriers when stimulated with either C. pneumoniae or C. trachomatis (25). In a recent article, Rupp and colleagues described an association between the mutant allele and an increased susceptibility to chronic C. pneumoniae infection in coronary artery disease patients (26). Since the CD14 -260 C>T is functional (25) and is associated with C. pneumoniae infection (26), one could hypothesize that in C. trachomatis infection this polymorphism could influence the susceptibility to and severity (defined as subfertility and/or tubal pathology) of C. trachomatis infection in Dutch Caucasian women.

A cohort of women attending a STD clinic was used to assess the susceptibility to C. trachomatis infection, taking into account both C. trachomatis DNA and C. trachomatis IgG detection, symptoms
and coinfections. A cohort of subfertile women with or without clinically well-defined tubal pathology was used to assess the role of CD14 in the severity of sequelae of *C. trachomatis* infection.

**Material and Methods**

**Patient populations**

**STD cohort**

Women of Dutch Caucasian (DC) origin (n=576), under the age of 33 (range 14 to 33 years; median 22 years) and visiting the STD outpatient clinic in Amsterdam, The Netherlands, were included in this study (collection period: July 2001 – December 2004) (Table 1).

**Table 1**: Patient characteristics in the STD and subfertility cohorts.

| Abbreviations: CT: *C. trachomatis*; STD: sexually transmitted disease; TP: tubal pathology |
|---------------------------------|---------------------------------|---------------------------------|
| **STD cohort**                  | **Subfertility cohort**         |
| n                              | 576                            | 253                            |
| CT DNA (LCx)                   | +                              | 184                            |
|                                 | -                              | 392                            |
| CT IgG                         | +                              | 217                            |
|                                 | -                              | 359                            |
| CT IgG (MIF)                   | + (>32)                        | 41                             |
|                                 | - (<32)                        | 212                            |
| Coinfections                   | -                              | 401                            |
|                                 | +                              | 175                            |
|                                 | *C. albicans*                  | 160                            |
|                                 | *N. gonorrhoea*                | 7                              |
|                                 | *T. vaginalis*                 | 6                              |
|                                 | H. simplex virus 1             | 2                              |
|                                 | H. simplex virus 2             | 5                              |
| Symptoms                       | -                              | 335                            |
|                                 | +                              | 221                            |
|                                 | Vulvovaginal discharge         | 157                            |
|                                 | Abdominal pain                 | 81                             |
|                                 | Dysuria                        | 58                             |
|                                 | Bleeding during / after coitus  | 25                             |
| Age                            | Average                        | 23.6y                          | 30y                            |
|                                 | Range                          | 15 – 41y                       | 19 – 39y                       |
|                                 | Median                         | 23y                            | 31y                            |
| Tubal Pathology                | +                              | 50                             |
|                                 | -                              | 203                            |
Chapter 7

All 576 women were consecutively included as the first part of a large prospective study. For every CT-DNA positive woman two consecutive CT-DNA negative controls were included in the study. The women were asked to sign an informed consent and to fill out a questionnaire, regarding their complaints at that moment, varying from increased discharge, having bloody discharge during and/or after coitus, recent abdominal pain (not gastrointestinal or menses related) and/or dysuria. A cervical swab was taken for the detection of *C. trachomatis* DNA (CT-DNA) by PCR (COBAS AMPLICOR; Hoffman – La Roche, Basel, Switzerland) (27).

Peripheral venous blood was collected for the analysis of IgG antibodies against *C. trachomatis* (CT) (Medac Diagnostika mbH, Hamburg, Germany). A titre of $\geq 1:50$ was considered positive. Samples with grey zone values, e.g. cut off $\pm 10\%$, were repeated and considered positive when the result was positive or again within the grey zone. Infections with the microorganisms: *Candida albicans*, *Neisseria gonorrhoea*, *Trichomonas vaginalis*, Herpes simplex virus 1 / 2, may result in symptoms similar to *C. trachomatis* infection. Infection status for these microorganisms was recorded. HSV 1 / 2 was detected according the methods described by Bruisten et al. (28). *N. gonorrhoea* was detected according methods described by Spaargaren et al. (29). *T. vaginalis* was cultured on Trichosel medium according standard procedures (30) and detection of *T. vaginalis* was according the methods described by van der Schee et al.(31). *C. albicans* was cultured on Chrom agar and detection of *C. albicans* was performed according standard procedures (30).

Subfertility cohort

The study was performed in 253 consecutive Dutch Caucasian women who visited the department of Obstetrics and Gynaecology of the Academisch Ziekenhuis Maastricht, The Netherlands, between December 1990 and November 2000 because of subfertility(32). In these women a laparoscopy with tubal testing had been performed as part of their fertility work-up. Preoperatively blood was drawn from all patients for Chlamydia IgG antibody testing (CAT), and spare sera were cryopreserved. Two independent investigators, who were unaware of the CAT results, scored the laparoscopy reports to assess the grade of tubal pathology. Tubal pathology was defined as extensive peri-adenxal adhesions and/or distal occlusion of at least one tube at laparoscopy (33). Subfertile women who had no peri-adenxal adhesions and had patent tubes at laparoscopy served as negative controls. Based on these criteria, 50 women had tubal pathology and 203 women served as controls. IgG antibodies to *C. trachomatis* were detected with a species-specific MIF test (AniLabSystems, Finland), as described previously (32), with comparable sensitivity and specificity as compared to the IgG ELISA from Medac used for the STD cohort (34). A positive *C. trachomatis* IgG MIF test was defined as a titre $\geq 1:32$. Findings at laparoscopy were correlated with the MIF test results. Based on the MIF test, 41 women were found to be CT IgG positive, while 212 were CT IgG negative. Of the
CT IgG positive women 28 (68.8%) had tubal pathology, while 22 women (10.4%) of the CT IgG negative women had tubal pathology.

Healthy controls
A healthy Dutch Caucasian control group (n=170) was included to assess the general frequency of the CD14 -260 genotypes in the Dutch Caucasian population.

Immunogenetic analyses

DNA Extraction

STD cohort
Eukaryotic DNA from PBMC was isolated using the isopropanol isolation method. In short: 100 µl PBMC in PBS were added to 600 µl L6 (Nuclisens Lysisbuffer, Organon Teknika, Boxtel, The Netherlands) and 1 µl glycogen (Roche Molecular Diagnostics, Almere, The Netherlands). The samples were incubated for 30 minutes at 65°C and left to cool at room temperature. An equal volume of cold (-20°C) isopropanol was added to the samples. The samples were then centrifuged (20 min at 20,000G). The supernatant was discarded and the pellets were washed twice in 75% EtOH. The pellets were dissolved in T10 overnight (O/N) at 4°C and then stored at –20°C until further analysis.

Subfertility cohort
Genomic DNA was extracted out of the cryopreserved sera using High Pure PCR Template Preparation Kit (HPPTP kit) according to the manufacturer’s instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Healthy controls
Blood was collected in EDTA-tubes and stored at room temperature until the genomic DNA was extracted from peripheral blood leukocytes (PBMC) according to an in-house DNAzol (Invitrogen, The Netherlands) isolation procedure.

CD14 –260C>T gene polymorphism
The C>T substitution in the proximal CD14 promoter GC box at position –260 from the translation start site (NCBI SNP CLUSTER ID:rs2569190) results in a HaeIII restriction site. We developed a PCR assay using the primers, 5’ TCA CCT CCC CAC CTC TCT T 3’ (sense) and 5’ CCT GCA GAA TCC TTC CTG TT 3’ (antisense) (Invitrogen Life Technologies, Breda, The Netherlands), flanking this restriction site. Amplification was performed using a thermal cycler Perkin-Elmer 9700 (Applied Biosystems, Foter City, CA, USA). The parameters were an initial denaturation at 95°C for 5 min,
Chapter 7

followed by 35 cycles: denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 1 min. The final elongation was at 72°C for 7 min followed for a cooling to 4°C. The 107-bp fragments were digested overnight at 37°C with HaeIII (Invitrogen, The Netherlands) resulting in fragments that either were cut in two fragments of 83-bp and 24-bp (allele C) or were not restricted (T allele). These fragments were analyzed by electrophoresis on 4% low melting agarose gels (Tebu-Bio, The Netherlands) stained with ethidium bromide.

Statistical analyses
All groups were tested for Hardy-Weinberg equilibrium to check for Mendelian inheritance. Statistical analyses were performed using Instat Graphpad and SPSS version 11 (SPSS Inc., Chicago, IL, USA). Fisher exact and \( \chi^2 \) tests were used to test for differences in CD14 allele / genotype / carrier frequencies between the (sub)groups and p-values <0.05 were considered statistically significant.

Results
All genotype distributions assessed were in Hardy-Weinberg Equilibrium. The CD14 -260 C>T SNP was assessed in the STD, subfertility and control cohorts.

CD14 –260 in the susceptibility to C. trachomatis infection
To determine the effects of CD14 –260 C>T on the susceptibility to C. trachomatis infection, the prevalence of CD14 –260 C>T genotypes were assessed in the STD cohort (table 2). The overall genotype distribution was 28.1% CC, 50.7% CT, 21.2% TT.
Table 2: CD14 genotype distribution in the Dutch Caucasian STD cohort.

*C. trachomatis* IgG positive and negative patients, divided in CT DNA (LCx) positive and negative and subdivided in coinfection with other microorganisms (*N. gonorrhoea, T. vaginalis, C. albicans, H. simplex virus 1 & 2*), symptoms (vulvovaginal discharge, abdominal pain, dysuria, bleeding during / after coitus) and lower abdominal pain. Abbreviations: CT: *C. trachomatis*; MO+: microorganism positive; LAP+: lower abdominal pain positive; Symp: symptoms positive

<table>
<thead>
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<th></th>
<th>Total</th>
<th>1.1 (CC)</th>
<th>CD14 –260 C&gt;T</th>
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<th>2.2 (TT)</th>
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<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
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<tr>
<td><strong>Total</strong></td>
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<td>28.2%</td>
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</table>

This distribution was comparable to the healthy controls (Figure 2). The distribution was 28.8% CC, 50.0% CT, 21.2% TT in *C. trachomatis* DNA positive women, while in *C. trachomatis* DNA negative women the distribution was 27.8% CC, 51.0% CT, 21.2% TT. In women with or without serological *C. trachomatis* responses the distribution was 30.4% CC, 49.3% CT, 20.3% TT and 26.7% CC, 51.5% CT, 21.7% TT, respectively. No differences could be observed in women with or without symptoms. Coinfection with other microorganisms or combinations of these four variables (*C. trachomatis* DNA, *C. trachomatis* serology, symptoms and microorganisms) did not introduce statistically significant differences or trends in CD14 genotype distributions.
CD14 -260 in the severity of sequelae of *C. trachomatis* infection

The effect of CD14 –260 C>T on the severity of sequelae of *C. trachomatis* infection was assessed in a cohort of subfertile women with clinically well-defined tubal pathology. The overall genotype distribution in the cohort was 27.7% CC, 49.0% CT and 23.3% TT (figure 2). The genotype distribution in women with tubal pathology was similar to the distribution in women without tubal pathology (28.0% CC, 48.0% CT, 24.0% TT and 27.6% CC, 49.3% CT, 23.2% TT respectively) and to the distribution in the healthy controls (table 3).

**Table 3:** CD14 genotype distribution in the Dutch Caucasian subfertility cohort. Distribution in the total cohort and subdivided in women with or without tubal pathology, and *C. trachomatis* IgG positive women with or without tubal pathology. *C. trachomatis* positivity defined as a titre ≥ 1:32 (MIF). Abbreviations: TP: tubal pathology; CT: *C. trachomatis*.

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>1.1 (CC)</td>
</tr>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Total</td>
<td>253</td>
</tr>
<tr>
<td>TP+</td>
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</tr>
<tr>
<td>TP-</td>
<td>203</td>
</tr>
<tr>
<td>CT IgG+ TP+</td>
<td>28</td>
</tr>
<tr>
<td>CT IgG+ TP-</td>
<td>13</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>170</td>
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</tbody>
</table>
Introduction of *C. trachomatis* IgG serology, with special attention to *C. trachomatis* positive women who did develop tubal pathology as compared to those who did not develop tubal pathology, did not alter the observed genotype distribution.

**Discussion**

We did not find an association between the functional upregulating CD14 –260 C>T polymorphism and the susceptibility to or subsequent severity of sequelae of *C. trachomatis* infection, as assessed in the STD and subfertility populations (Figure 2). However, these results do not exclude that a still unknown CD14 expression decreasing SNP may influence the course of *C. trachomatis* infection. Recent studies have shown that Chlamydia LPS is capable of inducing an inflammatory response through CD14 (35,36), although the potency to induce an inflammatory response was 100 – 1000 times less when compared to the responses induced by *S. minnesota*, *N. gonorrhoea* (35) and the enterobacteria *S. enterica* and *E. coli* (36). Heine et al. demonstrated that the CD14 associated inflammatory response was TLR4 but not TLR2 mediated (36). These results are corroborated by studies showing the role of the CD14-TLR4-MD2 complex in intracellular signalling by LPS (13,37) and studies showing the dependency on CD14 of phagocytosis of Gram negative bacteria (38).

The absence of an association between CD14 and susceptibility to *C. trachomatis* infection might be explained by the compartmentalisation of TLR4. The differential expression of TLR4 has been described in immortalised cell-lines derived from the female urogenital tract (39) and recently demonstrated in cells isolated from patients by Pioli (40) and Fazeli (41). TLRs 1 – 6 were found to be expressed in the epithelia of the female urogenital tract. TLR2 and TLR4 were the only Toll like receptors with a clear differential expression. Low expression in the lower urogenital tract and high expression in the upper genital tract (40,41). The expression remained similar in all subjects irrespective of age or status of the reproductive cycle (41). It is hypothesized that through this expression pattern TLR4 modulates immunological tolerance in the lower genital tract and induces host defence against ascending infection in the upper genital tract (41). In the upper genital tract, Fazeli and colleagues found TLR4 positive vacuole like structures that seemed to be secreted from endocervical glands (42). A secretory form of TLR4 has been described in mice, where the soluble TLR4 appears to inhibit LPS mediated signals, while at the same time sTLR4 mRNA is upregulated by LPS (43). This may represent a feedback mechanism to prevent excessive responses to LPS in the endocervix, which can be seen as a boundary between the lower and upper genital tract. Further evidence for the regulation of immune responses to LPS by TLR4 is provided by the study of Harju et al., who demonstrated the intrauterine expression of TLR4 and endotoxin responsiveness in mice in the perinatal period (44). mCD14 is expressed on human endometrial stromal cells but not on endometrial epithelial gland cells. The epithelial cells are dependent on sCD14 for LPS recognition (45). Soluble CD14 is present in the cervical mucosa and may be present in the endometrium (46).
Combining the aforementioned studies with the knowledge that CD14 can signal through TLR4, it might be hypothesized that the absence of an association between the CD14 –260 SNP and the susceptibility to *C. trachomatis* infection might be due to the low expression or absence of TLR4 in the lower urogenital tract. In the upper genital tract, strict regulation of immune responses to LPS by TLR4 may inhibit CD14 signalling through TLR4 (43,44), thus limiting the influence of CD14 on the development of tubal pathology.

However, this hypothesis does not take into account the ability of CD14 to signal through TLR2 (47), nor does it take into account that the study of Netea et al. which demonstrated that non-LPS components of *Chlamydia pneumoniae* can stimulate cytokine production through TLR2 dependent, CD14 independent pathways (48) and that a similar mechanism may exist and stimulate *C. trachomatis* induced cytokine production in urogenital infections.

Since TLR2 is involved in Chlamydia-induced TGF-beta, an anti-inflammatory cytokine with an important role in fibrosis, and thus very likely in post-infection tubal pathology, it might explain why CD14 polymorphisms may not severely impact the development of tubal pathology (49).

Darville et al. have demonstrated that TLR2 is an important mediator of innate immune responses in *C. trachomatis* infection in mice and plays an important role in early production of immune mediators and development of tubal pathology (50,51). In a recent publication by Pitz et al. it was shown that *C. pneumoniae* is capable of activating endothelial cells by TLR2 as initial extracellular *C. pneumoniae* receptor, whereas NOD1 was shown to be a potent intracellular immune receptor for *C. pneumoniae* in endothelial cells. Further research may extend these results to *C. trachomatis* infections. Overall, the recognition of bacterial LPS involves a complex system of multiple receptors and a complex orchestration of protein-protein interactions (52).

**Conclusions**

Our study showed that the functional up-regulating CD14 -260 C>T SNP did neither influence the susceptibility to nor the severity of late sequelae of *Chlamydia trachomatis* infection. However, this does not exclude a prominent role for CD14 in the course of an active *C. trachomatis* infection and not yet described CD14 expression decreasing SNPs may affect the course of *C. trachomatis* infection profoundly. Further studies on the immunogenetics of *C. trachomatis* infection will provide more insight in the clear differences in the clinical course that this microorganism induces in individuals and lead to potential vaccine candidates.
Acknowledgements

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The authors are indebted to Prof. Cathrien Bruggeman, head of the department of Medical Microbiology, Academisch Ziekenhuis Maastricht, Maastricht, The Netherlands, for the serological testing of the subfertility cohort.

The ICTI consortium (Integrated approach to Chlamydia trachomatis Infections[53]) provides a broad specialized network for the multidisciplinary studies described.

References


PART 2

LYMPHOGANULOMA VENEREUM
Aims and outline
LYMPHOGRANULOMA VENEREUM

Aims and outline

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by invasive LGV serovars of *C. trachomatis* and endemic in Africa, India, Southeast Asia, South America, and the Caribbean and occurs up until recently as a sporadic disease elsewhere. However, in 2004 the first report out of Rotterdam, The Netherlands was published concerning an unusual and possible outbreak of LGV infections among men having sex with men (MSM) all with a rectal LGV infection while no urogenital LGV infection was observed.

In this Part 2 of the thesis we describe this outbreak in The Netherlands from an epidemiological, clinical en pathogenetic perspective.

**Chapter 8** Can this LGV outbreak also be demonstrated in Amsterdam and since how long and if so, does this LGV strain differ from known LGV strains? Using stored rectal *C. trachomatis* DNA positive patient samples we determined the presence of LGV infection and when a LGV infection was demonstrated we characterized the LGV strain in more detail by sequencing. In addition, we looked at the *C. trachomatis* IgG specific serological responses and the clinical presentation of these patients.

**Chapter 9** Is it possible to develop a sensitive and specific real-time PCR assay to diagnose patients with LGV more rapidly?
Given the role of classical, labour-intensive diagnostic tests e.g. Restriction Fragment Length Polymorphism and sequencing, in diagnosing patients with probable LGV, it is valuable to develop a quick and easy to perform real-time PCR method, as has been developed for a lot of other infectious diseases e.g. *M. tuberculosis*, *L. pneumophila*, 2 other intracellular microorganisms.

**Chapter 10** What are the risk factors and clinical predictors of LGV to determine implications for clinical practice? Multivariate logistic regression was used to assess risk factors and clinical predictors by in MSM infected with LGV, as compared to MSM infected with serovars A-K, and thus with a non-LGV anorectal chlamydia, and MSM reporting receptive anorectal intercourse but without anorectal Chlamydia.
Chapter 11 Is it possible to demonstrate that the identification of LGV patients beyond the developed world is part of a new outbreak or that the infection has been there all along and that the physicians did not recognize the clinical presentation and missed it all those years?

Recently a number of LGV infections have been demonstrated in Europe and in the USA. Whether this is a classical outbreak of an infection to be traced back to one or more index patients or a continuous circulation of an known infection with e.g. a seasonal expansion was the principal theme of this study. Therefore we assessed retrospectively the presence of LGV in samples till 2000 in Amsterdam and we looked at LGV positive samples obtained from the ’80 among MSM from San Francisco.
Chapter 8

New lymphogranuloma venereum Chlamydia trachomatis variant, Amsterdam

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Emerging Infectious Diseases 2005;11(7):1090-1092.
Chapter 8

Abstract

We retrospectively conducted a study of men who have sex with men who visited the Amsterdam, the Netherlands, sexually transmitted diseases clinic from January 2002 to December 2003 and had rectal *Chlamydia trachomatis* infections. We found that symptomatic (73%) as well as asymptomatic (43%) patients were infected with a new *C. trachomatis* LGV variant.
Introduction

In December 2003, an unusual symptom of early lymphogranuloma venereum (LGV) in a patient infected with HIV-1, who also had proctitis, was reported in Rotterdam, the Netherlands (1). In the same city, an outbreak of LGV with similar symptoms, such as proctitis and constipation, subsequently was identified in men who have sex with men (MSM) (2). Here we report 32 patients with, and 13 patients without, mucous membrane abnormalities in MSM with confirmed LGV in 2002–2003.

LGV is a systemic disease caused by the *Chlamydia trachomatis* serovars L1 to L3. More invasive than disease caused by the urogenital serovars (D–K), LGV can manifest as 1) an inguinal syndrome, with genital ulceration and inguinal lymphadenopathy (buboes) and subsequent suppuration, and 2) an anogenitorectal syndrome, with proctocolitis and hyperplasia of intestinal and perirectal lymphatic tissue. Both syndromes can be accompanied by systemic symptoms including fever, malaise, chills, anorexia, myalgia, and arthralgia. If left untreated, the infection can lead to fistulas, strictures, genital elephantiasis, frozen pelvis, and infertility (3). LGV is endemic in Africa, Southeast Asia, and the Caribbean; it is a sporadic disease in Europe and North America.

Study population

For this study, we selected MSM who were treated at our sexually transmitted disease (STD) clinic (≈20,000 new consultations per year) in 2002 and 2003 with *C. trachomatis* proctitis confirmed by a positive polymerase chain reaction (PCR), COBAS AMPLICOR (Hoffman-La Roche Ltd., Basel, Switzerland). Upon proctoscopic examination by 1 medical practitioner, patients were designated into 2 groups: 1 group with mucous membrane abnormalities (MMA+, n = 44) when mucopurulent anal discharge or bloody, ulcerative rectal lesions were found, and 1 group without MMA (MMA–, n = 30) when those symptoms were not found. Samples were taken by proctoscopic examination. During the study, *C. trachomatis* proctitis was diagnosed in some patients at separate times. Those follow-up samples were excluded from the study. Calculations are based on the number of patients in whom *C. trachomatis* proctitis was diagnosed during their first visit. Patients were treated with a single dose of 1 g azithromycin, the consensus treatment for uncomplicated urogenital *C. trachomatis* infections at that time.
Laboratory methods

Purified *C. trachomatis* DNA obtained from the rectal samples of these 74 patients was used to assess *C. trachomatis* serovars identified by PCR, based on restriction fragment length polymorphism (RFLP) analysis of the *ompA* gene as described previously (4,5). In addition, we sequenced the complete *ompA* gene to identify possible changes at the nucleotide level (ABI 310 automated sequencer, PE Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The exact sequence methods and primers are described by Morré et al. (5). In short, *ompA* nucleotide sequence analysis was performed in several sequence reactions generating the complete 1.1-kbp order. DNA sequencing was performed in both directions and analyzed by automated DNA sequencing on an ABI 310 sequencer. Sequences were aligned with the BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA, USA). Reference sequences were derived from GenBank (http://www.ncbi.nlm.nih.gov/GenBank).

Serum samples from these patients, taken at consultation and stored at –20°C, were used to measure *C. trachomatis*-specific immunoglobulin (Ig) G. This *C. trachomatis*-IgG peptide enzyme-linked immunosorbent assay (pELISA) (Medac Diagnostika mbH, Hamburg, Germany) is based on a synthetic peptide from an immunodominant region of the major outer membrane protein and was performed according to the manufacturer's instructions, as described previously (6). A titer of both ELISAs of ≥1:50 was considered positive, and an arbitrary differentiation was made between low (1:50–1:200) and high titers (>1:200).

Results

Genotyping the *ompA* gene by RFLP of these 74 patients showed that 45 samples were positive for *C. trachomatis* all type L2 (Table). Sequencing of the *ompA* gene demonstrated that all L2-positive samples contained a new (based on the National Center for Biotechnology Information BLAST queries) *C. trachomatis* genovariant (Figure), which we designated L2b.
Figure: Schematic representation of the *Chlamydia trachomatis* ompA gene. In detail variable segment 2: nucleotide and amino acid sequence comparison of the prototypes L1, L2, L2', L2a, and L3 and the newly identified LGV strain which we designated L2b. Conserved nucleotides in VS-2 for all LGV strains are shown in red. The nucleotide substitutions in L2b as compared to all LGV strains is indicated by arrows. All amino acids encoded by the substitution combinations are indicated.

VS= variable segment; CS= constant segment; omp= outer membrane protein; CT= *Chlamydia trachomatis*; aa= amino acid; LGV= lymphogranuloma venereum

The novel sequence was deposited in GenBank (accession no. AY586530).

When the *ompA* sequences of these patients were compared to the prototype sequences of L2 and its variants L2a and L2', besides 2 already described changes, a new base pair change was found. One change in variable segment 2 was deducted from L2a and L2', and one from L2. The third change has not been described before. All nucleotide changes resulted in amino acid substitutions. The fourth change was found in constant segment 2 (CS-2) at amino acid 157: the third nucleotide is G in L2b and L1, C in L2, and A in L3. As expected, this mutation is conserved, and all combinations encode for the amino acid glycine. Combining the sequence data with the RFLP typing showed that 32 of 44 samples from MMA+ and 13 of 30 samples from MMA– patients were L2b. In the MMA+ patient group, a positive chlamydia serologic test results mainly an IgG titer $\geq 200$, correlated well with the LGV diagnosis. Approximately 80% of all LGV patients had high titers; in the MMA– group,
Chapter 8

Species-specific *Chlamydia trachomatis* serologic test results did not correlate with LGV. The patients' characteristics are shown in the Table.

**Table:** Patient characteristics of 44 MMA+ (2002 and 2003) and 30 MMA- LGV and non-LGV MSM included in the retrospective study at the STD outpatient clinic in Amsterdam, The Netherlands.

* Two sera were not available for testing
** One serum was not available for testing

- Serogroup B: n=5; Intermediate serogroup: n=5; Serogroup C: n=2
- Serogroup B: n=10; Intermediate serogroup: n=1; Serogroup C: n=6

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<tr>
<td></td>
<td>LGV: L2b</td>
<td>non-LGV</td>
</tr>
<tr>
<td>Number of patients</td>
<td>32</td>
<td>12(^a)</td>
</tr>
<tr>
<td>Anal discharge reported</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Genital ulcers</td>
<td>3 (L2b)</td>
<td>1</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Mean number of previous/present STDs</td>
<td>8.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Known HIV+</td>
<td>24</td>
<td>6</td>
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<tr>
<td>Gram staining; &gt; &gt; 10 WBC</td>
<td>26</td>
<td>3</td>
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<tr>
<td>CT lgG ≥ 200</td>
<td>22</td>
<td>2</td>
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<tr>
<td>CT lgG &lt; 200</td>
<td>8(^*)</td>
<td>10</td>
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</table>

Median age of the 45 men with samples positive for *C. trachomatis* was 35.8 years (range 25.9–47.6) compared with 38.1 years (range 25.8–58.2) for the men with samples negative for *C. trachomatis*. All *C. trachomatis* positive patients lived in the Netherlands, most in Amsterdam, and most were of Dutch ethnic background.

Anal discharge was reported by 15 of 20 patients with LGV. Genital ulcers (all localized to the perianal area) and inguinal lymphadenopathy were found in only a few patients. Ulcers in the 2 patients infected with a non-LGV *C. trachomatis* strain were caused by herpes simplex virus 2 and Treponema pallidum. In the 3 ulcers found in the MMA+ patients, the L2b *C. trachomatis* strain was found.

The mean number of previously documented sexually transmitted infection episodes was 8.3 among the MMA+ LGV patients in contrast to 5.8 episodes in the non-LGV patients. Twenty-four of 30 of
the MMA+ LGV patients and 7 of 14 of the MMA– LGV patients were HIV-infected. All patients with a retrospective diagnosis of LGV were contacted and offered reexamination. If the L2b strain persisted, the patients received doxycycline, 100 mg twice daily for 3 weeks, the consensus treatment for LGV.

Conclusions

We conclude the following: 1) the outbreak of LGV among MSM in the Netherlands expands beyond the cluster reported earlier in Rotterdam and can be traced back to at least January 2002; 2) the outbreak in Amsterdam and possibly the one in Rotterdam was caused by a newly identified L2b variant; 3) both MMA+ and MMA– men are infected with C. trachomatis and most of them are HIV-positive; 4) species-specific serology can help support the LGV diagnosis when clinical symptoms are present but cannot be used to detect LGV-infected persons who are asymptomatic. Although based upon a small, select population, our results justify additional study of high-risk core groups who transmit this LGV genovariant to determine transmission risk factors and diagnostic criteria. The outbreak of LGV is ongoing; we currently see 1–2 new patients per week at our STD clinic.

Acknowledgments

We thank Judith Merks for performing all the laboratory experiments, and Lucy Phillips for editorial review of the manuscript.

Submission to GenBank

The novel sequence is deposited in this public database.
The accession number is 153611 AY586530.
References


Chapter 9

Real-time polymerase chain reaction to diagnose lymphogranuloma venereum

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### Introduction

An outbreak of rectal lymphogranuloma venereum (LGV) has been detected in the Netherlands among men who have sex with men (1–4). More cases of LGV in other European countries such as Belgium, France, and the United Kingdom have been reported, and the first cases have been detected in the United States as well. This infection is encountered not only by clinicians who treat sexually transmitted diseases but also by gastroenterologists. Both the European Surveillance of Sexually Transmitted Infections (http://www.essti.org) and the Centers for Disease Control and Prevention (http://www.cdc.gov) are working on outbreak warning and response systems to increase the awareness and the direct management of the LGV outbreak (5,6).

Different approaches have been described to diagnose LGV infections (Figure).

![Diagnosis of LGV](image)

**Figure:** Diagnosis of lymphogranuloma venereum. MIF, microimmunofluorescence; STI, sexually transmitted infection; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; PAA, polyacrylamide; BLAST, basic local alignment search tool.

The first 3 approaches have serious disadvantages: cell culture is rarely available in routine diagnostic settings, polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) analysis (usually nested PCR approaches are used) needs post-PCR restriction enzyme profiling, and sequencing requires additional analyses of sequence data to identify the *Chlamydia trachomatis* serovar responsible for infection. In addition, all 3 techniques are time consuming (at least 1–4 days to
get a result), laborious, and require specially trained personnel in a sophisticated laboratory setting. Therefore, we developed a real-time PCR approach (TaqMan and Rotorgene) that can easily identify LGV strains in 2 hours with equipment that is available in almost all diagnostic settings.

**Laboratory methods**

**Real – time PCR**

We used the polymorphic membrane protein H gene (pmp gene) as a PCR target because it has a unique gap in LGV strains of *C. trachomatis*, compared to other serovars, which makes it highly specific. The following primers and probes were selected: LGV-F 5´ CTG TGC CAA CCT CAT CAT CAA 3´, LGV-R 5´ AGA CCC TTT CCG AGC ATC ACT 3´, and LGV MGB-probe 6-FAM-CCT GCT CCA ACA GT. Real-time PCR conditions (20-µL format) for TaqMan were as follows: 2× TaqMan Universal Mastermix (Applied Biosystems, Foster City, CA, USA), 18 pmol each primer, 0.2 µmol/L probe, and 2 µL (LGV L2) DNA or clinical sample; 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. Conditions for Rotorgene were as follows: 10× buffer (Hoffman-La Roche Ltd, Basel, Switzerland), 10 pmol each primer, 0.04 µmol/L probe, 2 µL (LGV L2) DNA or clinical sample; 2 min at 50°C, 10 min at 95°C, and 45 cycles of 15 sec at 95°C and 1 min at 60°C. By using a previously described serial dilution of LGV L2 (7), sensitivity was assessed as 0.01 inclusion-forming units for both real-time PCR assays.

**Validation**

To determine specificity, we tested different *C. trachomatis* serovars and serovariants A, B, Ba, C, D, Da, D-, E, F, G, Ga, H, I, Ia, I-, J, Jv, K, L1, L2, L2b, L3, *C. muridarum* (MoPn), *C. pneumoniae*, *C. pecorum*, *C. psittaci*, and 32 other microorganisms that normally reside in the human perianal and urogenital region and in the oropharynx. These organisms included gram-positive and gram-negative bacteria and yeast: *Acinetobacter baumannii*, *Campylobacter jejuni*, *Candida albicans*, other yeast, *Enterobacter agglomerans*, *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus* spp., *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Mycoplasma* spp., *Neisseria meningitidis*, *Pasteurella* spp., *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Shigella sonnei*, *Staphylococcus aureus*, and others. Only LGV strains L1, L2, L2b, and L3 tested positive in both the TaqMan and Rotorgene assays, which shows the analytical specificity of real-time PCR.

Subsequently, we determined in a blinded setting the presence of LGV in a selected group of patients (clinical spectrum and epidemiology described elsewhere (8) according to *C. trachomatis* positive rectal swab (Chlamydia 2SP Collection & Transport Kit [Quelab] by commercially available PCR (COBAS AMPLICOR, Hoffman-La Roche Ltd). By using the 2 reference standard techniques to type *C. trachomatis* serovars (PCR-based RFLP of the omp1 gene or sequencing the variable segment 2 [VS-2] of the omp1 gene) (9,10) with DNA isolated from rectal swab specimens (standard
isopropanol DNA isolation method), we identified 28 of 125 men as LGV-positive. These 28 samples were also positive in both the TaqMan and Rotorgene assays.

Discrepancy analysis
We also identified 2 additional LGV infections, which were initially typed and then retested as single-strain infections with serovars E and D by both PCR-based RFLP analysis and VS-2 sequencing. This discrepancy is most likely due to a double infection, which will, in most cases, result in the preferential amplification of 1 strain in the *ompA* PCR and PCR-based sequencing methods; in the TaqMan and Rotorgene assays, only LGV strains can be amplified.

Future investigation
Whether this outbreak is partially technically driven must be assessed in the future by retrospectively investigating the presence of these LGV infections in men who have sex with men and the presence of the L2b strain in the past, since at present only LGV infections from 2003 to 2005 have been investigated.


Chapter 10

Diagnostic and clinical implications of anorectal lymphogranuloma venereum in men who have sex with men: a retrospective case-control study

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a equal authorship

Clinical Infectious Diseases 2006;42:186–94
Abstract

**Background:** Recently, outbreaks of anorectal lymphogranuloma venereum (LGV) have occurred among men who have sex with men (MSM). This study identifies risk factors and clinical predictors of LGV to determine the implications for clinical practice.

**Methods:** The *Chlamydia trachomatis* serovars for all MSM who had anorectal chlamydia diagnosed at a sexually transmitted infection clinic in Amsterdam, The Netherlands, in 2002 and 2003 were retrospectively typed; 87 persons were infected with *C. trachomatis* serovar L2b and received a diagnosis of LGV. MSM infected with *C. trachomatis* serovars A–K and who thus had non-LGV anorectal chlamydia (np377) and MSM who reported having receptive anorectal intercourse but who did not have anorectal chlamydia (np2677) served as 2 separate control groups. Risk factors and clinical predictors were analyzed by multivariate logistic regression. Receiver operating characteristic curves were used to determine clinical relevance.

**Results:** HIV seropositivity was the strongest risk factor for LGV (odds ratio for patients with LGV vs. those with non-LGV chlamydia, 5.7 [95% confidence interval, 2.6–12.8]; odds ratio for patients with LGV vs. control subjects without chlamydia, 9.3 [95% confidence interval, 4.4–20.0]). Proctoscopic findings and elevated white blood cell counts in anorectal smear specimens were the only clinically relevant predictors for LGV infection (area under the curve of the receiver operating characteristic curve, 1.071). Use of these 2 parameters and HIV infection status provided the highest diagnostic accuracy (for MSM with anorectal chlamydia, the area under the curve was 10.82; sensitivity and specificity were 89% and 50%, respectively).

**Conclusions:** LGV testing is recommended for MSM with anorectal chlamydia. If routine LGV serovar typing is unavailable, we propose administration of syndromic LGV treatment for MSM with anorectal chlamydia and either proctitis detected by proctoscopic examination, 110 white blood cells/high-power field detected on an anorectal smear specimen, or HIV seropositivity.
Introduction

Lymphogranuloma venereum (LGV) is a sexually transmitted infection (STI) caused by Chlamydia trachomatis serovars L1, L2, and L3. It is endemic in parts of Africa, Asia, South America, and the Caribbean, but it has been rare in industrialized countries. However, in 2003, a cluster of cases of LGV among men who have sex with men (MSM) was reported in Rotterdam, The Netherlands (1). Since then, there have been other reports on similar outbreaks in large cities in western Europe and the United States (2–7). In contrast to urogenital chlamydia infections that are caused by C. trachomatis serovars A–K and characterized by mild and often asymptomatic infection, LGV can cause severe inflammation and invasive infection, often with systemic symptoms. Depending on the site of inoculation, LGV can manifest either as an inguinal syndrome with a unilateral painful inguinal lymphadenopathy (buboes) or as an anorectal syndrome with hemorrhagic proctocolitis and hyperplasia of intestinal and perirectal lymphatic tissue. LGV responds well to extensive antibiotic treatment, but when untreated, it may cause chronic or irreversible complications, including fistulas, strictures, genital elephantiasis, frozen pelvis, or infertility (8, 9). All of the recently reported cases of LGV infection presented as anorectal syndrome among MSM, and case reports suggest that the recent outbreak of LGV has been concentrated in sexual networks of MSM and is associated with attendance at sex parties and with HIV seropositivity (1, 3, 5). However, predictors of LGV in the present outbreak have not been studied systematically. Therefore, we conducted a retrospective case-control study to identify risk factors for and clinical and diagnostic signs of anorectal LGV infection in MSM, to determine the implications for clinical practice.

Methods

Study setting and procedure.
The STI clinic of the Municipal Health Service in Amsterdam, The Netherlands, offers free of charge examination and treatment for STIs, and ≈ 55%–60% of all recorded STIs in The Netherlands are diagnosed there (10). Every MSM client is routinely screened for urethral C. trachomatis infection by PCR (Cobas Amplicor; Hoffman–La Roche), for urethral and anorectal gonorrhea by culture tests (Becton Dickinson Biosciences), and for syphilis using a Treponema pallidum particle agglutination assay (Fujirebio). The Venereal Disease Research Laboratory test (Wellcome) and the fluorescent treponemal antibody absorption test (Trepo-spot IF; bioMérieux) are used to confirm and classify syphilis infection. All MSM clients, both symptomatic and asymptomatic, who have reported receptive anal intercourse in the past 6 months undergo proctoscopic examination using a proctoscope and a clinical examination lamp. During proctoscopic examination, anorectal swabs are obtained for
Gram staining and for the aforementioned chlamydia and gonorrhea tests (11). Clinical diagnosis of proctitis is based on the presence of mucosal tissue that is red or swollen or that easily bleeds upon manipulation and/or the presence of mucopurulent or purulent anal discharge. Gram-stained smears are evaluated by a light microscope (magnification, x 1000) to determine the WBC count. The mean number of WBCs were calculated for 3 separate microscopic fields and were categorized into the following groups: < 10 WBCs/high-power field (hpf), 11–50 WBCs/hpf, and >50 WBCs/hpf. Gram staining of rectal mucosa smear samples obtained with the use of a proctoscope to demonstrate the presence of leukocytes has proven to be an objective method to detect an infectious or inflammatory disease (12, 13). HIV antibody testing (Axzym; Abbott Laboratories) is not routinely performed but is offered to all clients. Reactive samples are confirmed by line immunoassay (Inno-Lia HIV I/II Score; Innogenetics). In cases of genital ulceration, dark-fieldmicroscopy and PCR tests are performed on ulcer swabs for \textit{T. pallidum}, \textit{Haemophilus ducreyi}, and herpes simplex virus (HSV) type 1 and 2 (14). If these PCR tests have negative results, clients are checked again at weeks 3, 6, and 12 for seroconversion of syphilis antibodies. If no seroconversion occurs, the ulceration is considered to be an ulceration without a laboratory-confirmed etiological diagnosis. All information used in this study, such as the clients’ characteristics and histories (including symptoms, sexual behavior, and previous STI diagnoses), clinical findings, laboratory results, current diagnoses, and therapies, are routinely recorded in an electronic database.

\textbf{Study population.}

To identify cases of anorectal LGV, we selected all MSM diagnosed with \textit{C. trachomatis} infection on the basis of anorectal swab findings from 2002 and 2003, and we retrospectively typed all anorectal samples for their \textit{C. trachomatis} serovar, as described previously (15, 16). For the control population, we included (1) all MSM with anorectal chlamydia caused by a non-LGV serovar strain, and (2) all MSM who attended the clinic in the same period who reported having receptive anorectal intercourse but who did not have anorectal chlamydia. For case patients and control subjects who attended the clinic more than once, we only included data from their first consultation in 2002 or 2003.

\textbf{Statistical analyses.}

We analyzed differences between case patients and control subjects univariately using \textit{x}2 and Student’s \textit{t} tests. We performed multivariate logistic regression for risk factors and clinical predictors separately, using backward selection to identify significant variables. Variable selection was based on the likelihood ratio test. We composed receiver operating characteristic (ROC) curves to determine the clinical relevance of the significant predictors. The ROC curve is a plot of the sensitivity, or true-positive rate, to the false-positive rate. The area under the curve (AUC) measures discrimination, which is the ability of a model to correctly classify persons with and without disease. The closer a ROC curve is to the upperleft hand corner of the graph, the more accurate it is (AUC=1). A ROC
curve close to the reference line corresponds to a test result that is positive or negative only by chance (AUC=0.5). A ROC curve that has an AUC of >0.75 is considered to be accurate. We defined statistical significance as a $P$ value of <.05.

Results

Study population.
We identified 87 patients with anorectal LGV; in all cases, the *C. trachomatis* serovar was L2b. Threehundred seventy-seven MSM with non-LGV anorectal chlamydia and 2677 MSM without anorectal chlamydia were included as control subjects. Compared with the 2 control groups, patients with LGV engaged more often in unprotected sex when having receptive anal intercourse with casual partners (41% vs. 20% and 17%; $P < .001$), were more often coinfected with HIV (60% vs. 23% and 12%; $P < .001$), and had more previous STIs than did control subjects (54% vs. 31% and 20%; $P < .001$) (table 1).
Table 1. Demographic and clinical characteristics of 87 men who have sex with men (MSM) who have anorectal lymphogranuloma venereum (LGV; case patients), 377 MSM who have non-LGV anorectal chlamydia (control group 1), and 2677 MSM who do not have anorectal chlamydia (control group 2).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases N=87</th>
<th>Control 1 N=377</th>
<th>Control 2 N=2677</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± SD</td>
<td>37.6 ± 6.5</td>
<td>35.0 ± 8.6</td>
<td>35.9 ± 9.3</td>
</tr>
<tr>
<td>Hard drug usage a</td>
<td>10 (12)</td>
<td>20 (5)</td>
<td>151 (6)</td>
</tr>
<tr>
<td>Engagement in unprotected receptive sexual anal contact with casual partners</td>
<td>36 (41)</td>
<td>76 (20)</td>
<td>448 (17)</td>
</tr>
<tr>
<td>HIV infection status at visit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tested HIV negative</td>
<td>9 (10)</td>
<td>143 (38)</td>
<td>1248 (47)</td>
</tr>
<tr>
<td>Known to be HIV-positive or tested HIV-positive</td>
<td>52 (60)</td>
<td>88 (23)</td>
<td>329 (12)</td>
</tr>
<tr>
<td>Unknown</td>
<td>26 (30)</td>
<td>146 (39)</td>
<td>1100 (41)</td>
</tr>
<tr>
<td>Concurrent anorectal gonorrhoea infection</td>
<td>23 (26)</td>
<td>74 (20)</td>
<td>174 (7)</td>
</tr>
<tr>
<td>Concurrent ulcerative STI b</td>
<td>18 (21)</td>
<td>20 (5)</td>
<td>131 (5)</td>
</tr>
<tr>
<td>Previously diagnosed STI c</td>
<td>47 (54)</td>
<td>115 (31)</td>
<td>529 (20)</td>
</tr>
<tr>
<td>Self-reported anorectal pain</td>
<td>2 (2)</td>
<td>1 (0.3)</td>
<td>9 (0.4)</td>
</tr>
<tr>
<td>Self-reported anorectal discharge</td>
<td>2 (2)</td>
<td>2 (0.5)</td>
<td>10 (0.4)</td>
</tr>
<tr>
<td>Physical examination findings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anorectal ulcer</td>
<td>8 (9)</td>
<td>5 (1)</td>
<td>39 (2)</td>
</tr>
<tr>
<td>Enlarged lymph nodes</td>
<td>21 (24)</td>
<td>40 (11)</td>
<td>185 (7)</td>
</tr>
<tr>
<td>Proctitis upon proctoscopic examination d</td>
<td>41 (47)</td>
<td>64 (17)</td>
<td>198 (7)</td>
</tr>
<tr>
<td>WBC count in Gram-stained anorectal smear specimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 cells/hpf</td>
<td>33 (38)</td>
<td>272 (74)</td>
<td>3334 (88)</td>
</tr>
<tr>
<td>11-50 cells/hpf</td>
<td>15 (17)</td>
<td>69 (19)</td>
<td>241 (9)</td>
</tr>
<tr>
<td>&gt;50 cells/hpf</td>
<td>38 (44)</td>
<td>29 (8)</td>
<td>74 (3)</td>
</tr>
</tbody>
</table>

Note. Data are no. (%) of subjects, unless otherwise indicated. All subjects were clients of a sexually transmitted infection (STI) clinic in Amsterdam, The Netherlands, in 2002 and 2003. hpf, High-power field.

- Defined as use of injection or noninjection drugs >3 times/week in the last 3 months.
- Primary syphilis, anogenital herpes, or anorectal ulceration without confirmed laboratory diagnosis.
- Previously diagnosed syphilis, chlamydia, or gonorrhoea.
- Red, swollen, and/or easily bleeding mucosa and/or mucopurulent or purulent discharge.

Risk factors for LGV infection.

HIV seropositivity was the strongest significant independent risk factor for anorectal LGV infection among MSM, irrespective of the control group used (OR for comparison with control subjects who had non-LGV chlamydia, 5.7 [95% CI, 2.6–12.8]; OR for comparison with control subjects who did not have chlamydia, 9.3 [95% CI, 4.4–20.0]). Other independent risk factors were concurrent ulcerative disease, previously diagnosed STI, and unprotected sex when having receptive anal intercourse with casual partners (table 2).
Table 2. Risk factors for anorectal lymphogranuloma venereum (LGV) in 87 men who have sex with men (MSM) who have LGV (case patients), 377 MSM who have non-LGV anorectal chlamydia (control group 1), and 2677 MSM who do not have anorectal chlamydia (control group 2), as determined by multivariate logistic regression analysis.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Case patients vs. control group 1</th>
<th>Case patients vs. control group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV infection status at visit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tested HIV-negative</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Known HIV-positive or tested HIV-positive</td>
<td>5.7 (2.6-12.8)</td>
<td>9.3 (4.4-20.0)</td>
</tr>
<tr>
<td>Unknown HIV status</td>
<td>2.7 (1.2-6.2)</td>
<td>2.6 (1.2-5.6)</td>
</tr>
<tr>
<td>Concurrent anorectal gonorrhoea (yes vs. no)</td>
<td>&gt;.05</td>
<td>4.4 (2.5-8.0)</td>
</tr>
<tr>
<td>Concurrent ulcerative STI (yes vs. no) a</td>
<td>4.0 (1.8-8.9)</td>
<td>.001</td>
</tr>
<tr>
<td>Previous syphilis infection (yes vs. no)</td>
<td>2.1 (1.1-4.5)</td>
<td>.04</td>
</tr>
<tr>
<td>Previous gonorrhea (yes vs. no)</td>
<td>&gt;.05</td>
<td>2.0 (1.1-3.4)</td>
</tr>
<tr>
<td>Previous chlamydia infection (yes vs. no)</td>
<td>2.9 (1.7-5.1)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Engagement in unprotected receptive sexual anal contact with casual partner (yes vs. no)</td>
<td>1.8 (1.1-3.2)</td>
<td>.04</td>
</tr>
</tbody>
</table>

Note. All subjects were clients of a sexually transmitted infection (STI) clinic in Amsterdam, The Netherlands, in 2002 and 2003.

a Primary syphilis, genital herpes, or anorectal ulceration without confirmed laboratory diagnosis.

Clinical presentation of LGV.
A small number of patients with LGV presented with self-reported anorectal problems, with only 4 of these patients reporting anorectal pain or discharge. Physical examination revealed anorectal ulcers in 8 patients (9%), enlarged inguinal lymph nodes in 21 patients (24%), and proctoscopic signs of a proctitis in only 41 patients (47%). Microscopic examination of Gram-stained anorectal smear specimens revealed >10 WBC/hpf for 53 patients (61%), including 38 patients (72%) for whom >50 WBCs/hpf were present (table 1). Of the 52 HIV-positive patients, 28 (54%) reported use of antiretroviral therapy. These patients were compared with HIV-positive patients who did not receive antiretroviral therapy, and there were no significant differences in the prevalence of proctitis noted by proctoscopic examination (57% vs. 50%; P=.2) or of anorectal smears with >50 WBCs/hpf (50% vs. 52%; P=.7). Proctoscopic signs of proctitis, enlarged inguinal lymph nodes, and the presence of ≥1 anorectal ulcer upon examination were significant predictors of LGV infection, irrespective of the control group used (table 3). In addition, the number of WBCs in the Gram-stained anorectal smear specimen was a significant predictor of LGV infection; when evaluating MSM with chlamydia infection, those for whom >50 WBCs/hpf were present were 5.4 times more likely to be infected with an LGV strain than with a non-LGV chlamydia strain (95% CI, 2.7–10.7). When patients with LGV were compared with control subjects who did not have chlamydia, those for whom >10 WBCs/hpf
were present were 3.5 times more likely to be infected with LGV (95% CI, 1.8–6.7), and those for whom >50 WBCs/hpf were present were 13.9 times more likely to be infected with LGV (95% CI, 7.6–25.5). Because concurrent infections could confound the aforementioned associations, we also restricted analysis to patients without any infections other than LGV chlamydia and control subjects without any infection other than non-LGV chlamydia (table 4). This revealed similar strong associations between high WBC count, physical and proctoscopic findings, and LGV infection. Also in patients and control subjects with (co-)infections, proctoscopic findings and WBC counts were significantly associated with LGV (data not shown). In genito-urinary medicine clinics, syndromic management of STI requires immediate blind treatment of symptomatic patients before definite test results become available. To decide which patients should start with immediate treatment for LGV, we composed ROC curves to determine the relevance of the clinical predictors in the total population of MSM who visited the STI clinic and who reported having unprotected receptive anal intercourse (n=3141). Individually, only the number of WBCs/hpf on a Gram-stained anorectal smear specimen and proctoscopic findings were relevant predictors for LGV infection (AUC, 0.75 and 0.75, respectively). Combination of both predictors improved the clinical accuracy considerably (AUC, 0.83), as did additional combination that included the strongest independent risk factor, HIV infection status (AUC, 0.90). In primary care settings like a general practitioners’ office, a Gram-stained smear is not readily obtained, so these results are often unavailable for immediate diagnostic and therapeutic considerations. Therefore, we composed ROC curves using only those parameters ready at hand in most practices: HIV infection status, proctoscopic findings, and enlarged lymph nodes noted during physical examination. A model with proctoscopic findings and HIV infection status is as accurate in predicting anorectal LGV in an MSM population reporting receptive anal sex as a model with proctoscopic findings and the number of WBCs/hpf in an anorectal Gram-stained smear (AUC, 0.86 vs.0.83) (figure 1). Addition of findings from an inguinal lymph node examination, however, did not further improve accuracy for LGV (AUC, 0.87). As soon as chlamydia PCR test results become available, it is essential to differentiate between LGV and non-LGV chlamydia serovars for treatment reasons. HIV infection status, proctoscopic findings, and the number of WBCs/hpf on an anorectal Gram-stained smear specimen all had similar accuracy for predicting LGV infection among MSM with anorectal chlamydia (AUC, 0.71, 0.69, and 0.71, respectively). Use of both the number of WBCs/hpf on a Gram-stained anorectal smear specimen and proctoscopic findings improved accuracy (AUC, 0.76), and use of all 3 variables combined yielded the highest accuracy (AUC, 0.82). Application of these 3 variables in a population of MSM reporting receptive anal sex to identify the MSM who are most likely to have anorectal LGV results in an overall sensitivity of 89% and specificity of 94% (figure 2).
**Table 3**: Clinical predictors for anorectal lymphogranuloma venereum (LGV) in 87 men who have sex with men (MSM) who have LGV (case patients), 377 MSM who have non-LGV anorectal chlamydia (control group 1), and 2677 MSM who do not have anorectal chlamydia (control group 2), as determine by multivariate logistic regression analysis.

<table>
<thead>
<tr>
<th>Clinical predictor</th>
<th>Case patients vs. control group 1 (OR, 95% CI)</th>
<th>Case patients vs. control group 2 (OR, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-reported anorectal discharge (yes vs. no)</td>
<td>&gt;.05, 8.3 (1.1-64.4)</td>
<td>0.044, 8.3 (1.1-64.4)</td>
</tr>
<tr>
<td>Physical examination findings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enlarged lymph nodes (yes vs. no)</td>
<td>2.3 (1.1-4.6)</td>
<td>3.2 (1.7-5.9)</td>
</tr>
<tr>
<td>Anorectal ulcer (yes vs. no)</td>
<td>4.9 (1.2-19.6)</td>
<td>3.0 (1.1-7.9)</td>
</tr>
<tr>
<td>Proctoscopic examination findings</td>
<td>.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>No physical signs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proctitis (^a)</td>
<td>3.4 (1.8-6.6)</td>
<td>6.5 (3.6-12.0)</td>
</tr>
<tr>
<td>Other proctal signs</td>
<td>1.4 (0.7-2.9)</td>
<td>1.7 (0.9-3.3)</td>
</tr>
<tr>
<td>WBC in Gram-stained anorectal smear specimen</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>≤10 cells/hpf</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10-50 cells/hpf</td>
<td>1.4 (0.7-2.9)</td>
<td>3.5 (1.8-6.7)</td>
</tr>
<tr>
<td>&gt;50 cells/hpf</td>
<td>5.4 (2.7-10.7)</td>
<td>13.9 (7.6-25.5)</td>
</tr>
</tbody>
</table>

**Note.** All subjects were clients of a sexually transmitted infection (STI) clinic in Amsterdam, The Netherlands, in 2002 and 2003. hpf, High-power field.

\(^a\) Red, swollen, and/or easily bleeding mucosa and/or mucopurulent or purulent discharge.
HIV seropositivity, proctitis noted by proctoscopic examination, and WBC count in a Gram stained anorectal smear specimen can be used to identify which MSM are most likely to have anorectal LGV. Systematically performed proctoscopic examination with determination of WBC count in Gram-stained anorectal smear specimens in a large group of MSM who attended a low-threshold STI clinic enabled us to perform this first systematic study on risk factors for and clinical predictors of LGV. It provides a simple strategy for LGV testing and/or (syndromic) treatment of MSM. Because a substantial proportion of all STIs in The Netherlands are diagnosed at our clinic, this study is well suited to describe the spectrum of LGV in MSM (10). Limitations of this study are that we did not know the HIV serostatus for a proportion of patients (30% of the case patients and 41% of the control subjects) and that data collection took place during a routine STI consultation. Because we did not specifically elicit data on risk behavior, symptoms, and/or medication use, underreporting of these risk factors may have occurred. This could explain, to some extent, the low proportion of complaints by patients with LGV in our study. The first LGV cases in the Rotterdam outbreak were reported in February 2003 (1). We describe 87 patients with LGV, the first of whom received a retrospective diagnosis in February 2002. This supports the presumption that LGV has been present for some time among MSM and may be far more common than was previously assumed (17, 18). Diagnosis of LGV was delayed or missed because it requires tests that are not routinely performed, such as C. trachomatis serovar typing using specialized, infrequently used nucleic acid amplification tests (NAATs) or serological tests for C. trachomatis. In addition, LGV has been presumed to be an ulcerative STI presenting with inguinal lymph nodes (buboes) and systemic involvement, whereas currently, LGV in MSM mainly presented as anorectal proctitis without lymph node enlargement (17, 19). As in previous reports (1, 3), we also found a strong association between anorectal LGV and HIV seropositivity. There are several possible explanations for this. First, sexual risk behavior has increased among HIV-positive MSM since the widespread introduction of HAART in the western world (20). Serosorting (i.e., when HIV-positive men choose to have unprotected sex with seroconcordant partners) could have created selective “high-risk” sexual networks for STI transmission, facilitating the spread of LGV within the group of HIV-positive MSM. Second, HIV infection could operate as a biological susceptibility factor for LGV. Third, immune restoration inflammatory syndrome (i.e., clinical manifestation of a previous asymptomatic infection after the commencement of HAART (21) may have had an effect on the sudden onset of this LGV epidemic. However, in our study, the clinical presentation of LGV was not associated with use of antiretroviral therapy in the HIV positive LGV group. Finally, the association between LGV and HIV infection may be explained by the ulcerative character of LGV, which could facilitate transmission and acquisition of HIV (22, 23). Because resources for LGV serovar typing are limited, LGV surveillance and treatment in Europe and other western countries is based on a probable case definition, which includes
confirmed anorectal chlamydia and clinical signs resembling the anorectal or inguinal syndrome, or confirmed anorectal chlamydia and a sexual partner with confirmed LGV (24, 25).

**Table 4.** Clinical predictors of anorectal lymphogranuloma venereum (LGV) infection, excluding possibly concurrent gonorrhea, syphilis, or ulcerative sexually transmitted infection, in 53 men who have sex with men (MSM) who have anorectal LGV (case patients), 287 MSM who have non-LGV anorectal chlamydia (control group 1), and 2380 MSM who do not have anorectal chlamydia (control group 2), as determined by multivariate logistic regression analysis.

<table>
<thead>
<tr>
<th>Clinical predictor</th>
<th>No. (%) of cases</th>
<th>Case patients vs. control group 1</th>
<th>OR (95% CI)</th>
<th>Case patients vs. control group 2</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-reported anorectal discharge (yes vs. no)</td>
<td>2 (4)</td>
<td>&gt;.05</td>
<td>11.7 (1.4-95.6)</td>
<td>.022</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enlarged lymph nodes noted by physical examination (yes vs. no)</td>
<td>10 (19)</td>
<td>2.4 (1.0-6.0)</td>
<td>.055</td>
<td>3.0 (1.2-7.2)</td>
<td>.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proctoscopic examination findings</td>
<td></td>
<td>.004</td>
<td>&lt;.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No physical signs</td>
<td>16 (30)</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proctitis *</td>
<td>26 (49)</td>
<td>3.9 (1.7-9.1)</td>
<td>6.4 (3.0-13.6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other proctal signs</td>
<td>11 (20)</td>
<td>1.2 (0.5-2.7)</td>
<td>1.4 (0.6-3.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC in Gram-stained anorectal smear</td>
<td></td>
<td>.001</td>
<td>&lt;.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10 cells/hpf</td>
<td>17 (32)</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-50 cells/hpf</td>
<td>12 (23)</td>
<td>1.8 (0.8-4.2)</td>
<td>5.2 (2.4-11.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50 cells/hpf</td>
<td>24 (45)</td>
<td>5.6 (2.3-13.6)</td>
<td>18.9 (8.6-41.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** All subjects were clients of an STI clinic in Amsterdam, The Netherlands, in 2002 and 2003. hpf, High-power field.

* Red, swollen, and/or easily bleeding mucosa and/or mucopurulent or purulent discharge
However, our study shows that a substantial proportion of patients with LGV are asymptomatic. Signs of a clinical proctitis were visible in only 47% of the patients, and a microscopic proctitis was present in 61% of the patients. Therefore, the current case definition, which is based on clinical symptoms, may not be sufficient for surveillance and syndromic management, because asymptomatic cases will be missed. On the basis of our treatment algorithm (figure 2), syndromic management of LGV in MSM who engage in receptive anal sex should preferably be based on (1) signs of proctitis upon proctoscopic examination, and (2) one of the following findings: >10 WBCs/ hpf for an anorectal Gram-stained smear specimen or HIV seropositivity.

Therefore, in addition to standard STI screening and appropriate treatment procedures, we suggest immediate administration of blind antibiotic treatment for LGV (doxycycline), pending test results, for these specific groups of MSM. This approach does not imply widespread and unnecessary treatment of MSM with a non-LGV proctitis. According to the 2002 guidelines for STIs of the Centers for Disease Control and Prevention (CDC), all patients with proctitis should be prescribed doxycycline (100 mg orally twice per day for 7 days) plus ceftriaxone (125 mg given im), pending the results of laboratory tests (26). If anorectal chlamydia is confirmed, doxycycline therapy should be continued for at least 7 days, which is adequate for non-LGV chlamydia. In addition, we recommend LGV serovar typing for all MSM with a confirmed anorectal chlamydia infection found during routine STI screening. We advise commencement or continuation of the LGV treatment regimen, pending serovar type data, for MSM with confirmed anorectal chlamydia and one of the following findings: proctoscopic proctitis, >10 WBCs/hpf in a Gram-stained anorectal smear, and HIV seropositivity. If LGV is confirmed, doxycycline therapy should be administered for a minimum of 21
days or for as long as anorectal symptoms persist. If LGV serovar testing is unavailable, blind LGV treatment is advisable in the aforementioned situations (figure 2). The strategy above also involves standard HIV testing of MSM who are at risk for LGV and other STIs. Because case reports suggest that LGV facilitates transmission of hepatitis C virus (27), hepatitis C virus infection should also be considered in all MSM with LGV. According to our proposed algorithm, detection of \textit{C. trachomatis} in rectal swabs is the first step in LGV screening, and LGV serovar typing for all MSM with confirmed anorectal chlamydia is the second. In many microbiological laboratories in continental European countries, NAAT technology is used for the detection of chlamydia in rectal swab specimens after “in house” validation (7, 13). Recently, the Roche Amplicor has been validated for the detection of \textit{C. trachomatis} in rectal specimens (11); this will further incorporate the use of NAATs for the diagnosis of anorectal chlamydia. Subsequently, determination of the serovar for \textit{C. trachomatis}–positive anorectal specimens could be offered by a network of appointed laboratories that specialize in LGV. The initiation of a network of specialized laboratories for LGV diagnostics was proposed during an European Surveillance of Sexually Transmitted Infections satellite workshop on LGV research during the International Society for Sexually Transmitted Diseases Research meeting in LGV in MSM Amsterdam on 10–13 July 2005. Our recently developed LGV-specific, RT-PCR–based Taqman test could be helpful for the incorporation of LGV diagnostics into more microbiological laboratories (28). However, compared with most European countries, in the United States, it will be more problematic to incorporate LGV diagnostics into laboratory testing, because both US Food and Drug Administration (FDA) and Clinical Laboratory Improvement Amendments (CLIA) regulations apply. The use of rectal specimens has not been evaluated for the different NAATs that have been approved by the FDA for laboratory diagnosis of \textit{C. trachomatis} for use with other urogenital specimens (i.e., endocervical, male urethral, and vaginal swab specimens and urine samples obtained from men and women); thus, none of these NAATs have been approved by the FDA for use with rectal specimens (for a list of FDA-approved NAATs, see the 510K database [http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm; product code: MKZ]). Consequently, the CDC does not recommend the use of NAATs for rectal chlamydia screening until performance of the different NAATs has been evaluated with this specimen type and any specific NAAT is approved by the FDA for a rectal swab indication. However, the CDC has initiated studies to support FDA approval of NAATs for \textit{C. trachomatis} detection with rectal swab specimens to alleviate the burden on individual laboratories for validating (under CLIA regulations) the NAAT that they may choose to use for testing of rectal samples. LGV infection is a serious concern for the MSM community in Western Europe and other industrialized countries. Awareness of, screening for, and prompt treatment of LGV is crucial for the individual patient and to prevent further transmission in the wider (MSM) community. With this in mind, future research on prevalence, natural history including complications, and straightforward diagnosis and treatment are necessary.
Figure 2. Proposal for management of lymphogranuloma venereum (LGV) in men who have sex with men (MSM) who report having had receptive anal intercourse. This flow chart can be used to assess syndromic treatment for LGV in MSM and is based on sensitivity (true-positive rate), specificity (true-negative rate), and positive and negative predictive test values. The following suggestions apply. (1) For all MSM reporting receptive anorectal intercourse, rectal chlamydia screening is recommended. (2) If chlamydia test results are not yet unavailable (yellow box), for MSM reporting receptive anorectal intercourse who have proctitis noted by proctoscopic examination and a WBC count of 110 cells/high-power field in an anorectal Gram stain specimen or who have proctitis noted by proctoscopic examination and HIV seropositivity, treatment with doxycycline (100 mg twice per day) is advised until chlamydia test results are available, with a minimum duration of treatment of 7 days; for all other MSM, the caregiver should await the results of chlamydia tests. (3) If the anorectal chlamydia test result is negative (green box), no treatment should be administered or doxycycline treatment should be stopped after a minimum of 7 days. (4) If the anorectal chlamydia test result is positive and LGV testing is available (green box), doxycycline treatment (100 mg twice per day) should be started or continued until LGV is rejected or LGV test results are available, with a minimum treatment duration of 7 days. A 7-day course of doxycycline is effective for treatment of a non-LGV chlamydia. Therapy should be continued until 21 days after confirmation of LGV. If the anorectal chlamydia test result is positive and if LGV testing is unavailable (green box), doxycycline treatment (100 mg twice per day for up to 21 days) should be started or continued for MSM who meet one of the following criteria: proctitis noted during initial proctoscopic examination, 110 WBCs/high-power field in the initial Gram-stained anorectal smear specimen, or HIV seropositivity. For all other MSM, doxycycline treatment (100 mg twice per day) should be started or continued for up to 7 days.
Acknowledgments

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References


Chapter 11

Slow epidemic of lymphogranuloma venereum L2b strain

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Chapter 11

Abstract

We traced the *Chlamydia trachomatis* L2b variant in Amsterdam and San Francisco. All recent lymphogranuloma venereum cases in Amsterdam were caused by the L2b variant. This variant was also present in the 1980s in San Francisco. Thus, the current "outbreak" is most likely a slowly evolving epidemic.
Introduction

Since the end of 2003, an ongoing lymphogranuloma venereum (LGV) proctitis outbreak has been reported in industrialized countries, first in the Netherlands, followed by neighboring European countries and the United Kingdom, and now in the United States and Canada (1–4). We recently identified a new LGV variant designated L2b (GenBank accession no. AY586530) in all our cases in 2002 and 2003 that suggests this LGV outbreak was new (5). Until now, only men who have sex with men (MSM) are affected, and most are HIV co-infected. Although these infections, which can be caused by LGV serovars L1, L2, L2a, and L3, are often characterized by severe inflammatory symptoms, delayed or incorrect diagnosis has increased both the risk for transmission and the development of severe sequelae. Successful treatment of LGV proctitis requires a 3-week course of doxycycline followed by a test of cure, whereas in the case of *Chlamydia trachomatis* proctitis caused by serovars D–K, a 1-week course will suffice.

In a recent article on this LGV outbreak (3), 2 issues were stressed: 1) the lack of an easy diagnostic tool and 2) whether lymphogranuloma venereum is a new problem or whether it has been present but undiagnosed. Indeed, among the obstacles to the correct diagnosis of LGV is the lack of a commercially available assay to specifically distinguish between *C. trachomatis* infections caused by LGV serovars and infections caused by less invasive *C. trachomatis* serovars. A definitive diagnosis of LGV is currently made with nucleic acid sequence-based tests, like polymerase chain reaction (PCR)–based restriction fragment length polymorphism (RFLP) analysis, which are only available in a few specialized laboratories. We recently developed a real-time PCR (TaqMan and RotorGene) that can specifically distinguish LGV infections from infections with other *C. trachomatis* serovars, which facilitates diagnosis (6).

We used this new diagnostic tool to determine whether the LGV outbreak and its cause are a new phenomenon or whether LGV *C. trachomatis* serovars have been present much longer but have gone undiagnosed. First, we determined if the newly identified Amsterdam L2b variant was already present in the MSM population before 2002 by using stored samples collected from MSM with and without proctitis who sought treatment at the sexually transmitted infections (STI) outpatient clinic in Amsterdam. Second, we performed the same analysis on archived specimens from MSM in San Francisco, California, collected 20–25 years ago.
The Study

From MSM who attended the Amsterdam Municipal Health Service STI Outpatient Clinic in 2000 and 2001, randomly selected stored specimens of *C. trachomatis* DNA–positive (as assessed by ligase chain reaction, Abbott Laboratories, Chicago, IL, USA) rectal samples were tested for the *C. trachomatis* variant by real-time PCR (6). From 2002 to 2005, MSM with symptomatic proctitis (i.e., purulent discharge, rectal ulceration, bleeding, or edematous mucosa) and MSM without symptoms were included.

From the San Francisco region, 51 LGV–positive isolates from symptomatic MSM were analyzed (7). The isolates were collected in medical clinics (e.g., ambulatory care, emergency room, screening, acute care) from 1979 to 1985 (Table).

LGV was assessed at the time of collection, according to phenotypic properties observed during cell culture. Although the growth characteristics of LGV serovars can be distinguished from serovars D–K, cell culture for *C. trachomatis* is no longer available in most clinical settings.

*C. trachomatis* serovar typing was performed as described previously (5). Briefly, amplification of the *ompA* gene (1.1 kb) was performed in a nested PCR format. Serovars and variants were initially identified by their RFLP patterns after polyacrylamide gel electrophoresis. The *ompA* nucleotide sequences were subsequently analyzed by automated DNA sequencing on an ABI 310 sequencer (PE Biosystems, Foster City, CA, USA). The sequences obtained from *C. trachomatis*–infected MSM in 2000 and 2001 in Amsterdam and from MSM in San Francisco were compared to the recently identified L2b variant to determine if the strain was present earlier.
Results

The table presents the results of this analysis.

<table>
<thead>
<tr>
<th>Location</th>
<th>Year*</th>
<th>No L2b</th>
<th>No. samples tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsterdam</td>
<td>2000</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>2001</td>
<td>4</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>2002</td>
<td>40</td>
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<td>52</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td>2005</td>
<td>26</td>
<td>161</td>
</tr>
<tr>
<td>San Francisco</td>
<td>1979</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1980</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1981</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
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<td>29</td>
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<tr>
<td></td>
<td>1985</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*In 2002 and 2003, 45 LGV L2b variants of 109 isolates have been described in detail (5).

In the Amsterdam *C. trachomatis* DNA–positive rectal samples, LGV strains were detected by real-time PCR in 2 of 67 samples in 2000 and in 4 of 28 samples in 2001. Sequencing showed that in all 6 LGV strain–positive samples, the L2b variant was present. Also in 2002 and 2003, 109 L2b-positive samples of 403 *C. trachomatis* DNA–positive rectal samples were identified, of which 45 were strain L2b, and these have been described in a previous publication (5).

All 51 San Francisco specimens (from 51 patients) were positive for LGV variants by real-time PCR. By sequencing variable segment 2 of the *ompA* gene (VS-2), we identified 15 as serovar L1, 18 as serovar L2 prototype, and 18 as the L2b variant. We sequenced the complete *ompA* gene of 5 of these 18 L2b variants that originated in San Francisco; all were identical to the recently described L2b variant circulating in Amsterdam. Four nucleotide changes were found when compared to reference serovars L2, L2a, and the variant L2', including 1 change that encoded the previously undescribed change at amino acid 162, AAT→AGT (5).
Conclusions

The L2b LGV variant identified as the cause of all the LGV proctitis in the recent outbreak among MSM in Amsterdam appears to have been circulating in Amsterdam in 2000. Moreover, we showed that this L2b variant was present in the 1980s in San Francisco with exactly the same mutations in the complete ompA gene. However, since we only sequenced the ompA gene, and although the sequence was identical in old and new L2b strains, we cannot exclude the possibility that it could involve different strains of *C. trachomatis* that differ in other parts of the genome, although this is unlikely. Since LGV causes potentially severe infections with possibly irreversible sequelae if adequate treatment is not begun promptly, early and accurate diagnosis is essential. Sequence-based nucleic-acid tests that can discriminate between LGV serovars and less invasive *C. trachomatis* species can help detect cases and prevent further transmission of LGV.

In conclusion, our results suggest that we are dealing with the same LGV variant >25 years later, and the current LGV outbreak in industrialized countries has most likely been a slowly evolving epidemic with an organism that has gone unnoticed in the community for many years and is now being detected by new technologies. The numbers detected in 2005 in Amsterdam suggest that a considerable reservoir exists, which emphasizes the need for ongoing public health awareness.

References

General discussion
GENERAL DISCUSSION

*Chlamydia trachomatis* is the most prevalent bacterial sexually transmitted agent worldwide. Most infections run an asymptomatic course, in women around 70%, and subsequently will be transmitted unknowingly and left untreated. In women this might result in severe complications like pelvic inflammatory disease (PID), ectopic pregnancy and tubal infertility. When symptoms do occur, abnormal vaginal discharge, dysuria, and postcoital bleeding are mostly reported. Symptoms of chlamydial PID, which may be subtle, include pelvic, uterine, and/or adnexal pain. It is still mostly unclear why some women develop symptoms and why others do not, why in some women upper genital tract progression occurs with in some cases detrimental results and why in others the infection seems to clear rapidly, apparently without any clinical consequences. However, it is likely, that, as for other infectious agents, differences in the susceptibility to and severity of infections are due to a complex interaction between bacterial, environmental and host factors.

In the first part of this thesis we addressed these three factors using an integrated approach to understand the different course of *C. trachomatis* infections.

Since the Municipal Health Service in Amsterdam has as one of its main goals the study of all sexually transmitted infections (STD) to provide clinically based general health care Part 2 of this thesis describes a recently identified, still ongoing, lymphogranuloma venereum (LGV) outbreak among men having sex with men (MSM) in Amsterdam to determine the implications for clinical practice. Retrospective and prospective epidemiological studies have been used to to diagnose these LGV infections. This part of the thesis describe the characterization of the LGV strain identified in MSM and algorithms to identify LGV positive patients.

**Part 1: Urogenital Chlamydia trachomatis infections**

It is still unclear why the majority urogenital *C. trachomatis* infections run an asymptomatic course in women and clear an infection rapidly and why in others upper genital tract progression occurs with destructive complications. In order to better understand the pathogenesis of the infection and the subsequent disease caused by this micro organism different approaches were used. The focus was on the analysis of bacterial, environmental as well as host factors, separate or combined, in order to explain the differences in the susceptibility to and the severity of infections. The research performed in this thesis was in most part done on patient samples obtained voluntarily from relative young women attending the STD clinic in Amsterdam. A well defined study group of women visiting the Amsterdam STD clinic was established to better define prognostic and/or diagnostic variables to identify women at risk for long-term sequelae. Also the results are presented of patient samples obtained from gynaecologically well defined group of women with confirmed (based on hysterosalpingography or laparoscopy) tubal pathology.
1A: Bacterial factors

To consider bacterial factors in the susceptibility to and severity of urogenital *C. trachomatis* infections we first undertook a retrospective study on the serovar distribution in The Netherlands published ever and compared the results with our prospective collected data at the STD clinic. The determination of *C. trachomatis* serovar distribution in time could give insight in this possible relationship. The most recent serovar distribution in The Netherlands during 2000 – 2002 was stable in time and no significant shifts were found.

Also no significant shifts in serovar distribution could be demonstrated in The Netherlands from 1986 through 2002. However, when the serovar distributions were compared to each other based upon the two geographically distinct locations from which these serovar distributions were obtained it appeared that serogroup C was found more frequently in Rotterdam as compared to Amsterdam of which the most prominent serovar difference was serovar K. The Intermediate-serogroup was found less frequently in Rotterdam of which the most prominent serovar difference was serovar F. Serogroup B was stable between the two cities (Chapter 1). However, this can be explained in part by differences in study size and population composition like different ethnic compositions, age and extent of co-infection status of the studied cohorts or other confounding factors between Rotterdam and Amsterdam. It may be the result of the chance that possible differences cannot be distinguished at the serovar level. In future better typing methods might be developed in order to discriminate in more detail.

Recent studies in large well defined patient groups on serovar/serogroup distributions and clinical outcome are sporadic. Serotype G has been associated with symptomatic infections and upper genital tract infections and was also associated with the development of cervical carcinoma (1). In addition others have shown differences between serovars, the presence or absence of clinical symptoms, specific clinical symptoms, and gender. Differences were most striking among the less frequent *C. trachomatis* serovars Ga, Ia and K.; thus, serovar Ga was associated with dysuria in men, serovar Ia was detected only in asymptomatically infected men and women and serovar K was associated with abnormal vaginal discharge (2). From a microbiological standpoint it might be speculated that specific *C. trachomatis* serotype(s) are more virulent than others and less sensitive to appropriate antimicrobial therapy, and relevant to carcinogenesis. This is supported by experimental work in a lower genital tract animal model by Ito and Lyons (3,29). They have shown that there are differences among serovars both in duration of infection and in the ability to establish upper tract infection, indicating that there might be virulence factors or antigens that correlate with typing antigens *in vivo*. More research is needed to elucidate these differences in humans. In particular, new molecular methods to address the genomic differences of serovars and strains of *C. trachomatis* could resolve this in more detail.

Martin *et al.* (4) applied sequence analysis of the *ompA* gene to study the distribution of genotypes from the early 1980s through the mid of 1990s in New Orleans. No statistical shifts of *C. trachomatis*...
ompA genotypes were found over a period of nearly 20 years. Dean and co-workers have suggested to use genetic variation in polymorphic membrane proteins (PMPs) for strain identification. (5). By applying genetic, genomic and molecular analyses of several of those polymorphic membrane proteins of *C. trachomatis* it might be conceivable that antigenic polymorphism on the bacterial surface promotes a set of different receptors involved in host cell adhesion and thus differences in tissue tropism are proposed (6).

Chapter 2 describes the expression and the relationship between IncA sequence types and the clinical outcome of a urogenital *C. trachomatis* infection in Dutch Caucasian female patients. Two percent of the isolates were non-fusogenic, indicating that nonfusogenicity of *C. trachomatis* isolates is rare among the female STD populations studied. The two non-fusogenic strains, which comprise the 2%, were isolated from symptomatic female patients, while in general IncA sequence types were randomly distributed among symptomatic and asymptomatic patients.

The finding that nonfusogenicity of *C. trachomatis* isolates is rare among a female STD population (Chapter 2) is supported by the study performed by Suchland et al. in the USA (7). In a study on patient material from over 7000 women attending a STD clinic 1.5% of all independent strains were nonfusogenic. Sequence analysis of the *incA* gene on a limited number of isolates showed slight nucleotide differences leading to amino acid changes with possible modification of the hydrophobicity profile. Speculations were formulated about the association of *IncA* gene mutations with the phenotype of nonfusogenicity and the clinical significance of this finding. Suchland et al. report an association between the production of secondary inclusions via *incA*-loaded fibers. This allows chlamydiae to generate an expanded intracellular niche in which they can grow and may provide a means for continuous infection within progeny cells following cell division. Isolates of serovar G seemed to produce secondary inclusions at the highest frequency. The possibility that different *incA* sequence types may generate differences in secondary progeny, in number as well as in function, can be subject of further study (10, 29).

Associations with different serovars should be taken into account in this respect. The finding that *incA* sequence polymorphisms were not involved in the clinical outcome of a urogenital *C. trachomatis* infection is not supported by the study of Geisler and others (8,9). These investigators showed, by using quantitative culture techniques, that in women greater inclusion-forming unit counts were associated with inflammation, diagnosis of mucopurulent cervicitis and PID. Thereby, Eckert *et al.* (11) found in a large STD population that sex, age, race and serovar class were independently related to the number of inclusion forming units, with counts being highest among young white women infected with B-class serovars. Although they did not perform sequence analysis it can be speculated that differences in inclusion-forming unit counts correlate with different *incA* sequence types. Further studies on fusogenicity and how the local and systemic immune responses influence the length or persistence of infection are necessary.
1B: Environmental factors and epidemiological studies

To study the influence of the local urogenital co-infection status on the susceptibility to a *C. trachomatis* infection we performed a population based screening study in young adults (Chapter 3). The results from the population based study justified the omission of determining gonococcal infection in *C. trachomatis* DNA positive patients. The results suggest that in low prevalence areas a routine *N. gonorrhoeae* screen would not be required. Proper risk-assessment accomplished by the physician appears to be more efficient. The few persons tested positive for *N. gonorrhoeae* in our study were all young women (17, 18 year) with a high risk profile ( > 6 lifetime partners, no condom use during last sex and two had Surinamese/Antillean ethnicity). All reported in the questionnaire subjective complaints.

Our results correspond well with a Chlamydia screening programme in the UK. In STI clinics the prevalence of gonorrhoea among *C. trachomatis* positives was 4.6% for women and 6.3% for men but via community screening only 0.2% for women and 1.2% for men in the *C. trachomatis* -positives found (12). In the US, a nationally representative prevalence study, found a *C. trachomatis* prevalence of 4.2% and a low infection rate for *N. gonorrhoeae* (0.43%) and prevalence of co-infection was only 0.3% (13). Also substantial racial/ethnic disparities in prevalence of both infections were reported.

We investigated the role of *C. pneumoniae* in women with self reported lower abdominal pain (not menses nor gastrointestinal related), and a urogenital *C. trachomatis* infection (Chapter 4). The determination of *C. pneumoniae* IgG responses in those women with a urogenital *C. trachomatis* infection and compare the results in women without a urogenital *C. trachomatis* infection would give some insight in a possible (auto-) immune regulatory mechanism in relation to the severity of an acute urogenital *C. trachomatis* infection as has been proposed in the murine model (14). We showed, although the studied number of women was low, an association between the presence of lower abdominal pain in *C. trachomatis* DNA positive women and the presence of *C. pneumoniae* IgG antibody response. Two other studies (14,15) support our data. The results from a mouse model and the results from a tubal infertility study population showed an association between the severity, e.g. late upper genital tract disease, of a *C. trachomatis* infection and the frequency of *C. pneumoniae* IgG antibodies. To elucidate our results in more depth we consider starting with lymphocyte stimulation tests. Additional animal experiments, including both combinations of active and previous *C. pneumoniae* and/or *C. trachomatis* infections and the effect of IgA and IgM responses should be performed.
1C: Host factors

To study the influence of host factors in the susceptibility to and severity of C. trachomatis infections two types of host responses were studied: serological responses to infection with C. trachomatis and the influence of functional genetic variation in genes that can potentially modulate the inflammatory response of the host to this micro-organism. Chapter 5 describes the recently developed C. trachomatis specific hsp60 serology test employed to study the clinical relevance of the test in women with proven late complications caused by C. trachomatis. We showed a clear difference in anti-cHSP60 responses between women with and without procedure confirmed tubal pathology, while an intermediate prevalence was observed in pregnant women. Although the concordance between C. trachomatis IgG and cHSP60 positively was high, a clear different subgroup of women could be identified since only 40% of the C trachomatis IgG positive women had a cHSP60 response. Three recent studies support our first results (16-18) and showed an association between the grade of tubal pathology and the presence and the level of, among other serological tests, cHSP60 antibodies. With the availability of this new test, which can lead to standardization and comparison between different laboratories, large prospective follow-up studies can generate important information about the clinical value of the results. Identification of women at risk for tubal pathology and subsequent infertility may be identified by employing cHSP60 serology or in combination with C. trachomatis specific IgG and C-reactive protein (17) in the work-up in diagnosing C. trachomatis induced infertility.

To identify immunogenetic traits of the host that influence the susceptibility and course of infection and the immunity that develops in response to a urogenital tract infection with C. trachomatis we used the candidate gene approach in a well defined female cohort attending the STD clinic in Amsterdam. We searched for functional genetic variation in the Interleukin-1 receptor antagonist (Chapter 6) and showed that the carriership of the IL-1RN 2 allele was significantly decreased in C. trachomatis DNA positive women compared to C. trachomatis DNA negative women. Neither the serology status nor the co infection status or symptomatology altered the results. This appears to be relevant because it has been previously shown that allele 2 is associated with decreased expression of IL-1ra and a pro-inflammatory immune response (19). Whether our finding is linked to the susceptibility for repeated infections or persistence of infection needs further and prolonged investigation. Since the cytokine cascade comprises different and redundant cytokines and chemokines that are induced as part of the innate response and acute–phase reaction, DNA and RNA microarray technology may be necessary to elucidate and understand the complexity of the inflammatory response. Previous studies have shown that the carriage of the IL-1RN 2 allele is associated with protection against infection related pre-term birth (20-23). However, no associations between IL-1RN and the late complication of C. trachomatis infection, tubal pathology have been demonstrated. This indicates that genetic variation in IL-1RN might protect against primary C. trachomatis infections but may not influence strongly the development of late complications, reflecting complex underlying pathogenic mechanisms, and
indicating the potential existence of other factors influence the risk for tubal pathology. From these data it can be hypothesized that immunogenetic intervention might be a step in reducing long term sequelae due to urogenital C. trachomatis infections in women.

Chapter 7 describes the results of the study of the role of the CD14 –260 C>T polymorphism in the susceptibility to and severity (defined as subfertility and/or tubal pathology) of C. trachomatis infection in Dutch Caucasian women. The functional polymorphism -260 C>T in the LPS sensing TLR4 co-receptor CD14 gene is known to enhance the transcriptional activity and results in a higher CD14 receptor density on the infected cell membrane (24, 28). By assessing the different CD14 –260 C>T genotypes with PCR-based RFLP analysis in women attending a STD clinic, in women with subfertility, and in an ethnically matched control cohort no difference was found in the genotype distributions indicating that this polymorphism on itself is most likely not involved in the susceptibility to or severity of sequelae of C. trachomatis infection.

Using the knowledge that CD14 can signal through TLR4, it might be hypothesized that the absence of an association between the CD14 –260 SNP and the susceptibility to C. trachomatis infection might be due to the low expression or absence of TLR4 in the lower urogenital tract or that CD 14 is perhaps not a strong regulator in this respect. In the upper genital tract, strict regulation of immune responses to LPS by TLR4 may inhibit CD14 signalling through TLR4, thus limiting the influence of CD14 on the development of tubal pathology.

However, this hypothesis does not take into account the ability of CD14 to signal through TLR2, nor does it take into account that Netea et al. (25) demonstrated that non-LPS components of Chlamydia pneumoniae can stimulate cytokine production through TLR2 dependent, CD14 independent pathways (26). Similar mechanisms may exist and stimulate C. trachomatis induced cytokine production in urogenital infections.

TLR2 is involved in Chlamydia-induced TGF-beta, an anti-inflammatory cytokine with an important role in fibrosis, and thus very likely to have a role in post-infection tubal pathology. This might explain why CD14 polymorphisms are not important in the development of tubal pathology (27). The recognition of bacterial LPS involves a complex system of multiple receptors and a complex orchestration of protein-protein interactions and more work needs to be done to fully understand the role of host factors in modulating the response to C. trachomatis infection.

**Future perspectives**

The results of the studies reported in this thesis illustrate the necessity of an integrated approach in unravelling the cause(s) of different clinical courses of urogenital C. trachomatis infections. Large well-defined patient populations and a new technology are necessary to fully understand the evolution of C. trachomatis infection in order to provide the clinician with tools to be able to predict the course of the infection. Advances in biotechnology of DNAChips and RNA microarray will probably be helpful in answering these questions.
References


Part 2: Lymphogranuloma venereum

The LGV outbreak versus ongoing spread
Lymphogranuloma venereum (LGV) is a sexually transmitted infection (STI) caused by Chlamydia trachomatis serovars L1, L2, and L3. It is endemic in parts of Africa, Asia, South America, and the Caribbean but has been rare in industrialised countries (1,2,3). However, in 2003, a cluster of LGV cases among men who have sex with men (MSM) was reported in Rotterdam, the Netherlands (4,5). Since then, other reports have appeared on similar outbreaks in large cities in Western Europe (6,7,8,9,10,11,15,16) and the United States (6,12) and Canada (13). Eventually by December 2005 a total of 179 confirmed cases of LGV were reported in The Netherlands, of which 65 were identified retrospectively (6). The Amsterdam STD clinic reported an additional 4 confirmed cases until April 2006 (personal communication H.J. de Vries, MD Amsterdam STD clinic).

When the spread of the disease was found in many countries the logical question to answer was whether this was really a new outbreak. The answer is essential for the management of this disease. We have investigated this issue and showed that the “outbreak” first reported in December 2003 in Rotterdam, was also present in 2000 around Amsterdam and elsewhere and was caused by a newly identified L2b variant which could be identified in both MSM with and without disease of the rectum. Also our extension to analyse samples from the ’80’s in San Francisco showed clearly that this is not a new outbreak (Chapter 11). Our data support strongly that an old ‘friend’ is reappearing or has been undiagnosed for a very long time (17).

LGV diagnostics
The management of the “outbreak” was severely hampered by quick and easy identification of LGV infection. To circumvent the labour intensive and time-consuming amplification of the ompA gene (nested format) and restriction fragment length polymorphism a real-time PCR technique was developed (Chapter 9) which can now be used in clinical practice. Based upon the amplification of the pmpH (19) gene a definite distinction can be made between LGV positive and LGV negative patient samples within 2 hours. However, direct genotyping of C. trachomatis NAAT positive rectal samples have yet to be developed and validated. Moreover, diagnosing rectal C. trachomatis infections using NAAT, a technique which has been validated extensively and approved by the FDA for urogenital patient material, could not legitimate be used for rectal swabs.

The diagnostic approaches have been extended not only to detection of LGV infection but also to strain characterization and resulted in the identification of the AMSTLGV L2b genovariant (accession number is 153611  AY586530) (Chapter 8). This strain was also identified in recent patient samples from France (9). It is possible that amino acid changes which cause conformational changes in this strain have immuno-modulating consequences for the host.
General discussion

**LGV diagnosis**
Initially recognition of the infection was hampered due to the unfamiliarity with the disease in MSM by western physicians. Primary ulcers may have been misdiagnosed as HSV or *T. pallidum* and treated as such. In addition, since cases were also characterized by severe anorectal infections with long-lasting symptoms including rectal pain, tenesmus, rectal discharge and constipation, it has been mistaken with Crohn’s disease of the rectum (14,18) and patients have been directed to gastroenterologists instead to venereologists.

Rapid recognition of the infection and disease at consultation is now supported by implementing additional anamnestic questions such as the HIV status of the patient and his sexual behaviour since associations were found with a variety of sexual risk behaviours with numerous anonymous sex partners and HIV positivity. Easy to perform during the consultation at the STD clinic is the determination of the WBC count of more than 10 WBC’s in a smear of the rectal mucus. If one of these answers is positive, as we have shown (Chapter 10), the diagnosis LGV will be strengthened by 80% and blind LGV treatment is justified. Clinical recognition needs still to be improved, updated and made available through the professional literature and circuit.

**Future perspectives**
In April 2005 a scientific meeting was held by the European Surveillance of Sexually Transmitted Infections (ESSTI) network and aimed to facilitate information exchange and defining management guidelines following the emergence of the current LGV outbreak (20). Published studies and preliminary data, including the work presented in this thesis, were discussed in sub-groups and used to define the following recommendations to enhance LGV prevention and control efforts across Europe. Clinical recognition still needs to be improved by increasing awareness among STD and general physicians.

The conclusions and recommendations of the April 2005 ESSTI meeting were:

- Updated clinical information and guidance for investigation (including questionnaires), diagnosis and management should be placed on the ESSTI website (www.essti.org/).
- There is a need for comparable case definitions. The ESSTI working group on LGV should prioritise harmonization of case definition as quickly as possible.
- International comparison of strains and lab routine procedures are required. Further collaboration among European microbiologists would be beneficial.
- International recommendations for uniform diagnosis of LGV are required.
- ESSTI should make available a list of reference laboratories available for LGV diagnosis confirmation. Outbreak investigation questionnaires should be standardised.
- The ESSTI working group on LGV could play a role in developing a standard instrument for use.
Consideration should be given to establishing internet-based reporting of LGV cases (real-time surveillance).

Consideration should be given to establishing a multi centre study on epidemic characteristics and clinical features of LGV proctitis among MSM.

Since all European countries are not equally affected by the LGV epidemics, a tiered response to managing local incidents was recommended.

Components of this approach could include:

- in countries with no or few cases at present (Level 1), the basic recommendation would be to raise awareness about LGV in the MSM community and the medical profession about the clinical features of LGV, diagnostics and methods of reporting. These countries should begin to identify pathways in which laboratory diagnosis, through networking with regional reference centres, could be organised.

- in countries with a number of reported cases (Level 2), active surveillance should be instituted and the transmission risk factors and clinical features need to be investigated thoroughly. International collaboration will increase the power of descriptive and analytic investigations.

Based on the above recommendations, the ESSTI working group on LGV is preparing a work plan of potential activities for 2006/7 which will also include steps to obtain additional funding to implement the recommended activities. At the ISSTDR meeting in July 2005 in Amsterdam on behalf of the ESSTI board a Satellite workshop on LGV research collaboration was organized to further discuss the recommendation made previously and include the most recent findings presented by both the workshop members and those who presented their most recent data at the ISSTDR meeting. ESSTI network grant has been renewed by the EC during 2005; funding has now been guaranteed until the end of 2008. A new board has been put in place and at present is working on organizing new activities and events on this topic.

References


SUMMARY

*Chlamydia trachomatis* infections impose a significant burden on humans globally. It is the most prevalent sexually transmitted bacterial infection and it can, if left untreated, lead to major complications like pelvic inflammatory disease, ectopic pregnancy, infertility, and infant pneumonia.

**The aim of the thesis was twofold:**

In **Part 1** we addressed the role of bacterial factors, amongst others *C. trachomatis* serovars (Part 1A), environmental factors and epidemiological variables (Part 1B), and host factors (Part 1C), among others serological responses and immunogenetic responses (candidate gene approach based), to assess their role, individual or combined, in the susceptibility to and severity of *C. trachomatis* infections to provide a basis for an integrated study approach.

In **Part 2** we described a recently identified, still ongoing, lymphogranuloma venereum (LGV) outbreak among men having sex with men in Amsterdam. To characterize and describe this current clinically relevant topic we used retrospective and prospective epidemiological studies, we addressed different approaches to diagnose these LGV infections, characterized the LGV strain identified in these men, and looked at algorithms to identify LGV positive patients.

In order to control its transmission and evolvement to severe complications it is vital to identify and screen patients at-risk and determine bacterial, environmental as well as host factors that can predict the course of infection. It is hypothesized that interaction between those factors will ultimately determine the patients outcome in the long term.

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**Part 1**
Summary

1A: Bacterial factors that contribute to the pathogenesis of *Chlamydia trachomatis* female genital tract infection

The role of two potential bacterial factors encoded by either *ompA* gene encoding a Chlamydial membrane protein, or the *IncA* gene, encoding a Chlamydial inclusion membrane protein were studied.

In Chapter 1 we show the results of the largest and most recent study on the serovar distribution in The Netherlands and compared them to all published Dutch serovar distributions. No major differences in serovar distributions in time (1986-2002) were observed in The Netherlands, but different stable geographical-based serovar distributions could be demonstrated. These findings suggest that the prevalence of urogenital serovars isolated from the female genital tract is stable in time using RFLP typing technique.

Chapter 2 summarizes the results of a study towards the variation in *incA* gene among Dutch *Chlamydia trachomatis* isolates in relation to the symptom presentation of an urogenital infection. In our study, 2% (2/88) of the isolates were non-fusogenic and isolated from symptomatic female patients. Among the fusogenic types multiple sequence types were found and those were randomly distributed among symptomatic and asymptomatic patients.

1B: Environmental factors and epidemiological studies to describe aspects of the susceptibility for and severity of an acute urogenital *Chlamydia trachomatis* infection

One of the aspects of studying infectious diseases is to apply epidemiological methods and analyses to assist in the understanding of disease (immuno) pathogenesis. Important issues are describing patterns of infection and disease in populations and identification and characterization of factors in the chain of infection that might contribute to agent transmission and development of disease. The aim of the two studies described below was to investigate the influence of a co-infection (*Chlamydia pneumoniae*) on the course of a *C. trachomatis* infection and to investigate the potential relevance of testing for *N. gonorrhoeae*, in *C. trachomatis* positive persons participating in a general screening programme.

Chapter 3 outlines the basis for testing for gonorrhoeae if chlamydia infection is found among asymptomatic persons. Four gonococcal infections were found among 166 participants with *C. trachomatis* infection (2.4%). All four had several risk factors and reported symptoms. Among 605 *C. trachomatis*-negative persons, no infection with *N. gonorrhoeae* could be confirmed. Although the
number of patient samples available was low, we showed that during population based screening, the population prevalence of *N. gonorrhoeae* among *C. trachomatis* positive patients is too low to include in screening programmes.

In Chapter 4 we studied the prevalence of *Chlamydia pneumoniae* IgG antibodies in women with self reported lower abdominal pain (LAP) and an urogenital *Chlamydia trachomatis* infection. Differences were observed between women with *C. pneumoniae* IgG and women without *C. pneumoniae* IgG antibodies. From the 170 *C. trachomatis* DNA positive women 152 had IgG antibodies against *C. pneumoniae* and from those 22% reported LAP while from the 18 women without antibodies to *C. pneumoniae* none reported LAP (p=0.025). These data provide new insight in the immunopathological mechanisms of *C. trachomatis* associated symptomatology.

**1C: Host factors that influence the susceptibility for and severity of an acute urogenital Chlamydia trachomatis infection**

Sensing of a pathogenic bacterium by the host is the first step in elimination. A number of signalling pathways have been identified that might play a role in determining the course, outcome and immune response to *C. trachomatis* female genital tract infections. Genetic variation could influence the inflammatory response modulated through NF-kB activation. The Toll-like receptors (TLRs) recognize components of the infectious agents and can trigger an inflammatory cascade that results in the expression of the array of cytokines, chemokines and other molecules. Interleukin-1 (IL-1), a proinflammatory cytokine, in balance with its antagonist interleukin-1 receptor antagonist with antinflammatory activity, are important, among others, in stimulating an immune respons of the host.

In Chapter 5 we assessed the clinical relevance of a commercially available serological assay that detects antibodies to chlamydial heat shock protein 60 (cHSP60). Three gynaecologically well defined groups of women were included in the study; 1) women without tubal pathology, 2) pregnant women and 3) women with confirmed and proven tubal pathology. The results of the study show that cHSP60 responses seems to be associated with one of the longterm complications of a *C. trachomatis* infection.

In Chapter 6 we describe the results obtained in young Dutch Caucasian women infected with *C. trachomatis*, demonstrating that the *IL-1RN* +2018 T>C polymorphisms of the interleukin 1 receptor antagonist is associated with the susceptibility to urogenital *C. trachomatis* infections. The results show that, after correction for other microorganisms and ethnical background, carrierness of the *IL-1RN* 2 allele was significantly decreased in *C. trachomatis* DNA positive women (39%) compared to *C. trachomatis* DNA negative women (50%) (p: 0.0005).
Summary

Chapter 7 presents the role of genetic variation in the LPS sensing Toll-like receptor 4 (TLR4) co-receptor CD14 in 3 different human cohorts of women: 1) women attending a STD clinic, 2) women with subfertility, and 3) a control cohort. The CD14 –260 C>T genotypes were identical in all three cohorts, showing that this polymorphism does not play a significant role in the susceptibility to or severity of *C. trachomatis* infection.

Conclusions Part 1

The results obtained and described in the first part of the thesis show that bacterial factors, environmental factors and epidemiological variables, and host factors, individual or combined are linked to the susceptibility to and severity of urogenital *C. trachomatis* infections in a STD population. This integrated approach has provided us with a starting point for future research in order to extend our knowledge and understanding on the pathogenesis of *C. trachomatis* infections and to predict with more certainty the ultimate clinical outcome of a urogenital *C. trachomatis* infection in an individual patient.
Part 2

Lymphogranuloma venereum (LGV), caused by *C. trachomatis*, is rare in developed countries. Recently, an outbreak was reported in Rotterdam, The Netherlands. Clinically these patients had severe proctitis and constipation. Epidemiologically the patients were predominantly men who had sex with men, were HIV positive and reported a variety of sexual risk factors. In order to understand this finding more thorough and to characterise the patients in more detail we started with a retrospective study in Amsterdam. Our hypothesis was that beyond Rotterdam, LGV infected patients could be identified in other parts of The Netherlands as well and potentially outside The Netherlands. Furthermore a validated real-time PCR detection method for LGV was necessary to be developed in order to identify patients adequately. Finally, we determined clinical variables to identify patients most likely to be LGV positive.

Chapter 8 describes the presence of LGV infected MSM in Amsterdam determined by restriction fragment length polymorphism and sequencing. In this retrospective study (2002-2003) LGV infections could be demonstrated in *C. trachomatis* DNA positive rectal samples from MSM with and without mucous membrane abnormalities locally attending the STD outpatient clinic. Sequencing of the *ompA* gene isolated from these men showed that all of the men tested positive were infected with a new LGV genovariant, designated L2b. These results indicate that the outbreak in Rotterdam is an outbreak which at least extends beyond Rotterdam and is caused by a newly identified strain.

Chapter 9 presents the results of the development of an *in-vitro* real-time PCR technique to diagnose LGV serovars directly from specimens to allow timely and sensitive detection. The laboratory diagnosis of LGV is normally performed using direct detection of *C. trachomatis* specific DNA in rectal specimens followed by amplification of the *ompA* gene using a nested PCR followed by RFLP to distinguish the different serovars. In order to overcome some important drawbacks of this procedure (the detection of *C. trachomatis* DNA in rectal samples is not validated by the FDA, RFLP is labour intensive and can only be performed in some reference laboratories) a sensitive and specific real-time PCR was developed. By using quantified cultured EB’s the sensitivity of the Taqman as well as the Rotorgene analysis was determined: 0.01 IFU. The specificity was extensively determined using 32 common gastro-intestinal as well as urogenital microorganisms. No cross-reactions was found for either detection method. In routine practice the application of this real-time technique can gain a result within 2 hr as compared to at least 2 days using the classical techniques and due to its simplicity it can easily be embedded in most routine diagnostic settings.

Chapter 10 describes, in a well defined two-year retrospective case-control manner, risk factors and clinical and diagnostic signs for rectal LGV among MSM. By defining cases as MSM proven infected
Summary

rectally with serovar L2b and the controls as infected with non-LGV genotypes or MSM reporting receptive anorectal intercourse but without anorectal chlamydia we observed that HIV-seropositivity was the strongest risk factor for LGV. Irrespective of the control group used, proctoscopic findings and white blood cell (WBC) count in Gram-stained anorectal smears were the only clinical relevant predictors for LGV infection, indicating that whenever routine LGV serovar typing is not available, blind LGV treatment in MSM with anorectal chlamydia is advised when additionally proctitis upon proctoscopic examination is localized or more than 10 WBC in an anorectal Gram-stained smear is found or when the patient is HIV positive.

Chapter 11 presents the results of a retrospective study on the presence of the *C. trachomatis* L2b genovariant in Amsterdam. Analyzing stored samples (available till 2000) we were able to identify the L2b genovariant back to 2000 in Amsterdam. To put these LGV results in a more global perspective we were able to analyse 51 stored samples from the 80s from our collaboration with Prof. Julius Schachter (SF, USA). The rectal samples were from men and collected in medical clinics (ambulatory care, emergency room, screening and acute care) in San Francisco, between 1979-1985. At that time these samples were determined positive culture. When send to Amsterdam in 2004 all resulted positive for *C. trachomatis* by NAAT indicating that storage at –20°C did not have had any influence on the presence of *C. trachomatis* in the US samples. In addition, sequencing of the strains showed 18 L2b; the current circulating Amsterdam strain. Taken together these results show that the new strain identified in The Netherlands has been circulating before, possibly on a low endemic level. which has not been recognized.

Conclusions Part 2

The results obtained from this part of the thesis demonstrate that the current outbreak of LGV is found among MSM in Amsterdam too. The infection is not exclusively found among MSM with clinically proven mucous membrane abnormalities. Also asymptomatic MSM can be infected and may play an important role in the transmission. At test locations without the facility of genotyping, physicians are supported in their diagnosis and can start antibiotic therapy when besides a positive *C. trachomatis* NAAT rectal sample a Gram stain is positive for more than 10 WBC in the rectal mucous or when the patient is HIV positive. New up to date laboratory tests, e.g. real-time PCR and sequencing, demonstrate that the causing agent, the L2b Amsterdam genovariant, is involved in the current so-called outbreak in The Netherlands as well as in the samples from the 80s obtained from MSM in San Francisco. These rectal LGV infections have thus been missed in daily STD practice for many years in the developed countries. Due to the association with concurrent HIV infection one might speculate that HIV infection and/or the combination with HAART will down regulate the individual immune
response and will diminish the clinical presentation by the patient so physicians will miss at least the primary presentation of a LGV infection, e.g. small ulcus, and the inguinal bubo formation. Thus by developing a real-time PCR method to diagnose LGV patients and utilizing the identified patients characteristics described in this thesis the starting point for further study and manage this slow evolving epidemic is deposited.
Summary
SAMENVATTING
Samenvatting
SAMENVATTING

Chlamydia trachomatis infecties leiden wereldwijd tot grote morbiditeit. Het is de meest voorkomende seksueel overdraagbare infectie, die, indien er geen behandeling met antibiotica plaats vindt, kan leiden tot ernstige complicaties zoals ‘pelvic inflammatory disease’, buiten baarmoederlijke zwangerschap, onvruchtbaarheid en pneumonieën bij neonaten.

Het doel van dit proefschrift was tweeledig:

Er zijn duidelijke verschillen in het klinisch beloop van C. trachomatis infecties tussen verschillende individuen. In deel 1 bestudeerden we daarom de rol van bacteriële factoren, waaronder de C. trachomatis serovars (deel 1A), omgevingsfactoren en epidemiologische variabelen (deel 1B), en gastheerfactoren (deel 1C), waaronder serologische- en immunogenetische responsen (gebaseerd op de zgn. ‘candidate gene approach’) om na te gaan wat de rol van deze factoren is, hetzij individueel hetzij gecombineerd, ten aanzien van de gevoeligheid voor en de ernst van C. trachomatis infecties.

Eén van de taken van de GGD van Amsterdam is het beschikbaar stellen van basisgezondheidszorg op het gebied van seksueel overdraagbare aandoeningen ten behoeve van m.n. de Amsterdamse bevolking. Vanuit deze achtergrond werd in deel 2 van het proefschrift de recent ontdekte en de nog steeds voortdurende uitbraak van Lymfogranuloma Venereum (LGV) onder homoseksuele mannen in Amsterdam beschreven. Om dit klinisch relevante thema nader te onderzoeken en in kaart te brengen is er gebruik gemaakt van zowel retrospectieve- als prospectieve epidemiologische onderzoeken, zijn er diverse benaderingswijzen toegepast om de diagnose LGV infectie te stellen, is de in Amsterdam circulerende LGV stam gekarakteriseerd en is er tenslotte gezocht naar diagnostische algoritmes om LGV positieve patiënten te identificeren.

Om nu de overdracht van C. trachomatis te beteugelen en de ontwikkeling naar ernstige complicaties als gevolg van een C. trachomatis infectie nader te onderzoeken is het noodzakelijk om patiënten, die risico lopen te identificeren en te screenen en zowel bacteriële-, omgevings- als gastheerfactoren in kaart te brengen, die het verloop van een infectie zouden kunnen voorspellen.
Deel 1

1A: Bacteriële factoren, die van invloed kunnen zijn op de verschillen in pathogenese van een *Chlamydia trachomatis* infectie in de tractus urogenitalis van de vrouw

De rol van 2 bacteriële factoren, die potentieel een rol zouden kunnen spelen in dit proces, werd bestudeerd. Bestudeerd zijn het *ompA* gen, dat codeert voor een Chlamydia membraan eiwit en het *IncA* gen, dat codeert voor het Chlamydia inclusie eiwit A.

In Hoofdstuk 1 hebben we de resultaten beschreven van de grootste en meest recente studie over serovar distributies in Nederland en vergeleken deze met alle in Nederland gepubliceerde serovar distributies. In de periode 1986 – 2002, zijn er geen grote verschillen in serovar distributies in Nederland waargenomen. Echter verschillende serovar distributies konden worden aangetoond in 2 geografisch locaties. Deze resultaten suggereren dat de prevalentie van urogenitale serovars, die geïsoleerd zijn uit de tractus urogenitalis bij vrouwen stabiel is in de tijd.

Hoofdstuk 2 vat de resultaten samen van de studie naar de variatie in het *incA* gen bij *C. trachomatis* isolaten, die verkregen zijn bij Nederlandse vrouwen, die de geslachts-ziektepolikliniek in Amsterdam bezocht hebben, in relatie tot de symptoompresentatie van hun urogenitale infectie. Uit onze studie bleek dat 2% (2/88) van de isolaten zgn. niet-fusogeen waren en dat deze isolaten geïsoleerd waren bij vrouwen met een symptomatische presentatie van deze infectie. Onder de fusogene types werden multipele sequentie types gevonden, die willekeurig verdeeld waren onder de symptomatiche- en asymptomatische patiënten.

1B: Omgevingsfactoren en epidemiologische studies, die aspecten beschrijven van de vatbaarheid voor en de ernst van een acute urogenitale *Chlamydia trachomatis* infectie

Eén van de aspecten, die van belang is bij het bestuderen van infectieziekten in het algemeen, is het toepassen van epidemiologische methoden en analyses om de (immuno) pathogenese beter te begrijpen. Belangrijk daarbij is o.a. de beschrijving van patronen van overdracht van de desbetreffende infectie en de daaraan gerelateerde ziekte in bepaalde bevolkingsgroepen. Het doel van de 2 studies, die hieronder beschreven worden was om te onderzoeken wat de invloed was van co-infectie (*Chlamydia pneumoniae*) op het beloop van een *C. trachomatis* infectie en wat de mogelijke relevantie is van het testen op *N. gonorrhoeae*, in *C. trachomatis* positieve personen, die meedoen aan een algemeen screeningsprogramma.

Hoofdstuk 3 beschrijft de basis voor het al dan niet verantwoord testen op gonorroe wanneer een chlamydia infectie is aangetoond bij personen, die geen symptomen vertonen van een dergelijke
chlamydia infectie. Uit de studie bleek dat er bij 166 deelnemers met een *C. trachomatis* infectie 4 mensen waren bij wie ook een gonococcen infectie aangetoond kon worden (2,4%). Bij de 605 deelnemers zonder een *C. trachomatis* infectie kon geen enkele infectie met *N. gonorrhoeae* worden aangetoond. Ondanks het relatieve kleine aantal suggereren deze resultaten dat, tijdens een populatie screening, de prevalentie van *N. gonorrhoeae* infecties laag is en het niet zinvol is om bij *C. trachomatis* positieve deelnemers *N. gonorrhoeae* detectie te includeren dit in tegenstelling tot symptomatische patiënten die gezien worden op een SOA poli.

In Hoofdstuk 4 bestudeerden we de prevalentie van *Chlamydia pneumoniae* IgG antistoffen bij vrouwen, die zelf met de klacht buikpijn op de SOA polikliniek komen en bij wie door middel van laboratoriumonderzoek een urogenitale *C. trachomatis* infectie is aangetoond. We konden verschillen aantonen tussen vrouwen met en vrouwen zonder *C. pneumoniae* IgG antistoffen: van de 170 *C. trachomatis* DNA positieve vrouwen hadden er 152 IgG antistoffen tegen *C. pneumoniae* en van deze groep vrouwen rapporteerden er 22% pijn in de onderbuik, terwijl van de 18 vrouwen zonder antistoffen tegen *C. pneumoniae* er geen één pijn in de onderbuik rapporteerde (p=0.025). Wellicht kunnen deze gegevens een toevoeging zijn ten aanzien van de visie op de immunopathogenetische mechanismen van urogenitale *C. trachomatis* infecties en de daaraan gerelateerde symptomatologie.

1C: Gastheerfactoren, die van invloed zijn op de vatbaarheid voor en de ernst van een acute urogenitale *Chlamydia trachomatis* infectie

Het waarnemen van een pathogeen door de gastheer is de eerste stap bij het uit de weg ruimen daarvan. Een aantal van dergelijke zgn. signaleringsmechanismen zijn geïdentificeerd en zouden ook een rol kunnen spelen in het beloop, de uitkomst en de immuunresponse bij urogenitale *C. trachomatis* infecties bij vrouwen. Genetische variatie hierin zou de ontstekingsresponse, die gemoduleerd wordt via NF-κB activatie, kunnen beïnvloeden. De Toll-like receptoren (TLRs) zijn in staat zijn om delen van micro-organismen te herkennen en deze herkenning om te zetten in activatie van een intracellulaire ontstekingscascade, die uiteindelijk resulteert in de expressie van verschillende boodschappermoleculen als cytokines en chemokines. Eén van de cytokines is interleukine 1 (IL-1) dat een pro-inflammatoire rol heeft in dit geheel en in staat is om een immuunresponse te stimuleren. De balans tussen IL-1 en de interleukine 1 receptor antagonist, dat een anti-inflammatoire werking heeft, bepaalt uiteindelijk het fysiologisch effect.

In Hoofdstuk 5 bepaalden we de klinische relevantie van een commercieel verkrijgbare serologische test, die antistoffen bepaald tegen chlamydia ‘heat shock’ eiwit 60 (cHSP60). Daarvoor hebben we gebruik gemaakt van 3 gynaecologisch goed gedefinieerde groepen; groep 1: vrouwen zonder
Samenvatting

tubapathologie, groep 2: zwangeren, en groep 3: vrouwen met bewezen tubapathologie. De resultaten van de studie toonden aan dat de cHSP60 IgG responsen correleerden met de aanwezigheid van tubapathologie.

In **Hoofdstuk 6** laten we zien dat het **IL-1RN**+2018 T>C polymorfisme van de interleukine 1 receptor antagonist geassocieerd lijkt te zijn met de vatbaarheid voor een urogenital *C. trachomatis* infectie bij jonge Nederlandse vrouwen. De resultaten tonen aan dat, na correctie voor andere micro-organismen en etnische achtergrond, dragerschap van het **IL-1RN**2 allele significant minder voorkomt bij *C. trachomatis* DNA positieve vrouwen (39%) in vergelijking met *C. trachomatis* DNA negatieve vrouwen (50%; p=0.0005).

**Hoofdstuk 7** beschrijft de rol van genetisch variatie in de LPS onderscheidende Toll-like receptor 4 (TLR4) co-receptor CD14 in 3 verschillende cohorten vrouwen: 1) vrouwen, die de SOA kliniek bezoeken, 2) vrouwen met subfertiliteit, en 3) controlegroep. Uit onze studie is gebleken dat de CD14 –260 C>T genotypen gelijk voorkwamen in al deze 3 cohorten, hetgeen aangeeft dat dit polymorfisme geen grote rol speelt in de vatbaarheid voor of de ernst van een *C. trachomatis* infectie.

**Algemene conclusies deel 1**

De resultaten, die verkregen en beschreven zijn uit studies in het eerste deel van dit proefschrift hebben aangetoond dat bacteriële factoren, omgevingsfactoren en epidemiologische variabelen, en gastheerfactoren, individueel of gecombineerd onderling samenhangen in relatie tot de vatbaarheid voor en de ernst van urogenitale *C. trachomatis* infecties in een SOA populatie en/of een subfertiliteits populatie. Deze samenhangende benadering van onderzoek zou een basis kunnen betekenen voor toekomstig onderzoek om de kennis en het begrip rondom de pathogenese van *C. trachomatis* infecties uit te breiden, waarbij het uiteindelijk mogelijk zou kunnen worden om in iedere individuele met *C. trachomatis* geïnfecteerde patiënt te kunnen voorspellen wat of het klinisch beloop zal gaan worden.
Deel 2
Lymphogranuloma venereum (LGV), is in de westere wereld een zeldzame seksueel overdraagbare aandoening, die veroorzaakt wordt door *C. trachomatis*. Recent werd een uitbraak gerapporteerd in Rotterdam. De patiënten uit de uitbraak hadden ernstige proctitis en constipatie. Epidemiologisch onderzoek toonde aan dat het uitsluitend homoseksuele mannen betrof, die HIV positief waren en verschillend risico gedrag vertoonden. Om deze bevinding beter te kunnen begrijpen en om deze patiënten meer gedetailleerd te kunnen gaan beschrijven zijn we begonnen met een retrospectieve studie in Amsterdam. Onze hypothese was dat LGV patiënten niet alleen in Rotterdam konden worden geïdentificeerd en ook in andere delen van Nederland en wellicht daarbuiten voorkwamen. Om dit onderzoek uit te kunnen voeren was het noodzakelijk om een gevalideerde real-time PCR methode voor LGV op te gaan zetten om patiënten adequaat en snel te kunnen identificeren. Tot slot brachten we klinische variabelen bij patiënten in kaart die gerelateerd waren aan het hebben van een LGV infectie.

**Hoofdstuk 8** beschrijft de aanwezigheid van LGV geïnfecteerde homoseksuele mannen in Amsterdam, waarbij gebruik gemaakt is van ‘restriction fragment length polymorphism’ en sequentieanalyse. Deze retrospectieve studie (2002 – 2003) toonde aan dat LGV infecties aangetoond konden worden in *C. trachomatis* DNA positieve rectum monsters van homoseksuele mannen, die zich zowel klinisch presenteerden met als ook zonder lokale slijmvliesafwijkingen. Sequentieanalyse van het *ompA* gen van deze patiëntmaterialen toonde aan dat deze mannen allen geïnfecteerd bleken te zijn met een nieuw LGV genovariant, die we L2b genoemd hebben. Deze resultaten toonden aan dat de uitbraak van Rotterdam ook buiten deze plaats voorkomt en dat deze veroorzaakt lijkt te zijn door een nieuw geïdentificeerde variant.

In **Hoofdstuk 9** staan de resultaten van het ontwikkelen en valideren van een real-time PCR methode ten behoeve van de snelle en gevoelige diagnostiek van LGV serovars direct uit patiëntenn materiaal. Laboratoriumdiagnostiek van LGV vindt gewoonlijk plaats door middel van directe detectie en amplificatie van *C. trachomatis* specifiek DNA in patiëntenn materiaal zoals een rectumuitstrijk. Bij een positief resultaat volgt amplificatie van het *ompA* gen volgens een ‘nested format’ en RFLP om de verschillende serovars van elkaar te kunnen onderscheiden. Omdat deze opzet een groot aantal nadelen heeft nl. de detectie van *C. trachomatis* DNA (COBAS Amplicor Roche) in rectummateriaal is niet gevalideerd door de FDA, RFLP is arbeidsintensief en kan slechts in een beperkt aantal gespecialiseerde laboratoria worden uitgevoerd, werd er een sensitieve en specifieke real-time PCR methode ontwikkeld. De sensitiviteit van zowel de Taqman als van de Rotorgene analyse werd bepaald aan de hand van gekwantificeerde, gekweekte EB’s en was voor beide analysemethoden 0.01 IFU. De specificiteit werd nagegaan aan de hand van 32 gangbare gastrointestinale- en urogenitale
Samenvatting

micro-organismen. Geen enkele kruisreactie werd waargenomen; noch met de Taqman noch met de Rotorgene. De tijdswinst met deze real-time methode, die ongeveer 2 uur in beslag neemt ten opzichte van de tijdsduur van de klassieke RFLP methode, die minstens 2 dagen duurt, is duidelijk. Vanwege de eenvoud van uitvoeren van deze techniek maakt dat deze methode uiterst eenvoudig te implementeren valt in de meeste routine diagnostische laboratoria.

**Hoofdstuk 10** beschrijft een retrospectieve case-control studie van 2 jaar waarin nagegaan is wat de risicofactoren en de klinische- en diagnostische aanwijzingen zijn voor een rectale LGV infectie bij homoseksuele mannen. De cases werden gedefinieerd als homoseksuele mannen met een bewezen rectale infectie met het *C. trachomatis* genovariant L2b. Als controles werden 2 verschillende groepen gekozen; groep 1: homoseksuele mannen rectaal geïnfecteerd met non-LGV genotypes, groep 2: homoseksuele mannen, die receptief anorectaal geslachtsgemeenschap hadden maar niet rectaal geïnfecteerd waren met *C. trachomatis*. We namen waar dat HIV seropositiviteit de sterkste risicofactor op LGV was. Ongeacht de toegepaste controlegroep waren de proctoscopische bevindingen en de telling van het aantal leukocyten in het Gram preparaat van het anorectaal verkregen slijm de enige klinisch relevante voorspellende factoren op een LGV infectie. Dit lijkt in te houden dat wanneer LGV serovar typering niet voorhanden is op het laboratorium een blinde LGV behandeling geoorloofd is wanneer additioneel het beeld van een proctitis bij proctoscopisch onderzoek wordt gevonden of wanneer er meer dan 10 leukocyten per gezichtsveld worden waargenomen of wanneer de patiënt HIV positief is.

**Hoofdstuk 11** staan de resultaten van een retrospectieve studie over de aanwezigheid van de *C. trachomatis* genovariant L2b in Amsterdam. Door gebruik te maken van bewaarde rectum uitstrijkjes en deze te analyseren bleek dat mogelijk was terug te gaan tot het jaar 2000 en aan te tonen dat de L2b genovariant al voorkwam in 2000 in Amsterdam. Vanwege ons samenwerkingsverband met Prof. dr. Julius Schachter (SF, USA) kregen we de beschikking over 51 rectum uitstrijkjes uit San Francisco uit de jaren 80 (1979-1985). We konden aantonen dat in alle materialen *C. trachomatis* DNA aantoonbaar was en dat transport en opslag bij −20 °C geen invloed heeft gehad. Met behulp van sequenti-analyse is duidelijk geworden dat van de 51 uitstrijkjes er 18 waren met de genovariant L2b; de huidige in Amsterdam circulerende stam. Deze resultaten suggereren dat de LGV al veel eerder circuleerde onder homoseksuele mannen mogelijk op een laag endemisch nivo zonder dat het herkend is.
Algemene conclusies deel 2

De resultaten, die verkregen en beschreven zijn uit studies in het tweede deel van dit proefschrift, hebben aangetoond dat de huidige uitbraak van LGV ook bij homoseksuele mannen in Amsterdam gevonden is. De infectie blijkt ook alleen bij homoseksuele mannen zonder slijmvliesafwijkingen aantoonbaar te zijn. Op locaties waar men niet de beschikking heeft over genotyperingsmethoden van *C. trachomatis* kunnen clinici ondersteund worden bij de diagnostiek en antibiotica starten als er naast een positieve *C. trachomatis* DNA test een Gram preparaat van het rectumslijm hebben met meer dan 10 leucocyten per gezichtsveld of wanneer de patiënt HIV positief is. Nieuwe laboratoriumtechnieken, zoals real-time PCR en sequentieanalyse, hebben aangetoond dat de L2b Amsterdam genovariant betrokken is bij de zgn. uitbraak in Nederland als ook in de patiënten uit de jaren 80 uit San Francisco. Samenvattend lijkt het erop dat deze rectale LGV infecties jarenlang gemist zijn in de dagelijkse praktijk van een SOA kliniek in de Westerse landen. Speculerend zou het zo kunnen zijn dat gezien de associatie met HIV positiviteit dat een eventuele combinatie met HAART de individuele immuun response onderdrukt en daardoor de klinische presentatie vertroebeld en minder duidelijk maakt zodat clinici tenminste de primaire presentatie van een klein ulcus en een beginnend inguinaal bubo kunnen missen. De ontwikkeling en de toepassing van een real-time PCR methode om LGV patiënten te diagnosticeren en het implementeren van bepaalde patiëntencarakteristieken in de klinische diagnostiek, zoals hierboven beschreven zijn, zouden de basis kunnen vormen voor naderestudie en het hanteren van de zich langzaam ontwikkelende epidemie.
Samenvatting
ADDENDUM
Addendum
Dankwoord

In 1999, bij het begin van mijn baan als arts-microbioloog bij het Streeklaboratorium voor de Volksgezondheid en Bijzonder Instituut voor de Virologie in Amsterdam, beseft ik al wel dat dit een goede, vruchtbare plek zou kunnen zijn om (toegepast) wetenschappelijk onderzoek te doen dat wellicht tot een proefschrift zou kunnen leiden.

In mijn tijd als O.I.O. op de afdeling Bloedstolling van het toenmalig Centraal Laboratorium van de Bloedtransfusiedienst (hoofd: dr. J.A. van Mourik) had ik de nodige ervaring opgedaan als promovendus en wist dat een dergelijke exercitie nooit een éénmans actie zou kunnen zijn. Zonder de hulp van velen, waaronder allen die vrijwillig hebben deelgenomen aan de verschillende studies, zou dit proefschrift er nu niet liggen. Ik wil dan ook eenieder, die op enigerlei wijze heeft bijgedragen aan het feit dat dit proefschrift er nu ligt, bedanken.

Een aantal mensen zou ik graag persoonlijk willen noemen.


Beste Roel, bedankt voor de gelegenheid die je me bood om aan het cluster Infectieziekten te kunnen promoveren. Het was zoals ik me had voorgesteld: een zee van mogelijkheden. Hoewel het niet altijd makkelijk was om de dagelijkse hectiek van het Streeklaboratorium het hoofd te bieden en daarnaast de voortgang van het Chlamydia trachomatis onderzoek te bewaken en daar ook voor voldoende diepgang te zorgen heb jij mij toch altijd inhoudelijk gestimuleerd om door te gaan.

Beste Salvador, ik ben je erkentelijk voor de mogelijkheden, die je me hebt geboden om, samen met Servaas, urogenitale infecties met Chlamydia trachomatis vanuit een immunogenetisch oogpunt te beschouwen. Het heeft het onderzoek in een veel breder perspectief gezet.

Daarnaast ben ik veel dank verschuldigd aan mijn copromotor, dr. S.A. Morré.

Beste Servaas, met veel plezier heb ik de afgelopen jaren aan het Chlamydia onderzoek gewerkt. Je hebt me geïntroduceerd bij de BoBo’s in het veld en mij laten delen in de credits van vele initiatieven en publicaties.

De leden van de promotiecommissie, prof.dr. O.P. Bleeker, prof. dr. J.D. Bos, mevr. Prof. Dr. C.A. Bruggeman, dr. J.M. Ossewaarde, mevr. Dr. Y. Pannekoek, mevr. Prof. Dr. C.J.M.E. Vandenbroucke-Grauls, wil ik hartelijk danken voor de tijd en aandacht die ze aan dit proefschrift besteed hebben.
Speciaal wil ik ook stilstaan bij de analisten, studenten en stagiaires, die veel voor mij gedaan hebben: Judith Ursem-Merks, je was de eerste analiste, die ik op het Streeklaboratorium heb aangenomen en tot het laatst toe heb je je met het onderzoek bemoeid en gezorgd dat zaken voor elkaar kwamen. Het CHLJJ-onderzoek draagt zelfs je naam. Ook Sonja Mooij wil ik expliciet noemen: jij hebt ervoor gezorgd dat het kweken van *C. trachomatis* min of meer routine werd. Met gemak haalde je een kweekpercentage van 85% met monsters van de SOA polikliniek. Op het VUmc ben ik met name Sander Ouburg en Jolein Pleijster erkentelijk. Beste Sander, bedankt voor je werk op het gebied van de immunogenetica van *C. trachomatis* infecties. Ook je kundigheid op automatiseringsgebied is onmisbaar gebleken; het CHLJJ bestand is nu tenminste ‘veilig’. Beste Jolein, ik zal nooit vergeten hoeveel patiëntenmonsters er door jouw handen zijn gegaan; ze gingen met auto’s vol naar het VUmc. Ook jou wil ik bedanken voor het uitvoeren en ordenen van een groot aantal experimenten.

Bij een aantal onderzoeken hebben de bijdragen van de onderzoekers van de afdeling HIV en SOA onderzoek tot meer epidemiologische- en statistische diepgang geleid. Twee mensen wil ik hier dan ook met name noemen: Colette Smit en Akke van der Bij. Beste Colette bedankt voor je uitleg, advies en inspanningen, altijd op een kalme rustige manier. Ik ben er heel wat wijzer van geworden. Beste Akke, bedankt voor de samenwerking die tot een mooi, waardevol stuk in CID heeft geleid. Door jou heb ik als medicus ingezien dat het wel degelijk mogelijk is om enig epidemiologisch inzicht te ontwikkelen.

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Kees, voor jou was het niet altijd even gemakkelijk de laatste tijd; op onze ‘think-tank’ kamer op zolder is er veel de revue gepasseerd. Je bent altijd in de afronding van het proefschrift blijven geloven. Dank je voor alles en nu ben jij aan de beurt. XXXX. JJ.
Curriculum Vitae


Aansluitend werkte zij ruim 6 jaar als analist op de afdeling Bloedstolling van het toenmalige Centraal Laboratorium van de Bloedtransfusiedienst, eerst als analist op de afdeling stollingsdiagnostiek, later op de afdeling stollingsresearch, waar zij betrokken was bij fundamenteel wetenschappelijk onderzoek naar de pathogenese van arteriële trombose en arteriosclerose. Tijdens deze periode heeft zij in de avonduren de vervolgstudie Laboratoriumonderwijs (HBO-B) gevolgd en met goed gevolg in 1983 afgerond.


Met ingang van 1 oktober j.l. werkt zij met 5 andere collega’s bij het microbiologisch laboratorium Twente Achterhoek in Enschede.
Addendum

Publications

Published, in press or submitted


