Detection of *Chlamydia trachomatis* and *Mycoplasma genitalium* by genetic and serological methods
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Abstract


Chlamydia trachomatis infections are associated with a spectrum of clinical diseases including urethritis, prostatitis and epididymitis among men and cervicitis and pelvic inflammatory disease (PID), with an increased risk of infertility and ectopic pregnancy (EP), among women. In the search for other pathogens causing urethritis, Mycoplasma genitalium was isolated from urethral specimens from two men with acute urethritis (1980). Mycoplasma bacteria are extremely difficult to isolate by culture, and clinical studies have been possible only after the advent of the first PCR-based detection method. M. genitalium has been found to be associated with lower genital tract infections in both men and women. Finding evidence for a connection between M. genitalium and upper genital tract infections in women is still of major importance.

The aim in papers I and II was to develop a PCR method for genetic characterization of clinical C. trachomatis isolates by sequence analysis of the omp1 gene, and to study the distribution of genotypes within sexual networks and determine if genotyping would improve partner notification. The method was used to determine the genotypes of C. trachomatis in 237 positive urogenital and/or urine specimens from men and women attending the STD Clinic in Örebro during one year. Sequence analysis of the omp1 gene revealed that the most prevalent genotypes corresponded to C. trachomatis serovar E (47%), followed by F (17%), and K (9%). There were 161 networks found and specimens were sequenced from at least two patients in 47 networks. In seven of these 47 networks there were discrepant genotypes. In the largest network comprising 26 individuals two different C. trachomatis genotypes were found, and one partner had urethritis due to a Mycoplasma genitalium infection but was C. trachomatis negative.

The need for a new method for M. genitalium DNA detection was one reason for study III. An existing conventional PCR protocol for detection of M. genitalium DNA was further developed into a real-time PCR (RT-PCR) with hybridisation probes. In order to evaluate the RT-PCR assay with clinical material, specimens from 398 men and 301 women attending the STD Clinic in Örebro were analysed, using the RT-PCR assay, and also by the well established conventional PCR in Copenhagen. Using the conventional PCR method as “gold standard”, the sensitivity for the RT-PCR assay was 72.2% and 68.2% and the specificity was 99.7% and 98.6%, respectively, in urogenital specimens from men and women.

The aim in paper IV was to adapt a Triton X-114 extracted Lipid-Associated Membrane Protein (LAMP) Enzyme Immuno Assays (EIA) method to detect antibodies against M. genitalium and to evaluate the association between M. genitalium and PID and EP, using sera sampled in Örebro during the 1980s, and also to compare the number of sera having M. genitalium antibodies against those having C. trachomatis antibodies, using a commercial anti-Chlamydia trachomatis EIA assay. No statistical significant association could be demonstrated between M. genitalium antibodies and PID or EP in our serum material. However, a slight trend toward association was found when focusing on younger individuals. Antibodies against C. trachomatis were found to be significantly associated with PID and EP.

Keywords: Chlamydia trachomatis, genotyping, contact tracing, sexually transmitted diseases, Mycoplasma genitalium, PCR, and serology

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Sammanfattning


Syftet i arbete I och II var att utveckla en PCR metod för att amplifiera genen som kodar för ett yttermembranprotein (*omp1* genen) i klamydiabakterier för att sedan gå vidare med DNA-sekvensering för att se vilka genotyper som förekom i kliniska prover från alla män och kvinnor som sökte på STD-mottagningen i Örebro under ett år, samt att se om utfallet av typningen skulle kunna förbättra smittspårningen vid genital klamydia infektion. Metoden användes framgångsrikt för att genotypa 237 kliniska klamydiastammar och av dessa var de flesta genotyp E (47 %), F (17 %) samt K (9 %). Sammanlagt hittades 161 sexuella nätverk vid smittspårningen och i 47 av dessa var prover från minst två individer genotypade. I sju av dessa 47 nätverk hittades diskrepanta genotyper. I studiens största sexuella nätverk kunde 26 individer identifieras, och två olika genotyper förekom. Dessutom hade en man uretrit på grund av en *Mycoplasma genitalium* infektion.

I arbete III utvecklades en ny realtids PCR (RT-PCR), utifrån en konventionell PCR metod, för att kunna diagnostisera *M. genitalium*. Metoden utvärderades genom att prover från 398 män och 301 kvinnor som sökte på STD-mottagningen i Örebro analyserades med RT-PCR samt skickades till Köpenhamn för analys av *M. genitalium* med en konventionell PCR metod. RT-PCR metodens känslighet var 72,2 % för analys av urinprover från män och 68,2 % för urogenitala prov från kvinnor, och specificiteten var 99,7 % respektive 98,6 %, jämfört med den konventionella PCR metoden som ”gold standard”.


List of papers

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>DFA</td>
<td>Direct immunofluorescence assay</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>EB</td>
<td>Elementary body</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immuno assay</td>
</tr>
<tr>
<td>EP</td>
<td>Ectopic pregnancy</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>HPF</td>
<td>High power field</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilo base pair</td>
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<tr>
<td>LAMP</td>
<td>Lipid associated membrane protein</td>
</tr>
<tr>
<td>LC-PCR</td>
<td>LighCycler PCR</td>
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<tr>
<td>LGV</td>
<td>Lymphogranuloma venerum</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccaride</td>
</tr>
<tr>
<td>Mg</td>
<td>Mycoplasma genitalium</td>
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<tr>
<td>MgPa</td>
<td>Mycoplasma genitalium adhesion protein</td>
</tr>
<tr>
<td>MIF</td>
<td>Micro-immuno-fluorescence</td>
</tr>
<tr>
<td>MOMP</td>
<td>Major outer membrane protein</td>
</tr>
<tr>
<td>MSM</td>
<td>Men who have sex with men</td>
</tr>
<tr>
<td>NAAT</td>
<td>Nucleic acid amplification test</td>
</tr>
<tr>
<td>NaCl</td>
<td>Natrium Chloride = sodium chloride</td>
</tr>
<tr>
<td>NCNGU</td>
<td>Non-chlamydial, non-gonoccoccal urethritis</td>
</tr>
<tr>
<td>NGU</td>
<td>Non-gonoccocal urethritis</td>
</tr>
<tr>
<td>omp1</td>
<td>Outer membrane protein – 1 gene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PID</td>
<td>Pelvic inflammatory disease</td>
</tr>
<tr>
<td>PMNL</td>
<td>Polymorph nuclear leucocytes</td>
</tr>
<tr>
<td>RB</td>
<td>Reticulate body</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>SARA</td>
<td>Sexually aquaired reactive arthritis</td>
</tr>
<tr>
<td>2SP/4SP</td>
<td>Sucrose phosphate medium</td>
</tr>
<tr>
<td>SSI</td>
<td>Statens serum institute, Copenhagen, Denmark</td>
</tr>
<tr>
<td>STATA</td>
<td>Software for statistical analysis. StataCorporation (<a href="http://www.stata.com">www.stata.com</a>)</td>
</tr>
<tr>
<td>STD</td>
<td>Sexually transmitted diseases</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually transmitted infections</td>
</tr>
<tr>
<td>VD</td>
<td>Variable domain</td>
</tr>
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</table>
Introduction

Background

Chlamydia trachomatis and Neisseria gonorrhoeae are known to be the most common bacterial sexually transmitted infections (STI) among young adults worldwide (23). A spectrum of clinical diseases is associated with these infections including urethritis, prostatitis and epididymitis among men, and cervicitis and pelvic inflammatory disease (PID) with an increased risk of infertility and ectopic pregnancy (EP), among women (4, 37, 40). However, in many patients with symptomatic non-chlamydial, non-gonococcal urethritis (NCNGU) no etiological agent has been found.

Several clinical studies have indicated a role for Ureaplasma urealyticum and Mycoplasma hominis in NCNGU, but the importance of these bacteria remains controversial, since they have been isolated with the same frequency from patients with and without urethritis in several studies (14, 29, 84). In the search for other pathogens causing urethritis, Mycoplasma genitalium was first isolated in 1980 from urethral specimens from two men with acute urethritis (90).

Chlamydia trachomatis

Biological characteristics

The Chlamydia species are distinguished from all other microorganisms by a unique growth cycle, and are placed in their own family (Chlamydiaceae). They are gram-negative obligate intracellular bacteria that replicate within the cytoplasm of host cells and the elementary body (EB) is adapted for extracellular survival and for initiation of infection. The EB is metabolically inactive but is initially involved in the attachment to the host cells. When the EB adheres to the eukaryotic cell, it enters the cell by endocytosis and stays in that intracellular vacuole, called an inclusion, through its entire lifecycle. This EB changes to a metabolically active and dividing form called the reticulate body (RB), which is adapted for intracellular multiplication. The RBs (diameter, 0.5 to 1 mm) are able to synthesize their own RNA, DNA, and protein inside the inclusion with the help of the ATP produced by the host cell. This entire cycle takes about 18 to 24 hours, and as the RBs not are stable outside the host cell, some of them re-organize into the infectious EB. After about 72 hours, the host cells rupture and there is a release of the infectious EBs to start a new attachment (71, 73). Chlamydiaceae are highly complex organisms with genus, species and serovar specificity, and the most
easily detected antigen is the group antigen, shared by all members of the genus. The major genus specific antigen has been identified as lipopolysaccharide (LPS), and is expressed on the surface of Chlamydia organisms. Some recent genome sequence studies have described new understandings about the Chlamydiaceae family. Phylogenetic analyses of the 16S and 23S rRNA genes from different Chlamydia species pathogenic to humans show two distinct lineages: the genus *Chlamydia* (*C*) with *C. trachomatis* and *Chlamydophila* (*Cp*) consisting of *Cp. psittaci* and *Cp. pneumoniae* (17).

The first sequenced *C. trachomatis*, serovar D, genome consisted of a 1,042,519–base pair chromosome (GenBank accession no. AE001273) and a 7,493–base pair plasmid. Counterparts of enzymes characterized in other bacteria were identified in *C. trachomatis* to account for the minimal requirements for DNA replication, repair transcription and translation (70, 77). The chlamydia organism encodes a major outer membrane protein (MOMP) that is surface exposed and is used for classification of different sero- and genotypes. The gene *omp1 (omp A)* that is coding for the major outer membrane protein in *C. trachomatis* and contains five conserved domains and four variable domains (VDI to VDIV) varies considerably between the various chlamydial species (78, 98).

**History**

Human diseases caused by *C. trachomatis* were described as long ago as in Egyptian papyri (the eye disease trachoma) but were first visualized in 1907 by Halberstaedter and Prowazek. They were able to see the typical intracytoplasmic inclusions in stained conjunctival scrapings from orangutans that had been inoculated with human trachomatous material. Shortly thereafter, similar inclusions were identified in conjunctival scrapings from infants with trachoma. At the same time, the same types of inclusions were found in the genital tracts of mothers of the affected infants and also in the urethras of the fathers. These inclusions were associated with nongonococcal urethritis. A tissue culture method was developed in 1965 (24), and it was shown in several studies that nearly half the cases of non-gonococcal urethritis in adults were chlamydial infections (67).

**Clinical manifestations**

Several hundred million people in developing countries are currently being infected with trachoma and 6–8 million have been blinded because of the disease. The disease is called inclusion conjunctivitis and is mainly caused by
C. trachomatis serovar A, B and C. Genital infection with C. trachomatis is the main cause of preventable sexually transmitted bacterial infection in the world, in both men and women (66). These infections are often characterized by no symptoms or mild symptoms and are therefore often found when screening young sexually active adults. Microscopic examination of gram or methylene blue stained smears from the distal urethra of men and women with urethritis show an increased number of polymorphnuclear leucocytes (PMNL). The most accepted and utilized definition is more than four PMNL per high power field (HPF; 1000 x magnification) in at least five HPFs in men (80) and more than 10 PMNL in women (76). Untreated, there is a risk for PID in women, which can cause sequel such as ectopic pregnancy and tubal infertility (38). In men the infection can cause prostatitis, epididymitis and possible infertility. Chlamydia infections of the genital tract are primarily caused by serovars D to K. Ocular infections with these serotypes in adults are probably acquired by inoculation from genital infection. Infants exposed to Chlamydia by passage through the birth canal may also acquire pneumonia and/or conjunctivitis (76).

Lymphogranuloma venerum (LGV) is a sexually transmitted disease caused by C. trachomatis (serovar L1 to L3). It is endemic in East and West Africa, India and parts of Southeast Asia. Most of the reported LGV cases in nonendemic areas occur in sailors, soldiers and travellers who acquire the infection while visiting or living in an endemic area. A resent report (25) presented evidence for an outbreak of LGV infections in homosexual men in the Netherlands. After that report, several cases have been reported among men who have sex with men (MSM) from different parts of Europe and the US. In Sweden, two cases have been reported (6). When genotyped, most of the cases were found to be L2 serovars (22, 27). LGV is predominantly a disease of the lymphatic tissue and the inflammatory process lasts several weeks (63).

**Epidemiology**

In Sweden, it has been mandatory since 1919, under the Communicable Diseases Act, to report cases of gonorrhoea and syphilis, and since April 1988 it has been mandatory to report genital infection with C. trachomatis. The incidence of genital chlamydial infections declined in all Swedish counties up until 1996. It has been suggested that this decline was due to partner notification, screening and treatment of asymptomatic men and women (39). However, there is some doubt about the efficacy of partner notification, and data from the Swedish Institute for Infectious Disease Control show that
genital infection due to \textit{C. trachomatis} has increased by about 10\% each year since 1997. The incidence was 172 cases per 100,000 inhabitants in 1998 and the reported number of cases in 2005 was 367 per 100,000 inhabitants (1).

\textbf{Diagnostics}

\textbf{Tissue culture.} Development of a tissue culture method was described in which the clinical specimens were inoculated to cycloheximide treated McCoy or HeLa cells and incubated at +37° C for 48 to 72 hours (68). Cycloheximide inhibits host cell protein synthesis, while the chlamydia organisms are able to replicate in the host cell. The RBs grow inside an inclusion body and can be visualized either after staining with iodine, which reacts with the glycogen accumulated in the inclusion body, or by staining with a fluorescein-conjugated antibody directed against the \textit{C. trachomatis} antigens exposed on the cell surface. Since the inclusion body is highly characteristic, cell culture is considered to have a specificity of 100\% (26). However, even with the use of fluorescein-conjugated antibodies, the sensitivity is not optimal, partly because of inhibition of the EBs during transportation and storage (72). Tissue culture is also time consuming and laborious, and it was replaced by antigen detection in many routine laboratories as early as the late 1980s.

\textbf{Antigen detection.} The currently commercially available antigen detection methods for identifying \textit{C. trachomatis} comprise direct immunofluorescence assays (DFA) and enzyme immuno assays (EIA).

In DFA, specimen material is placed directly on a slide. Fluorescein-conjugated antibodies directed against either the LPS or the MOMP react with the Chlamydia surface and are visualized by fluorescence microscopy (81). This method requires a trained microscopist who can distinguish between fluorescing chlamydial particles and nonspecific fluorescence, and it is used almost exclusively as a confirmatory test in some routine laboratories. Monoclonal antibodies against LPS will stain all \textit{Chlamydia} species, while antibodies that are prepared against \textit{C. trachomatis} MOMP will only stain \textit{C. trachomatis} i.e. they are species specific (11).

The EIAs for detection of \textit{C. trachomatis} were first described in the mid 1980s, and most of them use antibodies against the LPS as detecting antibodies, and can therefore theoretically detect all chlamydiae (9). The EIAs detect \textit{C. trachomatis} by adding the clinical specimen to a microtiterplate that is coated with a polyclonal antibody (against chlamydial LPS). A second antibody (monoclonal) against \textit{C. trachomatis} that is linked to an enzyme is
added, which generates a colour change on addition with a substrate and is measured as optical density. Most EIAs are less sensitive than tissue culture and have a specificity of about 90–95% without a confirmation test, such as blocking tests or DFA (9, 72). With EIAs it was possible for the first time to analyse first void urine samples (FVU) (58). In many routine laboratories these methods have now been replaced by nucleic acid amplification assays that have higher sensitivity.

**Nucleic acid amplification tests (NAATs).** The polymerase chain reaction (PCR), developed by Mullis and Faloona in 1987, was the first described amplification method (53). Several DNA amplification tests for detection of *C. trachomatis* have been developed and are often designated as “in house” PCRs.

In short, two primers (consisting of 15–30 nucleotides) are constructed to complement each of two conserved and specific regions of the target DNA of interest (template). The template DNA is mixed with heat-stable DNA Taq-polymerase, deoxynucleotides and the primers in a Tris-buffer containing MgCl₂. The PCR is subsequently carried out, by repeating three reaction steps at different temperatures (Figure 1). First, the double stranded target DNA is denatured into two single strands, by heating to about 95°C. At about 50°C the two primers anneal to their respective regions on each of the single stranded target DNA strands. In the presence of the four nucleotides (dATP, dTTP, dCTP, and dGTP), new DNA is generated from each end (in 5’→ 3’ orientation) of the primer by the heat-stable DNA Taq-polymerase (at 72°C), generating two double stranded DNA strands. In the second cycle this reaction will be repeated and generates four strands. By repeating this about 30–50 times, one target DNA will be amplified to millions of copies (64). The sample preparation to make the DNA accessible and to remove compounds that inhibit the activity of the Taq-polymerase, is also very important with respect to the PCR result. DNA is stable (in the absence of DNA grading enzymes), and if *C. trachomatis* organisms have lost their infectivity during transportation and storage they may be detected by PCR, which therefore also has a sensitivity higher than that of a routine culture. If the annealing of primers is unique for *C. trachomatis*, the DNA amplification tests have a high specificity, although their high sensitivity may also increase the risk of contamination of samples with either native or amplified DNA.
Figure 1. **Polymerase chain reaction** (PCR). A DNA fragment is copied for identification of a specific gene (after gel electrophoresis) or used as template for DNA sequencing. Each cycle is composed of three steps. 1. DNA is denatured to single stranded (ss) DNA. 2. The primers (15–30 DNA nucleotides) anneal to their specific sites. 3. A heat stable enzyme copies the fragments by inserting complementary nucleotides (dATP, dTTP, dCTP and dGTP). After 30–40 cycles million of copies have been made.

The conventional method of PCR product detection is gel-electrophoresis (Figure 2). The amplified products are visualized after electrophoresis through agarose gel containing ethidium bromide (EtBr), a fluorescent dye that intercalates between base pairs in ds DNA. EtBr emits fluorescence when excited by ultraviolet light. A Molecular Weight Marker containing DNA fragments of known sizes is included in each electrophoresis run.

**Figure 2. Gel electrophoresis.** PCR products or other DNA fragments (1–4) can be visualized by gel electrophoresis. The DNA is loaded into the wells and the negatively charged DNA migrates to the positive anode. By using ethidium bromide (EtBr), a fluorescent dye that intercalates between base pairs in DNA, the DNA bands can be visualized by ultraviolet light and photographed. Large fragments and linear molecules migrate more slowly in the agarose gel than small fragments and compact molecules because they are retarded by the agarose particles. A molecular weight marker (MW), fragments of known sizes, is run together with the samples to establish the fragments sizes.
Several DNA amplification kits are currently commercially available, and some of them allow simultaneous detection of *C. trachomatis* and *Neisseria gonorrhoeae* in a single patient specimen (52).

The most commonly used commercial NAATs use the cryptic plasmid as target molecule for amplifying *C. trachomatis* DNA in clinical samples (57). The plasmid is unique for *C. trachomatis*, is well conserved within the species, and is present in about 10 copies in each organism (28, 75). Using the plasmid as target DNA should theoretically lower the detection limit compared to a single chromosomal gene for example the MOMP gene, which has been confirmed in different studies (46). However, some studies suggest that plasmid-free variants of *C. trachomatis* are present in clinical samples (2), and these will not be detected if the plasmid is used as target DNA.

**Characterization**

**Conventional phenotypic characterization.** Characterization of *C. trachomatis* strains can provide valuable information about the variants circulating in the community, and with better knowledge of the epidemiology of Chlamydia infections efforts against spread can probably be more effective. The MOMP is the major structural protein exposed on the surface of the infectious EB and RB. Serovariant-specific epitopes are associated with the MOMP of *C. trachomatis* and conform to serovars determined by micro-immunofluorescence (79). Prototypic serovars designated A to K and L1 - L3, as well as additional immunovariants (Ba, Da, Ia, etc.), have been identified. Since a cell culture is necessary for this method, the viability of the cells and the number of typeable organisms are of great importance. This method is only used in a minority of laboratories.

**Genotypic characterization.** By using MOMP directed (*omp1* gene) primers in a PCR, which covers the variable domains VDI to VDIV, it is possible not only to detect but also to distinguish between the various types of *C. trachomatis* in non-cultured clinical samples (98). Amplification of the *omp1* gene by PCR has made it possible for further characterization either by restriction fragment length polymorphism (RFLP) or by DNA sequence analyses. There is good agreement between RFLP and serotyping by monoclonal antibodies (50), but it has also been shown that DNA sequencing is a more reliable epidemiological tool as compared to RFLP (62).

The DNA sequencing procedure involves extraction of nucleic acids, PCR-mediated amplification, sequence determination, and computer-aided analysis. Direct sequencing of amplicons provides a high-resolution method for
studying organism variation and molecular epidemiology. Sequencing by this approach is performed with instruments made by several manufacturers. In this thesis the Perkin Elmer Biosynthesis sequencing analysis system was used and it will be described in more detail.

The amplified DNA from the MOMP-PCR is mixed with one of two primers in separate reactions together with ABI PRISM® BigDye™ Terminator Cycle Sequencing ready reaction reagent. The reagent consists of AmpliTaq polymerase, FS, which is a variant of *Thermus aquaticus*. The mixture also contains the four nucleotides, dATP, dTTP, dCTP, and dGTP in a Tris-HCl-buffer, MgCl₂ and fluorescent dye-labelled dideoxynucleotide chain terminators (ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit, Perkin-Elmer Applied Biosystems). As shown in Figure 3, a cycle sequencing PCR is run according to the manufacturer's instructions.

![DNA Sequencing Diagram](image)

**Figure 3. DNA Sequencing.** Each of the four terminators (nucleotides) is tagged with a different fluorescent dye. Thus, when the template is copied, the growing chain is simultaneously terminated with a labelled base. This cycle is repeated 25 times to get detectable amounts. The fragments are then separated by capillary electrophoresis (separates fragments of one base different in length) and each fragment is registered by its laser excited dye. The order of bases can be read.

The cycle sequencing product is purified and transferred to tubes in a tray and placed in the ABI PRISM 310 machine, which then will automatically inject each sample into a capillary filled with polymer by electro kinetic injection, and current is applied to separate the nucleotides (electrophoresis). The separation of the different nucleotides is analysed with a laser, that excites the different fluorescent dye labels, and the results enter a computerised workstation (Figure 4).
Figure 4. The nucleotides are separated in a capillary filled with polymer in an ABI PRISM 310 machine. The emitted specific fluorescences of the laser-excited dyes are detected, and the results will enter a computerised workstation (manual of cycle sequencing Perkin-Elmer Applied Biosystems).

The individual consensus sequence is sent to a database, available at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov), to establish the *C. trachomatis* genotype, corresponding to the isolate.

**Antibody detection**

The diagnostic method of choice for measuring antibodies against bacterial infections when no infectious agent can be detected is serology. Since *C. trachomatis* infection often is asymptomatic, this infection can lead to severe sequel such as PID and EP (48, 65). *C. trachomatis* serology is often used for both diagnostic purposes and epidemiological studies. The microimmunofluorescence (MIF) assay is regarded as the “gold standard” and is performed on pre-treated object glasses, which are inoculated with MOMP antigen from three different chlamydia bacteria. In short, the serum is diluted and one drop is added to the antigen on the object glass and incubated according instructions in the kit. After a washing procedure, a fluorescent anti-human antibody is added and after incubation and wash, the reaction is read using fluorescence microscopy. However, the MIF assay is laborious and the reading of the specific fluorescence requires a trained microscopist.

In the past, several user-friendly EIAs were developed using LPS or reticulate bodies as antigen, and thus often showing cross reactivity with *C. pneumoniae* (58). Nowadays, however, there are several commercially available EIAs that have been developed using specific synthetic peptides based on the MOMP of *C. trachomatis*. They have been evaluated against the MIF assay by different researchers who have found limited or no cross reactions (5, 42, 51). The EIA assay is performed in a 96-well microtiterplate that is coated with the synthetic peptide, and the sera is diluted according to instructions in the kit and is added to the well. After incubation and a washing
procedure, an enzyme marked anti-human antibody is added, which generates a colour change upon addition with a substrate, and the coloured end product is measured as optical density (Figure 5).

**Figure 5.** The Enzyme Immuno Assay (EIA) is performed in a 96-well microtiterplate that is coated with an antigen. The patient’s serum is diluted to a suitable dilution and added to the well. After incubation and following a washing procedure, an enzyme marked anti-human antibody (conjugate) is added, which generates a colour change upon addition with a substrate if the serum contains antibodies against the antigen, and no coloured end product if the serum is negative. The intensity of the colour is proportional to the increasing amount of antibodies in the serum and is measured as optical density in a spectrophotometer.

**Mycoplasma genitalium**

**Biological characteristics**

Mycoplasmas, bacteria with the smallest known genomes, are found among members of the Mollicutes (mollis = soft; cutis = skin) class. This class also presently comprises the genera Acholeplasma, Anaeroplasma, Asteroleplasma, Mycoplasma, Spiroplasma and Ureaplasma. The common characteristics of the class are the complete lack of a bacterial cell wall, osmotic fragility, and colony shape (fried egg). Mycoplasmas were initially mistaken for viruses because they can pass through 0.25 μm filters. The relatively close phylogenetic relationship of these genera was measured by comparative sequence analysis of the 5S and 16S ribosomal RNA (rRNA). The rRNA sequence analyses also revealed that the Mollicutes are developed by degenerate evolution from gram-positive bacteria with a low guanine and cytosine content of DNA, the Lactobacilllus group containing Lactobacilllus, Bacillus, Streptococcus and two Chlostridium species. The complete genome size is as
low as 580 to 2,200 kbp as compared to 2,500 to 5,700 kbp long genomes of their ancestor bacteria. There are several mycoplasmas that have been isolated from humans and most of the species indicate constitutive parts of the normal flora (41, 54, 92).

Mycoplasmas usually exhibit organ and tissue specificity. Thus, *M. pneumoniae* is found preferentially in the human respiratory tract and *M. genitalium* is found primarily in the urogenital tract, but they share several structural properties such as their flask shape and their terminal tip-like structure. They both have adhesive properties, and the major adhesin of *M. genitalium* (MgPa) is a 140 kDa protein, which differs from that of *M. pneumoniae* (170 kDa). The complete nucleotide sequence of *M. genitalium* (G37) is 580,070 bp while *M. pneumoniae* (M129) consists of 816,300 bp (82). The recent mycoplasma genome projects have revealed a remarkable scarcity of genes in mycoplasmas involved in biosynthetic pathways. For example, both *M. genitalium* and *M. pneumoniae* lack all genes involved in amino acid synthesis, which is one of the explanations for difficulties with *in vitro* cultivation (93).

**History**
*M. genitalium* was first isolated after prolonged incubation of 13 urethral specimens from men with urethritis. The specimens were inoculated in a special medium (4SP) and were mistakenly forgotten in the incubator for about a month. An acidic colour change had occurred in two of the cultured specimens and electron microscopic examination revealed a bacterium, later termed *M. genitalium* (90). One of these *M. genitalium* strains (G37) was inoculated intraurethrally in four chimpanzees. Two of the animals became persistently infected and developed an antibody response after five weeks, and a urethral inflammatory response was noted (88, 91). Despite repeated attempts by conventional culture techniques, urogenital isolates have been extremely rare and only a few isolates have been described (30, 33). Because of the failure of traditional procedures such as culture and serology for diagnosing *M. genitalium*, studies indicating that *M. genitalium* is a cause of STI had to await the development of the PCR (35, 82).

The first two PCR based studies of *M. genitalium* in patients with nongonococcal urethritis (NGU) were reported in the early 1990s (34, 61), and several publications during the 1990s and 2000s have shown a strong association between *M. genitalium* and NGU, independent of *C. trachomatis* infection (30).
Clinical manifestations
Genital infections with M. genitalium as is the case with C. trachomatis infections are often characterized by no symptoms, or mild symptoms like discharge and/or pain during urination, in both men and women (18, 19). In microscopic examination of gram or methylene blue stained smears from the distal urethra from men and women with urethritis, there are an increased number of PMNL (as described previously).

M. genitalium is undoubtedly an important cause of urethritis in men, but there are not enough studies to support the contention that the bacterium can cause epididymitis and prostatitis (30). In women the number of publications concerning the role of M. genitalium as a cause of lower genital tract infections is limited, but there is some recent evidence that it may cause endometritis, PID and ectopic pregnancy or tubal infertility (10, 12, 13, 74).

The findings of M. genitalium in joints (87) may raise speculations concerning the role of this bacterium in sexually acquired reactive arthritis (SARA).

Epidemiology
Few epidemiological studies have been conducted following the discovery of M. genitalium because of difficulties in culturing the organism. However, further clinical studies were made possible due to the development of PCR assays. The meta analysis performed by J. S. Jensen showed that the prevalence of M. genitalium infections among men seems to be lower than that of C. trachomatis infections (30). In a recent study of STD Clinic attendees in Örebro, Sweden, the prevalence of C. trachomatis infections was found to be 12% among men while the prevalence of M. genitalium infection was 7%, and among female STD Clinic attendees these figures were 10% and 6%, respectively (18, 19).

Diagnostics
Culture. The dependence of mycoplasmas on their host for many nutrients explains the great difficulty in cultivating the bacteria in the laboratory. The complex media (4SP) for mycoplasma culture consists of peptone, yeast extract and serum, which provide fatty acids and cholesterol for mycoplasma membrane synthesis (93). To prevent overgrowth of fast growing bacteria that usually accompany mycoplasmas in clinical materials, antibiotics are added. When mycoplasmas grow in this medium they produce acid, causing a colour change in the medium after several weeks. As M. genitalium is extremely difficult to cultivate, this method is not feasible in clinical practice (41).
Nucleic acid amplification tests (NAATs). Due to the lack of reliable culture and serological methods, the role of *M. genitalium* in NCNGU has been difficult to establish (34, 83), although the progress of molecular techniques like the PCR has made it possible to detect the bacterium in urogenital specimens. PCR based assays have been developed by several research groups but most of them are labour intensive and none of them are commercially available (15, 32, 35, 82).

One of the PCR methods for detecting *M. genitalium* in urogenital specimens was developed by Jensen and co-workers and is used as a routine assay at the Mycoplasma Laboratory, Statens Serum Institut (SSI), Copenhagen, Denmark and is also widely used in other laboratories in Scandinavia. The primers used are designed to cover a 427 bp fragment of the 16S rRNA gene (1,490 bp) of the *M. genitalium* G-37 type strain, that has the least homology with the same gene of *M. pneumoniae*, in order to prevent cross reaction. Included in this assay is also an internal process control, detecting a 100 bp longer amplicon from the phage lambda, in order to detect the presence of DNA polymerase inhibitors (32). Routinely, all positive results with this PCR assay are confirmed by the MgPa-1-MgPa-3 PCR assay detecting the MgPa adhesion gene (35). The last step in both PCR assays needs detection of the product by the use of gel-electrophoresis, as described previously. These methods are labour intensive and a more automated method would be desirable, but so far no commercial kits are available.

Antibody detection. As mentioned before, *M. genitalium* and *M. pneumoniae* share several antigen properties, and the relationship between the two mycoplasma species has hampered diagnostic serology like the Complement fixation test and EIAs because of serological cross reactions (43, 86).

Using micro-immunofluorescence (MIF) Taylor-Robinson *et al.* found a significant rise in antibodies against *M. genitalium* in four of 14 patients with NGU, but also in two of 17 without urethritis (85), but only one specimen was identified by culture. A promising serological EIA assay was developed by Wang *et al.* using Triton X-114 extracted lipid associated membrane proteins (LAMPs). The correlation between a positive *M. genitalium* PCR test and detection of *M. genitalium* antibodies was highly statistically significant and no crossreactions were found (95, 96). As antibodies are slow to develop after genital infections, this method is not feasible in clinical practice, for diagnosis of an acute *M. genitalium* infections but it is the method of choice when no infectious agent can be detected.
Aims
The major aims of this thesis were:

- To develop a PCR method for genetic characterization of clinical \textit{C. trachomatis} isolates in a Swedish STD Clinic population by sequence analysis of the \textit{omp1} gene, and to study the distribution of genotypes within sexual networks and to determine if genotyping would improve partner notification (I & II).

- To further develop an existing conventional PCR protocol for detection of \textit{M. genitalium} DNA into a real-time PCR with hybridization probes, and to evaluate it as a method for detecting \textit{M. genitalium} in first void urine and endocervical specimens (III).

- To adapt a Triton X-114 extracted Lipid-Associated Membrane Protein (LAMP) Enzyme Immuno Assays (EIA) method to detect antibodies against \textit{M. genitalium} and to evaluate the association between \textit{M. genitalium} and pelvic inflammatory disease (PID) and ectopic pregnancy (EP), using a unique serum material sampled from patients hospitalised for acute PID and EP during the 1980s, and also to compare the number of sera having \textit{M. genitalium} antibodies against those having \textit{C. trachomatis} antibodies, using a commercial anti-\textit{Chlamydia trachomatis} EIA assay (IV).
Material and methods
Patients and Controls
In papers I-IV, urogenital and/or urine samples for diagnosis of C. trachomatis were prospectively obtained from all new attendees (n = 2195) at the Outpatient STD-Clinic, Örebro Medical Centre Hospital, Sweden, during a one-year period (March 1, 1999 to February 29, 2000). The mean age for men (n = 1141) was 28.5 (range 14 to 68) years and the mean age for women (n = 1054) was 25.7 (range 13 to 59) years. Twenty-four patients, who were strongly epidemiologically suspected of having a C. trachomatis infection, but with negative diagnostic tests for C. trachomatis, were also included in the study. The inclusion period was elongated for some individuals because they belonged to a sexual network and were sexual partners of C. trachomatis infected patients included earlier in the study. One negative patient sample per every C. trachomatis- positive sample was randomly selected each day, and these were used as negative controls in the study.

In paper III, endocervical specimens and/or first void urine samples were obtained from all new attendees (n = 699) at the Outpatient STD-Clinic at the Örebro University Hospital, Örebro, Sweden, between May 27 and September 2, 2002. The mean age for men (n = 398) was 28 (range 17–58, median = 27) years and the mean age for women (n = 301) was 26 (range 15 – 56, median = 23) years.

In paper IV, a total of 303 sera from 194 patients with a clinical diagnosis of PID and 104 sera from 83 women with a clinical diagnosis of EP, who were patients at the Department of Obstetrics and Gynaecology, Örebro Medical Centre Hospital, Örebro, Sweden, were obtained during a 25-month period from February 1984 to April 1986, and were stored at -20°C. The median age was 23 (range 15–50) years for the PID patients and 29 (range 18–42) years for the EP patients. As control material, sera from healthy pregnant women (three women for each women with EP, matched for age) were obtained in 1988 and stored at -20°C. A control group consisting of 150 sera from female blood donors (age 18–50 years) were obtained and stored at -20°C during 2002 and were used as negative controls. A collection of acute phase sera from 99 men attending a STD-Clinic in Copenhagen, Denmark, with known results for their urogenital specimens from the Mycoplasma genitalium – PCR performed in the Mycoplasma laboratory, Statens Serum Institut (SSI), Copenhagen, Denmark (32), was used to evaluate the results from the LAMP EIA regarding sensitivity and specificity.
Sampling and diagnostics (I–IV)

In papers I–II, urethral or endocervical specimens for tissue culture were obtained from males and females, respectively, using sterile Dacron swabs. Swabs were placed into transport medium containing 2 ml of 2SP-medium (sucrose-phosphate buffer, 5 % fetal bovine serum (v/v), and antibiotics) and were directly transported to the laboratory and stored at -70°C until processed for tissue culture. At the same examination, first void urine (FVU) samples from both men and women were collected and stored at +2 - 8°C in sterile screw cap plastic tubes until analysed by the Chlamydia trachomatis Amplicor™ PCR test as described in paper I. All C. trachomatis positive patients were treated with appropriate antibiotics and requested to return for a follow up visit 4–5 weeks after the initial sample was obtained. A total of 240 specimens were found to be C. trachomatis positive by tissue culture and/or by the Amplicor™ PCR or COBAS Amplicor™. Two samples were lost, and 238 were stored at -20°C until used in the study, as were 24 specimens from patients strongly suspected of being C. trachomatis infected, but with negative diagnostic tests for C. trachomatis. One negative patient sample per every C. trachomatis-positive sample was randomly selected each day and used as negative controls in the study.

In paper III, endocervical specimens were obtained from females (n = 321) using four sterile Dacron swabs. The first and second swabs were placed in one polypropylene tube and the third and fourth swabs were placed into another tube, both containing 2 ml of 2SP-medium. The specimens were randomly (by a die) assigned to be sent to Statens Serum Institut, Copenhagen, Denmark (SSI), or directly transported to the Department of Microbiology, University Hospital Örebro (UHÖ) and stored at -70°C until used for isolation of DNA to detect M. genitalium and for cultivation of C. trachomatis in McCoy cell cultures as previously described. The first void urine samples were collected at the clinical examination and were divided into two sterile screw cap polypropylene tubes; one was sent to SSI and the other was sent to UHÖ to detect M. genitalium. Likewise, first void urine samples were obtained from men (n = 398) attending the STD Clinic. All urine samples (from women and men) were also analysed by the COBAS Amplicor™ Chlamydia trachomatis Test.

In paper IV, sera were obtained from women hospitalised and treated for PID (n = 194) and EP (n = 83), during a 25-month period from February 1984 to April 1986, at the Department of Obstetrics and Gynecology,
Örebro Medical Centre Hospital, Örebro, Sweden. The PID diagnosis was based on clinical criteria, i.e. pain in the lower abdomen of not longer than 3 weeks duration with palpable adnexal mass and/or motion tenderness, fever > 38.0 °C and objective signs of lower genital tract infection defined as pus from the cervical ostium macroscopically and/or microscopically (more polymorph nuclear leucocytes than epithelial cells). In total 60–65% of the cases underwent laparoscopy for direct visual diagnosis of acute salpingitis (37).

Patients with suspected ectopic pregnancy (EP) had clinical signs of subjective symptoms of early pregnancy together with a positive urine human Chorionic Gonadotropin (hCG) test and low abdominal pain. Some of the patients had a previous history of PID or EP, had undergone surgery of the lower abdomen or the fallopian tubes, used an intrauterine contraceptive device or progesterone-only preventive pills, or had clinical evidence of vaginal bleeding. When clinical evidence was uncertain, the diagnosis was confirmed by laparoscopy (38).

**Isolation of DNA (I–III)**

DNA was isolated directly from the clinical samples and the reference strains using Chelex 100 Resin (Bio-Rad Laboratories). Chelex is a chelating resin with a high affinity for polyvalent metal ions, and is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions. It has been proposed that the presence of Chelex during boiling prevents degradation of DNA by chelating metal ions that may act as catalysts in the breakdown of DNA at high temperatures (94). In papers I–II, a volume of 100 ml from the clinical specimens (in 2SP medium) for tissue culture, or a pellet from 1000 ml urine, was washed in distilled water for 30 minutes at room temperature and micro centrifuged for 5 min at 18,000 g. The pellet was resuspended to a final volume of 200 ml in distilled water and mixed with 2 ml of 10 mg/ml proteinase K (SIGMA) and incubated for one hour at +37°C. The tubes were then micro centrifuged for 5 min at 18,000 x g, and the pellet was resuspended in 200 ml of 5% Chelex 100 Resin, thoroughly mixed and incubated at +56°C for 30 minutes, mixed again and incubated in boiling water for 8 minutes. In paper III, a volume of 100 ml from urogenital specimens (in 2SP medium) was added to 1000 ml saline, or 1800 μl urine was directly transferred to eppendorf tubes and was micro centrifuged for 15 min at 20,000 g. The pellet was resuspended in 300 μl of 5% Chelex 100 Resin in distilled water (w/v), thoroughly mixed and incubated at 99°C for 10 minutes. The cell debris was pelleted by centrifugation at 10,000 g for 3 minutes and the
supernatant containing the DNA was withdrawn and stored at +4°C until used (I–III).

**PCR (I–III)**

In papers I–II, the *C. trachomatis* reference strains A-K, L1-L3 were used for optimization of the *omp*1-PCR and DNA sequencing, and a *C. trachomatis* strain serotype E was used as positive control in each PCR-run. All the reference strains were originally from the Institute of Ophthalmology, London, UK.

As described in detail in paper I, the DNA preparation was added to the PCR mixture containing the primers $P_1 = 5^{\prime}>$ATG AAA AAA CTC TTG AAA TCG G $<3^{\prime}$ and $OMP_2 = 5^{\prime}>$ACT GTA ACT GCG TAT TTG TCT G $<3^{\prime}$. The PCR was run with the hot start technique using AmpliTaqGold at +94°C for 10 min to reduce non-specific amplification, followed by 40 cycles at +94°C for 30 sec, +55°C for 30 sec and +72°C for 1.5 min. At the end of the final cycle, an extension at +72°C for 7 min was included before storage at +4°C.

In paper III, a conventional PCR designed to amplify a 427 bp fragment of the 16S rRNA gene of *M. genitalium* was used as reference (performed at SSI) and for verification (at UHÖ). The primers used were MG16-45F ($5^{\prime}$-TAC ATG CAA GTC GAT CGG AAG TAG C-3’) and MG16-447R ($5^{\prime}$-AAA CTC CAG CCA TTG CCT GCT AG-3’) and an internal process control was also included in the reaction mix. All primers used in papers I–III were purchased from Scandinavian Gene Synthesis (SGS) AB, Köping, Sweden.

The amplification product was visualized after electrophoresis through an agarose gel containing ethidium bromide (papers I–III). At SSI (III), all positive results were confirmed by an independent PCR amplifying a fragment of the MgPa gene as previously described (32).

**Sequencing (I–II)**

The PCR products were purified by the High Pure PCR Product Purification Kit (Roche diagnostics), according to the manufacturer's instructions, prior to the cycle sequencing PCR. The amplified DNA from the *omp* 1-PCR was mixed with one of two primers ($S_1 = 5^{\prime}>$TTG AGT TCT GCT TCC TCC T<3’ or $OMP_2$) in separate reactions together with ABI PRISM® BigDye™ Terminator Cycle Sequencing ready reaction reagents, according to the manufacturer’s instructions (PE Applied Biosystems, Warrington, UK). The sequence of the *omp*1 gene was determined by using an ABI PRISM 310.
Genetic Analyser (PE Biosystems). Each PCR product was sequenced twice in each direction, and this gave an overlap of about 200 bp in the middle of the \textit{omp1}-gene. The individual consensus sequences of the isolates were sent to a database available at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) to establish the \textit{C. trachomatis} genotype, corresponding to the isolate. Phylogenetic tree analyses were used to illustrate the relationships between clinical isolates and the reference strains of \textit{C. trachomatis} as shown in paper I.

\textbf{Partner notification and sexual networks (II)}

In Sweden, it has been mandatory since April 1988, under the Communicable Diseases Act to report \textit{C. trachomatis} infections. In the present study, partner notification regarding \textit{C. trachomatis} infection was done according to standard Swedish procedures (16). The index patient (\textit{C. trachomatis} infected) was obliged to facilitate the notification, and in most cases the index patient contacted all recent partners. Otherwise a letter was sent to the partners by the contact tracer, but in that case the identity of the index patient was not revealed. The partners were asked to attend the Örebro STD Clinic, but they were free to choose another clinic.

Partner notification was done by the social worker at the STD Clinic. She always met the patient at the follow-up visit, but this was also done at the first visit if a \textit{C. trachomatis} infection was suspected.

In this study the term network was used for designation of the anamnestically revealed part of the total sexual network including all individuals and their known sexual links in the community. We also used the term “chain” to describe the transmission of a \textit{C. trachomatis} strain between individuals within the networks. Network is synonymous with the term component, proposed by Wylie and Jolly, and consists of two or more persons and an identified path from one person to every other person (97). In the beginning of the study period, the specimens for routine analysis from \textit{C. trachomatis} positive patients were frozen during the period of optimisation of the \textit{omp-1} PCR and genotyping. Therefore, there was a delay in constructing a network based on the anamnesis from the index patient, and hence the data were provisional. Later in the study the genotyping was performed shortly after the routine analysis, and hence it was possible to compile the network with known genotypes.

\textbf{Real-time PCR (III).} In the present thesis, real-time PCR amplifications with hybridization probes were performed in a LightCycler (LC) PCR system.
The principle for the Hybridization Probe format is that primers used for conventional PCR are used together with specially designed, sequence specific oligonucleotides (probes) labelled with fluorescent dyes (e.g. Fluorescein and LC-Red 640). The primers of the LC-PCR assay were identical to those designed to amplify a 427 bp fragment of the 16S rRNA gene of *M. genitalium* used in the conventional PCR (32) and were used together with the sequence specific oligonucleotide probes (TIB MOLBIOL, Syntheselabor, Berlin, Germany): Mg16S-137-probe (LC-red 640 AAT TCA TGC GAA CTA AAG TTC TTA TGC GGT ATT AGC T - phosphate) and Mg16S-169-probe (AAT AAC GAA CCC TTG CAG GTC CTT TCA ACT T – fluorescein) labelled with fluorescent dyes. The reaction mix was added to the glass capillary together with the template DNA from the clinical specimen. Included in each run was a positive *M. genitalium* control and water as a negative control. The PCR-program started with a pre-incubation for activation of the FastStart enzyme at 95°C for 10 min, followed by amplification for 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 16 sec, and at the end of the protocol there was a cooling-program (40°C for 30 sec). The sequences of the two probes are designed so that they hybridise to the amplified DNA fragment in a head to tail arrangement, and so that they can hybridize to adjacent regions of the template DNA separated by one nucleotide (Figure 6). When the two dyes are in close proximity the first dye (fluorescein) is excited by the LightCycler’s light emitting diode, and the emitted energy excites the LC Red 640 attached to the second hybridization probe that emits red fluorescent light. This energy transfer (Fluorescence Resonance Energy Transfer = FRET), is dependent on the specific binding of the probes in close space (1-5 bp). The fluorescence measurement is performed after the annealing step and the increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the PCR process.
**Figure 6.** LightCycler PCR using hybridization probes (Roche Diagnostics). **A.** The reaction mix contains FastStart DNA Master Hybridization Probes, with FastStart Taq DNA polymerase, reaction buffer, dNTP mix and primers used for the conventional PCR together with specially designed, sequence specific oligonucleotides (probes) labelled with fluorescent dyes e.g. Fluorescein (Oligo 1) and LC-Red 640 (Oligo 2). The sequences of the two probes are designed such that they hybridize to the template DNA fragment in a head to tail arrangement, separated by one nucleotide. **B.** When the two dyes are in close proximity, the first dye (fluorescein) is excited by the LightCycler’s light emitting diode, and the emitted energy excites the LC Red 640 attached to the second hybridisation probe that emits red fluorescent light. The intensity of the emitted light is proportional to the increasing amount of DNA generated during the ongoing PCR process (Pictures from www.rochediagnostics.com).

**Antibody detection against M. genitalium (IV).** In order to analyse antibodies against *M. genitalium* in a unique serum material from PID and EP patients, an EIA method was adapted using a Triton X-114 extracted and purified fractionation containing the LAMP of *M. genitalium* as previously described (96). The reference strain *M. genitalium* G37\(^T\) and a Danish patient strain designated M2341 were used as antigen. They were grown in modified Friis FF medium (33) containing horse serum in 175 cm\(^2\) tissue culture flasks (Nunc A/S, Roskilde, Denmark) until the late log phase. The adherent cells were washed with saline and subsequently scraped off and harvested by centrifugation and stored at -80°C. The pellet was resuspended in a 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and 1 mM EDTA (Buffer A) and the protein concentration was adjusted to 2 mg/mL. The cells were lysed by adding 10% Triton X-114 to a 2% (v/v) concentration, and the lysates were sonicated for 30 seconds, five times, with a cooling period on ice between times. The NaCl concentration was then adjusted to 0.5 M with a 5 M NaCl stock solution, and was mixed and stored on ice for two hours followed by centrifugation at 21,000 g for 20
minutes at +5°C to remove insoluble materials. The supernatant was removed to new tubes on ice, and the first cycle of Triton X-114 phase fractionation was started by incubation of the cleared lysates in a +37°C waterbath for ten minutes to induce the condensation of Triton X-114. The lysates were then transferred to a preheated (+30°C) centrifuge and were centrifuged for ten minutes at 5,200 g, and the upper aqueous layer was removed while the heavier detergent-enriched phase was incubated on ice. An ice-cold 50 mM Tris-HCl buffer containing 0.5 M NaCl and 1 mM EDTA (pH 8.0) was added up to the original volume, mixed by vortexing, and the fractionation procedure was repeated two more times. After the third cycle of Triton X-114 phase fractionation, Buffer A was added up to the original volume, vortexed and stored on ice for 30 minutes and centrifuged at 21,000 g for 20 minutes at +5°C. The supernatant containing the Triton X-114 extracted LAMP antigen was transferred to a new tube and the protein concentration was determined with Bio-Rad, DC Protein assay kit, and was stored at -80°C until used in the LAMP-EIA.

The LAMP-EIA was performed using 96-well microtitre plates (Maxisorb, Nunc), which were coated with a mixture (100 μL) of the two antigen preparations (1μg/mL) and incubated overnight at room temperature (RT). After the wells were blocked with 5% nonfat milk (Biorad) in PBS + 0.05% Tween 20 (PBST) for two hours at RT, 100 μL of sera diluted 1/50 in blocking solution was added to each well in duplicate and incubated for two hours at RT. The plates were washed four times in PBST after each step. Diluted (100 μL) peroxidase – conjugated Goat-Anti-Human IgG (Fc-fragment, Sigma, A0170) was added (100 μL) and incubated for another two hours at RT, washed six times and followed by 30 minutes development with 100 μL SIGMA FAST™ OPD (o-phenylenediamine dihydrochloride). The reaction was stopped using 50 μL 1.5 M HCl and the optical density was measured at 492 nm (Figure 5).

Serum from a patient with a Mycoplasma genitalium – PCR positive result in the urogenital specimen was used as a positive control and pooled sera from blood donors were used as a negative control in each run. Rabbit antiserum against different Mycoplasma species and acute phase sera from patients with M. hominis or Ureaplasma urealyticum positive cultures from urogenital tract specimens were tested during the developing procedure and no cross reactivity were found. Furthermore, 10 human sera from adults with a high antibody titer against M. pneumoniae in a complement fixation tests were negative in the LAMP–EIA assay.
Antibody detection against *C. trachomatis* (IV). In order to control for confounding from *C. trachomatis* infection in PID and EP, but also to compare the number of sera with *M. genitalium* antibodies with those having *C. trachomatis* antibodies, the sera were analysed with a well-documented commercial anti-*Chlamydia trachomatis* IgG EIA (Ani Labsystems Ltd. Oy, Finland) according to the manufacturer’s instructions. To avoid cross reactions against *Chlamydia pneumoniae*, the antigen in this EIA assay is based on synthetic peptides derived from the *Chlamydia trachomatis* specific variable domain of the outer membrane protein (55).

Results and discussion

**Genotyping of *C. trachomatis* and partner notification (I–II)**

All tested prototype isolates of serovars A to L3 of *C. trachomatis* were successfully amplified in the *omp1*-PCR. The *omp1*-PCR showed high concordance with the diagnostic tests for *C. trachomatis*, and out of 238 *C. trachomatis* positive clinical samples analysed in the diagnostic tests, 235 were found positive in the *omp1*-PCR. Consequently, three clinical samples were found negative in the *omp1*-PCR, but positive in the routine PCR assay (COBAS Amplicor™). Retesting in the routine PCR assay showed that two of the three samples were negative and thus considered false positive in Amplicor™ PCR. However, for one of these there was a clinically strong suspicion of *C. trachomatis* infection, since the patient had two sexual partners who were *C. trachomatis* positive. The remaining COBAS Amplicor™ positive sample was repeatedly negative in *omp1*-PCR. One reason for this false negative result could be that the *omp1* gene is only present in one copy per organism, whereas the plasmid, which is targeted by the diagnostic test, is present in approximately 10 copies per organism. However, the *omp1*-PCR was also positive in 2 of 24 diagnostic test negative samples from individuals strongly suspected, epidemiologically, of having a *C. trachomatis* infection. One of these two samples was positive after retesting with the diagnostic test. The remaining sample, which was found positive in the *omp1*-PCR only, could contain a plasmid-free variant of *C. trachomatis* (2).

All reference isolates were successfully sequenced and the sequence analysis of the *omp1* gene from amplified DNA from the 237 clinical strains revealed that the most prevalent genotypes corresponded to *C. trachomatis* serovar E (47%) followed by F (17%) and K (9%), and Ba, D, D/B, G, H, I, and J (0.4 – 6%). One woman appeared to have a mixed infection with two different *C. trachomatis* strains; F was found in the endocervical sample and E in the urine sample. The distribution of genotypes is shown in Figure 7.
Genotyping of samples collected at the follow-up visit showed that there were five patients still infected with *C. trachomatis*, see Table 3 in Paper I. The sequence analysis suggested that a re-infection had occurred in two of these five individuals. Thus, one woman was found to have *C. trachomatis* genotype K sequence in the initial endocervical sample and a genotype D/B-185 sequence one month later. Similarly, in the initial urogenital specimen from one man and also in the second sample (received two months later), *C. trachomatis* genotype E was found, while in the third urethral sample, five months after the initial sample, *C. trachomatis* genotype D/B 120 was found. No change in genotype was found in the specimens from the remaining three individuals, which suggests either treatment failure or re-infection with the same *C. trachomatis* genotype. (See table 3 in Paper I.)

There was a high level of conservation of the *omp1* gene in infections caused by *C. trachomatis* genotype E of which 106 out of 112 had 100% similarity with the reference strain. However, we found three genotype E strains with a mutation (G→A) in position 997 and this caused an amino acid change in the MOMP gene (Ala→Thr), see Table 2 in Paper I. The same strain was found in a study in a neighboring county the year after this study was performed (44). Five of six of the *C. trachomatis* genotype G sequences had identical substitutions, and strains with the same substitution were also found in the neighboring county, indicating the introduction of new genetic variants in this area. In contrast, four of ten clinical *C. trachomatis* genotype J strains were found to have one identical substitution, but were not found by Lysén et al.

![Figure 7. omp1 genotype distribution of 237 urogenital C.trachomatis strains isolated during one year, from patients attending the STD clinic, in Örebro](image-url)
Few studies have addressed the possible correlation between specific C. trachomatis serovars or genotypes and disease manifestations and severity of disease. However, in a Finnish serological study it was contended that serovar G could be associated with subsequent development of cervical squamous cell carcinoma (3). This finding is interesting and requires further study especially due to the fact that some genotype G sequences in our study differ from the prototype sequence by three amino acids.

Partner notifications were performed according to Swedish standard methods (16), and a sexual network with a time axis was outlined and the genotypes were established after partner notification (II). The omp1 gene from C. trachomatis in specimens from at least two patients in 47 of 161 networks was successfully sequenced. In seven of these 47 networks there were discrepant genotypes and in two of these seven networks three different genotypes were found, see Table 2 in Paper II.

Partner notification in the neighboring county showed that a man with genotype D/B-120 referred to a contact in Örebro, and two strains with the same substitution were found in this study (44). This suggests a link in a sexual network that could not be further traced. In a recent review article, the most common C. trachomatis genotypes in different parts of the world were shown to be D, E and F (21). In the present study, there was strong evidence that twenty-two patients probably contracted their C. trachomatis infection abroad, namely one in Australia (genotype D), two in Thailand (Ba, E), two in the United States (Ia), two in Austria (D/B-120, D), two in Bulgaria (E), three in Greece (2x F, E), four in the Netherlands (3x F, E), two in Norway (F, D), two in Spain (Ia, K), one in Switzerland (D), and one in the United Kingdom (E).

Most networks (n = 131, 81%) could not be completed because the index patient withheld or provided the contact tracer with insufficient information for partner identification. In our study, contact tracing was performed by a social worker at the STD Clinic and by specially trained midwives in the neighbouring county (59). The largest network in our study comprised of 26 individuals, and it took about 13 months until all partners were tested and treated (Figure 8).
Figure 8. A network comprising 26 individuals. Arrows indicate the believed transmission of infection; circles = women and squares = men; question marks indicate unknown individuals. The white E is the index patient who attended the STD Clinic in November 1999. His steady partner was also *C. trachomatis* genotype E-positive, and her second latest partner was *C. trachomatis* negative but had urethritis due to a *Mycoplasma genitalium* infection. Therefore, further partners were tested, and they were also *C. trachomatis* negative. The index patient's second latest partner was identified but did not attend the clinic until May 2000. During the eight months prior to attendance, she had had unprotected sexual intercourse with a further eight men, of whom five were traced and tested and three were found to be *C. trachomatis* genotype E-positive. Furthermore, six *C. trachomatis* positive individuals were found; three had a *C. trachomatis* genotype E infection, one had infection with a strain not sequenced, and one, a women attending the clinic in August 2000, was infected with genotype D/B-120. Her latest partner, a man who attended the STD Clinic three times between February and July 2000, was genotype E positive twice and the third infection was genotype D/B-120. Notes: * A women attending the clinic in May 2000. † A *C. trachomatis* negative man with urethritis due to an *M. genitalium* infection. ‡ A man attending the clinic three times.

One partner in that network († in figure 8) was *C. trachomatis* negative but had urethritis due to a *Mycoplasma genitalium* infection. There is a strong association between *M. genitalium* and NGU, independent of *C. trachomatis* infection and the need for a new method for *M. genitalium* DNA detection was one reason for study III in this thesis.
Evaluation of the real-time PCR assay for detection of *M. genitalium*

In paper III, a real-time (RT) LightCycler PCR assay with hybridization probes for detection of *Mycoplasma genitalium* in endocervical and first void urine specimens was developed and compared with a conventional PCR. The primers of both assays were identical and designed to amplify a 427 bp fragment of the 16S rRNA gene of *M. genitalium*. The LightCycler PCR assay (LC-PCR) had a detection limit of <5 bacterial genome per reaction when dilutions of genomic DNA from a type strain of *M. genitalium* were tested (Figure 9).

![Fluorescence vs Cycle Number graph](image)

**Figure 9.** The PCR amplification by the LightCycler PCR assay is shown by a series of 10-fold dilutions of genomic DNA from *M. genitalium* G-37T, containing 10^5 (A), 10^4 (B), 10^3 (C) and 100 genome per 1ml (D). E is the negative control (distilled water). The sample volume in each capillary is 2 μl.

In order to evaluate the LC-PCR assay with clinical material, specimens from men and women attending the STD Clinic were analysed at the local laboratory in Örebro, Sweden using the LC-PCR assay, and were also sent to the Mycoplasma Laboratory at SSI in Copenhagen where the conventional PCR was well established. Both laboratories applied confirmatory testing of positive results and although different approaches were chosen, we decided to consider a patient infected with *M. genitalium* if one specimen was found confirmed positive in one of the laboratories.

A total of 19 (4.8%) of 398 men were found positive for *M. genitalium* in one or both PCR methods when the first void urine specimens were analy-
Six men were still *M. genitalium* positive at the follow-up visit after initial antibiotic (azithromycin or tetracycline) treatment, but are not included in the evaluation. Using the conventional PCR method as “gold standard” the sensitivity for the LC-PCR assay was 72.2% with a 95% confidence interval (CI) of 46.5 – 90.3%, and the specificity was 99.7% (95% CI = 98.5 – 99.9%).

First void urine and endocervical specimens from 301 women were analysed, and 26 women were found to be *M. genitalium* DNA positive either in the swab or in the urine specimen by the LC-PCR assay and/or the conventional PCR assay at SSI. The prevalence of *M. genitalium* infected women in this material was found to be 8.6% (26/301). The LC-PCR assay detected 19 of the 26 women deemed *M. genitalium* positive. Consequently, the sensitivity of the LC-PCR assay was 68.2% (95% CI = 52.2–88.4%), and the specificity was 98.6% (95% CI = 96.4–99.6%), when using the conventional PCR method as “gold standard”. In order to increase the sensitivity in the LightCycler assay, a new RT-PCR with Taqman probes was adapted to the LightCycler system in collaboration with SSI and a neighbouring county. The primers and probes in this RT-PCR assay were designed to detect a 78 bp fragment of the *M. genitalium* MgPa gene (RT–MgPa–PCR) and were the same as used in a Taqman assay at SSI (31). Three different PCR assays; the LC-PCR, the conventional 16S PCR (at SSI) and the RT-MgPa-PCR, were compared in a clinical study performed in Karlstad, Sweden. A higher sensitivity was demonstrated in the RT-MgPa-PCR as compared to the 16S PCR and LC-PCR, and the results were presented by Andreas Edberg *et al.* in a poster at the European Society for Clinical Microbiology and Infectious Disease (ESCMID) meeting in Nice in 2006 (abstract No. P956).

Dual infection with both *M. genitalium* and *C. trachomatis* has been reported in several studies and was also found in the present study (8, 19, 34, 45, 89). Thirty-two women were *C. trachomatis* positive; three (9.3%) of them were simultaneously positive for *M. genitalium*, indicating that *M. genitalium* could be found in *C. trachomatis* positive women with approximately the same prevalence as in the remaining study population. In this study, the prevalence of Chlamydia infection was 10.6 % among women, while the prevalence of *M. genitalium* infection was 8.6 %. Out of 52 men found to be *C. trachomatis* positive, none were simultaneously *M. genitalium* positive. This is in accordance with the results reported in several studies reviewed by Uusküla *et al.* showing that men with NGU tend to harbour *C. trachomatis* or *M. genitalium* separately rather than together (92). In the
In the present study, the prevalence of Chlamydia infection in men was 12.6%, and the prevalence of M. genitalium infection was 4.6%. The reason for the larger difference in C. trachomatis and M. genitalium prevalences in men compared to women is not clear.

For a six-month period in 2000, Falk et al. reported the prevalences for C. trachomatis infection and M. genitalium infection among STD Clinic attendees in Örebro to be 12% and 7% respectively, among men, and 10% and 6% respectively, among women (18, 19). There are few cross-sectional studies published about the prevalence of these bacteria but it is likely that M. genitalium is less prevalent than C. trachomatis in Western countries (36, 47). Although patients found to be M. genitalium DNA positive received standard antibiotic treatment used for urethritis and cervicitis, four women and six men were still M. genitalium positive at the follow-up visit. Falk et al. demonstrated in a recent study that tetracyclines are inappropriate for treatment of M. genitalium infections (20).

Several clinical studies strengthen the evidence that M. genitalium is associated with persistent or recurrent infection among both women and men (7, 20, 30, 92). The aim of the present study was not to evaluate signs and symptoms among those infected with M. genitalium, but the findings in this study show that M. genitalium is frequently encountered in the female genital tract of women attending the STD Clinic in Örebro. The same findings were reported in a recent investigation about a year prior to this study (18). Included in that study were a control group of healthy women (n = 157) in the cervical cancer-screening program, and none were infected with M. genitalium.

Most investigations have concerned male urethritis patients, but establishing a connection between M. genitalium and upper genital tract infection among women is of major importance in determining the significance of the infection. Some studies have also shown a connection to PID and infertility (12, 49, 74), but there is a need for more evidence, and that is one of the reasons for paper IV in this thesis. A method for antibody detection was adapted and was used to analyse a unique serum material from women with the clinical diagnosis of PID or EP obtained in the 1980s when the incidence of PID in Sweden was high (39).

**Antibody detection against M. genitalium by LAMP – EIA (IV)**

We successfully adapted a Triton X-114 extracted LAMP-EIA assay to detect antibodies against M. genitalium. In our hands, the LAMP-EIA showed no cross reactivity when analysing sera from adult humans with high antibody
titers against *M. pneumoniae* in complement fixation tests and sera from patients with *M. hominis* or *Ureaplasma urealyticum* positive cultures from urogenital tract specimens, as well as rabbit antisera against other Mycoplasma species.

The negative cut off level was set at 0.3 OD$_{492}$ and was determined as 3 standard deviations above the negative control mean. The sensitivity and specificity were determined by comparing the *M. genitalium*-PCR results from 99 men attending a STD Clinic in Copenhagen, Denmark, with the LAMP–EIA results of their acute phase sera. Eight patients were found to be positive in both tests while 12 were only LAMP–EIA positive and five were only Mg - PCR positive. The sensitivity in the LAMP-EIA was found to be 62% with a 95% confidence interval (95% CI) of 31–86%, and the specificity was 86% (95% CI = 77–93%).

**The role of *M. genitalium* and *C. trachomatis* in pelvic inflammatory disease and ectopic pregnancy (IV)**

The sera in this study were obtained from women who were seen at the Department of Obstetrics and Gynecology, Örebro Medical Centre Hospital during a 25-month period from February 1984 to April 1986. They were obtained during a prospective study from 1970–1997 of patients hospitalized for acute PID. The incidence rates of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections, and the relationship between these infections and the annual number of PID patients during that time, were shown to decline in the late eighties (37). In Sweden, there has been mandatory reporting of gonorrhoea and syphilis since 1919, under the Communicable Diseases Act, and this has been the case for genital infections with *C. trachomatis* since April 1988. The technique for culturing *C. trachomatis* in tissue culture started during the observation time, and the serum material in this study was consequently obtained before the start of mandatory contact tracing, screening and treatment of asymptomatic men and women. In the present study, serological evidence of chlamydial infection was detected using a well-documented commercial anti-*Chlamydia trachomatis* EIA assay (CT-EIA). Sera from healthy pregnant women (three for each case of EP matched for age) were used as control material (Cp). The high numbers of women (Table 1) with antibodies against *C. trachomatis* among the patients (PID = 54% and EP = 58%) and the control group of pregnant women (36%) in our study are in good agreement with figures reported by other Scandinavian groups from the same period of time (48, 56, 60, 69). Among the control group of female blood donors from 2002 only 8.7% (13/150) were found
CT-EIA positive, perhaps due to the screening and treatment of asymptomatic women that is currently carried out in Sweden.

The same unique serum material from the PID- and EP patients was analysed in the *M. genitalium* LAMP–EIA assay. The optical density (OD) distribution is illustrated in Figure 10 A, B, and C.

Previous serological studies have shown discrepant results in regard to a change in titer in acute- and convalescence phase sera among PID patients (49, 74). In our study 68 of the PID patients and 16 of the EP patients provided both acute- and convalescence phase sera, and among the PID patients, 16 attended the clinic again during the study period with a new PID. In this study, only data from the first visit were analysed. In some cases we could see a seroconversion but in the most cases both acute – and convalescence phase sera were negative. See Table 1 in Paper IV.

**Table 1.** Number of pelvic inflammatory disease (PID) patients (n=194) and ectopic pregnancy (EP) patients (n=83) seropositive in the *M. genitalium* LAMP EIA and *Chlamydia trachomatis* IgG EIA (CT) as compared to results in the control group (Cp) of healthy pregnant women (three for each EP-case). Analyses were done by logistic regression, both univariate, with age included, and multivariate. The effect parameter is expressed as the odds ratio (OR) and its 95% confidence interval (CI).

<table>
<thead>
<tr>
<th>Age group</th>
<th>PID patients seropositive (%)</th>
<th>EP patients seropositive (%)</th>
<th>Pregnant (Cp) seropositive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>OR (95%CI)</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td>15-30*</td>
<td>26/143(18) 1.9 (0.8–4.2)</td>
<td>9/44(20) 2.0 (0.8–5.0)</td>
<td>15/132 (11) (0.7–2.2)</td>
</tr>
<tr>
<td></td>
<td>77/143(54) (2.2–7.9)</td>
<td>26/44(59) (1.7–7.1)</td>
<td>39/132 (29) (1.7–7.1)</td>
</tr>
<tr>
<td>31-50†</td>
<td>7/50 (14) 0.7 (0.3–1.8)</td>
<td>6/38 (16) 0.8 (0.3–2.2)</td>
<td>21/114 (18) (1.4–3.2)</td>
</tr>
<tr>
<td></td>
<td>27/50(54) (0.7–2.7)</td>
<td>21/38(55) (0.7–3.2)</td>
<td>51/114 (45) (1.4–3.2)</td>
</tr>
<tr>
<td>Total</td>
<td>33/193(17) 1.3 (0.7–2.2)</td>
<td>15/82(18) 1.3 (0.7–2.5)</td>
<td>36/246 (15) (1.4–3.2)</td>
</tr>
<tr>
<td></td>
<td>104/193(54) (1.6–3.8)</td>
<td>47/82(57) (1.4–3.9)</td>
<td>90/246 (37) (1.4–3.9)</td>
</tr>
</tbody>
</table>

* The age group for the EP patients and the control group is 18-30 years
† The age group for the EP patients and the control group is 31-42 years
‡ One serum sample from one patient in each patient material was not found
§ Results were equivocal in the CT EIA for 7 PID patients, 5 EP patients, and 5 in the control group of pregnant women
No statistically significant association between PID and *M. genitalium* antibodies in the LAMP–EIA could be found by logistic regression with the outcome PID or EP versus Cp controls in univariate or multivariate analysis. Among the PID patients, 17% (33/193) were *M. genitalium* LAMP-EIA seropositive. When analysing the different age groups in the PID material, 18% (26/143) of the LAMP-EIA positive patients were found in the group aged 15–30 years as compared to 11% (15/132) in the control group of pregnant women, which showed a trend toward an association between PID and *M. genitalium* antibodies (OR = 1.9, p = 0.123) in that age group, although it was not statistically significant (Table 1).

It was also the case for the EP patients, where 18% (15/82) were LAMP-EIA seropositive, that no association with *M. genitalium* LAMP antibodies could be found in the univariate analysis, OR 1.31 (95% CI 0.67–2.54), and this was even less so in the multivariate analysis, OR 1.01 (95% CI 0.51–2.02). However, there was a trend towards an association in the age group 18–30 years, where 20% (9/44) of the EP patients were LAMP-EIA seropositive (OR 2.01, p = 0.133) compared to the Cp control group. Surprisingly, we found that the older pregnant women (age group 31–50 years) had *M. genitalium* antibodies (18%) more often than the younger age group (11%). This situation was reversed in the PID and EP material, where a slightly higher seroprevalence was found in the younger age group (Table 1).

Among the control group of female blood donors from 2002, only 3% (5/150) were found to be *M. genitalium* seropositive and 9% (13/150) were found to be CT–EIA positive. The decreasing *C. trachomatis* antibody prevalence could perhaps be due to the screening and treatment of asymptomatic women but this does not explain the lower *M. genitalium* seroprevalence. Obviously, behavioural differences may also play a significant role. These findings strongly emphasize the need for careful selection of control material collected in the same time period as the study population, but attention should also be directed toward behavioural differences between the groups. In this study, it was not possible to control for differences in the number of lifetime sexual partners, previous STIs and other factors since this information was not available. Future studies should preferably address these issues.

Although the seroprevalence for both *M. genitalium* and *C. trachomatis* has decreased over the years, as demonstrated by the 3% prevalence of *M. genitalium* antibodies in the donor population collected in 2002, the two
year difference in collection time between the PID/EP populations and the Cp control group is not sufficient to explain this difference.

Dual infection with both *M. genitalium* and *C. trachomatis* as diagnosed by PCR has been reported in several studies (8, 13, 18, 34, 45, 89), but in most investigations the two bacteria have been found more often alone than together, indicating that they may act as separate causes of disease. In the present study, 23 (69%) of the 33 *M. genitalium* seropositive PID patients and 10 (67%) of the seropositive EP patients (n = 15) were found to be *C. trachomatis* seropositive. Although this could reflect dual infection with *M. genitalium* and *C. trachomatis*, it may also reflect subsequent infections in a population with a very high burden of STIs.

In summary, we successfully adapted a LAMP-EIA assay to detect antibodies against *M. genitalium*. Our findings did not indicate a connection between PID or EP and the presence of *M. genitalium* antibodies. However, there was a slight trend towards an association between PID and EP and *M. genitalium* LAMP antibodies in the younger age group 18–30 years, although this was not statistically significant.

Further studies are needed where serology and PCR are performed in parallel.
Figure 10. Distribution of Optical density value (OD) at 492 nm in the Mg LAMP-EIA assay among the PID patients (A), EP patients (B) and controls comprising pregnant women (Cp). The negative cut off 0.3 was determined as 3 standard deviations above the negative control mean.
Conclusions

- A sensitive and relatively simple method for detection and genotyping of *C. trachomatis* strains in clinical samples was established. DNA was successfully extracted from urogenital or urine samples using a Chelex-based method, and an approximately 1100 base pair fragment from the *ompT* gene was directly amplified and sequenced. Genotypes E and F were predominant in our Swedish material and the individual sequences were stable and showed limited variation (I).

- Genotyping was shown to be a valuable tool in determining whether a new infection had occurred or whether a new genetic variant had been introduced in those patients still *C. trachomatis* positive at follow up. The findings of discrepant strains within sexual networks would improve contact tracing by suggesting the presence of unmentioned contacts that are unknown to the contact tracer. The overall genetic stability of the different isolates hampered utility in a partner notification context. In unselected populations today, the low genetic variability and hence the benefits regarding partner notification, are too limited to recommend the use of sequence-based genotyping of *C. trachomatis* in routine work. However, genotyping of *C. trachomatis* could play an important role in special circumstances, such as sexual abuse (I–II).

- In paper III, adaptation of the PCR concept to a LightCycler real time PCR method using hybridization probes provided a specific method for detection of *M. genitalium*. This method has some advantages over conventional PCR assays, which are labour-intensive and generally more prone to contamination. The LC-PCR assay is easy to perform, and the simultaneous amplification and detection eliminates the need for further handling of PCR products and decreases the risk of contamination. However, in order to implement this method for routine diagnostics, the sample preparation method should be improved to minimize inhibition problems. The low load of *M. genitalium* DNA in many specimens necessitates a concentration step or larger template DNA volumes in the PCR assay in order to increase the sensitivity.
In paper IV, a Lipid associated membrane protein (LAMP)- EIA was successfully adapted to detect antibodies against *M. genitalium* in human sera. The LAMP-EIA was specific as determined from lack of cross reactivity with other Mycoplasma species. No significant association could be demonstrated between *M. genitalium* antibodies and PID or EP in our serum material using univariate and multivariate analysis. However, a slight trend toward an association was found when focusing on younger individuals. Antibodies against *C. trachomatis* were found to be significantly associated with PID and EP. Further studies are needed, in which serology and PCR are performed in parallel together with control for differences in the number of lifetime sexual partners, previous STIs and other factors.
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