Chlamydia trachomatis

Insights on genetics, molecular epidemiology, diagnostics and treatment, and host-pathogen interactions

Versteeg, A.J.

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Chapter 1

General Introduction
General introduction of *Chlamydia trachomatis*

**History and classification of *Chlamydia trachomatis***

Chlamydia is one of the oldest recognized human diseases, as references to chlamydial-like diseases of the eye (trachoma) were already described in ancient Egyptian and Chinese texts as early as 15 BC [1,2]. *Chlamydia trachomatis* was first identified in 1907 by Ludwig Halberstaedter and Stanilaus von Prowazek [3]. Using Giemsa staining, they observed intracellular inclusions in conjunctival scrapings from individuals infected with trachoma. Shortly after the discovery of the *C. trachomatis* pathogen, the neonatal form of *C. trachomatis* conjunctivitis was described and related to cervicitis in the mothers of affected infants causing it also to be recognized as an urogenital infection [3,4].

For many years, *C. trachomatis* was considered a virus since the infectious agent could only grow in living cells. In the 1960’s, *C. trachomatis* was confirmed to be a bacterium as it was discovered that it possesses a complex cell wall, both DNA and RNA, prokaryotic ribosomes, and metabolic enzymes that would permit an independent existence [2,5-7]. However, *C. trachomatis* lacks the mechanisms for the production of metabolic energy thereby restricting the bacterium to an intracellular existence [5-7].

**Epidemiology of *Chlamydia trachomatis***

*C. trachomatis* infections are a major public health problem, as it remains the primary cause of bacterial sexually transmitted infections (STI) worldwide and the leading cause of preventable blindness [8-10].

*C. trachomatis* is a Gram-negative obligate intracellular bacterium that comprises a 1.0 Mb chromosome and multiple copies of a 7.5 kb extra chromosomal plasmid which have been found to be highly conserved between strains. Moreover, *C. trachomatis* comprises three biovars associated with different disease phenotypes. These *C. trachomatis* biovars are associated with distinct tissue tropisms, as strains from each biovar infect specific cell types and tissues in the human body: the trachoma biovar infects conjunctival epithelial cells [11,12]; the urogenital biovar infects the epithelial layer of the genital and ano-rectal mucosa [13,14]; and the lymphogranuloma venereum (LGV) biovar is known to infect monocytes and macrophages which are cable of passing through the epithelial layer to invade the
underlying connective tissue and disseminate to regional lymph nodes causing LGV [15-17]. The epidemiology of *C. trachomatis* infections thus varies depending on these different biovars.

Trachoma inducing *C. trachomatis* strains are still endemic in poor and remote areas of Africa, Asia, Australia, and the Middle East affecting an estimated 21 million people [11,18]. Active infections are predominantly present among young children and are the result of inadequate hygiene resulting in exposure to ocular secretions. Repeat trachoma infections during childhood causes scarring, distortion of the eyelids, and the eyelids to fold inwards (trichiasis). This eventually causes the eyelashes to touch and damage the cornea leading to irreversible blindness [11]. Trachoma has disappeared in most parts of the world due to improvements in sanitation and hygiene. In 1997, WHO implemented a strategy, called SAFE, to eradicate trachoma by 2020 [19]. This SAFE strategy focuses on: Surgery for trichiasis, Antibiotics for infectious trachoma, Facial cleanliness to reduce transmission and Environmental improvements such as control of disease-spreading flies and access to clean water [19,20].

*C. trachomatis* infection, caused by strains from the urogenital biovar, is the most common bacterial STI worldwide with an estimated 128 million new cases annually [10]. Risk factors associated with *C. trachomatis* infections include being under 25 years of age but (this is) also (dependent) on sexual behaviour, changing to a new sexual partner and having multiple sexual partners [21-23].

In contrast to the urogenital biovar, the LGV biovar is mainly endemic in parts of Africa, Asia, Latin America and the Caribbean. However, since 2003 LGV infections have also emerged in high income countries [15,16]. Infections in high income countries are predominantly found among HIV-positive men who have sex with men (MSM), and are rarely reported in heterosexuals. LGV infections predominantly cause ano-rectal infections [15,16].

Many efforts have been made to reduce the prevalence of urogenital *C. trachomatis* infections. Control strategies for *C. trachomatis* transmission have mainly focused on active testing, partner notification and treatment [24]. However, due to the asymptomatic nature of *C. trachomatis* infections, many infections remain unnoticed, constituting a large reservoir of untreated individuals which causes a potential reservoir for on-going transmission.
Pathogenesis of Chlamydia trachomatis

*C. trachomatis* is characterized by a unique and complex biphasic developmental cycle that alternates between the infectious, metabolically inactive elementary bodies (EBs) during the extracellular phase and the non-infectious metabolically active reticulate bodies (RBs) during the intracellular phase [25,26]. The developmental cycle takes between 48 to 72 hours (Figure 1).

*C. trachomatis* infection is initiated by EBs that recognize host epithelial cells through ligand-receptor interaction [27]. After attachment, EBs are actively endocytosed by the host cell, upon which Chlamydia-derived vesicles mature into a specialized parasitophorous vacuole known as the inclusion [27-29]. Once this inclusion is formed, EBs will immediately start to differentiate into RBs, which are the replicating form of the pathogen. Replication of RBs occurs through binary fusion after which they differentiate back into EBs, which are subsequently released from the host cell to infect new cells and repeat this developmental cycle [25,26].

During the replication stage the bacterial developmental cycle can also reversibly transition from their normal developmental cycle into a state of persistence [30,31]. During this persistent stage, *Chlamydia* inclusions present as morphologically enlarged and non-dividing RBs. Moreover, the ability of these enlarged RBs to differentiate into EBs is inhibited [30]. This persistent state is often induced by exposure to antibiotics or the result of nutrient deficiency and can be reversed by removal of the particular inducer [31].

After invasion of the mucosal lining and establishment of a productive infection, cells of the innate immune system, such as macrophages, dendritic cells, neutrophils and natural killer cells are attracted to the site of infection. This subsequently leads to the production of pro-inflammatory cytokines that result in inflammation and triggering of an adaptive immune response, predominantly through the Th1 pathway [32-34]. Clinical manifestations probably represent the combined effects of fibrosis and tissue damage from inflammatory responses to chlamydial replication in infected host cells.

Clinical manifestations

*C. trachomatis* infections remain asymptomatic in up to an estimated 70% of infected women and up to 50% of infected men [35,36]. Symptoms of urogenital
Figure 1. The life cycle of *Chlamydia trachomatis*: EB, Elementary body; RB, Reticulate body.
C. trachomatis infection in women, when present, include abnormal vaginal discharge, dysuria, and intermittent intermenstrual or post-coital bleeding [13,37]. Long-term undetected and untreated infections can ascend into the upper genital tract and cause more severe complications such as pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility [37]. In men, symptoms of urogenital infection, when present, include urethritis, abnormal urethral discharge, dysuria, frequency and epididymitis [38]. Compared to urogenital infections, ano-rectal and pharyngeal infections caused by non-LGV strains are more often asymptomatic, in up to 90-100% of cases [39,40]. Pharyngeal and ano-rectal infections often occur asymptomatic, but pharyngeal infections can manifest as pharyngitis with a sore throat as the most frequently reported symptom. In contrast, ano-rectal infections can manifest as proctitis with rectal pain, discharge, and/or bleeding as most frequently reported symptoms [15-17].

**Laboratory diagnostics of C. trachomatis**

Decades ago, the traditional approach to laboratory diagnostic testing for C. trachomatis infections consisted of cell culture for the inoculation of clinical specimens [41]. Culture was the most sensitive diagnostic test for chlamydial infection until the introduction of NAATs [42,43]. However, culturing of C. trachomatis is labour intensive, has a rather low sensitivity and has a long turnaround time.

Today nucleic acid amplification tests (NAATs) are the most widely used technique for the detection of C. trachomatis infections, and have replaced culture as the method of choice to diagnose C. trachomatis. NAATs are designed to amplify and detect nucleic acid sequences (DNA or RNA) that are specific for C. trachomatis. NAATs are now also the recommended diagnostic tool due to their superior sensitivity, specificity, and speed to detect chlamydial infections [9,44]. The sensitivity of C. trachomatis cell culture compared to RNA and DNA-based NAATs is much lower and varies in direct comparisons from 36% in rectal infections [45] and 50 to 83% in urogenital infections [46-49]. However, the high sensitivity of NAATs is also a disadvantage since they are not able to discriminate between DNA or RNA from a viable or non-viable infection. As a result, nucleic acid amplification of non-viable C. trachomatis infections may result in an overestimation of the number of true viable C. trachomatis infections. Several studies already demonstrated the presence of DNA and RNA after treatment for up to 51 days after treatment [50-54].
Treatment

*C. trachomatis* is a curable STI and treatment is relatively simple and effective. The recommended treatment for urogenital and pharyngeal *C. trachomatis* infections consists of a single oral dose of 1000 mg azithromycin, or alternatively, doxycycline 100 mg twice daily for 7 days [9,44]. Both antibiotics have proven to be very effective and a recent randomized controlled trial did not show inferiority of azithromycin (97% effective) compared to doxycycline (100% effective) in urogenital chlamydia infections [55]. For ano-rectal *C. trachomatis* infection by non-LGV genovars, the recommended treatment consists of doxycycline 100 mg twice daily for 7 days [9,44]. However, for ano-rectal *C. trachomatis* infections by LGV genovars (L1 to L3), the recommended treatment consists of doxycycline 100 mg twice daily for 21 days [9,44].

Although current treatment methods are effective, there are a few documented reports of antibiotic resistance of *C. trachomatis* in clinical isolates associated with treatment failure [56-61]. All these resistant isolates were cultured, but they often had poor growth rates and showed altered inclusion morphology suggesting that these isolates had entered a persistent and metabolically inactive state. Eventually these isolates could not survive long-time passage or lost their resistant phenotype *C. trachomatis* [56-62]. So far, no natural and stable antibiotic resistant *C. trachomatis* strain has emerged among humans. However, it could be that *C. trachomatis* resistance to antibiotics occurs more frequently but is rarely recognized since most laboratories no longer perform *C. trachomatis* culture, which is the only method to assess reduced sensitivity to antibiotics [62]. Even though resistance has been reported as early as the 1980s, the exact mechanisms of *C. trachomatis* for antibiotic resistance still remain unknown [56,62].

A vaccine is expected to have better impact on the reduction of *C. trachomatis* infections worldwide than screening and treatment. Moreover, a vaccine is expected to be cheaper and more efficient than current screening programs and antibiotic treatments [63]. Over the last decades research has resulted in the identification of several antigens, adjuvants, and immunization routes that can induce significant protection against chlamydia infections in animal models [63]. Unfortunately, still no vaccine is available to prevent *C. trachomatis* infections, but at this moment a MOMP-based vaccine is tested in phase I clinical trials [63,64].
Molecular epidemiology, tissue tropism and infection models of *Chlamydia trachomatis*.

Typing of *Chlamydia trachomatis*

Typing of *C. trachomatis* is essential to improve understanding of the population structure and to gain further insight into the transmission of *C. trachomatis*, which helps to detect specific networks and populations at risk. Moreover, insight into the transmission of *C. trachomatis* and the populations at risk can help to improve screening and prevention campaigns aiming to reduce the number of infections [65-67].

Original strain typing of *C. trachomatis* was based on characterizing the major outer membrane protein (MOMP) using specific monoclonal antibodies to identify the serovar in an indirect micro immunofluorescence method, enzyme-immunoassay or radio-immunoassay [68-70]. With serotyping, 15 different serovars could be identified, serovars A to L [14,68-72]. A limitation of serotyping was that it could only be performed by propagation of *C. trachomatis* using cell culture, which is labour intensive, and that there was only a limited number of monoclonal antibodies available, resulting in low sensitivity and discriminatory power.

The development and rise of new molecular techniques, such as polymerase chain reaction (PCR) and restriction length polymorphism (RFLP), offered new and better opportunities for typing of *C. trachomatis* strains. The main advantage of these molecular techniques was that they could be applied on direct clinical samples without the need for an additional cell culture step. Molecular typing, also known as genotyping, of *C. trachomatis* is based on PCR amplification and subsequent RFLP or sequence analysis of the 1.2 kb long *ompA* gene encoding MOMP [73-79]. Genotyping of the *ompA* gene produces a genovar that is comparable with the letter-based nomenclature of traditional serotyping and the two techniques were demonstrated to have a high concordance [74,78,79]. Using *ompA* genotyping, 19 different genovars were identified which can be assigned to the *C. trachomatis* biovars [14,71,72]. Genovars A to C are responsible for the ocular infections from the trachoma biovar, genovars B and D to K are responsible for the sexually transmitted infections from the urogenital biovar whereas genovar L1 to L3 is responsible for the invasive LGV infections from the LGV biovar [14,71,72,80].
Performing typing of *C. trachomatis* strains based on only the *ompA*-gene is not recommended, as whole-genome-sequencing (WGS) data revealed that it is an unstable and unreliable target due to extensive recombination [81]. Numerous sets of WGS data have become available in the last couple of years for a variety of bacterial isolates including *C. trachomatis* [81]. Unfortunately, due to the complex intracellular lifecycle of *C. trachomatis* it remains technically challenging to perform WGS directly on clinical specimens. Until recently, WGS could only be performed on cultured samples that contain large and sufficient quantities of *C. trachomatis* genomes. However, cultivation of *C. trachomatis* is not successful for all strains resulting in potential biases. Several methods have been described to perform WGS directly on clinical isolates but the sensitivities of these methods still have to be improved to make them useful for large scale population studies [79,82-84].

Instead of WGS, several multilocus typing systems such as multilocus variable tandem repeat (VNTR) analysis (MLVA) and multilocus sequence typing (MLST) were successfully developed and validated for the genome of *C. trachomatis* to gain insight into the epidemiology and transmission of Chlamydia [85-92]. The MLVA typing method is based on the difference of the number of tandem repeats between strains and combines *ompA*-typing with analyses of three highly variable genomic targets CT1291, CT1299, and CT1335 [89-92]. MLVA-typing of *C. trachomatis* showed an increased discriminatory power compared to *ompA*-typing only [93].

In comparison to MLVA, MLST is based on the sequence variation in several genomic loci. Until now, three MLST schemes have been described for *C. trachomatis* [65,86-88]. Two of those are based on seven housekeeping genes which discriminate between the *Chlamydiaceae* on the level of the genus and can also discriminate between *C. trachomatis* strains that belong to the same genovar [86,88]. The third method is based on five highly polymorphic genetic regions of the *C. trachomatis* genome [87], which was later combined (by our group) with the *ompA*-gene and converted to a nested assay to allowed for testing on direct clinical specimens without the need for an additional cell culture step [65,85,87]. The discriminatory power of these multilocus typing methods is much higher compared to using *ompA* genotyping only [65,85-88,93].
Although both MLVA and MLST showed an increased and comparable discriminatory power compared to *ompA*-typing, MLVA is more error prone. MLVA is based on repeats in genomic regions, and one disadvantage of repeats in DNA is that it is often difficult to determine the actual number of repeats as a result of DNA polymerase errors during replication. This often results in ambiguities, which, in turn, can produce biases and errors when interpreting results. For *C. trachomatis* MLVA-typing, sequences of the MLVA genomic regions were also reported to be ambiguous, making the interpretation user dependent and error prone [85,93]. Since MLST is based on actual sequencing of genomic regions this offers a more consistent method which produces robust data. Moreover, an advantage of using multilocus typing schemes to type *C. trachomatis* strains is that it is a standardized and portable method, which can easily be used in different laboratories on a global basis [79,94-96].

**Tissue tropism of *Chlamydia trachomatis* infection**

Tissue tropism is associated with *C. trachomatis* biovars as *C. trachomatis* strains from each biovar infect specific anatomical locations, as described earlier. However, besides differences between the *C. trachomatis* biovars, serotyping and genotyping of *C. trachomatis* strains revealed nearly identical distributions among most populations world-wide with genovar D, E and F being most prevalent [97-99]. However, among MSM this distribution differs, as genovars D, G and J are predominantly observed [100-102]. Some studies also reported differences in serovar or genovar distributions among anatomical sites and suggested that genovar G infections were more commonly found in the ano-rectal tract because of tissue tropism [99,101,103,104].

Both MLST and MLVA-typing confirmed the existence of distinct strain distributions among MSM and heterosexuals [65,100,105]. However, in these population studies samples from MSM were primarily taken from the ano-rectal tract, whereas samples from heterosexuals were taken from the urogenital tract. Combined with previous reports on differences in serovar and genovar distributions among anatomical sites, this raised the hypothesis that the occurrence of these distinct distributions among MSM and heterosexuals might also be explained by tissue tropism among the urogenital biovar, causing different *C. trachomatis* strains to be preferentially associated with either the urogenital or ano-rectal tract.
Infection models to study *Chlamydia trachomatis* infections

Most of the research on host-pathogen interaction of *C. trachomatis* has been conducted using traditional epithelial cell lines to culture *C. trachomatis*. However, these cell culture based models do not fully reflect infections and their pathophysiology as occurring in native human tissue, since human tissue is more complex. Therefore, over the years several alternative infection models have been developed to mimic the infectious process as it occurs in the human host.

The most commonly used models to study host-interaction of *C. trachomatis* are animal models. Over the last decades, various animal models have been developed, including mouse [106-108], guinea pig [108-110], nonhuman primate [108,111,112], pig [108,113], rat [108,114], and rabbit [108,115] models. Besides studying host-pathogen interactions, these animal models also harbour a functional immune system making them essential for development and evaluation of potential vaccines.

The main disadvantage of animal models is that they do not fully represent the human host. Therefore, to gain more insight in to host-pathogen interactions, also some more advanced *in vitro* culture systems have been developed to mimic the human host. While these models do not harbour a functional immune system, they offer the possibility to study the effect of pathogens on the human tissue. One example of a more advanced *in vitro* culture system is the use of a microcarrier bead suspension culture system to grow polarized cell lines under three-dimensional (3D) conditions representing a more simplified human urogenital epithelial layer [116,117]. Besides only using cell lines, tissue engineering provides opportunities to culture 3D organotypic tissue models from human tissue. So far, various models have been constructed representing some of the anatomical locations that can be infected by *C. trachomatis*, including fallopian tubes [118-120], oral mucosa [121-124], and urethral tissue [125-127]. However, urethral models are mostly constructed of urothelium or oral mucosal cells instead of actual urethral mucosal cells which line the majority of the urethra [125]. Another model that recently has been developed is a 3D organoid culture from single epithelial stem cells which are stimulated to differentiate in organoids containing ciliated and secretory cells that mimic the polarization and folding of mucosal epithelium [128]. These organoids make fallopian tubes accessible to study host-pathogen interactions. Despite the fact that multiple advanced 3D *in vitro* organoids and organotypic models have become available, studies on *C. trachomatis* infections in these models are lacking.
Aim of this thesis

*Chlamydia trachomatis* is the commonest cause of bacterial STI worldwide [10]. Unfortunately, huge public health efforts, such as treatment, partner notification, and counselling, have not resulted in a reduction of the prevalence of *C. trachomatis* infections. These high prevalences of successful infections and their continuous transmission, in spite of implemented control strategies, are likely a combined result of *C. trachomatis* genetics, epidemiological and behavioural factors, diagnostics and (in)effective treatment, and host-pathogen interactions. To gain a better insight into the basics, epidemic and transmission of *C. trachomatis* infections, several studies were conducted and subdivided into four parts in this thesis.

Outline of this thesis

Part 1 of this thesis focusses on the genetics of *C. trachomatis* infections. Chapter 2 provides a comparison of two multilocus sequence typing (MLST) schemes that were used to compare the clustering of *C. trachomatis* strains derived from men who have sex with men (MSM) and heterosexuals. Chapter 3 provides an overall analysis of all *C. trachomatis* MLST sequence types (STs) that were deposited in the global hr-MLST database to examine STs with a global spread, and an evaluation of the phylogenetic capability of the MLST targets. Chapter 4 reports on the use of an *in silico* plasmid MLST scheme to investigate the clustering of *C. trachomatis* isolates in association with previously defined *ompA* biovars.

Part 2 of this thesis focusses on the molecular epidemiology of *Chlamydia trachomatis* infections. Chapter 5 describes the relationship of *C. trachomatis* strain types, defined by high-resolution MLST, with ethnicity and self-reported urogenital symptoms in a selected young urban screening population. Chapter 6 compares the distribution of *C. trachomatis* strains, defined by high-resolution MLST, found among women from a South African rural community, the Mopani district, and women from Amsterdam, the Netherlands.

Part 3 of this thesis focusses on diagnostics and effective treatment of *Chlamydia trachomatis* infections. Chapter 7 reports on the use of *C. trachomatis* cell culture to determine if a positive RNA- or DNA-based NAAT after treatment indicates the
presence of viable *C. trachomatis* bacteria. Chapter 8 reports on the positivity rate of urethral LGV among MSM and whether it indicates a contribution to the ongoing LGV epidemic.

Part 4 of this thesis focuses on *Chlamydia trachomatis* host-pathogen interaction. Chapter 9 describes whether the differences in strain distribution between MSM and heterosexuals are due to tissue tropism, which would cause different *C. trachomatis* sequence types, high-resolution MLST, to be preferentially associated with either the urogenital or ano-rectal tract. Chapter 10 reports on the development of a 3D in vitro reconstructed human urethra that can be used to study *C. trachomatis* infections in host tissue.

Finally, chapter 11 provides a general discussion in which the findings presented in this thesis, as well as future research possibilities are discussed with respect to recent literature.
General Introduction

References

Chapter 1


