Discussion and conclusions
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Anogenital *Chlamydia trachomatis* (Ct) infections are the most common bacterial sexually transmitted disease, with annually 92 million new infections worldwide of which approximately 60,000 infections in the Netherlands (12, 32). The majority of Ct infections remain asymptomatic in men (50%) and women (70%), contributing to further transmission and the development of late complications (38). Although men also have a risk for development of complications (i.e., prostatitis, epididymitis, possibly male infertility, and reactive arthritis), women are at higher risk for complications, due to an ascending cervical Ct infection that can lead to pelvic inflammatory disease, tubal adhesions, ectopic pregnancies, perihepatitis and infertility later in life (28). Also, it has been suggested that a Ct infection can contribute to the development of human papillomavirus (HPV) induced cervical cancer (1).

The species Ct can be divided in 3 serogroups (i.e., group B, C and intermediate) and 19 serovars (i.e., A, B, Ba, C, D, Da, E, F, G, Ga, H, I, Ia, J, K, L1, L2, L2a and L3), based on specific antibodies against epitopes on the major outer membrane protein (MOMP). In general, the serovars A–C are associated with eye infections, the serovars D–K with anogenital infections and the serovars L1–L3 with the disease lymphogranuloma venereum (LGV).

Early detection and treatment of (asymptomatic) Ct infections are pivotal in the prevention of Ct related complications. Nowadays, several Ct detection tests are commercially available that are highly sensitive, specific and easy to perform, giving perspective to the development of Ct screening programs (37). Nevertheless, those Ct detection tests can only detect a Ct infection, but cannot differentiate between the different Ct serovars.

Several (in-house) Ct genotyping techniques are available for differentiation between serovars, contributing to more information regarding serovar distribution, sexual networking and the relation of serovars with specific signs and symptoms (e.g., *OmpA* sequencing, Restriction Fragment Length Polymorphism or in house reverse line blots) (26). However, with the nowadays available testing platforms, large scale serovar specific screening programs are still time and cost consuming, due to technical limitations and difficulties regarding an adequate preselection of Ct positive samples. Since most serovar distribution studies were performed with different in-house sero/genotyping methods, non-homologue results are generated which are difficult to compare. Therefore, quick, easy to perform and commercially available Ct genotyping assays are required.

Urogenital Ct infections, independent of the serotype, are easily treatable infections. Until recently, serovar determination were considered as an
This discussion chapter consists of 3 parts. **Part 1** is a technical discussion in which two new Ct detection and Ct genotyping methods are evaluated. **Part 2** includes demographic data regarding the serovar distribution and evaluates the role of specific Ct serovars in relation with clinical signs and symptoms. In **Part 3**, the latest insights into the role of Ct in the development of cervical cancer are discussed.

**PART 1 (Chapter 2-5, 8, 11)**

**Ct detection methods**

Ct detection methods have become less laborious, more sensitive, more specific and cheaper over time since the sole availability of Ct culture. Currently, the reference methods for Ct detection are based on Nucleic Acid Amplification Tests (NAATs) due to their high sensitivity and specificity. Worldwide, the Aptima Combo 2 Ct assay® (Gen-Probe) and the COBAS TaqMan® and Amplicor® platforms (Roche Diagnostics) are the most frequently used detection assays. The COBAS TaqMan and Amplicor platforms are dual target assays, that simultaneously detect Ct genomic DNA and Ct plasmid DNA, while the Aptima Combo 2 assay is a Ct rRNA amplification assay. NAATs are more sensitive than DNA probe hybridization assays (e.g., PACE2 assay; Gen-Probe and Hybrid Capture 2; Qiagen), due to DNA amplification before probe detection.

Recently, we have developed a new Ct-DT detection (DEIA) assay (Labo Biomedical Products BV, Rijswijk, the Netherlands) (**Chapter 2**). Like the Cobas TaqMan and Amplicor platforms, the Ct DT DEIA uses a dual target PCR, making Ct detection not depending on a single target. This is an important feature, as single target assays are more vulnerable to genetic variants of Ct than the dual target assays, which can lead to false-negative test results. For instance, the Swedish O variant, with a 377 basepair deletion in the endogenous plasmid, have been led to under-diagnosis of Ct infections (30). Therefore, the COBAS TaqMan and Amplicor platforms have been upgraded to a dual target PCR, detecting both the endogenous plasmid and the chromosomal OmpA gene (31). In the Swedish variant, the Ct-DT assay can detect Ct DNA from the endogenous plasmid as well as the OmpA gene, because specific primers targeting the endogenous plasmid domain 2 are used, while the 377-bp deletion is observed in domain 1. Since unrecognized deletions/mutations may also occur in other regions as observed in the Swedish variant, we still have chosen a dual target detection assay, to minimize the chance of false-negative results with unknown variants.
The performance of the Ct-DT assay is compared to other assays, available in the market. The Ct-DT DEIA showed an excellent agreement with the COBAS TaqMan and was more sensitive than the HybriD Capture 2 assay (Chapter 2, 3), indicating that the Ct-DT assay is compatible with other second generation NAATs. The Ct-DT DEIA is also specific for Ct, since no cross-reaction is observed with other Chlamydia species and the common vaginal flora. Another advantage of the Ct-DT DEIA system, as compared to the other detection methods, is the possibility for Ct genotyping with the Ct-DT Reverse Hybridization Assay (RHA), directly following detection and using the same PCR products as the Ct-DT DEIA (Figure 1). Since only a single PCR is needed for both Ct detection and Ct genotyping, serovar determination becomes less time-consuming and laborious.

Figure 1 Algorithm of the Ct-DT Detection and genotyping (DT) assay. First DNA is isolated, followed by a PCR (Ct amplification step). The PCR products are tested for Ct positivity by the Ct-DT DEIA (Ct detection step). Finally, all Ct positive samples can be further genotyped with the Ct-DT RHA (Ct genotyping step), using the same PCR products as the Ct-DT DEIA.

Ct serovar identification methods
Several different methods have been described for Ct serovar determination, like serotyping by microimmunofluorescence or genotyping by OmpA sequencing, RFLP and reverse line blots (22, 26, 33, 44). Table 1 describes the key characteristics of these methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Target</th>
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<th>Comments</th>
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<td>OmpA</td>
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<td>Cross-reactivity due to genovariants</td>
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<tr>
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<td>OmpA</td>
<td>No</td>
<td>Detection of new OmpA genovariants</td>
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<tr>
<td>PCR+Probe hybridization</td>
<td>Genotyping</td>
<td>OmpA</td>
<td>Yes</td>
<td>Can not differentiate between genovariants</td>
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<tr>
<td>pmpH real-time PCR</td>
<td>Genotyping</td>
<td>pmpH</td>
<td>Partly*</td>
<td>Only LGV/non-LGV determination</td>
</tr>
<tr>
<td>PCR+MLST* sequencing</td>
<td>Genotyping</td>
<td>various</td>
<td>No</td>
<td>Subtyping serovars based on other genes</td>
</tr>
</tbody>
</table>

* This PCR can only detect mixed LGV/non-LGV infection but no double infections within the non-LGV or LGV group.

Until now, OmpA sequencing was the reference method for Ct serovar determination, due to high discriminating power that led to identification of new Ct genovariants. This high discriminating power makes OmpA sequencing highly appropriate for phylogenetic studies. Nevertheless, direct OmpA sequencing is not always available, is still a time-consuming method and will give a non interpretable result when multiple serovar infections are present (Chapter 4). Direct OmpA sequencing is therefore not appropriate for large epidemiological or clinical vaccine studies, in which large quantities of samples have to be tested and in which multiple serovar infections are important to recognize.

In the current thesis we evaluated two new commercially available Ct genotyping assays (i.e., the Ct-DT assay; Chapter 2, and the Ct-MS assay; Chapter 5) both based on the same broad spectrum multiplex PCR (Ct amplification step).
The two newly developed Ct genotyping assays can detect and differentiate the 14 major Ct serovar strains (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/ia, J, K, L1, L2/ L2a and L3). The genotyping step of the Ct-DT assay is a reverse hybridization assay (RHA) on a nitrocellulose strip, while the Ct-MS assay is a combined detection and genotyping assay based on a microsphere suspension RHA system using Luminex technology. The Ct-MS assay is a high-throughput system, because the combined detection and genotyping step saves time. This feature makes the Ct-MS assay an excellent method for mass Ct screening and serovar determination, although a Luminex platform needs to be available to perform the Ct-MS assay. The Ct-DT RHA can be performed manually, but is more time consuming. A preselection step of Ct positive samples with the relatively easy to perform and inexpensive Ct-DT DEIA was developed before applying the genotyping step with the Ct-DT RHA. This approach avoids unnecessary costs by spoiling RHA strips on samples which are negative for Ct, but takes more time compared to the Luminex based assay. At present, the only clinical indication for Ct genotyping is the differentiation between LGV and non-LGV Ct infections. Currently, some STI clinics use in-house developed real-time PCRs, targeting the pmpH gene for discrimination between LGV and non-LGV Ct infections. However, those assays are in-house tests and prescreening for Ct should be done with a commercially available standardized Ct detection method. For example, the STI clinic in Amsterdam, which detect LGV on a daily basis, has developed a specific algorithm for Ct LGV detection in rectal swabs from MSM (Figure 2). First, all rectal swabs were screened for Ct with the commercially available Aptima Combo 2 Ct assay. Secondly, all Ct-positive samples were further tested with an in-house pmpH real-time PCR, differentiating between LGV and non-LGV Ct infections (Chapter 4) (40). This testing algorithm for LGV infections was evaluated by using the Ct-DT RHA as a reference test (Chapter 8). The results showed that the algorithm used in the Amsterdam STI clinic is an effective and sensitive method to differentiate LGV infections from non-LGV Ct infections and double LGV/non-LGV Ct infections can be detected simultaneously. Although a good diagnosis is obtained with the Amsterdam STI clinic algorithm, the Ct-DT assay has some advantages. First, only a single PCR is needed for Ct detection with the Ct-DT DEIA and Ct genotyping with the Ct-DT RHA, enabling a faster diagnosis. Also, additional information for Ct monitoring and epidemiological purposes can be generated, since the Ct-DT RHA can identify all Ct serovars and not only discriminates between LGV and non-LGV serovars.

Although the Ct-DT RHA and Ct-MS assay are valuable in research settings as well as in clinical settings, both assays have limitations regarding the discriminatory power. Worldwide, Serovar E is by far the most prevalent serovar in urogenital samples obtained from heterosexuals, ranging between the 30 and 40% of the total serovar distribution. To reveal transmission patterns in sexual networks and to differentiate a persistent Ct infection from Ct re-infection, higher discriminatory levels are desirable than can be reached by serovar determination alone. For this reason, novel algorithms for Ct genotyping are under development to provide an optimal discriminatory capacity for Ct strains, by genotyping other parts of the Ct genome than the OmpA gene. The 3 new Ct genotyping methods that are now used in research settings are OmpA gene sequencing supplemented with three new variable number tandem repeats (VNTR), multilocus sequence typing on 7 housekeeping genes of Chlamydiaceae (MLST7) and multilocus sequence typing on the OmpA gene and the 5 most variable regions of the Chlamydial genome (MLST5) (15, 25, 27). The VNTR genotyping method is far more discriminative as OmpA sequencing alone, since the choice of 3 tandem repeats ensures a high degree of variation. The MLST7 is based on 7 housekeeping genes with minor variation. This makes MLST7 useful in analyzing evolutionary changes for the full Chlamydiaceae order rather than analyzing sexual networking for Ct. With the MLST5 typing method promising results have been obtained. This method allows differentiation between serovar E strains with identical OmpA, making it effective for sexual network studies.
All the three methods create the need for a new method-specific nomenclature for the Ct genovariants. It is of major importance to select the recommended MLST method that fits with the purpose of the study, since otherwise it is not possible to compare the study with previous studies all using different nomenclatures. At this moment, it would be recommended to use MLST5 for Ct networking studies. However, typing systems require easy interpretation and communication of data. This will be a challenge that needs to be resolved, since the nomenclature of all three methods is complex. Therefore more studies are needed and more research has to be performed before researchers will switch from OmpA genotyping to a new high resolution typing system and nomenclature.

PART 2 (Chapters 2, 3, 6-9)

Worldwide serovar distribution

Like mentioned above, serovar distribution studies might contribute to a better understanding of the natural course of a specific Ct serovar infection and might reveal associations with specific signs and symptoms. Worldwide, the three most common urogenital serovars are serovars E, F and D/Da, accounting for approximately 70% of the urogenital Ct infections in the general population (8, 14, 23, 29, 39). However, geographical serovar differences (2, 20, 35) and serovar differences between population groups (e.g., heterosexual vs. homosexual) are observed (3, 10, 18).

In this thesis, we determined the serovar distribution in samples collected from women in Uganda (Chapter 2), women in Costa Rica (Chapter 3), men and women in Russia (Chapter 6), men (partly MSM) and women with different ethnicities in The Netherlands (the Hague area; Chapter 7) and MSM in the Netherlands (Amsterdam area; Chapter 8). In Costa Rica, Russia and The Netherlands, serovar E is the most prevalent serovar, accounting for 30-40% of all Ct infections (Table 2). Remarkably, serovar G/Ga (47%) was the most prevalent serovar in Uganda. The observed difference in prevalence of serovar G/Ga can be explained by geographic factors or a selection bias due to a small selection of Ct positive samples (n=53) in a closed community of women all visiting the same health clinic. So we have to be careful with the conclusion that serovar G/Ga is most prevalent in Uganda. Since no extensive serovar distribution studies have been performed in Uganda, an expansion of the study will be necessary to confirm the observed serovar distribution in Uganda.

The study in Costa Rica represents the most stable serovar distribution study, since it consists a large homogenous sample group (n=806). The three most

<table>
<thead>
<tr>
<th>Country</th>
<th>N</th>
<th>Most prevalent serovar</th>
<th>2nd most prevalent serovar</th>
<th>3rd most prevalent serovar</th>
<th>% double-serovar infections</th>
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<tbody>
<tr>
<td>Uganda</td>
<td>53</td>
<td>G/Ga (47%)</td>
<td>E (25%)</td>
<td>K (13%)</td>
<td>4%</td>
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<tr>
<td>Costa Rica</td>
<td>806</td>
<td>E (30.7%)</td>
<td>F (21.3%)</td>
<td>D/Da (20.9%)</td>
<td>1.7%</td>
</tr>
<tr>
<td>Russia</td>
<td>181</td>
<td>E (33.7%)</td>
<td>G/Ga (24.9%)</td>
<td>J &amp; K (both 8.8%)</td>
<td>4.4%</td>
</tr>
<tr>
<td>The Hague (NL)</td>
<td>429</td>
<td>E (33.7%)</td>
<td>G/Ga (24.9%)</td>
<td>F (21.5%)</td>
<td>2.6%</td>
</tr>
<tr>
<td>Amsterdam (NL)</td>
<td>102</td>
<td>G/Ga (35.7%)</td>
<td>D/Da (22.5%)</td>
<td>J (14.7%)</td>
<td>1.0%</td>
</tr>
</tbody>
</table>
prevalent serovars in Costa Rica were serovars E, F and D/Da accounting for 73% of all serovars, which is in line with previous described distribution studies elsewhere in the world. The distribution study performed in The Hague (NL) revealed a slightly different serovar distribution (E, F and G/Ga accounting for 73%). The prominent third place for serovar G/Ga (14.6%) could be explained by the inclusion of rectal swabs from MSM and women (Chapter 7). Like observed in rectal samples from Amsterdam (Chapter 8), serovar G/Ga is more frequently observed in MSM.

As mentioned above, the rectal swabs from MSM (Chapter 8) have a completely different serovar distribution, which is dominated by the serovars G/Ga, D/Da and J (accounting for 72.4%). The differences in the serovar distribution between MSM and heterosexual males and females are most likely caused by sexual networking, but tissue tropism for the proctum might be an alternative explanation. For example, rectal swabs from heterosexual women (Chapter 7) showed a trend towards significantly more serovar G/Ga infections, as compared to urogenital samples from heterosexual women. Also, a recent study observed an association between polymorphisms of open reading frames within serovar G and rectal tropism (13). Still, more research has to be performed to confirm these hypotheses.

Multiple Ct serovar infections

Overall, the percentage of multiple serovar infections varied between 1-4% with the Ct-DT assay (Table 2). The observed prevalence of double infections is lower as observed in previous studies, performed in Columbia and Australia, where percentage ranging between the 8.7% and 13% were observed (21, 42). Nevertheless, the majority of double serovar infections in those previous studies were double infections that belong to the same serogroup (i.e., Columbia 4 out of 7 double infections and Australia 9 out of 11 double infections). Serovars that belong to the same serogroup have only a few nucleotide differences in the amplified part of the OmpA gene. Also, new genovariants of serovars, not described in GenBank, might mimic a double serovar infection, due to cross-reaction with other probes or overlapping RFLP patterns. Therefore, it is possible that the higher prevalences of double infections, as observed in previous studies, are caused by new genovariants or unspecified in-house genotyping methods. For this reason, the manual of the Ct-DT assay recommends to sequence all double serovar infections belonging to the same serogroup, to exclude the presence of new genovariants that cross-reacts with other probes. For example, sequence analysis revealed that the double infections with serovars H and K (both serogroup C), detected in Uganda, in fact represents a new genovariant of serovar K that cross-reacts with the probe for serovar H (Chapter 2). These "double infections" with serovar H and K were also observed in studies performed in Columbia and Australia and interpreted as double serovar infection. With the current knowledge, those double serovar infections are highly suspected for the new genovariant of serovar K. In the studies performed with the Ct-DT RHA, the majority of double serovar infections belong to different serogroups. It is not necessary to sequence double serovar infections that belong to different serogroups. Those double infections are directly confirmed by the more conservative serogroup probes, which are also included on the Ct-DT RHA strip.

We also determined the double serovar infections in rectal swabs obtained from MSM diagnosed with LGV Ct infections or non-LGV Ct infections.Remarkably, double serovar infections are less frequently detected in MSM infected with a non-LGV Ct type (1%), compared to the general population (2-4%). Whether this observation is due to the sample site (rectal) or due to a specific population (MSM) is unknown. However, co-infections with an urogenital (D-K) serovars were observed in 6.1% of the MSM infected with a LGV strain (majority HIV+) (Chapter 8). The increased incidence of double serovar infections in LGV positive MSM seems to be related to a more risky behavior in the MSM group infected with LGV strains, since in this specific patient group more co-infections with other STDs are observed as in MSM infected with anogenital serovar infections. However, we cannot fully exclude that the increased incidence of double serovar infections in the LGV group is caused by a suppressed immunity, due to more HIV infections among LGV positive patients. Therefore, it would be interesting to investigate rectal swabs from LGV infected HIV negative MSM, to observe whether those men also have more serovar D-K co-infections. Anyhow, selection of such a study population will be very difficult.

The association between Ct serogroups and clinical parameters

Previous studies revealed associations between specific serovars and clinical symptoms (8-9). It might be very difficult to determine an association between serovars and symptoms, since symptoms are a subjective measurement depending from person to person. This makes it extremely difficult to repeat and compare previous studies. For this reason, a more objective parameter was measured in patients infected with different Ct serovars, namely Ct IgG antibodies in the serum (Chapter 9). We observed a significantly higher Ct antibody response among patients with a serogroup B Ct infection. This result is partly in line with a previous result, suggesting that serovar E (serogroup B) induces a higher serological response (24). Also an increased serological
response for serovar D (serogroup B) was observed in an Indian population (36). Although this association is now observed in several studies, the etiology of the increased antibody response remains unclear. A possible explanation could be a more invasive behavior of serovars belonging to serogroup B, like the higher serological response as observed in patients infected with the invasive LGV serovars (41). Further research, also taking into account the patient's immune system, is warranted to confirm this possible explanation.

PART 3 (Chapters 10, 11)

The cofactor role of Chlamydia trachomatis in the development of cervical cancer

Infections with high-risk human papillomaviruses (HPV) are necessary for the development of cervical squamous cell carcinoma (SCC). Nevertheless, the majority of women will get a HPV infection during life without developing (pre)cancerous high-grade lesions (CIN2+). Cofactors (e.g., smoking, oral contraceptive use, and other infectious agents) can increase the risk for the development of malignant lesions. Previous studies observed that women with Ct antibodies in the serum have an increased risk for the development of cervical SCC (1, 16), which led to the common thought that Ct infections are a cofactor for the development of cervical cancer.

In the current thesis (Chapter 10), the supposed cofactor role of Ct in the development of cervical cancer is further investigated. We observed that the Ct status at enrollment was a risk factor for current and subsequent carcinogenic HPV infection. It is therefore possible that Ct is associated with cervical cancer because it is associated with HPV acquisition (either by a noncausal link through common risk factors such as sexual behavior, or oral contraceptive use), making Ct a marker for a carcinogenic HPV infection (Figure 3A). This observation is supported by the result that HPV negative/Ct DNA positive women at baseline are at increased risk for the incidence development of premalignancy during follow-up, possibly caused by a high-risk HPV infection required during follow-up.

However, we do not think that Ct infections affect HPV persistence and progression to cervical premalignancy, since we did not observe an association between Ct and the development of CIN2+ among carcinogenic HPV positive women (Figure 3B). Our results are in contrast with several previous studies, which have suggested that Ct is a cofactor for the development of cervical cancer. The majority of studies adjust for an HPV infection in which residual confounding cannot be ruled out because the serologic methods used to measure the HPV status were of limited sensitivity (serology) and/or DNA analyses include a small spectrum of carcinogenic HPV types. A recent study evaluated the use of polyclonal ELISA HPV serology as a biomarker for HPV exposure. It concludes that HPV serology can lead to misclassification when used as a biomarker for HPV exposure and can have major implications for studies investigating cofactors that possibly influence progression of HPV-infected cells to precancer and cancer (6).

Based on the current study design, we cannot exclude that Ct acts as a cofactor in invasion from cervical precancer to invasive cancer, since the sample size of CIN3+ is not sufficient (Figure 3C). However, when we restricted our analysis in HPV positive women to CIN3+ or even invasive cancers alone, no association with Ct DNA and antibodies is observed.

Previous studies only observed an association between Ct antibodies and SCC of the cervix while no association is observed for cervical adenocarcinomas (19). Since a Ct infection shows tropism for endocervical columnar cells over ectocervical squamous cells, an association between Ct antibodies and adenocarcinoma seems more likely than an association between Ct antibodies and SCC, because the cervical inflammation is located in the columnar cells from the endocervix (34). In Chapter 11, we investigate whether Ct DNA was detected in adenocarcinoma of the cervix. Since we have no serum available for Ct antibody determination, the observation remains restricted to Ct DNA in cervical adenocarcinomas. However, no Ct DNA was observed in those tumors, confirming the results of a previous study investigating the association between Ct DNA and adenocarcinoma of the cervix (43).
References:


32. RIVM 2008, posting date. Urogenitale Chlamydia trachomatis en lymfogranuloma venereum. RIVM. [Online.]