Comparison of three genotyping methods to identify Chlamydia trachomatis genotypes in positive men and women

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Comparison of three genotyping assays to identify *Chlamydia trachomatis* genotypes in positive men and women.

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Keywords: *Chlamydia trachomatis*, genotyping, reverse hybridization, Ct sequencing,
lymphogranuloma venereum, LGV
Abstract

Chlamydia trachomatis (Ct) comprises 3 serogroups and 19 serovars. Different genotyping methods are available to differentiate between the serovars. The aim of this study was to evaluate the sensitivity and discriminatory power of three genotyping methods, respectively Omp1 sequencing, the Ct-Detection and genoTyping (DT) assay and the pmpH PCR discriminating a LGV infection from a non-LGV infection. In total, 50 Aptima Combo 2 (AC2) Ct positive samples were selected and tested with the 3 genotyping methods. The Ct-DT assay detected 3 double Ct infections that caused a non interpretable result by Omp1 sequencing, while Omp1 sequencing has a higher discriminatory power that gave additional information about Ct genovariants. All three methods detected the 6 LGV samples. Although the pmpH PCR detected all LGV infections, a substantial amount (24%) of non-LGV infections were missed. The sensitivity compared to AC2 Ct detection was 0.8 (95% CI 0.67-0.89) for the Ct-DT assay, 0.72 (95% CI 0.58-0.83) for Omp1 sequencing and 0.64 (95% CI 0.50-0.76) for the pmpH PCR. In conclusion, the Ct-DT assay is appropriate for serovar distribution studies, epidemiological studies and differentiation between a LGV and non-LGV Ct infection, while Omp1 sequencing is more appropriate for phylogenetic studies. The pmpH PCR is suitable as second assay to differentiate for a LGV infection, but not as primary detection assay, due to its low sensitivity for non-LGV strains.

Introduction

Chlamydia trachomatis (Ct) is the most common bacterial sexually transmitted infection (STI) in men and women worldwide. A Ct infection can cause urethritis, cervicitis, proctitis and conjunctivitis depending on the anatomic site of infection. In approximately 50% of the men and 70% of the women a urogenital Ct infection remains asymptomatic (1-4). When a Ct infection remains untreated, severe complications like epididymitis and pelvic inflammatory disease may occur, leading to infertility in men and women (5,6). Several Ct detection methods are commercially available, providing information about the Ct status, but not on the Ct serovar type of infection.

Ct comprises 3 serogroups (serogroup B, C and Intermediate) and 19 serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2/L2a and L3), based on immunotyping of the Major Outer Membrane Protein (MOMP) epitopes. Besides the defined serovar, genovariants have been described including Ja and L2b (7,8).
The majority of serovars A, B and C are detected in conjunctival samples of patients in developing countries, while serovars D-K are mostly found in the urogenital tract and proctum and remain confined to the mucosal layer. The serovars L1, L2 and L3 in contrast invade the submucosal connective tissue layers and disseminate to locoregional lymph nodes causing lymphogranuloma venereum (LGV). In the developed countries, LGV Ct serovars are mostly detected in HIV-positive men who have sex with men (MSM). Because LGV Ct infections require longer antibiotic treatment, it is highly recommended to differentiate them from non-LGV serovars (9).

A number of reverse line blot assays were developed making genotyping faster and less laborious, compared to sequencing (10-12). The Ct-Detection genoTyping (DT) assay consists of a Ct amplification step (PCR), a Ct Detection step (DNA Enzyme Immuno Assay; DEIA) and a Ct genotyping step (Reverse Hybridization Assay; RHA). This assay is an alternative for Omp1 sequencing by differentiating between the 14 major serovars (12,13).

Besides genotyping of the Omp1 gene, a new real time PCR based on the pmpH gene (pmpH PCR) can differentiate between an LGV Ct infection and a non-LGV Ct infection (13). This assay is used routinely as a second assay after Ct screening with the Aptima Combo 2 Ct–RNA TMA assay (GEN-PROBE, San Diego, USA) in rectal swabs from high risk MSM visiting the Center for Public Health in Amsterdam. In this study, the sensitivity and discriminatory power of the Ct-DT assay and the pmpH PCR, were evaluated by a comparison with Omp1 sequencing.

**Materials and Methods**

**Clinical Specimen selection**

Fifty Aptima Combo 2Ct–RNA TMA assay (AC2) Ct positive samples were selected from STI outpatient PHS clinic visitors between 2007 and 2009. The Aptima Combo 2 was considered as reference Ct detection test and performed according to the manufacturer’s instruction. The 50 samples consisted of three urethral swabs, four first void urine samples, fourteen cervical swabs, four vaginal swabs and twenty-five rectal swabs. The rectal swabs were collected from MSM suspected for an LGV infection and from heterosexual women.

**DNA isolation**
The isolation of the DNA was performed in duplicate at the PHS in 2009. DNA was isolated from 200 μl transport medium (GEN-PROBE, San Diego, USA) by adding 500 μl lysisbuffer (bioMérieux, Boxtel, the Netherlands), 1 μl glycogen (20mg/ml, Roche Diagnostics, Almere, the Netherlands) and 700 μl isopropanol (-20°C). The precipitate was washed twice with 70% ethanol and subsequently dissolved in 50 μl 10mM Tris buffer (pH 8.0).

Ct Omp1 sequencing

The DNA isolates were amplified by a nested Omp1 PCR, using a C1000 PCR machine (Bio-Rad, Veenendaal, the Netherlands). The outer PCR was performed in a volume of 25 μl, containing 2 μl of isolated DNA, 0.63 U GoTaq polymerase (Promega, Leiden, the Netherlands), 2mM MgCl₂, 25 μM of each dNTP, 0.11 μM of the primer ompA OF (Table 1) and 0.13 μM of ompA OR, resulting in a PCR fragment of 1182 to 1194 bp, comprising the full Omp1 gene. The inner PCR was also performed in a volume of 25 μl, containing the same quantities of polymerase, MgCl₂ and dNTPs as the outer assay, but with 2 μl of the outer amplicon, 0.13 μM of the primers ompA NF and OMP6AS, resulting in a PCR fragment of 615 to 624 bp fragment, comprising the variable domain 1 and 2 of the Omp1 gene. Cycling conditions were: an initial step at 94°C for 3 minutes, followed by 35 cycles for the outer PCR and 30 cycles for the inner PCR and a final step at 72°C for 5 minutes. The cycles consist of 30 seconds at 93°C, 30 seconds at 57°C and 1 minute at 72°C. The amplified DNA was precipitated with 96% ethanol and sequenced in both directions with ABI BigDye Terminator v1.1 kit (Applied Biosystems, Nieuwerkerk a.d.IJssel, the Netherlands), using the primers from the inner PCR. Finally the labelled DNA was purified using a DyeEx spin kit (Qiagen) and analysed in an ABI 3130 genetic analyser (Applied Biosystems).

Ct-DT amplification, detection and genotyping

The Ct amplification (Broad spectrum-Multiplex-PCR), DEIA and RHA of the Ct-DT assay were performed according to the manufacturer’s instructions (Labo Biomedical Products BV, Rijswijk, The Netherlands) and as described previously (12,14). Briefly, amplification was performed with the Ct-DT-PCR, followed by Ct detection with the Ct-DT-DEIA. All Ct positive samples were further genotyped with the Ct-DT-RHA.

Ct-DT-PCR: A 10 μl aliquot of extracted DNA was used for each PCR reaction. The Ct PCR primer set was used to amplify all known serovars available in GenBank. Briefly, this multiplex primer set amplifies a small fragment of 89 base pairs from the endogenous plasmid and a fragment of 160/157 base pairs from the Variable Region 2 of the Omp1 gene. The
standard PCR program involves a 9-minute preheating step at 94°C, followed by 40 cycles of amplification (30 seconds at 94°C, 45 seconds at 55°C and 45 seconds at 72°C) and a final 5-minute elongation at 72°C.

**Ct-** DT-DEIA: The DEIA provides an optical density (OD) value at 450 nm. Each DEIA run contained separate titrated positive, borderline positive, and negative controls and a PCR-positive control containing isolated DNA from a cell culture of serovar E. Samples yielding OD values equal to or higher than the borderline were considered positive. The borderline positive samples are Ct positive samples that contained the lowest amount of Ct amplicon detectable with the Ct-DT assay. The OD value of the borderline range depends on the titrated borderline internal control and differs for every single run.

**Ct-DT-RHA:** All Ct-DT-DEIA positive samples were further genotyped with the Ct-DT-RHA, which contained probes for the endogenous plasmid, the Ct serogroups (B, C, and I) and the 14 serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2/L2a, and L3). One extra probe was added to detect a genovariant of serovar J that otherwise remains undetected.

**pmpH** real-time PCR

The selected samples were tested for LGV and non-LGV specific DNA with a real-time PCR adapted from Chen et al (15). The real-time PCR was performed in 20 μl, containing Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Breda, the Netherlands), 2 μl of isolated DNA, 4.3 mM MgCl₂, 0.40 μM of primer F3 LGV, 0.39 μM of primer F4 nonLGV and 0.92 μM of primer R2 LGV/nonLGV, 0.15 μM of probe LGVtotP and 0.21 μM of probe P4 nonLGV (Table 1). Cycling conditions for the real-time PCR were: uracil DNA glycosylase step at 50°C for 2 minutes and denaturation at 95°C for 2 minutes, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. All tests were performed on a Rotor-Gene 6000 (Qiagen, Venlo, the Netherlands).

**Statistical analyses**

The statistical analyses were performed using Graphpad software, calculating the sensitivity of the three genotyping methods. Differences between the genotyping methods were calculated with a McNemars test and Bonferroni correction was performed.

**Results**
Fourteen of the 50 samples were Omp1 negative by the nested PCR and could not be sequenced. From 36 samples the complete Omp1 gene was sequenced and analyzed (Table 2), containing 11 serovars E, 6 serovars L2, 6 serovars G, 5 serovars F, 3 serovars D, 3 serovars J, 1 serovar H and 1 serovar K. All L2 samples were detected in MSM rectal swabs. Omp1 revealed some extra information about the subserovars and genovariants. For example, all 6 L2 serovars consisted of the L2b variant and all serovars J were identified as serovar Ja. The serovars D included one genovariant identical to GenBank sequence X62920 and two genovariants identical to AF279587. The serovars G contained 2 genovariants identical to DQ287919 and 4 genovariants identical to AF063199. The sensitivity compared with the AC2 Ct detection was 0.72 (95% CI 0.58-0.83).

Ct-DT-RHA

42 of the 50 samples were Ct positive with the Ct-DT-DEIA and could be used for genotyping. No Ct DNA was amplified in the remaining 8 samples. All 8 samples were also negative by Omp1 sequencing. For 40 samples genotyping with the Ct-DT-RHA was possible, because two samples contained only endogenous plasmid DNA (Table 2). Three double infections (6%) were observed, containing the serovars E&G, F&K and J&E. One of the double infections was detected in an anal swab from a woman, one in a male’s first void urine sample and one in a cervical swab. By Omp1 sequencing, one double infection was determined as a single infection (serovar K) and 2 double infections were negative. In total 6 discrepant samples between the Ct-DT-RHA and Omp1 sequencing were observed. One sample was determined as serovar G by Omp1 sequencing, but only endogenous plasmid positive by the Ct-DT-RHA and 5 samples were positive with the Ct-DT-RHA assay, but negative by Omp1 sequencing (2 serovar D, 1 Serovar E, 1 serovars E&G and 1 serovars J&E). Overall a very good agreement of 0.875 (0.794 to 0.956) between both methods was observed (Table 3). The sensitivity of for genotyping with the Ct-DT-RHA assay compared with the AC2 Ct detection was 0.80 (95% CI 0.67-0.89; table 4).

pmpH real-time PCR

The pmpH PCR had a positive result in 32 of the 50 samples with 6 LGV and 26 non-LGV serovars (Table 4). Eight of the 18 negative pmpH PCR samples were determined positive with Omp1 sequencing and the Ct-DT-RHA, while another 8 pmpH PCR negative samples
were also negative with the other two methods. The remaining 2 *pmpH* PCR negative samples were determined positive with the Ct-DT-RHA for the endogenous plasmid. One double infection (J&E) was determined as Ct negative by the *pmpH* PCR. A sensitivity of 0.64 (95% CI 0.50-0.76) was observed comparing the *pmpH* PCR with the AC2 test. Significant more non-LGV samples were detected with the Ct-DT-RHA compared to the *pmpH* PCR (McNemar’s p=0.0133).
Discussion

The aim of this study was to evaluate the sensitivity and discriminatory power of three different genotyping methods. In this study, we showed that the Ct-DT-RHA is a rapid and simple alternative for Omp1 sequencing and is suitable for different clinical materials (first void urine, rectal swabs and urogenital swabs). The Ct-DT-RHA has the possibility to detect multiple serovars in clinical samples. Multiple infections will cause sequencing difficulties leading to a non-interpretable Omp1 sequence. In other studies 4-12% of the Ct infections contained multiple serovars (11,16), making the Ct-DT-RHA more suitable for serovar distribution studies and future Ct vaccine studies compared to Omp1 sequencing. All LGV strains detected by Omp1 sequencing and the pmpH PCR were recognized by the Ct-DT-RHA, indicating that the Ct-DT assay can also be used for LGV serovar detection.

The Ct positive samples were selected based on the AC2 test results. The AC2 test platform is considered the most sensitive RNA detection system (17). Eight samples were negative with all three genotyping methods possibly caused by a low Ct DNA load. The 8 samples were analyzed with another Ct detection method (COBAS TaqMan, Roche Molecular Systems, Branchburg, NJ). Seven samples were also negative with the COBAS TaqMan, implicating degradation of DNA due to storage, false positivity of AC2 test or an increased sensitivity of the AC2 test. The 2 Ct endogenous plasmid positive samples that could not be genotyped were also examples of a low DNA load (18,19).

While the Ct-DT-RHA is a rapid alternative in serovar distribution studies, the Ct Omp1 sequencing system has a higher discriminating power, because Ct Omp1 sequencing recognised most point mutations that were missed with the Ct-DT-RHA. This makes Omp1 sequencing more useful in networking studies and phylogenetical Ct studies.

The pmpH PCR detected all six LGV strains found with Omp1 sequencing. Nevertheless, the pmpH PCR has a low sensitivity for Ct typing of urogenital Ct strains compared with the Ct-DT-RHA and Omp1 sequencing. The low sensitivity may be the result of a less sensitive non-LGV PCR, sequence differences in the probe and or primer region relative to the circulating patient strains, a low bacterial load, and a 10-fold lower input in the PCR relative to the AC2 test. Because the highly sensitive AC2 test is used as first Ct detection method (before performance of the pmpH PCR) and the pmpH PCR detected all LGV variants, this algorithm can be used for differentiating between a urogenital Ct strain and a LGV Ct strain.

The LGV serovar samples were found in a population (MSM) that has a very high risk profile for other STI’s and multiple Ct infections that might be missed by Omp1 sequencing and the pmpH PCR. Possibly due to the small sample size, no double infections were observed in the
6 LGV positive samples with the Ct-DT-RHA. Other studies, containing larger sample
numbers of anal Ct infections in MSM, are needed to investigate the prevalence of multiple
infections among LGV positive MSM.

In conclusion, the Ct-DT-RHA is the most sensitive genotyping method, compared with
Omp1 sequencing and the pmpH PCR, making the Ct-DT-RHA appropriate for serovar
distribution studies, but also for differentiating between a LGV and Non-LGV infections.

Omp1 sequencing will determine additional information about point mutations in the Omp1
gene, while a multiple Ct infections can lead to a non interpretable sequence result. The new
pmpH PCR is suitable as second assay to detect LGV infections, but not as primary detection
assay, due to its low sensitivity for non-LGV strains.

Acknowledgements

We thank Dr. J. Lindeman for his contribution and the production of the Ct-DT assay.
The aims of the current study are in line with the aims within the European Sixth Framework
Programme through the EpiGenChlamydia Consortium (contract no. LSHG-CT-2007-
037637). See www.EpiGenChlamydia.eu
References


Table 1. Primers and probes used in the real-time PCR and nested *Omp1* PCR

<table>
<thead>
<tr>
<th>nested <em>Omp1</em> PCR</th>
<th>ompA OF ATGAAAAAACTCTTGAAATCGGT</th>
<th>ompA OR TTAGAAGCGGAATTGTGCAT</th>
<th>ompA NF CGCTTTGAGTTCTGCTTCTC</th>
<th>OMP6AS TGAGCGTATTGGAAAGAAGC</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><em>pmpH</em> real-time PCR</th>
<th>F3 LGV CACTGTGCAACCTCATCAT</th>
<th>F4 nonLGV CTATTGTGCCACATCGACTC</th>
<th>R2 LGV/nonLGV GACCCTTCCGAGCATCA</th>
<th>LGVtotP [6-FAM]-CTTGCTCAAAGT-[MGB]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4 nonLGV</td>
<td>[ROX]-AAAGAGCTTGGAAGCAGGAGCAGGAGC-[BHQ2]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Comparison of serovar distribution between Ct-DT-RHA and *Omp1* sequencing.

<table>
<thead>
<tr>
<th></th>
<th>N=50</th>
<th>Ct-DT Genotyping</th>
<th>Omp1 Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serogroup B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serovar D</td>
<td>5 (10%)</td>
<td>3 (6%)</td>
<td></td>
</tr>
<tr>
<td>Serovar E</td>
<td>12 (24%)</td>
<td>11 (22%)</td>
<td></td>
</tr>
<tr>
<td>Serovar L2</td>
<td>6 (12%)</td>
<td>6 (12%)</td>
<td></td>
</tr>
<tr>
<td>Serogroup C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serovar H</td>
<td>1 (2%)</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>Serovar J</td>
<td>3 (6%)</td>
<td>3 (6%)</td>
<td></td>
</tr>
<tr>
<td>Serovar K</td>
<td>0</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>Serogroup I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serovar F</td>
<td>5 (10%)</td>
<td>5 (10%)</td>
<td></td>
</tr>
<tr>
<td>Serovar G</td>
<td>5 (10%)</td>
<td>6 (12%)</td>
<td></td>
</tr>
<tr>
<td><strong>Double infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serovar E&amp;G</td>
<td>1 (2%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Serovar F&amp;K</td>
<td>1 (2%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Serovar J&amp;E</td>
<td>1 (2%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Not determined</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td>2 (4%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8 (16%)</td>
<td>14 (28%)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50 (100%)</td>
<td>50 (100%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Serovar specific agreement between Ct-DT-RHA and *Omp1* sequencing (n\(_{\text{samples}}=50\))

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Ct-DT-RHA and Omp1 Sequencing</th>
<th>Additional Ct-DT-RHA positive</th>
<th>Additional Omp1 Sequencing positive</th>
<th>Agreement (95% CI)</th>
<th>p-value ( ^# )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serogroup B</td>
<td>Serovar D</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0.727 (0.357 to 1.000)</td>
<td>0.4795</td>
</tr>
<tr>
<td></td>
<td>Serovar E</td>
<td>11</td>
<td>3</td>
<td>0</td>
<td>0.841 (0.666 to 1.000)</td>
<td>0.2482</td>
</tr>
<tr>
<td></td>
<td>Serovar L2</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Serogroup C</td>
<td>Serovar H</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Serovar J</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0.847 (0.549 to 1.000)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Serovar K</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Serogroup I</td>
<td>Serovar F</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0.898 (0.700 to 1.000)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>Serovar G</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0.811 (0.553 to 1.000)</td>
<td>1.000</td>
</tr>
<tr>
<td>Any serovar positive</td>
<td></td>
<td>35</td>
<td>8</td>
<td>1</td>
<td>0.875 (0.794 to 0.956)</td>
<td>0.045</td>
</tr>
</tbody>
</table>

\( ^\# \) McNemars test
Table 4. Comparison of the Ct-DT-RHA and the \textit{pmpH} PCR

<table>
<thead>
<tr>
<th></th>
<th>LGV</th>
<th>Non-LGV</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT-RHA lgV</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>DT-RHA non-lgV</td>
<td>0</td>
<td>26</td>
<td>8</td>
<td>34</td>
</tr>
<tr>
<td>DT-RHA not typable</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>26</td>
<td>18</td>
<td>50</td>
</tr>
</tbody>
</table>

McNemars p = 0.0133