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DOI

[10.1016/j.mcp.2010.04.007](https://doi.org/10.1016/j.mcp.2010.04.007)

Publication date

2010

Document Version

Submitted manuscript

Published in

Molecular and Cellular Probes

[Link to publication](#)

Citation for published version (APA):

Quint, K. D., Bom, R. J. M., Bruisten, S. M., van Doorn, L. J., Nassir Hajipour, N., Melchers, W. J. G., de Vries, H. J. C., Morre, S. A., & Quint, W. G. V. (2010). Comparison of three genotyping methods to identify *Chlamydia trachomatis* genotypes in positive men and women. *Molecular and Cellular Probes*, 24(5), 266-270.
<https://doi.org/10.1016/j.mcp.2010.04.007>

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Elsevier Editorial System(tm) for Molecular & Cellular Probes
Manuscript Draft

Manuscript Number:

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

Article Type: Original Research Paper

Keywords: Chlamydia trachomatis, genotyping, reverse hybridization, Omp1 sequencing, lymphogranuloma venereum, LGV

Corresponding Author: Drs Koen Dirk Quint,

Corresponding Author's Institution:

First Author: Koen D Quint, MD

Order of Authors: Koen D Quint, MD; Reinier J Bom, MSc; Sylvia Bruisten; Leen-Jan van Doorn; Nadia Nassir Hajipour; Willem Melchers; Henry de Vries, MD, PhD; Servaas Morre, PhD; Wim Quint, PhD

Suggested Reviewers: Berrit Anderson
ba@alm.au.dk

Lars Ostergaard
oes@sks.aaa.dk

Deborah Dean
ddean@chori.com

Cathy Ison
catherine.ison@hpa.org.uk

Scott Lamontagne
scott.lamontagne@gmail.com

Opposed Reviewers:

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

**K.D. Quint^{1*}, R.J.M. Bom^{2*}, S.M. Bruisten², L.J. van Doorn¹, N. Nassir Hajipour²,
W.J.G. Melchers³, H.J.C. de Vries^{4,5}, S.A. Morre⁶, and W.G.V. Quint¹**

***Both authors contributed equally to this manuscript**
DDL Diagnostics laboratory¹, Voorburg, the Netherlands; Public Health Laboratory², GGD Amsterdam, Amsterdam, The Netherlands; the Department of Medical Microbiology³, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; Department of Dermatology and Centre for Immunity and Infectious Diseases Amsterdam (CINIMA)⁴, Academic Medical Centre, University of Amsterdam, the Netherlands; STI Outpatient Clinic⁵, Public Health Service, Amsterdam, the Netherlands; the Department of Pathology, Laboratory of Immunogenetics⁶, VU University Medical Centre, Amsterdam, The Netherlands;

Corresponding author:
K.D. Quint, M.D.
Fonteynenburghlaan 7
2275 CX Voorburg
The Netherlands
Tel:+31(0)703401670
Fax:+31(0)703401671
Email: k.d.quint@gmail.com

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).

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70 The majority of serovars A, B and C are detected in conjunctival samples of patients in
 71 developing countries, while serovars D-K are mostly found in the urogenital tract and
 72 proctum and remain confined to the mucosal layer. The serovars L1, L2 and L3 in contrast
 73 invade the submucosal connective tissue layers and disseminate to locoregional lymph nodes
 74 causing lymphogranuloma venereum (LGV). In the developed countries, LGV Ct serovars are
 75 mostly detected in HIV-positive men who have sex with men (MSM). Because LGV Ct
 76 infections require longer antibiotic treatment, it is highly recommended to differentiate them
 77 from non-LGV serovars (9).

78 A number of reverse line blot assays were developed making genotyping faster and less
 79 laborious, compared to sequencing (10-12). The Ct-Detection genoTyping (DT) assay
 80 consists of a Ct amplification step (PCR), a Ct Detection step (DNA Enzyme Immuno Assay;
 81 DEIA) and a Ct genotyping step (Reverse Hybridization Assay; RHA). This assay is an
 82 alternative for *Omp1* sequencing by differentiating between the 14 major serovars (12,13).
 83 Besides genotyping of the *Omp1* gene, a new real time PCR based on the *pmpH* gene (*pmpH*
 84 PCR) can differentiate between an LGV Ct infection and a non-LGV Ct infection (13). This
 85 assay is used routinely as a second assay after Ct screening with the Aptima Combo 2 Ct–
 86 RNA TMA assay (GEN-PROBE, San Diego, USA) in rectal swabs from high risk MSM
 87 visiting the Center for Public Health in Amsterdam. In this study, the sensitivity and
 88 discriminatory power of the Ct-DT assay and the *pmpH* PCR, were evaluated by a
 89 comparison with *Omp1* sequencing.

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92

93 **Materials and Methods**

94 *Clinical Specimen selection*

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

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102 *DNA isolation*

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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

171 *Omp1* sequencing

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

183 *Ct-DT-RHA*

184 42 of the 50 samples were Ct positive with the Ct-DT-DEIA and could be used for
 185 genotyping. No Ct DNA was amplified in the remaining 8 samples. All 8 samples were also
 186 negative by *Omp1* sequencing. For 40 samples genotyping with the Ct-DT-RHA was
 187 possible, because two samples contained only endogenous plasmid DNA (Table 2). Three
 188 double infections (6%) were observed, containing the serovars E&G, F&K and J&E. One of
 189 the double infections was detected in an anal swab from a woman, one in a male's first void
 190 urine sample and one in a cervical swab. By *Omp1* sequencing, one double infection was
 191 determined as a single infection (serovar K) and 2 double infections were negative. In total 6
 192 discrepant samples between the Ct-DT-RHA and *Omp1* sequencing were observed. One
 193 sample was determined as serovar G by *Omp1* sequencing, but only endogenous plasmid
 194 positive by the Ct-DT-RHA and 5 samples were positive with the Ct-DT-RHA assay, but
 195 negative by *Omp1* sequencing (2 serovar D, 1 Serovar E, 1 serovars E&G and 1 serovars
 196 J&E). Overall a very good agreement of 0.875 (0.794 to 0.956) between both methods was
 197 observed (Table 3). The sensitivity of for genotyping with the Ct-DT-RHA assay compared
 198 with the AC2 Ct detection was 0.80 (95% CI 0.67-0.89; table 4).

200 *pmpH* real-time PCR

201 The *pmpH* PCR had a positive result in 32 of the 50 samples with 6 LGV and 26 non-LGV
 202 serovars (Table 4). Eight of the 18 negative *pmpH* PCR samples were determined positive
 203 with *Omp1* sequencing and the Ct-DT-RHA, while another 8 *pmpH* PCR negative samples

204 were also negative with the other two methods. The remaining 2 *pmpH* PCR negative samples
205 were determined positive with the Ct-DT-RHA for the endogenous plasmid. One double
206 infection (J&E) was determined as Ct negative by the *pmpH* PCR. A sensitivity of 0.64 (95%
207 CI 0.50-0.76) was observed comparing the *pmpH* PCR with the AC2 test. Significant more
208 non-LGV samples were detected with the Ct-DT-RHA compared to the *pmpH* PCR
209 (McNemar's $p=0.0133$).

210

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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

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

nested <i>Omp1</i> PCR	
ompA OF	ATGAAAAAACTCTTGAAATCGGT
ompA OR	TTAGAAGCGGAATTGTGCAT
ompA NF	CGCTTTGAGTTCTGCTTCCT
OMP6AS	TGAGCGTATTGGAAAGAAGC
<i>pmpH</i> real-time PCR	
F3 LGV	CTACTGTGCCAACCTCATCAT
F4 nonLGV	CTATTGTGCCAGCATCGACTC
R2 LGV/nonLGV	GACCCTTTCCGAGCATCA
LGVtotP	[6-FAM]-CTTGCTCCAACAGT-[MGB]
P4 nonLGV	[ROX]-AAAGAGCTTGAAGCAGCAGGAGC-[BHQ2]

Table2

Table 2. Comparison of serovar distribution between Ct-DT-RHA and <i>Omp1</i> sequencing.			
N=50		Ct-DT	<i>Omp1</i>
		Genotyping	Sequencing
Single infection			
Serogroup B	Serovar D	5 (10%)	3(6%)
	Serovar E	12 (24%)	11 (22%)
	Serovar L2	6 (12%)	6 (12%)
Serogroup C	Serovar H	1 (2%)	1 (2%)
	Serovar J	3 (6%)	3(6%)
	Serovar K	0	1 (2%)
Serogroup I	Serovar F	5 (10%)	5 (10%)
	Serovar G	5 (10%)	6 (12%)
Double infection			
	Serovar E&G	1 (2%)	0
	Serovar F&K	1 (2%)	0
	Serovar J&E	1 (2%)	0
Not determined	Plasmid	2 (4%)	0
	Negative	8 (16%)	14 (28%)
Total		50 (100%)	50 (100%)

Table3

Table 3. Serovar specific agreement between Ct-DT-RHA and <i>Omp1</i> sequencing (n _{samples} =50)						
		Ct-DT- RHA and <i>Omp1</i> Sequencing	Additional Ct-DT- RHA positive	Additional <i>Omp1</i> Sequencing positive	Agreement (95%CI)	p-value [#]
Serogroup	Serovar					
Serogroup B	Serovar D	3	2	0	0.727 (0.357 to 1.000)	0.4795
	Serovar E	11	3	0	0.841 (0.666 to 1.000)	0.2482
	Serovar L2	6	0	0	1.000	1.000
Serogroup C	Serovar H	1	0	0	1.000	1.000
	Serovar J	3	1	0	0.847 (0.549 to 1.000)	1.000
	Serovar K	1	0	0	1.000	1.000
Serogroup I	Serovar F	5	1	0	0.898 (0.700 to 1.000)	1.000
	Serovar G	5	1	1	0.811 (0.553 to 1.000)	1.000
Any serovar positive		35	8	1	0.875 (0.794 to 0.956)	0.045

McNemars test

Table 4. Comparison of the Ct-DT-RHA and the <i>pmpH</i> PCR					
		<i>pmpH</i> PCR			
		LGV	Non-LGV	Negative	Total
DT-RHA	LGV	6	0	0	6
	Non-LGV	0	26	8	34
	Not typable	0	0	10	10
	Total	6	26	18	50
McNemars p= 0.0133					