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

K.D. Quint [a,*,1], R.J.M. Bom [b,1], S.M. Bruisten [b], L.J. van Doorn [a], N. Nassir Hajipour [b], W.J.G. Melchers [c], H.J.C. de Vries [d,e], S.A. Morre [f], W.G.V. Quint [a]

[a] DDL Diagnostic Laboratory, Voorburg, The Netherlands
[b] Public Health Laboratory, GGD Amsterdam, Amsterdam, The Netherlands
[c] The Department of Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[d] Department of Dermatology and Centre for Immunity and Infectious Diseases Amsterdam (CINIMA), Academic Medical Centre, University of Amsterdam, The Netherlands
[e] STI Outpatient Clinic, Public Health Service, Amsterdam, The Netherlands
[f] The Department of Pathology, Laboratory of Immunogenetics, VU University Medical Centre, Amsterdam, The Netherlands

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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* real-time PCR discriminating an 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* real-time PCR detected all LGV infections, a substantial amount (24%) of non-LGV infections were missed. The sensitivity compared to AC2 Ct detection was 80% (95% CI 67–89%) for the Ct-DT assay, 72% (95% CI 58–83%) for *Omp1* sequencing and 64% (95% CI 50–76%) for the *pmpH* real-time PCR. In conclusion, the Ct-DT assay is appropriate for serovar distribution studies, epidemiological studies and differentiation between an LGV and non-LGV Ct infection, while *Omp1* sequencing is more appropriate for phylogenetic studies. The *pmpH* real-time PCR is suitable as second assay to differentiate between an LGV and non-LGV infection, but not as primary detection assay, due to its low sensitivity for non-LGV strains.

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1. 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

* Corresponding author. Tel.: +31 (0) 703401670; fax: +31 (0) 703401671.
E-mail address: k.d.quint@gmail.com (K.D. Quint).
[1] Both authors contributed equally to this manuscript.

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, differentiation between a LGV Ct infection and non-LGV Ct infection can be performed with a new real-time PCR based on the pmpH gene (pmpH real-time PCR) [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 real-time PCR, were evaluated by a comparison with an Omp1 nested PCR and sequencing.

2. Materials and methods

2.1. 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 a LGV infection and from heterosexual women.

2.2. 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 lysis buffer (bioMérieux, Boxtel, the Netherlands), 1 μl glycogen (20 mg/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 10 mM Tris buffer (pH 8.0).

2.3. 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), 2 mM $MgCl_2$, 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 1182–1194 bp, comprising the full Omp1 gene. The inner PCR was also performed in a volume of 25 μl, containing the same quantities of polymerase, $MgCl_2$ 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 615–624 bp fragment, comprising the variable domain 1 and 2 of the Omp1 gene. Cycling conditions were: an initial step at 94 °C for 3 min, followed by 35 cycles for the outer PCR and 30 cycles for the inner PCR and a final step at 72 °C for 5 min. The cycles consist of 30 s at 93 °C, 30 s at 57 °C and 1 min 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 same primers from the inner PCR. Finally the labelled DNA was purified using a DyeEx spin kit (Qiagen) and analyzed in an ABI 3130 genetic analyser (Applied Biosystems).

2.4. Ct-DT amplification, detection and genotyping

The Ct-DT amplification (Broad spectrum-Multiplex-PCR), Ct-DT detection (DEIA) and Ct-DT genotyping (RHA) 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.

2.4.1. 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-min preheating step at 94 °C for AmpliTaq Gold activation, followed by 40 cycles of amplification (30 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C) and a final 5-min elongation at 72 °C.

2.4.2. Ct- DT-DNA Enzyme Immuno Assay (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.

2.4.3. Ct-DT-Reverse Hybridization Assay (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.

2.5. 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 $MgCl_2$, 0.40 μM of primer F3 LGV, 0.39 μM of primer F4 non-LGV and 0.92 μM of primer R2 LGV/non-LGV, 0.15 μM of probe LGVtotP and 0.21 μM of probe P4 non-LGV (Table 1). Cycling conditions for the real-time PCR were: uracil DNA

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

Nested Omp1 PCR	
ompA OF	ATGAAAAAACTCTTGAAATCGGT
ompA OR	TTAGAAGCGGAATTGTGCAT
ompA NF	CGCTTTGAGTTCTGCTTCCT
OMP6AS [20]	TGAGCGTATTGGAAAGAAGC
pmpH real-time PCR	
F3 LGV	CTACTGTGCCAACCTCATCAT
F4 non-LGV	CTATTGTGCCAGCATCGACTC
R2 LGV/non-LGV	GACCCTTTCCGAGCATCA
LGVtotP [21]	[6-FAM]-CTTGCTCCAACAGT-[MGB]
P4 non-LGV	[ROX]-AAAGAGCTTGAAGCAGCAGGAGC-[BHQ2]

glycosylase step at 50 °C for 2 min and denaturation at 95 °C for 2 min, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. All tests were performed on a Rotor-Gene 6000 (Qiagen, Venlo, the Netherlands).

2.6. Statistical analyses

The statistical analyses were performed using online GraphPad software, calculating the agreement between the three genotyping assays. Kappa values were divided into 5 groups (0.0–0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.00) and respectively interpret as a slight, fair, moderate, substantial and very good agreement. The sensitivities and 95% CI were calculated with the clinical calculator 1 (http://faculty.vassar.edu/lowry/clin1.html). The AC2 detection test was performed as reference test for determination of the sensitivity for the three genotyping methods, since no reference genotyping methods is available. Differences between the genotyping methods were calculated with a McNemars test and Bonferroni correction was performed.

3. Results

3.1. Omp1 sequencing

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 of Omp1 sequencing was 72% (95% CI 58–83%), compared with the AC2 Ct detection assay.

3.2. Ct-DT-RHA

Forty-two 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, which were also negative by Omp1 sequencing. Genotyping with the Ct-DT-RHA was possible in 40

Table 2
Comparison of serovar distribution between Ct-DT-RHA and Omp1 sequencing.

N = 50		Ct-DT genotyping	Omp1 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%)

samples, since 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 ($\kappa = 0.875$, 95% CI = 0.794–0.956) between both methods was observed (Table 3). On serovar level, a substantial agreement ($\kappa = 0.727$, 95% CI = 0.357–1.000) was observed for serovar D, while a very good agreement was observed for the serovars E, L2, H, J, K, F and G (see Table 3 for kappa values). The sensitivity for genotyping with the Ct-DT-RHA assay compared with the AC2 Ct detection assay was 80% (95% CI 67–89%).

3.3. pmpH real-time PCR

The pmpH real-time PCR had a positive Ct result in 32 of the 50 samples with 6 LGV and 26 non-LGV serovars (Table 4). Eight of the 18 negative pmpH real-time PCR samples were determined positive with Omp1 sequencing and the Ct-DT-RHA, while another 8 pmpH real-time PCR negative samples were also negative with the other two methods. The remaining 2 pmpH real-time 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 real-time PCR. A sensitivity of 64% (95% CI 50–76%) was observed comparing the pmpH real-time PCR with the AC2 test. A substantial agreement was observed between the pmpH real-time PCR and the Ct-DT-RHA ($\kappa = 0.714$, 95% CI = 0.533–0.896), although significant more non-LGV samples were detected with the Ct-DT-RHA (McNemar's $p = 0.0133$). Also a substantial agreement between the pmpH real-time PCR and Omp1 sequencing was observed ($\kappa = 0.757$, 95% CI = 0.590–0.924, McNemar's $p = 0.4497$).

4. 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). Also, 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 than Omp1 sequencing for serovar distribution studies and future Ct vaccine studies. Although the Ct-DT-RHA can detect multiple Ct serovar infections, the Ct Omp1 sequencing system has a higher discriminating power. Omp1 sequencing can recognize most point mutations that were missed with the Ct-DT-RHA, making Omp1 sequencing more useful in networking studies and phylogenetical Ct studies, in which genovariants of Ct serovars are important to recognize.

The Ct positive samples were determined positive by the AC2 test. The AC2 test platform is considered the most sensitive and specific RNA detection system and therefore used as reference test [17]. Nevertheless, eight AC2 Ct positive samples were negative with all three genotyping methods. The discordant result between

Table 3
Serovar specific agreement between Ct-DT-RHA and *Omp1* sequencing ($n_{samples} = 50$).

Serogroup	Serovar	Ct-DT-RHA and *Omp1* sequencing	Additional Ct-DT-RHA positive	Additional *Omp1* sequencing positive	Kappa-value (95%CI)	*p*-value[a]
Serogroup B	Serovar D	3	2	0	0.727 (0.357–1.000)	0.4795
	Serovar E	11	3	0	0.841 (0.666–1.000)	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 (0.549–1.000)	1.000
	Serovar K	1	0	0	1.000	1.000
Serogroup I	Serovar F	5	1	0	0.898 (0.700–1.000)	1.000
	Serovar G	5	1	1	0.811 (0.553–1.000)	1.000
Any serovar positive		35	8	1	0.875 (0.794–0.956)	0.045

[a] McNemars test.

Ct detection by the AC2 and the 3 genotyping methods might be due to a low Ct-DNA load. To assure true Ct positivity another Ct detection method (COBAS TaqMan, Roche Molecular Systems, Branchburg, NJ) was used for the 8 discordant samples, but still 7 samples were Ct negative with the COBAS TaqMan. The discordant samples can be explained by degradation of DNA due to storage, false positivity of AC2 test or an increased sensitivity of the AC2 test, as previous described [17]. To exclude DNA degradation as possible explanation, we repeated the AC2 test for 4 of the 8 discordant samples and, again, a positive Ct result was obtained for all 4 samples. Not enough DNA was available to repeat the AC2 test for the remaining 4 samples.

The 2 Ct endogenous plasmid positive samples that could not be genotyped were also examples of a low DNA load. The endogenous plasmid is approximately $10–20\times$ more available per Ct bacterium as genomic DNA [18,19]. So if a sample contains a low Ct-DNA load, the possibility exist that only plasmid DNA is selected for PCR [13]. This phenomenon is known as sampling error and is also a possible explanation for the sensitivity differences between the Ct-DT-RHA and the other 2 typing methods, since the Ct-DT-PCR uses 10 μl DNA isolation while the *Omp1* sequencing and *pmpH* real-time PCR both use 2 μl.

All six LGV strains genotyped by *Omp1* sequencing were also recognized as LGV strain by the *pmpH* real-time PCR. Nevertheless, the *pmpH* real-time 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 primer and probe, sequence differences in the probe and or primer region relative to the circulating patient strains and a low bacterial load in combination with 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* real-time PCR) and the *pmpH* real-time PCR detected all LGV variants, this algorithm can be used for differentiating between a urogenital Ct strain and an LGV Ct strain. The Ct-DT assay is also a good alternative for routine screening with the *pmpH* real-time PCR, since all LGV strains detected by *Omp1* sequencing and the *pmpH* real-time PCR were recognized by the Ct-DT-RHA as serovar L2.

Table 4
Comparison of the Ct-DT-RHA and the *pmpH* real-time PCR.

		pmpH real-time 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

$K = 0.714$ (95%9 0.533–0.896), McNemars $p = 0.0133$.

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* real-time PCR. No double infections were observed in the 6 LGV positive samples with the Ct-DT-RHA, possibly due to the small sample size. 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* real-time PCR, making the Ct-DT-RHA appropriate for serovar distribution studies, but also for differentiating between an 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* real-time 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.

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