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Evaluation of a Novel *Chlamydia trachomatis* Microsphere Suspension Assay for Detection and Genotyping of the Different Serovars in Clinical Samples

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A novel *Chlamydia trachomatis* (Ct) microsphere suspension (MS) assay was evaluated for identification of the different serovars, using the same PCR primer set established for the Ct Detection and genoTyping assay. Both assays can detect and identify all 14 major serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2/L2a, and L3) and one genovariant of serovar J. The probe specificity for the Ct-MS assay was determined using 14 Ct reference strains and 1 clinical isolate from a genovariant of serovar J. Also, the Ct-MS assay and the Ct detection and genoTyping assay were compared in 712 Ct-positive clinical samples. The Ct-MS assay showed a highly specific reaction for all probes with the amplicons of the reference strains, giving a very low background median fluorescence intensity signal (median fluorescence intensity ≤ 10). An excellent overall agreement in the Ct detection (kappa = 0.947, 95% confidence interval, 0.89 to 0.999; McNemar's test, *P* = 1.000) and the Ct genotyping (kappa = 0.993, 95% confidence interval, 0.977 to 1.000; McNemar's test, *P* = 0.053) was observed between the Ct detection and genoTyping (DT) assay and the Ct-MS assay. In conclusion, the novel Ct-MS assay permits simultaneous detection and genotyping of Ct serovars, making the Ct-MS assay an excellent high throughput method. ***(J Mol Diagn 2011, 13:152–159; DOI: 10.1016/j.jmoldx.2010.11.017)***

Chlamydia trachomatis (Ct) is considered a global, public health problem, with an approximate 90 million new cases worldwide annually.[1] Ct can be divided in 3 serogroups (serogroup B, C, and intermediate) and 19 different serovars (A, B, Ba, C, D, Da, E, F, G, Ga, H, I, Ia, J, K, L1, L2, L2a, and L3) based on their reactivity with specific monoclonal antibodies targeting the major outer membrane protein.[2,3] The majority of anogenital Ct infections are caused by serovars D–K and may lead to infertility in both sexes.[4,5] Besides an infection in the anogenital region, some Ct serovars (A to C) can infect the conjunctiva leading to a follicular conjunctivitis and trachoma, which is still the major cause of blindness in developing countries.[6,7] Finally, some Ct serovars (L1–L3) invade to the submucosa of the proctum and the lymph nodes, causing the destructive disease lymphogranuloma venereum.[8,9] Clinically, differentiation between a lymphogranuloma venereum Ct infection and an A–K serovar Ct infection is necessary because a lymphogranuloma venereum infection needs prolonged antibiotic treatment.[10]

Nowadays, several in-house reverse line blots have been developed for Ct genotyping, often based on amplification of the *Omp1* gene, encoding for the major outer membrane protein.[11–14] Previously, we evaluated a commercially available Ct-detection and genoTyping (DT) assay, comprising Ct-detection by a DNA enzyme immunoassay (DEIA) and Ct-genotyping by a reverse hybridization assay (RHA) using the same amplicons.[14] An excellent

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agreement between the Ct-DT DEIA and other Ct detection assays [ie, Hybrid Capture 2 (Qiagen, Valencia, CA) and COBAS TaqMan tests (Roche Molecular Systems, Branchburg, NJ)] was observed.[13–14] Also, Ct genotyping with the Ct-DT RHA is a sensitive alternative for *Omp1* sequencing.[15] This algorithm of amplification, detection, and genotyping is a relatively fast method, easy to perform, and particularly suitable for study populations with a low Ct prevalence. At this moment, Ct genotyping with the Ct-DT RHA was performed in several epidemiological studies.[16–17]

Recently, a new Ct-microsphere suspension (MS) assay was developed that combines Ct detection and Ct genotyping, using the same amplification step as in the Ct-DT assay. The Ct-MS assay utilizes xMAP Microsphere Suspension Array technology (Luminex Corp, Austin, TX) that demonstrates great potential for large epidemiological studies in microbial and viral subtyping due to the high throughput scale.[18–20] In the present study, we evaluated the novel Ct-MS assay in a large number of clinical samples.

Materials and Methods

Reference Strains

Ct serovars reference strains (A/Sa-1, B/TW-5, C/UW-1, D/IC-CAL-8, E/DK-20, F/MRC-301, G/IOL-238, H/UW-4, I/UW-12, J/UW-36, K/UW-31, L1/440-L, L2/434-B, and L3/404-L) and one clinical sample containing a genovariant of serovar J (referred as J*) were selected. All strains were previously sequenced to confirm presence of the correct Ct serovar strain.[14] The DNA isolation was performed using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany), including pretreatment with proteinase K, according the manufacturer's instruction.

Clinical Samples

The DNA isolates (stored at −80°C) from 712 clinical samples were selected. The samples were determined as Ct positive in previous studies with different Ct detection assays [ie, an in-house based real-time PCR assay, the Aptima Combo 2 assay, or the PACE2 assay (Gen-Probe Inc., San Diego, CA)].[15,16,21] In short, 172 samples were collected from a Russian cohort (January 2006 to 2008), including 83 male urethral samples and 89 cervical samples. Those samples were all determined Ct positive with an in-house nucleic acid amplification test, described elsewhere.[22]

Another 50 samples were selected between January 2007 and 2009, from an outpatient sexually transmitted disease clinic (Amsterdam), consisting of 4 first void urines, 25 rectal swabs, 14 cervical swabs, 3 urethral swabs, and 4 vaginal swabs. The 25 rectal swabs were collected from men who have sex with other men. All 50 samples were determined Ct positive with the Aptima Combo 2 assay (Gen-Probe Inc.).

The remaining 490 samples were selected from a second outpatient sexually transmitted disease clinic (the Hague) in 2008, including 245 cervical swabs, 98 rectal swabs, 98 first void urines, 10 pharyngeal samples, and 39 urethral samples. All 490 samples were determined Ct positive with the PACE 2 assay (Gen-Probe Inc.).

In total, the 712 DNA isolates could be divided into 17.6% urethral samples, 49.4% cervical/vaginal samples, 17.3% rectal samples, 14.3% first void urines and 1.4% pharyngeal samples. All DNA isolates were transported on dry ice to DDL Diagnostic Laboratory and stored at −80°C for a maximum of 3 years.

The Amplification Step (PCR) for the Ct-DT Assay and Ct-MS Assay

The Ct-DT assay and the Ct-MS assay are comprised of a general PCR amplification step, which uses a Ct multiplex broad-spectrum PCR primer mixture, targeting both the *Omp1* VD2 region and the endogenous plasmid as previously described.[14,23] The Ct PCR primer set is designed to amplify all of the known serovars available in GenBank. Briefly, the multiplex primer set amplifies an 89 bp and a 160/157 bp fragment from the endogenous plasmid and the variable region 2 of the *Omp1* gene. The PCR mixture consists of 10 μL of isolated DNA, 2.5 mmol/L $MgCl_2$, 1 × GeneAmp PCR buffer II, 1.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.2 mmol/L deoxynucleoside triphosphates (GE Healthcare, Buckinghamshire, UK) and 15 pmol of each primer (Eurogentec S.A., Seraing, Belgium) in a total volume of 50 μL. The PCR program includes a 9-minute preheating step at 94°C for AmlipTaq Gold activation, followed by 40 cycles of amplification (30 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C) and a final 5-minute elongation step at 72°C. All PCR reactions were carried out in parallel with negative controls (water) and positive controls consisting of Ct serovar L2/434-B reference strain DNA. The primer specificity was previously determined for the other species: *Chlamydophila psittaci*, *Chlamydophila pneumoniae*, *Chlamydia muridarum, Enterococcus faecalis*, *E. coli*, *Gardnerella vaginalis*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. No DNA from the other species was amplified, indicating a Ct specific PCR.[14]

The Ct Detection and Genotyping Step of the Ct-DT Assay

The Ct-DT detection and genotyping step was performed according to the manufacturer's instructions (Labo Biomedical Products BV, Rijswijk, The Netherlands) and as previously described.[14] Briefly, the detection of Ct amplicons was performed by a DEIA. The PCR products were captured on streptavidin-coated microtiter plates, denaturated by alkaline treatment, and detected by a cocktail of digoxinenin-labeled probes. The DEIA provides an optical density value at 450 nm. Each DEIA run contained separate positive, borderline positive and negative controls and a PCR-positive control. Samples yielding optical density values equal to or higher than the borderline are considered positive.

Amplicons from the Ct-DT DEIA-positive samples can be further genotyped by the Ct-DT RHA. The biotin-labeled PCR amplicons hybridize to specific probes on a nitrocellulose strip. The strip contains a probe for the endogenous plasmid, probes for the three different Ct serogroups (B, C, and I), and probes for the 14 major 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.

The Combined Detection and Genotyping Step of the Ct-MS Assay

The Ct-MS assay (Labo Bio-medical Products BV, Rijswijk, The Netherlands) permitted simultaneous detection and identification of multiple Ct serovars in a single hybridization step. The Ct-MS assay contained a probe to detect the endogenous plasmid, probes to detect the 3 serogroups and 15 probes to detect the 14 major serovars (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2/L2a, and L3) and the genovariant J*. The probes were immobilized on color-coded Luminex beads (Luminex Corp). The Ct-MS genotyping kit was performed in the Luminex 100 IS system, according to the manufacturer's instruction. In short, 3B buffer (Labo Bio-medical Product BV) was added to the Ct bead mix to minimize background signals. Subsequently, 4 μL of Ct PCR products were added. Next, heat denaturation, hybridization under stringent temperature conditions (50°C), washing and incubation with streptavidin-conjugated R-phycoerythrin detection conjugate were followed by read-out according to the specified instrument settings, resulting in median fluorescence intensity (MFI) levels for the Ct endogenous plasmid probe, the Ct serogroup probes, and the Ct serovar probes for each specimen. The Ct-MS read-out was determined as Ct positive when the probe for the endogenous plasmid and/or the probes for genotyping were positive. The results with the Ct-MS assay were generated without knowledge of the serovar results obtained with the Ct-DT assay.

Dilution Series of Serovar L2 Amplicons

The PCR product of a clinical sample with serovar strain L2 was performed in a 10-fold dilution series. The results of the dilution series were analyzed by 3% agarose gel electrophoresis, the Ct-DT detection step (DEIA), the Ct-DT genotyping step (RHA), and the Ct-MS assay. Only a semiquantitive result can be reported because no exact copy number of amplicons was known per dilution.

Gel Electrophoresis

A total of 10 μL of PCR product was mixed with 2 μL ethidium bromide, and gel electrophoresis was performed on a 3% agarose gel for 225 to 300 volt-hours in 0.5X Tris borate-EDTA buffer.

Ct Omp1 *Sequencing*

Briefly, amplified DNA fragments were excised from the 3% Tris-borate-EDTA agarose gels and purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Purified amplicons were directly sequenced using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). The sequenced products were subsequently analyzed using the ABI 3100-Avant Genetic Analyzer (Applied Biosystems). The resulting DNA sequences were analyzed with the Vector NTI Advance 9.0 software (Invitrogen, Breda, The Netherlands) and compared with *C. trachomatis* serovars present in GenBank.

Contamination Prevention and Personnel Training

The overall risk of contamination with exogenous nucleic acid in our laboratory is limited to an acceptable level. Contamination is prevented by a set of measures, including a unidirectional workflow, physically separate areas for reagent preparation, nucleic acid extraction, and handling of postamplification products, decontamination of surfaces, and strict regulations concerning housekeeping, clothing, etc. The effectiveness of these measures is monitored on a continuous basis using blank controls and swabbing of critical surfaces. The actual risk of contamination is monitored by including blank controls in nucleic acid isolation and PCR runs. During the current study, no indications for contamination were observed.

Personnel involved are competent to perform the work, according to the requirements of ISO9001. In the current study, two qualified individuals performed the Ct-DT assay and the Ct-MS assay.

Data Analysis and Statistics

For analytical comparison of the Ct-MS assay with the Ct-DT DEIA/RHA, two threshold levels for positivity (30 MFI and 100 MFI) are presented in this study. The Ct-MS assay kit insert recommended a standard MFI threshold of 100 MFI. However, due to the low background, a lower threshold of 30 MFI was also evaluated to demonstrate the potentially higher sensitivity of the Ct-MS assay. The level of agreement between the Ct-DT DEIA/RHA and Ct-MS assay was determined using a Cohen's kappa, and a two-tailed McNemar's test was performed to investigate the differences between both assays. The level of statistical significance was set at $P < 0.05$. All analyses were performed in SPSS version 17.0 (Gorinchem, The Netherlands).

Results

Analytical Performance of the Ct-MS Assay

The specificity of the Ct-MS assay was evaluated with PCR products derived from a panel of 15 Ct reference strains. The endogenous plasmid probe showed a positive result for all reference strains. The probe for serogroup B showed a specific reaction with the amplicons from

Table 1. Type Specificity of the Ct-MS Assay for 15 Ct Reference Strains

	Chlamydia trachomatis genotypes/serovars														
	Group B					Group I		Group C							
Probe	B	D	E	L1	L2	F	G	A	C	H	I	J	J^{var}	K	L3
Conj	**8574**	**8572**	**7444**	**8795**	**8640**	**8273**	**8555**	**8227**	**7858**	**8234**	**8694**	**8224**	**9238**	**9187**	**8668**
Ct-CP*	**2624**	**2496**	**2511**	**2742**	**2916**	**2635**	**2666**	**2581**	**2376**	**2414**	**2663**	**2409**	**1931**	**2539**	**2465**
Serogroup probes															
Ct-gB	**1764**	**3290**	**1992**	**2480**	**1786**	1	1	1	0	1	1	1	1	1	10
Ct-gI	7	2	2	2	2	**3363**	**2888**	2	3	2	3	2	2	2	2
Ct-gC	2	2	2	2	2	2	2	**3574**	**3176**	**3017**	**4107**	**3204**	**2514**	**2073**	**2702**
Serovar probes															
Ct-B/Ba	**5250**	2	2	1	3	1	1	1	1	0	1	1	1	1	1
Ct-D/Da	1	**1980**	1	1	1	1	2	1	1	1	4	1	1	1	1
Ct-E	1	1	**1876**	1	1	1	1	1	1	1	1	1	1	1	1
Ct-L1	1	1	2	**2679**	2	1	2	2	1	1	1	2	1	1	2
Ct-L2/L2a	4	3	3	6	**3030**	3	3	5	4	3	3	3	2	3	4
Ct-F	2	3	3	2	3	**4712**	2	2	2	2	2	3	3	2	2
Ct-G/Ga	3	3	3	2	3	4	**1360**	3	3	3	3	3	2	3	2
Ct-A	2	2	2	2	2	2	2	**5645**	2	3	2	2	2	2	2
Ct-C	1	1	1	2	1	1	1	3	**1221**	1	1	2	2	1	4
Ct-H	1	1	1	1	1	2	2	2	1	**1979**	1	1	1	1	1
Ct-I/Ia	2	2	2	2	2	2	3	4	4	2	**2769**	4	2	3	3
Ct-J	2	2	2	2	2	3	2	3	4	2	2	**1732**	2	5	2
Ct-J/L3	2	2	2	2	2	2	2	3	2	3	4	3	**754**	2	**400**
Ct-K	2	2	2	2	2	2	2	3	2	2	2	2	2	**2447**	2
Ct-L3	2	2	1	1	2	1	2	1	2	1	2	1	2	2	**7129**

Analytical type specificity of the Ct-MS assay. Indicated are the MFIs read outs of the Ct-DT PCR amplicons generated from Ct reference strains (listed on top) in relation to the bead-bound capture probes (listed on the left). The conjugate control (conj.) serves as the positive control for correct incubation with the detection conjugate for each separate serovar.

Bold numbers indicate a positive MFI value.

*Probe for endogenous plasmid.

the reference strains B, D, E, L1, and L2, whereas the intermediate serogroup gave a specific reaction with the amplicons from serovars F and G. The serogroup C probe showed a specific reaction for the serovars A, C, H, I, J, J*, K, and L3. Amplicons of the different genotypes demonstrated probe specific hybridization with MFI values between 400 and 7129, whereas the background read-out varied between 0 and 10 (Table 1). No cross-reaction with untargeted serovars was observed for any of the probes. As with the Ct-DT RHA, differentiation between serovar J* and serovar L3 was possible by pattern recognition because a probe specific for serovar L3 was added.

The results of the serovar L2 amplicons 10-fold dilution series is reported in Table 2. No differences in the limit of detection (LOD) for the endogenous plasmid was observed between the Ct-DT DEIA (LOD: 10^{-3} dilution), Ct-DT RHA (LOD: 10^{-3} dilution), and the Ct-MS assay at a 30 MFI threshold (LOD: 10^{-3} dilution). However, the agarose gel (LOD: 10^{-1} dilution) was less sensitive. On genomic level (ie, serovar and serogroup), an LOD was observed for the Ct-MS assay (threshold, 30 MFI) at a 10^{-2} dilution, whereas the agarose gel shows an LOD at a 10^{-1} dilution. For the Ct-DT RHA, the lowest LOD was observed (LOD: 10^{-3} dilution).

Comparison between the Ct-MS Assay and the Ct-DT DEIA for Ct Detection

First a comparison was made with the Ct-DT DEIA for Ct detection because the Ct-MS assay combines Ct detection and Ct genotyping.

Table 2. Dilution Series of Serovar L2 Amplicons

Sample dilution	3% agarose gel		Ct-DT detection	Ct-DT genotyping (RHA)			Ct-MS assay (MFI value)		
	Plasmid (89 bp)	*Omp1* (160 bp)	(DEIA)	Plasmid	Group B	Serovar L2	Plasmid	Group B	Serovar L2
Undiluted	+	+	+	+	+	+	**6155**	**1643**	**2782**
L2 10^{-1}	+	+	+	+	+	+	**3958**	**387**	**905**
L2 10^{-2}	−	−	+	+	+	+	**1130**	**41**	**110**
L2 10^{-3}	−	−	+	+	+	+	**113**	3	8
L2 10^{-4}	−	−	−	−	−	−	9	1	3
L2 10^{-5}	−	−	−	−	−	−	2	0	2

Results were observed with a 3% agarose gel, the Ct-DT detection (DEIA) and genotyping (RHA) step and the Ct-MS assay (threshold 30 MFI).
Bold numbers indicate a positive MFI value.

Table 3. Comparison between the Ct-DT DEIA Assay and the Ct-MS Assay for Ct Detection (n = 712)

	Ct-MS assay	
Ct-DT assay	+	−
DEIA+	670	2
DEIA−	2	38

Kappa = 0.947; 95% confidence interval = 0.895 to 0.999; McNemar's test, P = 1.000.

In total, 712 clinical samples were analyzed with the Ct-DT assay and the Ct-MS assay. Overall, 670 samples were Ct positive with both the Ct-DT DEIA and Ct-MS assays and 38 samples were negative with both assays (Table 3). Four samples showed a discordant result, of which 2 were only positive with the Ct-MS assay and 2 were only positive with the Ct-DT DEIA. An excellent agreement between the Ct-DT DEIA and the Ct-MS assay was observed (kappa = 0.947; 95% CI = 0.895 to 0.999; McNemar's test, P = 1.000) (Table 3). No difference in Ct detection was observed when the MFI threshold was lowered from 100 MFI to 30 MFI.

Comparison between the Ct-MS Assay and the Ct-DT DEIA/RHA for Ct Genotyping

In the 712 clinical samples, the probe specific genotyping results revealed a complete concordance between both assays in respectively 690 (96.9%) or 695 (97.6%) samples, using the thresholds of 100 and 30 MFI, respectively.

The individual genotyping results of the 712 potentially Ct-positive samples are shown in Table 4. Genotyping agreement between the Ct-MS assay and the Ct-DT DEIA/RHA was dependent on the choice of the MFI threshold for Ct positivity with the Ct-MS assay. For all probes, the highest agreements were observed at an MFI threshold of 30 MFI. Overall, a high kappa value was observed between the Ct-DT RHA and Ct MS assay (30 MFI; κ = 0.993; 100 MFI; κ = 0.990), although the Ct-DT DEIA/RHA demonstrated significantly more overall probe positivity than the Ct-MS assay (30 MFI threshold, McNemar's test, P = 0.053; 100 MFI threshold, McNemar's test, $P < 0.001$). This effect is partly due to the identification of the *Omp1* amplicon on two separate levels (serogroup probe and serovar probe), so we exclude the serogroup probes from the analyses and focus only on the serovar probes. Still a statistical difference (McNemar's test, P = 0.003) was observed between assays when a threshold of 100 MFI was used, but not at a threshold of 30 MFI (McNemar's test, P = 0.780). Probe E and probe gC were significantly more positive with the Ct-DT DEIA/RHA, when a 100 MFI threshold was maintained for the Ct-MS assay (both P = 0.031). This significance disappears by lowering the MFI threshold to 30 MFI (P = 0.500 for probe E and P = 0.063 for probe gC). A positive probe reaction for serovar L3 was observed with the Ct-MS assay in 3 samples when a threshold of 30 MFI was used, whereas all 3 samples were negative at a threshold of 100 MFI. The three samples were positive for serovar I/Ia, indicating cross reaction of probe L3 with a genovariant of serovar I/Ia. No cross-reaction with probe L3 was observed with the Ct-DT RHA assay.

Comparison between the Ct-MS Assay and the Ct-DT DEIA/RHA for Ct Genotyping of Multiple Serovar Infections

A total of 20 multiple infections were detected with the Ct-DT DEIA/RHA. When a threshold of 100 MFI was used, 12 (60%) of the 20 multiple infections were detected with

Table 4. Comparison of Genotyping Findings of the Ct-DT Assay and the Ct-MS Assay in 712 Ct-Positive Samples

	MFI threshold 30						MFI threshold 100					
	MS and RHA positive	RHA positive only	MS positive only	MS and RHA negative	Kappa (95% CI)	McNemar's test, P value	MS and RHA positive	RHA positive only	MS positive only	MS and RHA negative	Kappa (95% CI)	McNemar's test, P value
Probe cp	668	1	2	41	0.962 (0.920–1.000)	1.000	668	1	2	41	0.962 (0.920–1.000)	1.000
Serogroup probes												
Probe gB	308	2	0	402	0.994 (0.986–1.000)	0.500	305	5	0	402	0.986 (0.973−0.998)	0.063
Probe gI	214	1	0	497	0.997 (0.990–1.000)	1.000	214	1	0	497	0.997 (0.990–1.000)	1.000
Probe gC	108	5	0	599	0.973 (0.950−0.997)	0.063	107	6	0	599	0.968 (0.942−0.993)	**0.031**
Serovar probes												
Probe B	10	0	0	702	1.000	1.000	10	0	0	702	1.000	1.000
Probe D	60	2	0	650	0.982 (0.957–1.000)	0.500	58	4	0	650	0.964 (0.928−0.999)	0.125
Probe E	231	2	0	479	0.994 (0.985–1.000)	0.500	227	6	0	479	0.981 (0.965−0.996)	**0.031**
Probe L1	0	0	0	712	—	—	0	0	0	712	—	—
Probe L2	6	0	0	706	1.000	1.000	6	0	0	706	1.000	1.000
Probe F	114	0	0	598	1.000	1.000	114	0	0	598	1.000	1.000
Probe G	100	0	0	612	1.000	1.000	99	1	0	612	0.994 (0.983–1.000)	1.000
Probe A	0	0	0	712	—	—	0	0	0	712	—	—
Probe C	0	0	0	712	—	—	0	0	0	712	—	—
Probe H	10	1	0	701	0.952 (0.857–1.000)	1.000	9	2	0	701	0.899 (0.758–1.000)	0.500
Probe I	10	0	0	702	1.000	1.000	10	0	0	702	1.000	1.000
Probe J	44	1	1	666	0.976 (0.943–1.000)	1.000	44	1	1	666	0.976 (0.943–1.000)	1.000
Probe J/L3	10	1	0	701	0.952 (0.857–1.000)	1.000	10	1	0	701	0.952 (0.857–1.000)	1.000
Probe K	33	0	0	679	1.000	1.000	32	1	0	679	0.984 (0.952–1.000)	1.000
Probe L3	0	0	3	709	—	—	0	0	0	712	—	—
Total probes	**1926**	**16**	**6**	**11580**	**0.993 (0.977–1.000)**	**0.053**	**1913**	**29**	**3**	**11583**	**0.990 (0.974–1.000)**	**<0.001**

Bold P values are considered significant.

Table 5. MFI Values of the Ct-MS Assay among the 20 Multiple Infections Determined with the Ct-DT Assay

	Multiple serovar Ct infections determined with the Ct-DT RHA																			
Probe	F&K*	E&gI	E&G*	E&K*	E&G	E&G	E&K	D&G	E&I	E&K	F&gC[†]	E&J	H&K[‡]	E&F	E&J	H&K[‡]	G&gC	E&J	F&K	E&G
Ct-CP	**5290**	**5041**	**5357**	**5337**	**5667**	**4876**	**5291**	**5298**	**5368**	**6515**	**6159**	**6044**	**5596**	**5621**	**5825**	**5643**	**4221**	**3272**	**6837**	**4142**
Serogroup probes																				
Ct-gB	1	**1427**	**53**	**84**	**826**	**103**	**154**	**165**	**1240**	**163**	1	**800**	1	**268**	**1863**	1	1	16[§]	1	22[§]
Ct-gI	**2575**	27[§]	**2245**	2	**1407**	**2219**	2	**1496**	3	2	**2976**	2	2	**1996**	2	2	**506**	2	**1346**	**224**
Ct-gC	**76**	1	1	**1900**	2	2	**2002**	2	**3441**	**1098**	**443**	**2240**	**1717**	2	**2600**	**1975**	2[§]	**155**	**1382**	2
Serovar probes																				
Ct-B/Ba	1	2	1	1	1	2	1	1	2	0	1	1	1	1	2	1	0	1	2	1
Ct-D/Da	0	1	1	1	1	1	1	**95**	3	1	1	1	1	1	1	1	1	1	1	1
Ct-E	1	**1501**	**30**	**44**	**454**	**55**	**79**	1	**1112**	**134**	2	**565**	3	**143**	**1995**	2	2	15[§]	5	20[§]
Ct-L1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1	2
Ct-L2/L2a	2	3	3	3	3	2	2	3	3	3	3	2	4	3	3	3	3	3	3	3
Ct-F	**3427**	2	2	2	2	3	2	2	2	2	2	2	2	**2409**	2	2	2	2	**1869**	2
Ct-G/Ga	4	9	**1019**	3	**675**	**879**	2	**624**	2	2	**3590**	3	2	4	2	2	**157**	3	3	**49**
Ct-A	3	3	3	2	3	4	3	3	3	4	3	4	4	4	3	4	3	5	7	2
Ct-C	1	2	1	1	1	2	1	2	1	1	2	2	1	2	5	2	1	2	1	2
Ct-H	2	1	1	1	1	2	1	1	2	2	1	1	**69**	2	1	2[§]	1	1	2	1
Ct-I/Ia	2	2	2	4	3	2	2	2	**1769**	3	3	3	3	2	5	3	2	3	4	2
Ct-J	2	2	3	4	1	2	4	2	3	4	**446**	**854**	5	3	**1624**	4	2	3	6	2
Ct-J/L3	1	2	2	2	2	2	2	2	2	2	3	2	2	2	3	2	2	12[§]	3	2
Ct-K	**69**	2	2	**1562**	2	3	**1664**	2	2	**2163**	2	2	**2042**	2	2	**1304**	2	1	**2467**	2
Ct-L3	1	2	2	1	1	2	1	2	3	1	1	1	2	2	2	2	2	2	2	1

Bold numbers indicate a positive MFI value.

*Recognized as double serovar infection at a threshold of 30 MFI, while not recognized as double infection at a threshold of 100 MFI. CI, confidence interval.

[†]Determined with the Ct-DT assay as a multiple infection with serovar F and serogroup C. The Ct-MS assay revealed serovar J, belonging to serogroup C.

[‡]Sequencing revealed a genovariant of serovar K. So, also for the Ct-MS assay, it is recommended to sequence multiple serovars belonging to one serogroup.

[§]Determined positive with the Ct-DT RHA, but negative with the Ct-MS assay at an MFI threshold of 30.

the Ct-MS assay. Lowering the threshold to 30 MFI resulted in the detection of 3 additional multiple serovar infections (Table 5). The remaining 5 undetected multiple infections consisted of double infection with serovars E and G, serovars E and J, serovars E and gI, serovars G and gC, and serovars H and K, according to the Ct-DT DEIA/RHA. Still, it was possible to recognize 3 of the 5 undetected multiple infections with the Ct-MS assay when a variable threshold (<30 MFI) was maintained. Differentiation between background and a positive probe reaction was still possible because the MFI values of the undetected serovars varied between 10 to 30 MFI and the background MFI was lower than 10 MFI. For one sample, additional serovar-specific information was obtained with the Ct-MS assay (serovar F and J), whereas the Ct-DT DEIA/RHA only revealed the serogroup of serovar J (serovar F and gC). A remarkable observation was that in almost all double infections a minority serovar had a 10- to 100-fold lower MFI value than the dominant serovar, indicating different serovar concentrations in the sample. Serovars H and K belong to the same serogroup. In these cases, the Ct-DT assay kit insert recommended sequencing to exclude cross reaction of new Ct genovariants not available in the GenBank.[14]

Discussion

The current study evaluates the Luminex platform-based Ct-MS assay, by determining the specificity in serovar reference strains and by comparing the Ct-MS assay with the Ct-DT assay in a large number of clinical samples. The Ct-MS assay differentiates between the 14 major serovars and the genovariant (J*) without any probe cross reactivity, using identical PCR products as the Ct-DT assay. Regarding the L2 amplicon dilution series, a slightly less sensitive LOD was observed for the Ct-MS assay, compared to the Ct-DT RHA on genotyping level (*Omp1*), whereas on the Ct endogenous plasmid detection level, the same LOD was observed for both assays. The less sensitive LOD for the Ct-MS genotyping might be caused by a lower amplicon input in this assay compared to the Ct-DT assay (4 μL versus 10 μL, respectively). However, the less sensitive LOD for the Ct-MS assay on genotyping level has minor consequences in the clinical samples. An excellent level of agreement in Ct detection ($\kappa = 0.947$) and Ct genotyping ($\kappa = 0.990$) was observed between the Ct-MS assay and the Ct-DT assay with a threshold of 100 MFI among the 712 clinical samples. By lowering the MFI threshold to 30 MFI, an increase in agreement between the Ct-DT assay and the Ct-MS assay was observed ($\kappa = 0.993$), without losing specificity. Therefore, a steady threshold of 30 MFI should be used in future studies.

A different way to approach the probe-specific MFI threshold is described by Schmitt et al,[24] in which the MFI threshold is calculated based on the background subtraction method ($>1.2 \times$ median probe MFI background $+ 5$ MFI $=$ threshold). This will lead in our study to extremely low thresholds, because the median background for each probe is <5 MFI. Because the probes can possibly give cross reaction with genovariants of other serovars, this approach will give rise to the detection of more multiple serovar infections, based on false-positive results. Nevertheless, the background substraction method showed an excellent agreement when + 9 MFI levels was used in the formula instead of + 5 MFI levels.

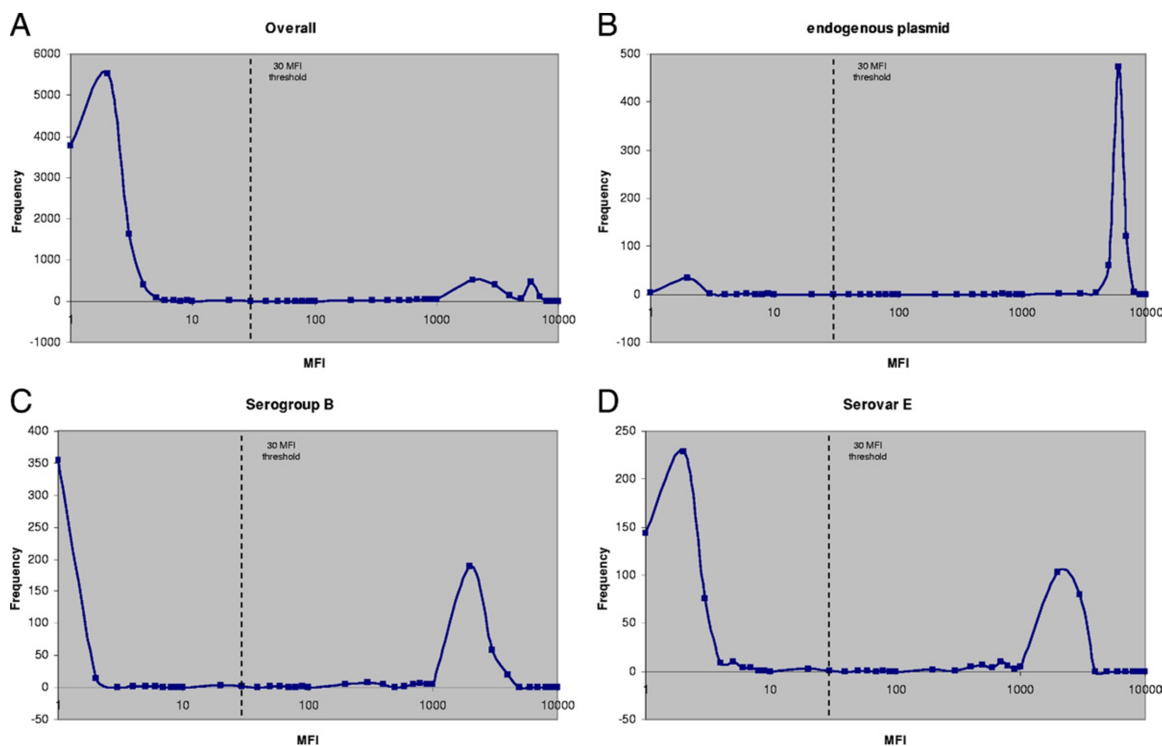

Figure 1. Frequencies of median fluorescence intensity (MFI) values plotted on a logarithmic scale (*x* axis) versus the frequency of each MFI in the clinical samples (*y* axis). The figure contains four graphs. **A:** A total of all 19 probes: Overall, a very low background MFI value was observed indicating that a 30 MFI threshold is a stable threshold with sufficient distance from the background MFI values. The first peak of true positive probe reaction was observed at 1000 MFI, indicating that the majority of positive probe reaction have an MFI value >1000. Nevertheless, in a few cases, also MFI values between 30 and 1000 MFI were observed. **B:** Endogenous plasmid level: Since almost all clinical samples were Ct positive, only a small peak of background MFI values for the endogenous plasmid probe was observed. Still, a 30 MFI threshold contains sufficient distance from the background MFI peak. **C:** Serogroup level: Serogroup B was taken as an example for the serogroup specific probes. Again, a 30 MFI value was sufficient to differentiate between background and true positive. **D:** Serovar level: Serovar E was taken as an example on serovar level, because serovar E was the most prevalent serovar. Also, a 30 MFI value was sufficient to differentiate between background and true positive.

Overall, we still prefer a stable threshold of 30 MFI, because the threshold is a 3- to 10-fold higher MFI value than the background MFI. This makes discrimination between background MFI and a true positive MFI value possible (eg, Figure 1). In addition, a stable threshold is easier to define than a variable threshold.

Another possible approach can be the usage of a gray zone. The background substraction method should then be used to determine true negative samples, whereas a stable MFI value is used for true positivity probe reaction. The MFI value between the true negative and true positive samples can be the gray zone, for which it would be recommended to repeat the PCR. This approach will be accomplished in future studies using the Luminex platform.

In three samples of serovar I, the Ct-MS assay showed a cross reaction with probe L3 at a threshold of 30 MFI, whereas this cross reaction was not observed with the Ct-DT RHA. This false-positivity problem can be solved by either increasing the MFI threshold for serovar L3 to 100 MFI (Table 4) or by sequencing all multiple serovars belonging to the same serogroup, such as that recommended for the Ct-DT assay.[14] When a noninterpretable result is obtained by sequencing, it could be assumed that a true-multiple serovar infection is present. Of course, it could also reveal a sequence of a new genovariant. For example, probe H showed cross reaction with a genovariant of serovar K (sequenced), leading to false positivity with the Ct-DT RHA, but also with the Ct-MS assay at a threshold of 30 MFI.

The Ct-DT DEIA/RHA system identified a total of 20 multiple infections, although only 15 multiple infections were observed with the Ct-MS assay (30 MFI threshold). In the majority of multiple infections, one of the serovars in a multiple infection has a 10- to 100-fold lower MFI value than the other serovar (Table 5). The difference in MFI level can be caused by a low DNA input of one serovar and/or competition in the PCR reaction between the serovars, leading to a lower amount of amplicons for one of the two serovars.

One of the five double infections that were only identified with the Ct-DT DEIA/RHA contained a cross reaction caused by the genovariant K (sequenced). In the remaining four samples, no double infections could be identified with the Ct-MS assay at a threshold of 30 MFI.

A total of 38 samples, previously determined to be Ct positive by different Ct detection methods, were negative with both the Ct-DT DEIA/RHA and the Ct-MS assay. This might be caused by a low DNA load (sampling variation) for the PCR or DNA degradation due to sample storage or the use of different Ct detection methods. Still, 94.4% of the previously determined Ct-positive samples were positive with both the Ct-DT DEIA/RHA and Ct-MS assay and 87.1% and 86.4%, respectively, could be genotyped with both the Ct-DT DEIA/RHA and the Ct-MS assay.

The Ct-MS assay is faster to perform than the Ct-DT DEIA/RHA because no separate detection step (DEIA) is performed to select the Ct-positive samples for genotyping with the RHA. The algorithm of DEIA followed by RHA was developed to reduce cost by avoiding testing Ct-negative samples on the RHA nitrocellulose strip. Because the Ct-MS assay is liquid-based, no expensive strips are used. This made Ct genotyping immediately after the PCR cost efficient, certainly in a population with a high Ct prevalence. However, a Luminex 100 IS system is necessary for the Ct-MS assay, whereas the Ct-DT DEIA/RHA can be performed manually with standard laboratory equipment. Overall, the Ct-MS assay showed great potential for serovar distribution studies and future Ct vaccine trials in which large quantities of samples need to be genotyped.

A limitation in the current study was that some rare serovars (such as A, C, L1, L3) were not detected in the clinical samples ($n = 712$). Still, the analytical specificity of the assay was confirmed by testing Ct reference strains.

In conclusion, the new Ct-MS assay is a sensitive and specific alternative for the Ct-DT DEIA/RHA. The Ct-MS assay is a highly efficient new genotyping method due to its high throughput, especially in Ct genotyping studies with a high Ct-positivity rate.

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