

Genotyping of *Chlamydia trachomatis* strains from culture and clinical samples using an *ompA*-based DNA microarray assay

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ARTICLE INFO

Article history:

Received 11 August 2010

Accepted 29 September 2010

Available online 8 October 2010

Keywords:

Chlamydia trachomatis

ompA gene

Serotyping

Genotyping

Diagnostic DNA microarray test

Testing of clinical samples

ABSTRACT

Current typing methods of *Chlamydia* (*C.*) *trachomatis* are mainly based on the diversity of the *ompA* gene, which is coding for the major outer membrane protein A. The present study aimed at facilitating genotyping of strains of this obligate intracellular human pathogen by developing a DNA microarray assay using the ArrayTube™ format for individual samples and the ArrayStrip™ format for higher throughput.

The new test is exploiting multiple discriminatory sites by involving a total of 61 oligonucleotide probes representing genotype-specific polymorphisms in variable domains 1, 2 and 4 of the *ompA* gene. After multiplex amplification of these domains using biotinylated primers, the sample is hybridized in the microarray vessel under highly stringent conditions. The resulting binding pattern is genotype specific, thus allowing direct identification. We were able to show that DNA from each of the currently accepted genotypes (serovars) yielded a unique, theoretically expected and distinct hybridization pattern. The assay was also shown to be highly sensitive as a dilution containing the equivalent of 1 inclusion-forming unit was still correctly genotyped. In addition, when 62 clinical samples were examined and compared to PCR-RFLP typing results, the genotype was correctly identified by the DNA microarray in all cases.

The present test is easy to handle and economically affordable, and it allows genotyping of *C. trachomatis* to be accomplished within a working day, thus lending itself for epidemiological studies and routine diagnosis.

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1. Introduction

Chlamydia (*C.*) *trachomatis* infection is the most prevalent sexually transmitted bacterial disease and, therefore, a significant global health problem. It is estimated that 90 million cases occur annually worldwide [1]. The number of infected people is likely to be much higher, because 50% of the urogenital infections in men

and 80% in women are asymptomatic [2]. In addition, *C. trachomatis* is the agent of trachoma, the major cause of preventable blindness, which still affects millions of people in developing countries [3].

As *C. trachomatis* strains are known to be divergent at both genome and proteome levels, typing can be an important tool to reveal transmission pathways and associations with different tissue tropisms and pathogenicity. Up to now, 17 human serovars have been generally accepted, i.e. A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, K, L1, L2, and L3 [4,5]. While serovars A to C are commonly associated with trachoma, serovars D to K primarily cause urogenital infections with associated sequelae, such as inflammatory disease, ectopic pregnancy and infertility, and the L1 to L3 serovars are the agents of lymphogranuloma venereum, a more invasive sexually transmitted infection.

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The various approaches to strain typing used in the past decades have been reviewed recently by Pedersen et al. [6]. Serotyping based on specific epitopes on the major outer membrane protein A (MOMP) was the first widely used methodology [7–9]. MOMP represents one of the immunodominant antigens and is encoded by the *ompA* gene, which consists of four variable domains (VD1–4) that are flanked and separated by five conserved domains [10]. The inherent drawbacks of serotyping, which include limited sensitivity due to the requirement of culture and difficulties in identifying newly emerging types, facilitated the development of DNA-based typing methods in the 1990s. The combination of *ompA* gene amplification by PCR with restriction enzyme analysis was found to reveal characteristic polymorphisms allowing the identification of genotypes equivalent to the established serotypes [11–13]. The approach is known as restriction fragment length polymorphism (RFLP). As DNA sequencing technology became more easily available, complete sequences of the approximately 1215-nt *ompA* gene have been increasingly used to genotype *C. trachomatis* strains [10,14,15]. While PCR-RFLP requires considerable amounts of non-fragmented DNA and is difficult to standardize, *ompA* gene sequencing is very precise and can be used as a gold standard. However, although accurate, both methods lack high sensitivity and rapidity and are difficult to conduct in a routine diagnostic setting.

Alternative methodologies that were suggested for genotyping include reverse dot blot [16], a combination of PCR-DNA enzyme immunoassay with reverse hybridization [17], multiplex real-time PCR [18] and a microsphere suspension array [19].

DNA microarray technology has recently emerged as a promising alternative in microbial diagnosis. While most of the commercially available equipment is still expensive and out of reach for normal diagnostic laboratories, the ArrayTube™ (AT) and ArrayStrip™ (AS) platforms have been shown to work with routine diagnostic applications [20,21]. Their key components are a 3 × 3-mm or 4 × 4-mm microarrays implanted on the bottom of a 1.5-ml standard plastic reaction vial or a standard microtiter plate strip of 8 wells, respectively. Recent applications include the identification of *Chlamydiaceae* species [22] and genotyping of *C. psittaci* [23].

It was the aim of the present study to develop a DNA microarray assay for identification of *ompA*-based genotypes of *C. trachomatis* strains.

2. Materials and methods

2.1. Chlamydial strains

The list of *C. trachomatis* strains used for reference hybridization experiments is provided in Table 1.

2.2. Sequencing of the *ompA* gene

All *C. trachomatis* strains used as serovar references were re-sequenced to cover the complete *ompA* gene in order to verify the identity to GenBank entries given in Table 1. The sequencing methodology was described in reference [24].

2.3. In silico analysis of *ompA* sequences

All available sequences of the *ompA* gene of *C. trachomatis* were downloaded for analysis from the database of the National Center for Biotechnology Information (NCBI) at <http://www.ncbi.nlm.nih.gov/Genbank/>. Among a total of 381 entries, 54 unique *ompA* sequences were found, of which 49 contained the complete coding sequence of the gene (Supplement 1). These 54 sequences were included in a global *ompA* sequence alignment using the program

Table 1
C. trachomatis strains used as genotype references.

Strain	Genotype (Serovar)	Class	<i>ompA</i> sequence (GenBank acc. no.)
TW5	B	B	AF304856
Apache 2	Ba		DQ064282
IC-Cal8	D		DQ064285
MT-566	Da		X62921
DK-20	E		X52557
440	L1		DQ064294
434	L2		M14738
Har-1	A	C	DQ064279
C3	C		EU040365
UW-4	H		AF304328
UW-12	I		DQ064290
I'	Ia		AF202456
UW-36	J (Ja)		AF202457
UW-31	K		DQ064293
404	L3		DQ064296
UW-57	G	Intermediate	DQ064299
MRC-301	F	Complex	X52080

E-INS-1 of the MAFFT package [25]. The alignment is provided in Supplement 2.

2.4. Probes and microarray design

Analysis of discriminatory regions of the global alignment led to the definition of 61 oligonucleotide probes binding within VD1, VD2 and VD4 of the *ompA* gene of *C. trachomatis*, which were specific for one or several genotypes. Nucleotide sequences of all probes are provided in Supplement 3. The oligonucleotides had an average size of 25 nt (min. 22, max. 30), a melting temperature of 60 ± 1 °C, and a G + C content from 32 to 65 mol-%. Each probe sequence was checked by local BLAST analysis against all known *C. trachomatis ompA* sequences to verify the specificity of detection and identify possible cross-reactions. Each genotype probe was spotted three-fold on the microarray. Biotinylated oligonucleotide probes were included as staining controls ($n = 12$), thus bringing the total number of spots used for genotyping of *C. trachomatis* to 195. Fabrication of the AT microarrays was described previously [22]. The same panel of probes was also printed on AS chips, alongside probe panels representing the 23S rRNA genes of all *Chlamydiaceae* spp. and the *ompA* gene of *C. psittaci*. (The latter probe sections are not relevant for this study, but they are part of a pan-Chlamydia array for simultaneous species identification and genotyping.)

2.5. DNA extraction

Cell cultured strains and tissue samples were subjected to DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany).

Table 2
Primers used for multiplex biotinylation PCR.

Primer designation	Sequence (5'-3')	Product size (bp)
Trach-VD1-fw	Biotin-ACC AAG CCT TAT GAT CGA C	326
Trach-VD1-rv	Biotin-AGA ATA CAT CAA AAC GAT CCC A	
Trach-VD2-rv	Biotin-TTG AGC ATA TTG GAA AGA AGC	572 ^a
Trach-VD4-fw	Biotin-CTT ACA TTG GAG TTA AAT GGT CT	231
Trach-VD4-rv	Biotin-CTA CTG CAA TAC CGC AAG	

^a Product of VD1-fw/VD2-rv.

2.6. Multiplex biotinylation PCR

Chlamydial DNA from culture and clinical samples was amplified and biotin labeled using a multiplex PCR protocol, which included five *ompA* primers covering three VDs, i.e. VD1, VD2 and VD4. Primer sequences are given in Table 2. Each

20- μ l reaction contained 1 μ l of each primer (10 pmol), 10 μ l Multiplex Mastermix (QIAGEN, Hilden, Germany) 1 μ l sample DNA extract and 4 μ l water. The temperature-time profile was as follows: initial denaturation at 95 °C for 15 min, 40 cycles of 94 °C/30 s, 50 °C/90 s, 72 °C/90 s, and final elongation at 72 °C for 10 min.

Table 3

Match characteristics: Number of mismatches between hybridization probes and individual genotypes or subtypes (more than one number indicates intra-genotype variation).

Probe	Serovar/Genotype																		
	A	B	Ba	C1	C2	D	Da	E	F	G	H	I	Ia	J	Ja	K	L1	L2	L3
VD1-11	0	0,1	0		2		0,1	0,1	0,1	2		2	2	0,2	0		0	0	1
VD1-12	2	2,3	2	2	0,2	2,3	1,2	2,3	2,3	0,3	2	0	0,2	0,2	2	3	2	2	3
VD1-13				0	0,2	0	3	3		1,2	0	2	0,2	2		1			3
VD1-21		2	2			0,2	2	3						3			2	2,3	3
VD1-22		0	0			1,2	0,1	1,2									0	0,1	1
VD1-31		0,1	1			2	0,2	2,3								1		1,2	
VD1-32		1,2	0			3	1,3	2,3								0		2	
VD1-33						0	0,1											3	
VD1-41																			0
VD1-42									0,1	0									
VD1-51				3	1,2						0,1	1	1	1	2	1,2			1
VD1-52				0	1,2						3	2	2,3	2	3	2			2
VD1-53				2	0,1						1,2	0	0,1	0	1	0,2			0
VD1-54				1	0,1						2,3	1	1,2	1	2	1			1
VD1-61		0	0			0	0	0,1									0	0	
VD1-71	0			0	0							0	0	0	0	0			0
VD1-81									0	0,2									
VD2-101									1,2	1,2									0
VD2-102									0,1	0,1									1
VD2-11	0			0	0						0	0	0	0	0	0			0
VD2-111		3	3			3	3	3	0,1,2	0	3			2		2	3	1	
VD2-112	0			0	0				1,3	3	0,1	0	0	0,2	0	0,1,2		3	0
VD2-21		0	0				0	0,1											
VD2-31									0	0							0	0	
VD2-41		0	0															3	
VD2-51						0	0,1												
VD2-52					3						0,2	3	2,3						
VD2-53					3						0,2	3	3						
VD2-54									0,1,2										
VD2-61								0,1									3		
VD2-62																		0,1	
VD2-63								3									0		
VD2-64						0	0,1	3											
VD2-71	0													2,3	3	1,2,3			3
VD2-72		0,1	1,2																
VD2-73		0,1,2	0,1																
VD2-74		0,1,2	1,2																
VD2-75	1			2	2					2,3	2	2,3	0,1,2	1,2	0,1,3				1
VD2-76	2			1	1					1,2,3	1	1,2	1,2,3	1,2	1,2	1,2			0
VD2-77	2			3	3					3	3	3	0,1	1,2,3	1,3				2
VD2-81	1			0	0,2					3	2	2,3	1,2	1,2	1,3				1
VD2-82	0			1	1					2,3	1	1,2	0,1	0,1	0,2				0
VD2-83	1			2	0,2					3	0	0,1	1,2	1,2	1,3				1
VD2-84										0	0								
VD2-91								0,1											
VD2-92	0			0	0				3		0	0	0,1	0,1	0,1	0,1,2			0
VD4-11	0	3	3	0	0						0	0	0	0	0	0		3	0
VD4-21						0,1	0	0,1									1		
VD4-31		0	0																
VD4-32	0																		
VD4-41		0	0															0	
VD4-42						0,1	0,1	0,1,2	3	3							0		
VD4-43		3	3			2,3	2,3	2,3	0,1	0						2	3		
VD4-44				0															
VD4-45	0				0,1						0	0	0	0	0	0,1			0
VD4-46	1				0,1						1	1	1	1	1	1,2			1
VD4-61									0	0,1									
VD4-71	0											0							
VD4-72				0	0,2						2		1,2	2	2	1,2			3
VD4-73				2	0,2						0,1		0,1,2	0,1,2	1,2	1			
VD4-81	0			0	0					3	0	0	0	0	0	0,2			0

2.7. AS hybridization

ArrayStrip units consisting of 8 connected plastic vessels in microtiter format, each carrying a microarray chip, were used. The AS hybridization reactions were performed using the Hybridization Kit (Clondiag GmbH, Jena, Germany) following the instructions of the manufacturer. Briefly, the AS vessels were conditioned by washing with 200 μ l each of deionized water and Hybridization buffer C1 at 45 °C for 5 min. All incubations were conducted upon slight shaking (550 rpm) on a heatable horizontal tube shaker (Bioshake iQ; Quantifoil Instruments, Jena, Germany). For denaturation, 1 μ l of the multiplex PCR product was mixed with 99 μ l hybridization buffer in a separate tube, heated at 95 °C for 5 min and put on ice. After transfer into the AS vessel, hybridization was allowed to proceed at 45 °C for 60 min. After removal of the supernatant, each vessel of the strip was washed twice with 200 μ l Wash buffer C2 at 45 °C for 10 min, followed by incubation with 100 μ l of 1 \times horseradish peroxidase (HRP) conjugate (1 μ l 100 \times C3 HRP Conjugate + 99 μ l C4 Conjugate buffer from the kit) at 30 °C for 10 min. Subsequently, the strip was washed twice with 200 μ l wash buffer C5 per vessel at room temperature. Finally, 100 μ l of the HRP Substrate D1 were added to each vessel and left at 25 °C for 5 min. The supernatant was discarded and hybridization signals were measured using an Array-Mate transmission reader (Clondiag).

Alternatively, hybridization can also be conducted using the AT format and following a previously published protocol [26].

2.8. Processing of AS hybridization data using the pattern match algorithm

Hybridization signals were processed using the Iconoclust software, version 3.3 (Clondiag). Normalized intensities of the spots were calculated automatically by the software using the following equation: $NI = 1 - (M/BG)$ (where NI – the normalized intensity, M – average intensity of the automatically recognized spot, and BG – intensity of automatically recognized local background). NI values would theoretically range from 0 (no signal) to 1 (maximum signal).

An algorithm integrated in the Partisan ArrayLIMS database software system (Clondiag) to compare the measured signals of a given sample with theoretically expected and/or experimentally obtained hybridization patterns of 17 reference strains representing all *ompA* genotypes/serovars of *C. trachomatis* was used for automatic assignment and identification. The final output is given as the matching score (MS), which represents the sum of numerical differences between corresponding signal intensities of sample and theoretical and/or practical reference hybridization patterns. Thus, the MS value is a measure of the overall similarity between two hybridization patterns. An ideal match of two patterns based on the same set of oligonucleotide probes will yield $MS = 0$, whereas values greater than 20 represent a poor match that does not allow reliable genotyping. The arithmetic difference between best and second best match, termed Delta MS [26], indicates the reliability of a given genotyping result. Values of $\Delta MS \geq 0.5$ were regarded as representing a sufficient degree of distinction between best and second best matches.

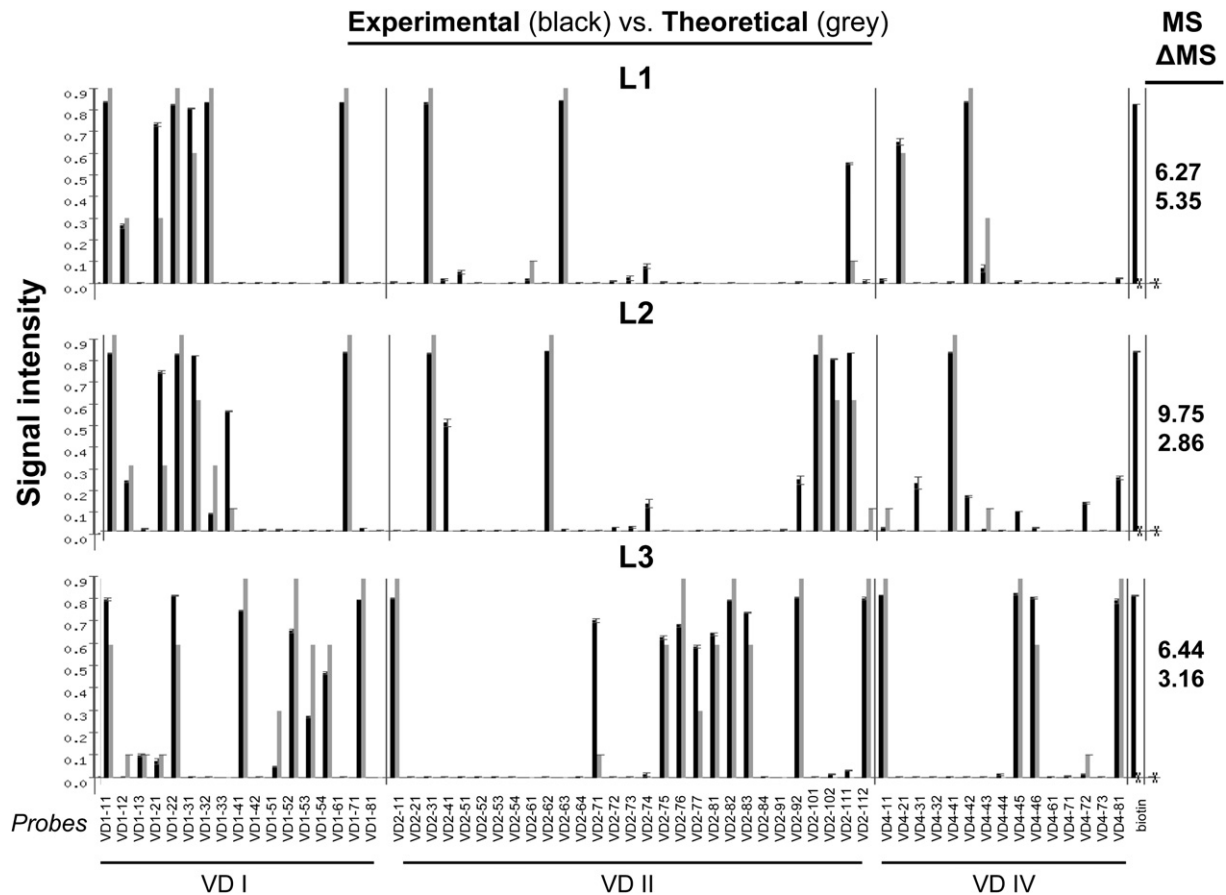


Fig. 1. Comparison of experimentally obtained (black bars) and theoretically constructed (grey bars) hybridization patterns of *C. trachomatis* genotypes L1 (strain 440), L2 (strain 434) and L3 (strain 404) using PatternMatch. The rightmost bar in each plot represents the signal of the internal staining control (biotinylated oligonucleotide probe). The matching score (MS) is a measure of similarity between sample and reference, and the Delta MS value represents the numerical difference between best and second best match.

2.9. Clinical samples

A total of 62 samples were examined. *C. trachomatis* strains isolated from consenting female Dutch Caucasian visitors of the Amsterdam STD outpatient clinic between 2001 and 2005 were propagated in eukaryotic HeLa cell cultures using standard techniques. The women were asked to fill out a questionnaire on urogenital complaints (i.e. vaginal discharge, contact bleeding, abdominal pain and dysuria; gastrointestinal and menstrual pain excluded). A selection of 58 strains representing the dominantly prevailing urogenital serovars was made for this study (designated A'dam 1 to 58).

In addition, three samples from women with asymptomatic genital infections and one man with unknown symptomatology were provided from Sweden (designated N59, N76, N19, and CV42, respectively).

2.10. PCR-RFLP

C. trachomatis typing was performed by amplification of the *ompA* gene (1.2 kbp) in a nested PCR using primers NLO/NRO (5'-ATG AAA AAA CTC TTG AAA TCG-3' and 5'-CTC AAC TGT AAC TGC GTA TTT-3) and sero1A/sero2A (5'-ATG AAA AAA CTC TTG AAA TCG G-3' and 5'-TTT CTA GA(T/C) TTC AT(T/C) TTG TT-3') as described previously for cervical and urethral swabs [11,27], as well as urine specimens [27]. The integrity of the PCR product was checked by

agarose gel electrophoresis. Subsequently, 10 μ l of the product was digested using different restriction enzymes (AluI, HinfI, EcoRI, DdeI, BstUI, CfoI). Serovars and variants were identified by their RFLP patterns after polyacrylamide gel electrophoresis [28].

3. Results

3.1. Analysis of *ompA* sequences and selection of hybridization probes

To identify potential target regions for the microarray typing assay we initially analyzed the diversity among naturally occurring *ompA* sequences. A GenBank search revealed a total of 381 entries with 54 unique sequences (Supplement 1). Based on the alignment of these sequences (Supplement 2), a split network graph illustrating the *ompA* sequence similarity among *C. trachomatis* genotypes (serovars) was constructed (Supplement 4). The graph confirmed the possibility of discriminating among the genotypes using the *ompA* gene locus.

The basic principles for probe selection included i) avoidance of cross-hybridization, ii) use of multiple probes per genotype, and iii) harmonization of biophysical parameters (melting temperature, G+C content, self annealing capacity). Analysis of the global alignment revealed both highly conserved and highly diverse sequence windows. The latter were located in VDs 1, 2 and 4 and served as focal points for probe design. In an iterative process,

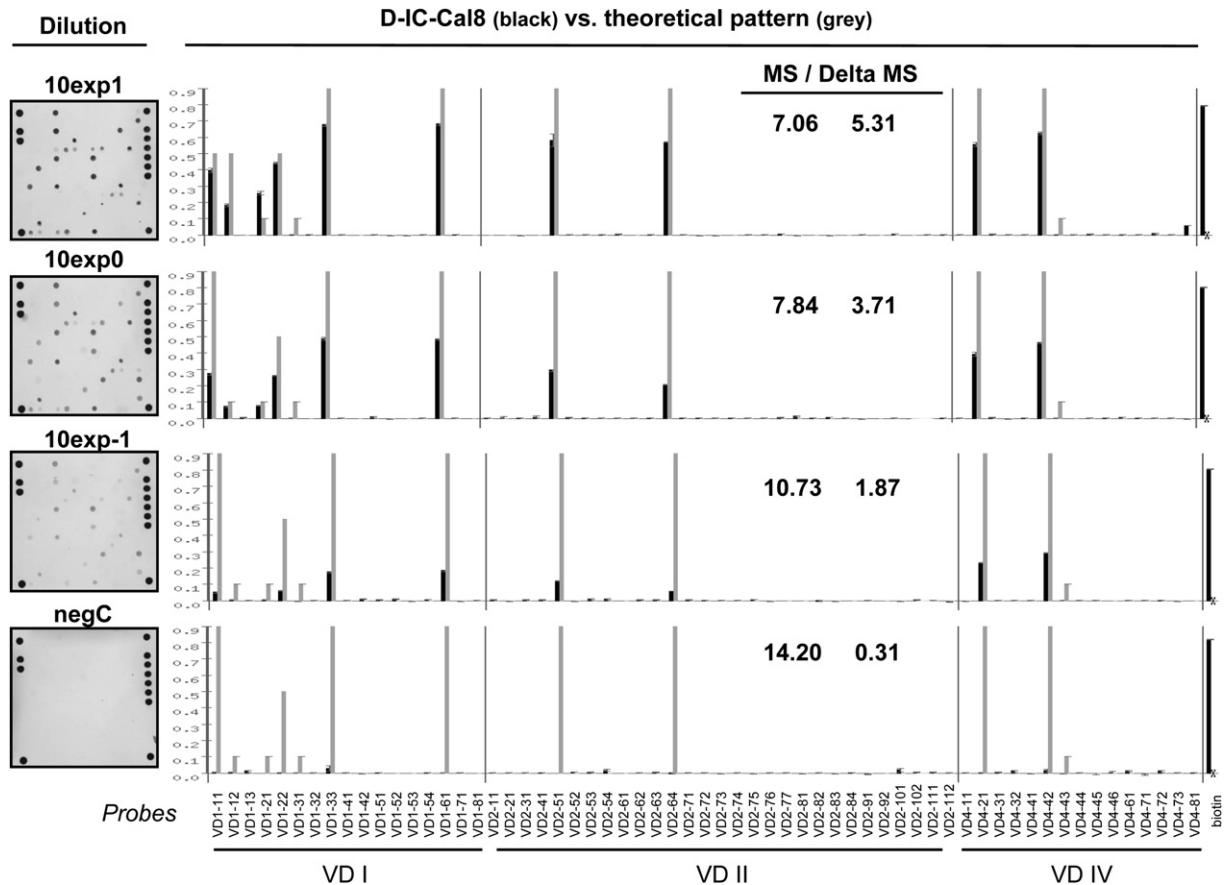


Fig. 2. Sensitivity of the microarray-based genotyping assay for *C. trachomatis*. DNA extracts of a decimal dilution series of *C. trachomatis* strain IC-Cal8 (serovar D) were amplified and biotinylated by multiplex PCR, hybridized, stained and processed using the Iconoclust program. For genotype identification, experimental hybridization patterns (black bars) are compared to theoretical reference strain patterns (grey bars; undiluted) using the PatternMatch.function. Signal intensities are given on the abscissa and denominations of the hybridization probes are shown on the ordinate.

a panel of 61 oligonucleotide probes binding to these segments was selected based on the alignment (Supplement 3).

A compilation showing the match characteristics, i.e. perfect match or number of mismatches, between hybridization probes and targets in the various genotypes is given in Table 3. These data illustrate the discriminatory capacity and can be used to predict signal intensities and construct theoretical hybridization patterns. As a result, we designed theoretical patterns of all genotype reference strains, where perfect target-probe matches were assumed to produce the strongest signals (100%), a single mismatch was assigned to a 60% signal, two mismatches to 30%, three to 10%, and more mismatches to the absence of a signal. Close similarity between theoretically expected and experimentally obtained hybridization patterns served as the central criterion in the optimization process of the microarray hybridization protocol.

3.2. Examination of type strains and processing of hybridization patterns

To validate the newly designed microarray, 17 strains representing all accepted serovars were examined (Table 1). Re-sequencing of the *ompA* gene locus of our strains confirmed the identity to GenBank entries in all instances (data not shown). The pre-microarray operations included multiplex amplification of VDs 1, 2, and 4 of the *ompA* gene using five biotinylated primers given in Table 2. The latter were labeled at the 5' end so that each newly synthesized strand carried two biotin residues, which are required for visualization. This approach ensured higher product yield (and, later on, higher sensitivity) as compared to simplex amplification of the whole *ompA* gene (data not shown). The PCR products were hybridized to the microarrays under stringent conditions, and the stained images were processed to yield bar diagrams. High stringency of hybridization was attained by using the Hybridization Kit (Clondiag) whose protocol had been optimized for temperature and salt concentrations at the hybridization and wash steps. Each genotype/serovar type strain was shown to generate a distinct hybridization pattern (Supplement 5).

The identification of genotypes from clinical isolates and tissue samples was based on comparison by superposition of hybridization patterns from sample and reference strains. To facilitate rapid processing of these complex patterns, the PatternMatch algorithm was used, which provides combined bar diagrams of sample and reference, as well as two numerical parameters to assess the similarity of two patterns and the accuracy of identification, i.e. the matching score (MS), and the Delta MS value, respectively (cf. Materials and Methods). We examined the possibility of using either experimentally obtained or theoretically constructed hybridization patterns of reference strains for the determination of genotypes in clinical samples. As the final results were the same in both approaches (data not shown) we decided to recommend the use of theoretical reference patterns for two reasons: i) theoretical patterns can be traced back directly to nucleotide sequences, and ii) in the case of newly emerging genotypes, it will be easy to add the corresponding hybridization pattern, even if a clinical isolate is not available. The high degree of similarity between theoretical and experimental hybridization patterns is shown in Fig. 1. It implies that in most cases hybridization signals were measured where predicted, i.e. at probes with 0 to 3 mismatches to their targets, while theoretical signal intensities deviated more often from experimentally obtained values because effects of secondary structure are more difficult to predict.

The analytical sensitivity of the present typing assay was evaluated by examining decimal dilution series of strains IC-Cal8 (serovar D, Fig. 2) and TW-5 (serovar B, not shown). The data show that, after PCR amplification, one inclusion-forming unit (ifu) was still typed correctly.

3.3. Examination of clinical isolates

To evaluate the performance of the microarray assay with real diagnostic samples, we blindly tested 62 clinical isolates that had been previously characterized by PCR-RFLP. Identification of the

Table 4

Comparison of test results on clinical *C. trachomatis* strains obtained from PCR-RFLP and ArrayStrip microarray genotyping.

Sample ID	Genovar PCR-RFLP	ArrayStrip (best match)	ArrayStrip (MS)	Delta MS	<i>ompA</i> sequence (acc. no)
A'dam 1	D	D	7.99	5.25	
A'dam 2	E	E	6.52	4.63	
A'dam 3	E	E	11.04	4.73	
A'dam 4	Ia	Ia	9.45 ^a	0.01	
A'dam 5	F	F	10.81	0.54	
A'dam 6	B	B	15.52	2.00	B (AF063208.1) ^b
A'dam 7	E	E	14.89 ^a	4.73	
A'dam 8	E	E	7.31	4.88	
A'dam 9	E	E	6.18	4.92	E (DQ064286.1) ^b
A'dam 10	E	E	10.27	4.71	
A'dam 11	E	E	7.11	4.92	
A'dam 12	E	E	6.29	4.92	
A'dam 13	E	E	9.03	4.92	
A'dam 14	F	F	5.41	1.80	
A'dam 15	E	E	13.23	5.38	
A'dam 16	E	E	5.54	4.99	
A'dam 17	F	F	6.43	1.92	
A'dam 18	Ia	Ia	7.53	2.23	
A'dam 19	E	E	8.68	5.05	
A'dam 20	E	E	11.60	4.46	
A'dam 21	E	E	9.55	5.04	
A'dam 22	F	F	6.81	1.78	
A'dam 23	E	E	11.20	2.99	
A'dam 24	E	E	12.38	4.79	
A'dam 25	E	E	5.52	5.69	
A'dam 26	E	E	12.61	4.67	
A'dam 27	E	E	9.70	4.99	
A'dam 28	F	F	7.29	1.13	
A'dam 29	E	E	13.60	4.11	
A'dam 30	E	E	10.45	4.53	
A'dam 31	E	E	6.37	5.26	
A'dam 32	F	F	6.12	2.15	
A'dam 33	F	F	6.17	1.96	
A'dam 34	E	E	12.94	3.98	
A'dam 35	E	E	7.09	5.38	
A'dam 36	F	F	14.51 ^a	1.74	
A'dam 37	F	F	5.50	2.12	
A'dam 38	F	F	6.22	1.68	
A'dam 39	J	J	7.27	2.54	
A'dam 40	F	F	6.29	2.01	
A'dam 42	I	I	9.35	2.40	
A'dam 43	H	H	6.58	2.32	H (AM179417.1) ^b
A'dam 44	I	I	8.01	1.84	
A'dam 45	K	K	10.62	0.48	
A'dam 46	E	E	15.10 ^a	0.56	
A'dam 47	Da	Da	7.40	2.36	D (DQ064285.1) ^c
A'dam 48	E	E	11.42	5.15	
A'dam 49	G	G	6.46	2.10	
A'dam 50	G	G	6.64	2.06	
A'dam 51	G	G	7.13	2.08	
A'dam 52	G	G	7.17	1.82	
A'dam 53	G	G	5.84	2.16	
A'dam 54	G	G	7.76	1.91	
A'dam 55	G	G	5.90	2.03	
A'dam 56	G	G	5.83	1.80	
A'dam 57	G	G	5.52	1.78	
A'dam 58	G	G	6.29	1.77	
N59	Ia	Ia	6.62	2.80	Ia (AF063201.2) ^b
N76	J	J	7.25	3.23	J (AF063202.2) ^b
N19	Da	Da	9.61	2.15	Da (X62921.1) ^c
CV42	L2b	L2a/b	16.30 ^a	1.33	L2b (AM884177.1) ^b

^a Low DNA concentration.

^b Sequence identical to GenBank accession number as indicated.

^c Single nucleotide mismatch with GenBank accession number as indicated.

genotypes was conducted using the PatternMatch algorithm. The results in Table 4 show that the microarray test correctly identified the genotypes in all cases.

In the case of sample A'dam4, the results of the microarray coincided with that of PCR-RFLP typing, but the Delta MS value of 0.01 indicated a high uncertainty, with the second and third best match being Ja and another Ia strain, respectively. Sample CV42 was also correctly identified, but, despite a Delta MS of 1.33, the high MS value of 16.30 would suggest a degree of uncertainty. However, it has to be noted that these two critical samples and three others were of relatively poor quality because they contained low concentrations of DNA (see Table 4). To show in principle that the assay was also working with clinical samples, DNA extracted from 12 urogenital swabs was PCR amplified and hybridized using the present protocol. The genotyping results were in complete agreement with *ompA* sequencing (data not shown). Altogether, the findings of this comparative study show the excellent performance of the newly developed genotyping test.

4. Discussion

The objective of our study was to facilitate *ompA* genotyping of *C. trachomatis* by developing a rapid and economical test based on a large number of discriminatory sites from the *ompA* gene. The present microarray carries a total of 61 oligonucleotide probes recognizing specific polymorphisms in VD1 (17 probes), VD2 (29) and VD4 (15). Upon hybridization, 5'-biotinylated amplicons of the respective domains are screened for their sequence homology to each individual probe. The resulting binding pattern is genotype specific, thus allowing direct identification.

Genotyping procedures based on DNA re-association have been used for some time. The first hybridization probe for *C. trachomatis* was published in 1989 by Dean et al. [29]. Membrane hybridization

tests were initially designed under the assumption that a single oligonucleotide probe per serovar located in one of the variable *ompA* domains was sufficient to identify and distinguish the various genotypes [16,17,30]. However, taking into account that Lysen et al. [15] found 34 polymorphic positions in a 990-nt coding region of this gene in a study of 188 specimens, it seems obvious that higher probe coverage is necessary to ensure accuracy of genotype identification. As the present microarray platform allows highly parallel screening for target sequences, we placed 3 to 5 probes for each serovar-equivalent genotype onto the array, so that not only the genotype involved can be identified unambiguously, but also intra-genotype sequence variations can be recognized. This approach is illustrated in Fig. 3, where the principle of discrimination between the frequently occurring genotypes D, E and F is shown.

Furthermore, several pairs and triplets of perfect-match and one-mismatch probes were added to the array. This was conceived to increase the accuracy of discrimination, because the probe pairs would always provide a high-intensity and a low-intensity signal, even if the absolute signal intensity values would vary depending on stringency of hybridization and binding efficiency. Examples of such probe counterparts include VD1-22/VD1-31/VD1-32, which was specifically designed to single out genotype E, VD2-72/73/4 (specific for B/Ba), VD2-101/102 (distinguishing F and G from the rest), and VD4-45/46 (identifying H, I/Ia, J/Ja, K and L3 as a group). The effect of nucleotide substitutions on signal intensity is also shown in Fig. 1. While probe VD1-32, which is perfectly matching its target in the L1 strain, shows a high-intensity signal (100%), its signal with L2 (two mismatches) is reduced to approximately 10%, and no hybridization is observed with the L3 strain (>3 mismatches).

As another crucial prerequisite for specificity of detection, the hybridized microarrays were processed at highly stringent conditions to distinguish up to single-nucleotide polymorphisms (SNPs).

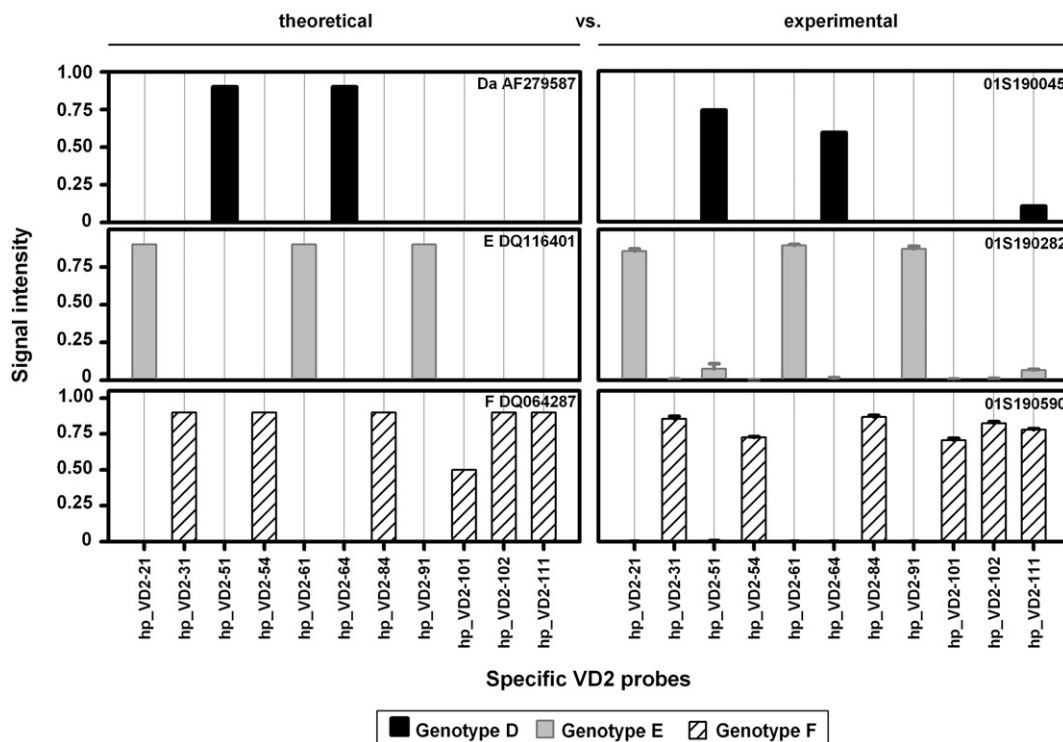


Fig. 3. Illustration of the principle underlying differentiation between genotypes D, E and F using the present microarray. Identification of these genotypes is based on the reaction of 2, 3 or 6 specific oligonucleotide probes, respectively, which were derived from VD2 of the *ompA* gene. The use of multiple specific probes ensures unambiguous assignment of hybridization patterns to *C. trachomatis* genotypes.

In the present protocol, the stepwise increase in stringency from one wash step to the next ensured the removal of non-specific targets that might have initially bound to the immobilized probes, because the hybridization itself was conducted at low stringency (45 °C). It is also pivotal that the required temperature is actually attained *inside* the hybridization vessel itself, which makes the choice of the thermal mixing device an important issue. The fact that experimentally obtained hybridization patterns closely matched the theoretically predicted patterns (Fig. 1 and Supplement 5), confirms the high level of stringency used and clearly illustrates the high specificity of detection. The detection limit of the present technology was previously shown to be comparable to that of real-time PCR, detecting 56 genome copies of *C. trachomatis* [31].

Simultaneous infections by two or more serovars of *C. trachomatis* are no rare events. Depending on the population studied and the detection method used, the proportion of these multiple infections was determined to be 2.4% [18], 3% [24], 8.7% [30], 13% [32] and even as high as 57% [33]. The present microarray assay has the potential to identify two individual serovars from a sample because the superposition of two different hybridization patterns will lead to unusually high values of the MS parameter, which in turn can be used as an indicator of mixed infection. Subsequently, direct graphical overlay of the sample's pattern with the reference patterns of suspected additional serovars will enable the investigator to also identify the second strain's genotype. To facilitate automatic identification, it is intended to extend the present PatternMatch algorithm by a function separating superimposed hybridization patterns. When we examined a series of mixed samples that contained different proportions of genotypes D and E or E and F using the microarray assay it was possible to identify both genotypes involved as long as their ratio was between 1:1 and 5:1 (data not shown). Preliminary data from an ongoing study in the corresponding author's laboratory indicate that the test also reliably identifies mixed serovar infections from real diagnostic samples.

In this context, the recently published microsphere suspension array [19] also represents a promising approach to genotyping mixed infections, because the probes coupled to microspheres can freely interact with their complementary targets in suspension. However, there seem to be limits to the degree of parallelity as only 8 genotypes of *C. trachomatis* were included. Likewise, although real-time PCR may be a promising choice to address mixed infections when the identity of the involved genotypes is known [18], its multiplexing capacity is limited to four or five channels (i.e. probes), and targets in natural intra-genotype variations can be missed causing false negative results.

Global comparison of the major performance parameters of the present genotyping assay with *ompA* gene sequencing and RFLP in Table 5 reveals rapidity, ease of operation and mixed serovar identification, as well as high throughput as its main assets.

The microarray platform used in the present study is open and flexible, so that iterative adaptation of probes and extension beyond the currently covered 17 serotypes will not pose a problem, for instance in the case of newly emerging types. In addition, the use of the pan-*Chlamydia* microarray will enable the diagnostician to simultaneously identify the *Chlamydiaceae* spp. involved (based on the 23S rRNA gene) and conduct genotyping of *C. trachomatis* (and *C. psittaci*, if applicable) based on *ompA* genes.

In view of the known limitations of *ompA*-based genotyping, recent studies recommended multi-locus sequence typing (MLST), i.e. typing schemes involving several genomic loci of *C. trachomatis* [34–36]. However, performing this procedure still requires considerable effort and there is no standardized MLST yet. In a future project, the present array could be extended to MLST format, thus yielding a more discriminatory tool for typing

Table 5
Comparison of *ompA* microarray genotyping with other established genotyping procedures.

Typing method	Time per assay	Major steps - Ease of operation	Specificity (Resolution of serovars)	Sensitivity (Sample material)	Cost per assay (Consumables)	Cost of equipment	Throughput	Detection of mixed serovar infection
RFLP	2–4 days (high proportion of hands-on time)	1. PCR 2. Enzymatic digestion 3. Acrylamide gel electrophoresis - Difficult to standardize	High (most serovars)	High, works with clinical samples, but may require more DNA if multiple digestions are required	High, due to multiple digestion and electrophoresis, as well as higher proportion of manual operations than other tests	Low	Low	Possible (for serovar ratios from 1:1 to 5:1)
<i>ompA</i> sequencing	2–4 days (if sent to commercial lab) 8h (in-house)	1. PCR 2. Sequencing 3. Data processing - Step 3 requires experience	High (all serovars, Gold standard)	Limited by integrity of sample DNA, but largely works with clinical samples	Moderate (ca. 15 €)	High	Potentially high	Possible, but requires experience
ArrayTube and ArrayStrip assays	6–8 h	1. PCR 2. Hybridization 3. Data processing - Easier than RFLP and sequencing	High (all serovars)	High, works with clinical samples	Moderate (ca. 20 €)	Approx. 25% of sequencing equipment	High (max. 32 per day per technician)	Possible (for serovar ratios from 1:1 to 5:1), best of the three tests

chlamydiae and differentiating strains according to tissue tropism and disease severity, which cannot be done with the present *ompA*-based version. The great potential of such a microarray-based MLST arises from the possibility to obtain hybridization signals, i.e. sequence homology data, from all target loci in a single run.

5. Conclusions

The main advantages of the present DNA microarray platform include the high parallelity in probing many different target regions, as well as rapidity (results available within one working day), ease of operation, compatibility with common laboratory equipment, and affordable costs. This *ompA* genotyping assay can easily be integrated in a routine diagnostic environment.

Conflict of interest statement

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the present paper.

Acknowledgments

The authors are grateful to Juergen Roedel, University of Jena, for providing reference strains of serovars A, B, Ba, C, E, F, G, H, K, L2, and L3. We acknowledge the contribution of Steffen Kube, who developed the PatternMatch algorithm. We thank Christine Gratzki, Simone Bettermann, Sabine Scharf, Ines Engelmann, Elke Müller, and Jana Sachtschal for excellent technical assistance. The study was supported by grant no. 01 KI 0720 of the German Federal Ministry for Education and Research (BMBF). The aims of this work are partially in line with the European EpiGenChlamydia Consortium, which is supported by the European Commission within the Sixth Framework Programme through contract no. LSHG-CT-2007-037637. In addition, part of the study was funded by the Sixth Framework Program of the European Commission, Proposal/Contract no.: 512061 (Network of Excellence 'European Virtual Institute for Functional Genomics of Bacterial Pathogens').

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.mcp.2010.09.004.

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