Genotyping of *Chlamydia trachomatis* serovars derived from heterosexual partners and a detailed genomic analysis of serovar F

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Abstract

Objective—To investigate *C trachomatis* serovars in contact-traced heterosexual partners.

Methods—Urogenital *Chlamydia trachomatis* isolates (n = 112) derived from 35 heterosexual patients (index patients) and their 37 chlamydia positive partners (contact patients) were differentiated into serovars by genotyping with restriction fragment length polymorphism (RFLP) analysis of the PCR amplified *omp1* gene. In order to investigate whether different strains within the frequently prevalent serovar F were transmitted, two pairs of serovar F (n = 4) were further analysed by genomic DNA fingerprinting with arbitrary primer PCR (AP-PCRs).

Results—Identical *C trachomatis* serovars were found in 31 of the 35 pairs, serovars E, F, D, and G being most prevalent. In the remaining four pairs different serovars (either D, E, F or G) were found between the index and the contact patients. By AP-PCR analysis the strains of serovar F were found to be identical between the index and the contact patients, but were different between the two pairs in all AP-PCRs used.

Conclusion—A majority of heterosexual partners, once traced positive for *C trachomatis* infections, are infected with identical serovars. Identical strains of serovar F found in partners as found by DNA fingerprinting confirms the sexual transmission of *C trachomatis*.

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Keywords: *Chlamydia trachomatis*; contacts; PCR

Introduction

Chlamydia trachomatis is very prevalent in urogenital infections and is transmitted through sexual contact. Fifteen major serovars have currently been classified: A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, and L3.¹ Serovars D to K are responsible for urogenital infections, of which serovars E, F and D account for up to 60-70% of these infections.²⁻⁵ To date epidemiological studies of *C trachomatis* infections in sexual contacts have been few,^{5a} which hampers the study of *C trachomatis* transmission, of the route of spread in a population, of chlamydial virulence factors and of insight into the associated risk factors of *C trachomatis* infections.

Typing of *C trachomatis* serovars has so far been limited to the identification with monoclonal antibodies (MAbs) (serotyping) of specific immunopeptides carried by the major outer membrane protein (Omp1).⁶⁻⁷ Analysis of the corresponding *omp1* gene has also been used for the typing of *C trachomatis* serovars.^{4-5 8-15} This was successfully achieved by using restriction fragment length polymorphism (RFLP) analysis,^{4-5 8-12} differential dot-blot hybridisation,¹³ or direct DNA sequencing of this *omp1* gene.^{14 15} The *omp1*-RFLP genotyping for differentiation of *C trachomatis* serovars was validated and found as a very reliable and easily-performed method as compared with the *omp1* serotyping.^{11 21} Epidemiological data have been obtained by using either serotyping or genotyping methods, whereby a number of *C trachomatis* substrains (serovariants or genovariants) have been identified.¹⁶⁻²¹

Amongst the most prevalent serovars D, E, and F, serovar D was found to possess extensive *omp1* genetic diversity, whereas serovars E and F had relatively stable *omp1* genes as shown by either RFLP⁴ or nucleotide sequence analysis of this gene.^{19-20 22} So far, these techniques have only been used for analysing the *omp1* gene. Techniques with greater discriminatory power capable of differentiating *C trachomatis* strains within a certain serovar are necessary for a higher resolution in transmission studies, particularly for the highly prevalent serovars D, E and F. With the advent of DNA fingerprinting technology, it is now possible to analyse genetic differences of closely related bacterial strains by studying genomic DNA.^{23 24} Recently, a highly sensitive DNA fingerprinting method by using arbitrary primer mediated amplification of the genomic DNA by polymerase chain reaction (AP-PCR) was introduced.²⁵ It was also shown that by using assay *C trachomatis* serovars could be differentiated into several groups according to DNA banding patterns generated, but the grouping was different from the current serovar grouping.²⁶ So far, this technique has not been used for epidemiological studies of chlamydial infections or for transmission studies of *C trachomatis* strains derived from the sexual partners.

In this study, *C trachomatis* strains isolated from heterosexual patients and their contacts, attending a sexually transmitted disease (STD) outpatient clinic, were analysed by using *omp1*-RFLP genotyping.¹²⁻¹³ To determine whether identical strains are transmitted between sexual partners, several serovar F iso-

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Table 1 List of primers used for AP-PCRs with chlamydial genomic DNA*

Code	Nucleotide sequence	Reference
ERIC1R	5'-ATGTAAGCTCCTGGGGATTAC-3'	23
ERIC 2	5'-AAGTAAGTGAAGTGGGGTGAGCG-3'	23
REP1RI	5'-XXXXCGXCGXCATCXGGC-3'	23
REP2I	5'-XCGXCTTATCXGGCCTAC-3'	23

*X represents nucleotide A, T, G or C.

lates were further analysed by chlamydial DNA fingerprinting using AP-PCRs.

Material and Methods

C trachomatis isolates

C trachomatis isolates (n = 112) obtained from 35 heterosexual patients (index patients) and their *C trachomatis*-infected partners (contact patients, n = 37), were included in this study. These patients²⁷ attended a STD clinic of Municipal Health Service, Amsterdam, between September 1986 and December 1988. All women were sampled from cervix, urethra and anus. All men were sampled from the urethra. HeLa 229 cell culture was used for *C trachomatis* detection in shell vials.²⁸ The index patients included 20 men and 15 women, and the 37 contact patients included 22 women and 15 men. The mean ages of women and men were 28 and 31 years, respectively. Homosexuals were not included in this study.

omp1-RFLP genotyping

The *C trachomatis omp1* gene was amplified by PCR as previously described,^{9,12} with primers SERO1A (5'-ATGAAAAAACTCTTGAAATCGG-3') and SERO2A (5'-TTTC-TAGA(T/C)TTCAT(T/C)TTGTT-3'). Briefly, 1 µl of *C trachomatis* isolate was heat denatured and pipetted into 49 µl of PCR reaction mixture, containing 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH8.3), 200 µM of each deoxynucleotide (dATP, dTTP, dGTP and dCTP), 50 pmol of each primer, and 1 U Taq polymerase (Amplitaq, Perkin Elmer, Branchburg, New Jersey). The PCR amplification was carried out in a thermocycler (Biomed, Theres, Germany) for 49 cycles of amplification, consisting of denaturation at 95°C for 1 min, annealing at 45°C for 2 min, and chain elongation at 72°C for 3 min. For RFLP genotyping the *omp1* PCR products were principally digested with AluI and analysed on a 7% polyacrylamide gel (acrylamide/bis-acrylamide: 29/1) to differentiate serovars Ba, D, E, F, G, K and C-complex serovars (C, J, H, I, L3). The C-complex serovars were further differentiated by digestion of the *omp1* PCR products with HinfI and the combination of EcoRI and DdeI. Serovar D was further differentiated into D₁ or D₂ by CfoI.¹²

Arbitrary primer PCR

C trachomatis genomic DNA was obtained after purification of the elementary bodies (EBs) with a slightly modified protocol as previously described.²⁸ *C trachomatis* isolates cultured in large quantities in HeLa229 cells in

six-well microtiter plates (Becton Dickinson, UK) were harvested in phosphate buffered saline (PBS). The cell suspension was sonicated 3 times for 10 s to break down the HeLa229 cells and subsequently centrifuged at 500 × g for 15 min to remove the cell debris. The supernatant was centrifuged for 30 min at 25000 × g at 20°C. The pellet was resuspended in PBS and purified by centrifugation for 45 min at 25000 × g at 20°C through a layer of 35% sodium diatrizoate (Sigma). The pellet containing the EBs was digested with DNase-I to eradicate HeLa229 DNA. After heat inactivation (10 min at 65°C) the EBs were lysed with proteinase K (50 µg/ml) and Tween 20 (0.45%).¹² The suspension was extracted twice with phenol/chloroform (24:25), and the chlamydial DNA was precipitated with ethanol and resuspended in Tris-HCl (pH8.3). The purity of chlamydial DNA was confirmed by failure to detect human β-globin with PCR.²⁹ Human liver DNA was used as a positive control for the β-globin PCR. Thereafter, a portion of 50 ng of purified chlamydial DNA was amplified in different AP-PCRs with different primers (Table 1). The AP-PCR was performed for 40 cycles of amplification, of which each cycle consisted of denaturation at 95°C for 1 min, annealing at 25°C for 1 min and chain elongation at 72°C for 3 min. The PCR reaction mixture was identical to that used for the *omp1* PCR, except that 2.5 mM MgCl₂ was used. HeLa229 DNA was used in order to ensure that HeLa229 DNA, if present in such a minimal amount that was not detected by β-globin PCR, was not interfering with the chlamydial DNA amplification in these low stringency AP-PCRs. The amplified fragments of DNA were analysed with 2% agarose gel electrophoresis.

Results

Thirty-five pairs of isolates from patients and contacts with chlamydial urogenital infections were investigated for their *C trachomatis* serovars. By *omp1*-RFLP genotyping identical serovars were found in 31 of the 35 pairs (Table 2 and pairs 1–3 in table 3). Among these 31 pairs, it was possible to trace a single partner who was chlamydia positive in 28 index patients. Of these 28 pairs, the cervixes of the female partners was positive in 27, the urethra and anus being variably positive, whereas in one *C trachomatis* infection was

Table 2 *C trachomatis* serovar distributions in 28 contact-traced heterosexual partnerships with identical serovars regardless of sampling sites

Serovar	No of serovars (in pairs)		
	Men as index	Women as index	Total
D	2	1	3
E	6	5	11
F	7	2	9
G		2	2
H		1	1
I	1		1
K	1		1
Total	17	11	28

Table 3 *C. trachomatis* serovar distributions in 7 heterosexual partnerships having i) the same serovar infections in one of the two partners (pair 1), ii) infections with identical serovars to only one of the two serovars (pairs 2–3), or iii) infections with different serovars (pairs 4–7)

Pair	Index patient			Contact 1			Contact 2		
	sex	site	serovar	sex	site	serovar	sex	site	serovar
i): 1	f	Cx/Ure	E	m	Ure	E	f	Cx/Ure	D
ii): 2	m	Ure	H	f	Cx	H			
3	f	Cx	F	m	Ure	D			
		Ure	E			F			
iii): 4	m	Ure	D	f	Cx/Ure/An	E			
5	f	An	E	m	Ure	F			
6	m	Ure	F	f	Cx	D			
7	m	Ure	F	f	Cx/Ure	G	f	Cx/Ure/An	G

f = female, m = male, Cx = cervix, Ure = urethra, An = anus.

only detected in the urethra and anus. Of the remaining 3 of the 31 pairs, one pair had two contact partners traced who were chlamydia positive but only one of the contact partners was infected with an identical serovar as the index patients (Table 3, case 1); in the other two pairs the women had two different serovar infections in the cervix and urethra, while the male partners were infected with the serovars identical to those found in the cervix (Table 3, pairs 2 and 3). These 3 cases (Table 3, pairs 1–3) were therefore considered as pairs infected with the same serovars. Of these 31 pairs (Table 2 and pairs 1–3 in table 3), serovar D was found in 3 pairs, E in 12, F in 10, G in 2, H in 2, J in 1, and K in 1. No serovar I or serovariants Da and D⁻ were found. When the men in the index group were analysed, the prevalence of serovar distribution was in a decreasing order of serovars F, E, D, and H/J/K. When the females in the index group were analysed, the prevalence of serovar distribution was in a decreasing order of serovars E, F, G, and D/H. In the remaining 4 of the 35 pairs, different serovars were found between the index patients and their contact partners. Either serovar D, E, F or G was involved in these 4 pairs. In these 4 pairs, 3 had single contact partners (Table 3, pairs 4–6) and 1 had 2 contact partners traced who were *C. trachomatis* positive (Table 3, pair 7).

Serovar F isolates derived from two pairs were analysed by DNA fingerprinting with 4 different AP-PCRs. For this purpose, EBs were isolated and the purified DNA was shown to be free of HeLa229 DNA contamination by a negative human β -globin PCR.³⁰ An additional *omp1*-RFLP genotyping of these purified chlamydial DNAs confirmed their initial genotyping results as serovar F. Genomic DNA fingerprinting by AP-PCRs showed that each of the primers (ERIC1, ERIC2, REP1, or REP2) gave rise to specific amplified DNA banding profiles in all isolates tested, which could be clearly distinguished from the profiles of HeLa229 control. The DNA banding profiles with either ERIC1 or ERIC2 primers in the PCR were more clearly visible and discriminative after gel electrophoresis than those generated by either REP1 or REP2 primers. The F strains belonging to the same pairs were identical in all AP-PCRs tested. In contrast, the F strains of the two confirmed but non-related pairs, showed two different DNA banding profiles in all AP-PCRs analysed. The figure shows a representative AP-PCR analysis of the serovar F isolates from these two pairs using primer ERIC2.

Discussion

This study showed that the majority of the heterosexual pairs (31 of the 35 pairs) with chlamydial urogenital infections were infected with identical serovars. Serovars E, F, and D were the most prevalent types found in these 31 pairs (Table 2 and pairs 1–3 in table 3). The frequency of the serovar distributions was similar to those found in STD populations in other European countries.^{2,3,5} The results found in this study are in agreement with those obtained by Viscidi *et al.*,^{5a} who found identical serovars (identified by nucleotide sequencing of a small fragment of the *omp1* gene) in a smaller group of 8 pairs. In addition, a slight difference in serovar F prevalence was observed between male index patients (7 of the 17 pairs in table 2) and female index patients (2 of the 11 pairs in table 2). In another report, serovar F was found to be associated with fewer clinical symptoms in women with cervicitis and pelvic

DNA banding profiles from a representative AP-PCR analysis with primer ERIC2 in two pairs of *C. trachomatis* serovar F strains (pair 1: lanes 2 and 3, pair 2: lanes 4 and 5) using purified EB DNA. The strains derived from the partners were identical (lanes 2 and 3, lanes 4 and 5), while the strains from the different pairs (pairs 1 and 2) were different. Lane 1 represents a molecular weight marker with pBR322 DNA digested with *Hinf*I. Lane 6 is an AP-PCR control using HeLa229, and lane 7 is a negative control. bp = base pair.

inflammatory disease.³⁰ In this study, in general all the male index patients were symptomatic whereas not all female index patients were found to have specific urogenital symptoms. Although the number of pairs in this study was small, these data may reflect the fact that the virulence of serovar F is different in men and women.

It is worth noting that different serovar infections were found in different anatomical sites in two female patients of two pairs (Table 3, pairs 2–3). One was a contact patient infected with serovar H in the cervix and D in the urethra, and the other one was an index patient infected with serovar H in the cervix and D in the urethra, and the other one was an index patient infected with serovar F in the cervix and E in the urethra. Interestingly, both of their male partners were infected with a single serovar identical to the one found in the female cervix. In addition, in those 28 pairs with identical serovar infections (Table 2), a large majority of the female partners (27 pairs) had *C trachomatis* identified in the cervix, no matter whether or not it was identified in urethra or anus. These data emphasise that *C trachomatis* transmission might be restricted to the site of actual contact, that is, the cervix of women and the urethra of men.

In this study, chlamydial infections with different serovars between the index patients and their contacts were observed in five pairs (Table 3, pair 1 (the second contact) and pairs 4 to 7). One explanation may be that in these pairs double serovar infections existed, but were missed possibly because of preferential growth of one serovar in the cell culture. Unfortunately, the original specimens were no longer available for PCR testing. It would be extremely interesting to determine whether double serovar infections exist in such patients because certain serovars might be more virulent than others resulting in preferential transmission, or one of the serovars might be more susceptible to the host immune response and could be eradicated after entering a new host. Alternatively, one of the members of the pair may have already been infected with another *C trachomatis* serovar which prevented infection by the newly introduced serovar.

Serovar F has been found to be one of the most prevalent types in different epidemiological studies.^{2–5} Since the genetic variation in its *omp1* gene was found to be very low,^{4,20,22} the analysis of the *omp1* either by RFLP or by nucleotide sequencing is not suitable for discriminating between different isolates. With AP-PCR, a technique with greater discriminatory power, it is shown in this study that the F strains derived from contact partners were identical, while F strains from the non-partners studied were genetically different. Although the genetic variation of F strains needs to be studied in more isolates, the AP-PCR results so far clearly show the sexual transmission from an individual strain between sex partners. The fact that the strains derived from partners were identical, also

demonstrates that the AP-PCRs were reproducible and suitable for analysing chlamydial genomic DNA. Whether the genetic differences found in serovar F isolates are related to differences in cell biology or in virulence in chlamydial infections needs to be further investigated. In future studies a more simplified test for individual fingerprinting should be developed, and strain variations in a larger group of serovar F isolates as well as other frequently prevalent serovars (such as D and E) need to be investigated. This could be of great value for *C trachomatis* contact studies.

In conclusion, this study shows that in urogenital *C trachomatis* infections the majority of heterosexual partners, when identified to be chlamydia positive, are infected with identical serovars. The transmission of a certain serovar might be more dependent on the anatomical site of the actual contact. The fact that the highly prevalent serovar F does possess genetic variation warrants a future contact study of *C trachomatis* molecular epidemiology based on genomic DNA analysis with AP-PCR.

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