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Distribution of *Chlamydia trachomatis ompA* genotypes in patients attending a sexually transmitted disease outpatient clinic in New Delhi, India

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***Background & objectives*: Limited data are available on the typing of *Chlamydia trachomatis* in India. Serovars D to K of *C. trachomatis* are chiefly responsible for urogenital infections. Thus, this study was conducted to determine the distribution of *C. trachomatis* serovars in patients with urogenital infections and to characterize *ompA* gene of the detected *C. trachomatis* isolates by sequence analysis. Presence of other co-infections was also evaluated.**

***Methods*: Endocervical swabs were collected from 324 women and urethral swabs/urine were collected from 193 men attending the sexually transmitted diseases outpatient clinic. The samples were screened for *C. trachomatis* by cryptic plasmid PCR and *ompA* gene PCR. Genotyping was performed by PCR-restriction fragment length polymorphism (RFLP) and sequencing of the *ompA* gene. Samples were screened for genital mycoplasmas, *Neisseria gonorrhoeae*, *Treponema pallidum* and human immunodeficiency virus (HIV).**

***Results*: *C. trachomatis* was found in 15.0 per cent men and 10.8 per cent women. Serovar D was the most prevalent followed by serovars E, F, I and G. Twenty two *C. trachomatis* isolates were selected for *ompA* gene sequencing. No mixed infection was found. Variability in *ompA* sequences was seen in 31.8 per cent cases. Both PCR-RFLP and *ompA* gene sequencing showed concordant results. The presence of *Ureaplasma* spp. and *Mycoplasma hominis* was observed in 18.7 and 9.5 per cent patients, respectively. Co-infection of *C. trachomatis* was significantly associated with *Ureaplasma urealyticum* and HIV.**

***Interpretation & conclusions*: The high occurence of *C. trachomatis* infections warrants its screening in addition to other sexually transmitted infections namely *U. urealyticum* and HIV. Genotyping of the *ompA* gene may provide additional information for vaccine development.**

Key words *Chlamydia trachomatis* - *ompA* gene - PCR-RFLP - sequencing - serovars

Chlamydia trachomatis urogenital infections are the most commonly reported bacterial sexually transmitted diseases (STDs)[1]. Genital infections with *C. trachomatis* (serovars D-K) are associated with urethritis, pelvic inflammatory disease and infertility[2]. *C. trachomatis* infection facilitates the transmission

of human immunodeficiency virus (HIV) and is often associated with other STDs[3].

The major outer membrane protein (MOMP) of *C. trachomatis* contains specific antigens that differentiate chlamydial strains by serovars (based on antigenic cross-reactivity on microimmunofluorescence) or genotype (based on nucleotide sequencing of the *omp*A gene)[4]. Whether different serovars of *C. trachomatis* demonstrate different virulence potential is unclear. Genotyping of *C. trachomatis* strains is important to monitor contact tracing, to enable thorough understanding of the pathogenesis and epidemiology of genital chlamydial infections[5]. The genital mycoplasmas include *Ureaplasma* spp. and *Mycoplasma hominis* which are potentially pathogenic species playing an aetiologic role in genital infections. Most colonized individuals remain asymptomatic, but there are considerable evidences that *M. hominis* and *Ureaplasma* spp. also cause disease[6].

Limited information is available on the typing of *C. trachomatis* from India. The aim of the present study was to determine the presence of *C. trachomatis* infection in patients with urogenital infections, to characterize the *omp*A gene of the detected *C. trachomatis* isolates by sequence analysis of DNA, to determine the occurrence of other aetiological agents (*viz*. *Ureaplasma* spp. and *M. hominis*) and to evaluate the presence of STD co-infections.

Material & Methods

All consecutive sexually active adults attending the STD Outpatient Clinic at the All India Institute of Medical Sciences, New Delhi, India, from February 2009 to February 2014, were included in the study. Patients who had been treated with antibiotics within the past four weeks and those who tested positive for bacterial vaginosis and *Candida* were excluded, whereas patients tested positive for *Niesseria gonorrhoeae*, *Treponema pallidum* and HIV were included in the study.

A total of 517 patients (324 women and 193 men) were eligible for enrolment. Ethical Committee approval for study protocol and written informed patient consents were taken for this study.

Three Dacron-tipped endocervical swabs from women and three urethral swabs were collected from men. Twenty millilitres of the first void urine (FVU) was also collected from men. The first swab was transported to the laboratory in 0.2 M sucrose phosphate buffer chlamydial transport medium (7.5 g sucrose, 0.052 g KH_2PO_4, 0.122 g K_2HPO_4, 72 g glutamine, 10 µg/ml gentamycin, 10 µg/ml amphotericin B) for *C. trachomatis* PCR assays. Two swabs were placed in two screw cap test tubes containing 2 ml pleuropneumonia-like organism (PPLO) broth for detection of *Ureaplasma* spp. and *M. hominis*. The PPLO broth for *Ureaplasma* spp. contained 2.1 g PPLO broth (Difco, USA), yeast extract (25%), horse serum (unheated), urea solution (50% w/v), penicillin solution (10^4 units/ml), trimethoprim (7.5 mg/ml), and phenol red (0.2 % w/v), and for *M. hominis* 2.1 g PPLO broth, yeast extract (25%), horse serum (unheated), arginine (20%), penicillin solution (10^4 units/ml), trimethoprim (7.5 mg/ml), thallium acetate solution (1/80 w/v), and phenol red (0.2% w/v). DNA was extracted from all the three swabs and FVU using QIAamp Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was stored at −20°C till further use. The reference strains from National Collection of Type Culture *Ureaplasma* (NCTC10177) and *M. hominis* (NCTC10111) were used as positive controls.

Cryptic plasmid PCR: *C. trachomatis* DNA was detected by PCR targeting a sequence of the cryptic plasmid using primers KL-1 and KL-2 as described by Mahony *et al*[7]. Template DNA of *C. trachomatis* serovar D ATCC VR-885 (also known as prototype strain D/UW3) was used as positive control in each PCR assay.

*Omp*A *gene PCR*: Samples positive for *C. trachomatis* by cryptic plasmid PCR were confirmed by a second PCR targeting the *omp*A gene using primers NLO and NRO, and nested *omp*A gene PCRs were run using 5 µl of *omp*A primary PCR products using primers NLI and NRI as described by Gao *et al*[8]. Template DNA of *C. trachomatis* serovar L2 was used as positive control in each PCR reaction.

Multiplex PCR for Ureaplasma spp. and Mycoplasma hominis: In addition to culture, multiplex PCR was performed for the detection of genital mycoplasmas with primers specific for urease gene of *Ureaplasma* and 16S rRNA gene of *M. hominis*[9]. All the isolates of *Ureaplasma* were further biotyped in a second PCR targeting the multiple banded antigen (MBA) gene[10]. PCR positive for biovar 1 was further subtyped into serovars as described earlier by De Francesco *et al*[11].

Restriction fragment length polymorphism (RFLP) and genotyping: Ten microlitres of the nested *omp*A PCR products were digested with 1 unit *Alu*I (New England Biolabs, MA, USA). Products were electrophoresed through a polyacrylamide gel (acrylamide/bisacrylamide, 29:1; 12 V/cm for 1.5 h) to enable the identification of serovars D to K (Figure A). Further, Serovars H and I were separated with 1 unit of *Hha*I (Figure B). The digested products were electrophoresed through a seven per cent polyacrylamide gel (acrylamide/bisacrylamide, 29:1; 1× tris-borate-EDTA; 5 V/cm, 1.5 h) to differentiate serotypes.

Sequencing of the ompA gene: The sequencing of the *omp*A gene was carried out on ABI PRISM 310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDye DNA sequencing kit (PE Biosystems) using primers NLI: 5'-TTTGCCGCTTTGAGTTCTGCT-3' and NRI: 5'-CCGCAAGATTTTCTAGATTTC-3'according to the manufacturer's instructions[8].

Phylogenetic analysis: Sequences were manually aligned and adjusted to prototype sequences. Phylogenetic analysis was performed by using the maximum likelihood method implemented in MEGA6 program[12].

Statistical analysis: All statistical analyses were performed using STATA version 11.2 software (STATA Corp LP, College Station, TX, USA). The difference in the clinical features and sociodemographic data was analyzed by multivariate analysis.

Results

C. trachomatis was detected in 12.3 per cent (64/517) of patients with urogenital infections, of whom 10.8 per cent (35/324) were women and 15.0 per cent (29/193) were men. Of the 38 couples enrolled in the study, 11 were found to be infected; the infection involved partners in 54.5 per cent (6/11) of couples, only the male partner in 36.4 per cent (4/11) and only the female partner in 9.1 per cent (1/11) of couples.

The presenting symptoms and demographic characteristics of patients with urogenital infections in relation to *C. trachomatis* status are given in Table I. The mean age of the patients was 31.59±8.64 yr. In women, *C. trachomatis* infection was significantly associated with vaginal discharge (P=0.007), abdominal pain (P=0.04), low back pain (P=0.02), burning micturition (P<0.05) and unprotected sex (P=0.008).

Of the 517 patients, 18.7 per cent (97/517) were positive for *Ureaplasma* spp., 9.5 per cent (49/517) for *M. hominis*, 1.2 per cent (6/517) for *N. gonorrhoeae*, 0.6 per cent (3/517) for *T. pallidum* and 4.6 per cent (24/517) were HIV seropositive. Co-infection with *Ureaplasma* and *M. hominis* was detected in 6.2 per cent (32/517) of patients by culture and/or PCR. *U. parvum* (biovar 1) was detected in 84.0 per cent (81/97) and *Ureaplasma urealyticum* was detected in 16.5 per cent

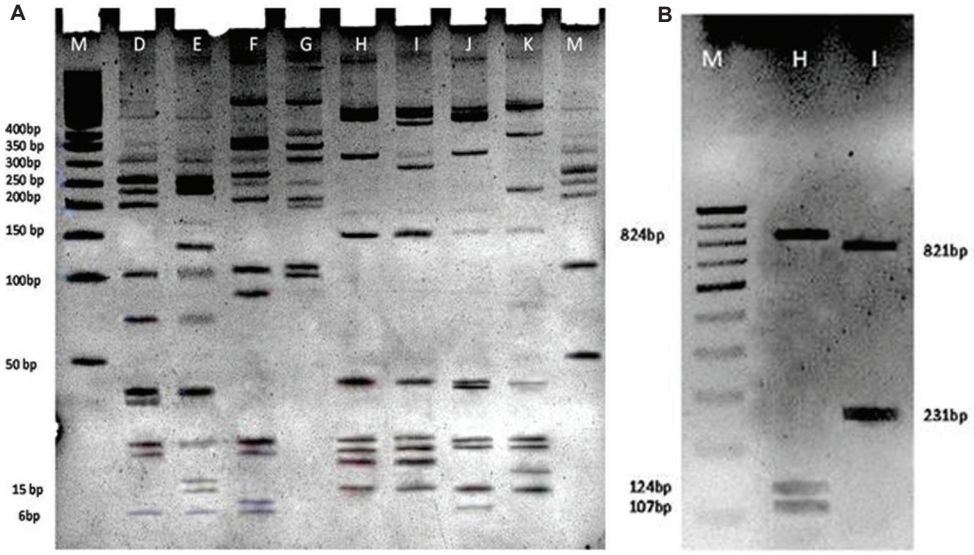

Figure. (**A**) Restriction fragment length polymorphism patterns of *omp*A gene after restriction with *Alu*I for differentiating serovars D to K. (**B**) Restriction fragment length polymorphism patterns of *omp*A gene after restriction with *Hha*I for differentiating serovars H and I.

Table I. Demographic and clinical characteristics in patients with (n=64) and without (n=453) *Chlamydia trachomatis* infection from patients with urogenital infections

Characteristics	*C. trachomatis* positive (%), (n=64)	*C. trachomatis* negative (%), (n=453)	*P*	Crude OR	95% CI
Demographic characteristics					
Sex					
Male	30 (46.9)	163 (36.0)	0.14	0.67	0.39-1.15
Female	34 (53.1)	290 (64.0)			
Age group (yr)					
<30	28 (43.8)	202 (44.6)	0.7	1.21	0.62-2.35
30-34	16 (25.0)	96 (21.2)			
35+	20 (31.3)	155 (34.2)			
Education level					
Illiterate	13 (20.3)	78 (17.2)	0.67	0.85	0.38-1.97
Primary	20 (31.3)	141 (31.1)			
High school	29 (45.3)	157 (34.7)			
University	2 (3.1)	77 (17.0)			
Clinical characteristics					
Cervicitis*					
Yes	34 (53.1)	290 (64.0)	0.6	1.11	0.65-1.88
No	30 (46.9)	163 (36.0)			
Urethritis†					
Yes	28 (43.8)	149 (32.9)	0.08	1.58	0.93-2.69
No	36 (56.2)	304 (67.1)			
Vaginal discharge*					
Yes	27 (42.2)	290 (64.0)	0.007	5.76	1.37-24.09
No	37 (57.8)	163 (36.0)			
Abdominal pain*					
Yes	9 (14.1)	31 (6.8)	0.04	2.22	1.0-4.92
No	55 (85.94)	422 (93.2)			
Low back pain*					
Yes	3 (4.7)	67 (14.8)	0.02	0.28	0.05-0.91
No	61 (95.3)	386 (85.2)			
Burning micturition					
Yes	43 (67.2)	188 (41.5)	<0.05	2.88	1.65-5.02
No	21 (32.8)	265 (58.5)			
Pruritis					
Yes	29 (45.3)	172 (38.0)	0.25	1.35	0.77-2.37
No	35 (54.7)	281 (62.0)			
Dyspareunia*					
Yes	11 (17.2)	43 (9.5)	0.06	1.97	0.96-4.07
No	53 (82.8)	410 (90.5)			

Contd...

Characteristics	*C. trachomatis* positive (%), (n=64)	*C. trachomatis* negative (%), (n=453)	*P*	Crude OR	95% CI
Risk factors associated					
Protection					
Yes	2 (3.1)	70 (15.5)	0.008	0.18	0.02-0.69
No	62 (96.9)	383 (84.5)			
Sexual history					
One partner	50 (78.1)	365 (80.6)	0.4	1.02	0.51-2.06
Two partners	11 (17.2)	78 (17.2)			
>Two partners	3 (4.69)	10 (2.2)			
Source of infection					
ME	51 (79.7)	362 (79.9)	0.9	1.01	0.52-1.94
EME+PME	13 (20.3)	91 (20.1)			

*Female patients only; †Male patients only. ME, marital exposure; EME, extramarital exposure; PME, premarital exposure; *C. trachomatis, Chlamydia trachomatis*

Table II. *Chlamydia trachomatis* co-infection with genital mycoplasmas, *Treponema pallidum*, HIV and *Neisseria gonorrhoeae*

	C. trachomatis infection					
Co-infecting pathogens	Positive (%) (n=64)	Negative (%) (n=453)	Total (n=517)	*P*	OR	95% CI
Ureaplasma spp.						
Yes	11 (22.4)	38 (77.6)	49 (100)	0.02	2.27	0.98-4.86
No	53 (11.3)	415 (88.7)	468 (100)			
Mycoplasma hominis						
Yes	14 (14.3)	84 (85.7)	98 (100)	0.52	1.23	0.60-2.39
No	50 (12.0)	369 (88.0)	419 (100)			
Treponema pallidum						
Yes	3 (100)	0	3 (100)	_	_	_
No	61 (11.9)	453 (88.1)	514 (100)			
HIV						
Yes	12 (50.0)	12 (50.0)	24 (100)	0.001	8.48	3.27-21.68
No	52 (10.5)	441 (89.5)	493 (100)			
Neisseria gonorrhoeae						
Yes	2 (100)	0	2 (100)	_	_	_
No	62 (12.0)	453 (88.0)	515 (100)			

(16/97) of patients. None of the patients were infected with both biovars. *U. parvum* isolates were further subtyped into different serovars. Serovar 3/14 (58.0%; 47/81) was the most frequent isolate followed by serovar 1 (27.2%; 22/81) and serovar 6 (14.8%; 12/81).

Multivariate analysis was done to find the possible association of *C. trachomatis* co-infection with other STDs (Table II). The rate of co-infection of *C. trachomatis* with HIV [18.8% (12/64); *P*=0.001; OR=8.48; 95% CI=3.27-21.68] and *Ureaplasma* spp. (17.2% (11/64); *P*=0.02; OR=2.27; 95% CI=0.98-4.86) was significantly associated, whereas no significant association was found between *M. hominis* (21.9%; 14/64), *N. gonorrhoeae* (3.1% ; 2/64) and *T. pallidum* (4.7% ; 3/64 with *C. trachomatis* infection.

Distribution of C. trachomatis serovars by PCR-RFLP: Of the 64 *omp*A *C. trachomatis*-positive samples, serovars D (48.4%; 31/64), E (32.8%; 21/64), F (7.8%; 5/64), G (1.6%; 1/64) and I (9.4%; 6/64) were the most

Table III. Mutation in seven clinical samples from patients with urogenital infections at baseline compared with ATCC reference strains

Serial number	Serovar	Number of nucleotide changes	Nucleotide change	Position (bp)	Accession number
1	D	2	C---T	841	KP015820
			C---T	961	
2	D	2	T---A	681	KP015821
			A---G	729	
3	D	3	A---T	678	KP015822
			T---A	704	
			T---G	738	
4	E	1	A---G	622	KP015823
5	E	2	A---T	622	KP015819
			T---A	740	
6	I	2	T---A	750	KP015818
			A---G	941	
7	I	2	G---A	1006	KP015824
			T---A	1031	

common *C. trachomatis* serovars accounting for 53 and 47 per cent of the infections in women and men, respectively. Serovars in women and men, respectively, included serovars D (26.6%; 17/64 vs. 21.9%; 14/64), E (15.6%; 10/64 vs. 17.2%; 11/64), F (4.7%; 3/64 vs. 3.1%; 2/64), I (6.3%; 4/64 vs. 3.1%; 2/64) and G (0.0% ; 0/64 vs. 1.6% ;1/64) (Figure A and B).

An analysis was done to identify the possible relationship between *C. trachomatis* serovars with demographic and clinical manifestations. However, no significant association was found except for the serovar E with lower abdominal pain (P=0.01) in women and serovar D with smoking (P=0.03) in men.

Sequencing of ompA gene: Optimization of the DNA sequence analysis was performed using reference DNA for *C. trachomatis* serovars D, E, F, G, H, I, J and K. Of the 64 *C. trachomatis*-positive cases, 31 (48.4%) isolates were randomly selected for sequencing. Of which, 22 (71.1%) *C. trachomatis* isolates were successfully sequenced and analyzed. The sequence data showed no sign of mixed infections. Variability in *omp*A sequences was seen in seven isolates (31.8%). Of these seven *C. trachomatis* isolates, five (71.4%) resulted in point mutations (one sample of serovar D and serovar I and two samples of serovar E). Insertion was observed in one (12.5%) of serovar I and silent mutation was observed in two isolates (25%) of serovar D (Table III).

On the basis of sequence similarity of *omp*A gene, phylogenetic analysis was performed to find out the evolutionary relationship among 22 *C. trachomatis* isolates. In B-complex, among 12 *C. trachomatis* isolates of serovar D, eight clinical samples were identical to the reference strain D/UW-3/CX, whereas the other four were closely related, showing 98 per cent of identity. Among the three isolates of serovar E, two were identical to reference strain E/UW-5/CX, showing 98 per cent of identities, whereas the other isolate showed 94 per cent of identity which was closely related. In F/G group, one isolate of serovar F and one strain of serovar G were identical to the reference strain F/IC-CAL3 and G-UW57, showing 97 per cent of identity. In C-complex, only four clinical isolates of serovar I were identified and all were closely related to the reference strain I/UW-12, showing 95 per cent of identity.

Discussion

Genitourinary tract infections due to *C. trachomatis* are a major cause of morbidity in sexually active individuals[13], and women carry the major burden of the disease[14]. In the present study, *C. trachomatis* was found in 12.3 per cent samples by PCR assays; similar detection rates of *C. trachomatis* in patients with urogenital infections have been reported in previous studies from developing countries[15]. In our study, the highest rate of chlamydial infections was found in

≤30 yr of age group. This is the sexually active group and more vulnerable to sexually transmitted infection (STI) acquisition[16,17]. A multivariate analysis showed that the most common presenting symptoms which were significantly associated with *C. trachomatis*-infected women were vaginal discharge, low back pain and dysuria. The clinical presentation was similar to those described in previous studies[18,19].

Ureaplasma spp. and *M. hominis* have been implicated in a variety of clinical conditions primarily related to lower genital tract colonization and infection. In the present study, 28.2 per cent of patients with urogenital infections were infected by genital mycoplasmas. Similar rates of infection have been reported by others[20,21]. Among the *Ureaplasma* isolates, *U. parvum* (Biovar 1) was the most prevalent and serovar 3/14 was the most frequent serovar detected, suggesting a possible pathogenic role of *U. parvum* serovar 3/14 [11].

The non-ulcerative STIs caused by *C. trachomatis* and genital mycoplasmas, namely *Ureaplasma* spp., and *M. hominis,* potentially increase the susceptibility of acquiring and transmitting HIV[22]. Our study highlighted the importance of early laboratory diagnosis and specific treatment of these agents as these increase the risk of transmission many folds when exist together.

To develop epidemiological data and to detect multiple serovar infections, accurate and specific typing of *C. trachomatis* isolates is required[23]. Sequencing of the amplified *omp*A gene, which encodes the MOMP, is currently considered to be more sensitive, and more specific methods are available for identifying *C. trachomatis* serovars[24].

In our study, serovar D was found to be the predominant serovar followed by serovars E, F, I and G. A similar distribution of *C. trachomatis* serovars has been reported[23,25], whereas others have shown serovar E as the most prevalent one worldwide[26]. In a study by Gita *et al*[27], serovar D was found to be the most predominant serovar amongst patients with urogenital infections, followed by E, F and I. However, in another study, serovar E was found to be the most predominant followed by genovars D and F in patients with infertility[28]. In our study other serovars H, J and K of *C. trachomatis* were not found which have been reported by other workers[26,29].

Clinical symptoms were analyzed for possible associations with particular *C. trachomatis* serovars. Serovar E was significantly associated with lower abdominal pain in women, which was in contrast with previous studies which reported that women infected with intermediate group F/G genotypes more often complained of lower abdominal pain[4,25]. Most studies investigating the association between *C. trachomatis* serovars and clinical symptoms of infection showed contradictory results[30].

Sequencing for a subset of 22 *C. trachomatis* isolates was performed and mutations were observed. However, no visible trace of recombination was found in *omp*A in our genetic variants compared to the respective prototype strain, suggesting that the genetic variability observed in our study was strictly a consequence of the occurrence of point mutations. A minor sequence variation was observed within the genotypes in our clinical isolates. Single-nucleotide variation was observed in one isolate of serovar E, which made it difficult to exclude the possibility of sequencing artefacts. However, two to three nucleotide substitutions were observed in five isolates, which indicated that these were accurate. Two (28.6%) of the seven *C. trachomatis* isolates were synonymous (*i.e.* silent), which suggested that these were evolutionary neutral.

In conclusion, patients infected with *C. trachomatis* have a significant risk of being infected with other STIs, namely *U. urealyticum* and HIV, suggesting screening of these agents along with *C. trachomatis*. Genotyping of the *omp*A gene of *C. trachomatis* isolates could be useful for epidemiological characterization of circulating *C. trachomatis* strains in the community and could provide additional information for vaccine development.

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***Conflicts of Interest*:** None.

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