

Genotyping of *Chlamydia trachomatis* from endocervical specimens in Shiraz, Iran

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Summary

The objective of this study was to determine the prevalence and distribution of *Chlamydia trachomatis* genotypes in Shiraz, Iran. Two hundred twelve cervical swab samples were collected from women attending Shahid Motahari Polyclinic in Shiraz, Iran. The endocervical specimens were screened for *C. trachomatis* by plasmid PCR. Genotyping was performed in *C. trachomatis*-positive samples by nested PCR amplification and sequencing of 571 fragment encompassing VD1 and VD2 of *omp1* gene. The overall prevalence rate of *C. trachomatis* in endocervical specimens determined by plasmid nested PCR was 8%. The deduced serovars found, in descending order of prevalence, were F (46.6%), E (33.3%), and D (13.3%), and serovar G was found in a single sample. Sequence mutation analysis by BLAST search against GenBank reference sequences identified 4 genetic variants. This study can be considered a contribution to increasing knowledge on *C. trachomatis* genotype distribution and sequence variations within each genotype in Shiraz. Further studies are needed to better define molecular epidemiology of *C. trachomatis* serovars and to investigate its genotype variations in Iran.

Key words: *Chlamydia trachomatis*, Endocervical specimen, Genotyping, Iran, Shiraz

Introduction

Chlamydia trachomatis is one of the most common pathogens of sexually transmitted diseases worldwide (Molano *et al.*, 2004). Chlamydiae can grow in wide range of the animal origin cells. The role of *C. trachomatis* in bovine abortion was investigated which imply that the microorganism is not specifically human biovar. As such, it can also emphasize a novel rout of transmission for *C. trachomatis* infection (Ozbek *et al.*, 2008).

The urogenital infections with *C. trachomatis* in women have a clinical course varying from asymptomatic *C. trachomatis* infections to ascending infections leading to pelvic inflammatory disease associated with late ectopic pregnancy and tubal infertility (Lan *et al.*, 1995). When symptoms do occur, they most commonly consist of vaginal discharge and dysuria. Postcoital

bleeding is often reported. Symptoms of chlamydial pelvic inflammatory disease (PID), which may be subtle, include pelvic, uterine, or adnexal pain. Asymptomatic chlamydial infections are an important cause of PID and ectopic pregnancy (Black, 1997).

In pregnant women, *C. trachomatis* is a recognized agent of preterm labor and premature rupture of membranes. However, its role in miscarriage is unclear (Mårdh, 2002). It has been demonstrated that *C. trachomatis* human specific biovars are possible infectious agents in the aborted bovine fetuses (Ozbek *et al.*, 2008).

The major outer membrane protein (MOMP), a key constituent of the cell wall of this obligate intracellular bacteria, is encoded by the *omp1* gene and contains 4 variable domains (VDs) interspaced with five highly conserved regions. Three of these VD's are surface exposed and allow serovars classification. Currently, there are 19

serovars recognized for *C. trachomatis*, all of them with their genotypic profile well established (Lima *et al.*, 2007; de Jesús De Haro-Cruz *et al.*, 2011). Serovars A-C are most often associated with trachoma and serovars L1-L3 are associated with lymph granuloma venereum, while serovars D-K are primarily associated with urogenital and neonatorum infections (Ngandjio *et al.*, 2004). Characterization of *C. trachomatis* strains can provide important epidemiological knowledge and contribute to improved control measures (Lysén *et al.*, 2004). To study the epidemiology of *C. trachomatis* infections, different laboratory techniques for identification of *C. trachomatis* serovars have been developed in recent years. These techniques include standard MOMP serotyping, restriction fragment length polymorphism (RFLP) analysis of the PCR amplified *omp1* gene, and nucleotide sequencing of the *omp1* gene (Molano *et al.*, 2004). It was shown that sequence determination of *omp1* provided a higher resolution than serotyping and RFLP, and at present, the state-of-the-art method for typing is sequencing (Pedersen *et al.*, 2009). The submitted genetic variations within serovars may reflect the power of DNA sequencing to detect subtle differences between isolates, differences which may not be detected by antibody-based methods or restriction fragment length polymorphism analysis (Poole and Lamont, 1992). In this study we aimed to determine the distribution and prevalence of *C. trachomatis* serovars in Shiraz, Iran based on *omp1* gene amplification and sequencing.

Materials and Methods

Sample collection

Endocervical swab specimens were collected from 212 women attending the Gynecology Department of Shahid Motahari Polyclinic in Shiraz, Iran. The endocervical swabs were collected using a sterile Dacron-tipped plastic swab, and transferring it into a sterile tube containing 1-2 ml of PBS. Samples were kept on ice and immediately transferred to the laboratory for processing.

DNA Extraction

Chlamydia trachomatis DNA extraction was performed using Cinnapure DNA kit based on manufacturer's instruction (Cinnagen, Iran). Briefly, the endocervical swab was removed from the vial and the sample was centrifuged at 8000 rpm for 10 min. The supernatant was discarded and the pellet was vortexed and transferred to a sterile 1.5 ml microtube. Two hundred microliter of lysis buffer and 40 μ l of proteinase k from the Cinnapure DNA kit were added and incubated at 65°C for 15 min. The DNA was further purified and resuspended in 60 μ l elution buffer according to the manufacturer's instruction.

β -globin PCR

To test DNA sample quality, the β -globin gene was amplified using primer set PCO₃/PCO₆ (Table 1). The PCR was performed on 2 μ l of the extracted DNA sample in a final reaction mixture of 25 μ l. The final reaction mixture contained 2.5 mM MgCl₂, 50 mM KCl PCR buffer, 200 μ M

Table 1: Primers for β -globin, plasmid and MOMP PCR

Name	Sequence	Amplicon size (bps)
PCO3	5' -ACACAACCTGTGTTCACTAGC-3'	326 bp
PCO6	5' -CATCAGGAGTGGACAGATCC-3'	
T1	5' -GGACAAATCGTATCTCGG-3'	517 bp
T2	5' -GAAACCAACTCTACGCTG-3'	
T3	5' -ATTAACCCTCACTAAAGGGA-3'	320 bp
T4	5' -GCCATGTCTATAGCTAAAGC-3'	
CT1	5' -GCCGCTTTGAGTTCTGCTTCCTC-3'	1085 bp
CT5	5' -ATTACGTGAGCAGCTCTCTCAT-3'	
CTIF	5' -CTGCTGAACCAAGCCTTAT-3'	571 bp
CTIR	5' -TGAGCATATTGGAAAGAAGC-3'	

deoxynucleoside triphosphates (dTTPs), 50 pmol of each primer, and 1 U *Taq* DNA polymerase. The PCR consisted of 40 cycles, comprising denaturation at 94°C for 1 min, primer annealing at 55°C for 40 s, and primer extension at 72°C for 40 s. Before the PCR assay, the tubes were incubated at 94°C for 4 min, and after PCR, they were incubated at 72°C for 4 min. The expected PCR product of 326 bp was visualized on a 1.5% agarose gel after ethidium bromide staining (Refaat *et al.*, 2009).

Plasmid nested-PCR

Detection of *C. trachomatis* was done using initial primers T1/T2 for cryptic plasmid of *C. trachomatis* and secondary primers (T3/T4) internal to the initial primers (Table 1) (Claas *et al.*, 1990; Hosseinzadeh *et al.*, 2004). PCR was performed with a final volume of 25 µl containing final concentrations of 50 mM KCl, 10 mM Tris-HCl (pH = 8.3), 1.5 mM MgCl₂, 200 µM each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 25 pmol of each outer primer, and 1 U of *Taq* DNA polymerase (Vivantis, Malaysia). The thermal condition consisted of initial denaturation at 94°C for 7 min followed by 35 cycles of denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min and elongation step at 72°C for 1 min. In the second round PCR, 1 µl product from the first PCR step and 0.4 µM each T3/T4 inner primer pair was added to the final volume of 25 µl. PCR conditions were as described above. Serotypes I and E were used as positive control. A negative control was included in each run. Finally, 10 µl of the PCR product was analysed using 1.5% agarose gel electrophoresis.

Omp1 PCR and sequencing

The cryptic plasmid positive samples were subjected for *omp1* genotyping. Primary amplification of an approximately 1085-bp fragment encompassing VD1-VD4 of *omp1* was performed using the outer primer pair CT1/CT5 (Table 1) (Rodriguez *et al.*, 1991). One set of primers targeting VD1 and VD2 regions of *C. trachomatis* was designed for second round nested-PCR,

based on the published *omp1* sequences of 15 reference serovars of *C. trachomatis*. The first PCR step was carried out in a final reaction volume of 25 µl, containing 0.4 mM each CT1/CT5 outer primer pair, 5 µl extracted DNA, 2.5 µl of 10 × PCR buffer (200 mM Tris-HCl [pH = 8.4], 500 mM KCl), 1.5 µl of MgCl₂ (50 mM), and 1 U of Platinum *Taq* DNA polymerase (Invitrogen, Grand Island, USA).

The amplification conditions consisted of initial polymerase activation at 94°C for 15 min; 35 cycles of 94°C for 60 s, 55°C for 60 s and 72°C for 80 s; and a final elongation step at 72°C for 10 min. In the second PCR step, 1.5 µl product from the first PCR step and 0.4 mM each CTIF/CTIR (Table 1) inner primer pair were added to a final volume of 25 ml. The PCR conditions were: 94°C for 10 min; 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; and a final elongation step at 72°C for 10 min. The amplified products were electrophoresed through a 1.5% agarose gel and visualized by staining with ethidium bromide.

DNA sequencing

The 571-bp *omp1* fragments successfully amplified from the *C. trachomatis*-positive samples were purified using a GF-1 Gel DNA recovery KIT (Vivantis, Malaysia), and sequenced (Source BioScience LifeSciences, Nottingham, UK). The primer sets used for sequencing were CTIF/CTIR.

BLAST analysis and alignments

The consensus sequences were compared to sequences of known *C. trachomatis* strains by BLAST searching GenBank (www.ncbi.nlm.nih.gov/GenBank). The sequences were assembled into alignments using reference sequences derived from GenBank: B/IU-1226 (AF063208), B/B-16 (AY950630), D/B-120 (X62918), Da/TW-448 (X62921), E/Bour (X52557), F/ICCa3 (X52080), G/UW57 (AF063199), H/Wash (H/UW4) (X16007), J/UW36 (AF063202) and K/UW31 (AF063204). The sequence data of new variants were submitted to NCBI and have the following accession numbers: F1

(JX893029), F2 (JX893030), E (JX893031), and G (JX893032).

Results

All samples were successfully screened by β -globin PCR and no inhibition was detected. Out of the 212 endocervical swabs, 17 were found to be positive for *C. trachomatis* plasmid nested-PCR. The *omp1* gene amplification and sequencing was successfully performed with 15 *C. trachomatis*-positive samples (Fig. 1).

Sequence analysis of the 571 bp fragment of the *omp1* gene, comprising VD1-2 showed that the most prevalent *omp1* sequences corresponded to serovars F (7,

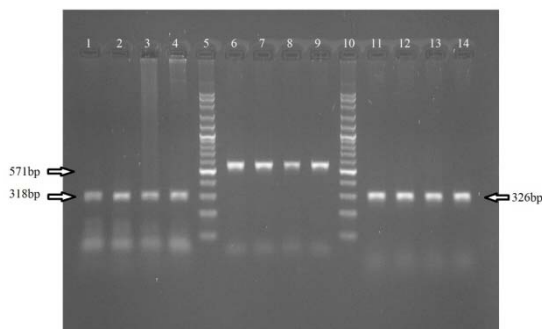


Fig. 1: Agarose gel electrophoresis of PCR products of the nested-plasmid, nested-*ompA* and β -globin. Lane 5 and 10: 100 bp Plus DNA ladder (Vivantis, Malaysia), Lane 1-4: PCR product of nested plasmid PCR, Lane 6-9: PCR product of nested-*ompA* PCR, and Lane 11-14: PCR product of β -globin

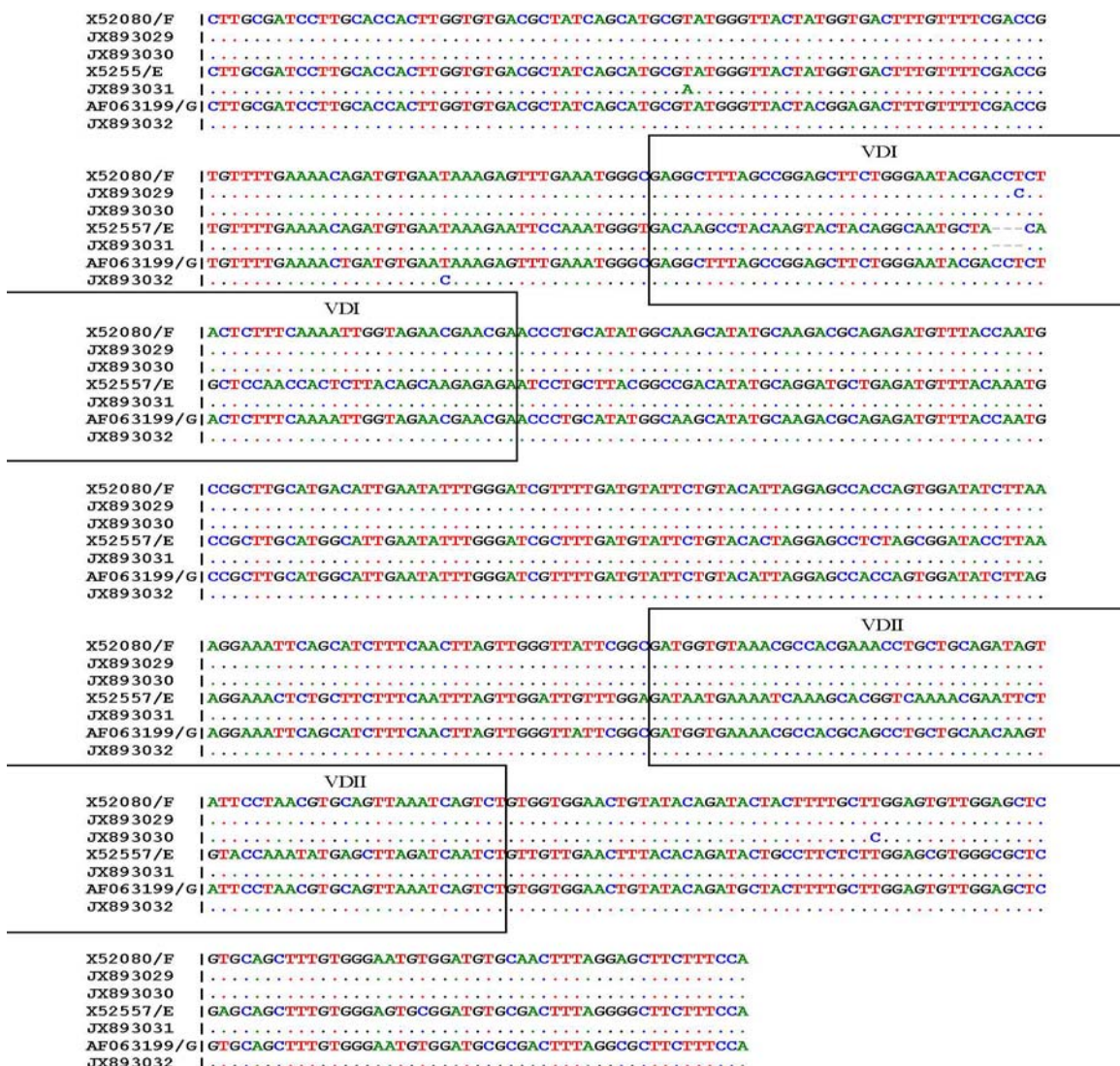


Fig. 2: Partial nucleotide sequence of *omp1* showing variable regions that permitted the determination of each genotype. Reference sequences were used as a prototype

Table 2: Mutations found in clinical specimens compared to strains in GenBank

Genotype	No. of mutations	Nucleotide change	Position	Amino acid change	GenBank accession No.
F	1	T to C	290	Ser to Pro	JX893029
F	1	T to C	580	Trp to Arg	JX893030
E	1	T to A	183	Arg to Arg	JX893031
G	1	T to C	237	Asn to Asn	JX893032

46.6%) and E (5, 33.3%); serovar D was found in two samples (13.3%) and a strain of serovar G was found in a single sample (6.6%).

Detailed analysis revealed that there were limited sequence differences within genotypes (Table 2). Majority of the *omp1* gene sequences from the clinical samples had sequences that were identical to those reported for the respective reference isolates. Out of the 15 samples, eleven had *omp1* sequences identical to sequences in GenBank, and the other four samples had nucleotide variations, all differed by one nucleotide only (Fig. 2). Genotype D did not show any variation and were identical to the reference strain D/B-120. In genotype E one strain displayed a silent T to A substitution at position 183 (compared to E/Bour). For genotype F, 2 out of 7 samples (28.5%) differed from the reference strains F/ICCa13. The nucleotide substitutions at positions 290, and 580, changing base pair T to C, and T to C, respectively, caused the amino acid to change from serine to proline, and tryptophan to arginine, respectively. Genotype G had a point mutation at position 237, which showed a T to C variation, and caused the amino acid to change from asparagine to asparagine (silent).

Discussion

The 8% prevalence rate of *Chlamydia trachomatis* infection among our study population is similar to that of other studies in Iran (Bakhtiari and Firoozjahi, 2007; Chamani-Tabriz *et al.*, 2007). A recent study conducted in Shiraz by Hadi *et al.* (2010) showed a *Chlamydia* infection rate of 8% in women who attended health centers. Studies in the United Kingdom showed prevalence rates of 6 to 8% (Gopalakrishnakone *et al.*, 2009). A study conducted in Singapore among sub-fertile women showed a prevalence rate of 8%.

To the best of our knowledge, this is the

first study on genotype distribution of *C. trachomatis* in Shiraz, Iran. We found four different *C. trachomatis* serovars among 212 collected specimens. Genotype F was the most common genotype (46.6%), followed by genotypes E (33.3%), D (13.3%), and G (6.6%).

According to our result, F and E were the most prevalent genotypes in Shiraz. These results are consistent with those in studies of urogenital chlamydial infection elsewhere, although there are some differences in relative proportions in different geographic areas. For example, in Sweden, Australia, Japan and Korea, serovar E was the most commonly identified one (Jurstrand *et al.*, 2001; Hsu *et al.*, 2006; Bandea *et al.*, 2008). However, the distribution pattern of genotypes in Shiraz, where serovar F is the predominant genotype, is more likely to be similar to the distribution in Mexico, Thailand and southwestern China. In Thailand, F (25-60%) was the most prevalent genotype followed by E (9-20%) and D (7-23%). Differences in genotype prevalence were found in different study populations (Banda *et al.*, 2001; Gao *et al.*, 2007; de Jesús De Haro-Cruz *et al.*, 2011).

Sequence analysis of *omp1* and other genes has been used previously as a tool to differentiate *Chlamydia* strains and gain epidemiological knowledge. The variability in *omp1* sequences in previous studies, as a genetic variant as a proportion of all *Chlamydia* cases, has ranged between 16-81% in various studies (Pedersen *et al.*, 2009). However, in these studies the study populations were recruited from high-risk groups (Lysén *et al.*, 2004). The results of this analysis indicated only limited sequence differences between the reference sequence and within genotypes. We found 4 genetic variants among the 15 *omp1* sequences. Three out of four variations were found within CDs. Two of the CDs nucleotide variations were silent. In Genotype E there

was variation in just one of the cases. These findings are in agreement with those of other studies which described few variants for serovar E (Rodriguez *et al.*, 1993; Lysén *et al.*, 2004).

It has been demonstrated that high level of conservation of MOMP seems to be associated with a higher ecological success. Nunes *et al.* (2010) speculated that the low MOMP amino acid differences among genotypes may confer E and F antigenic profiles better fitted to deal with the host immune system.

All genetic variants were detected only in a single sample, which was different from the substitutions reported previously.

The main immunoreactive parts of the MOMP that induce neutralizing antibodies are found in VD1, VD2 and VD4. VD4 is the largest of the variable regions within the *omp1* gene and with VD1, the segment we sequenced, encodes the most immunoreactive domains of the MOMP (Zhong and Brunham, 1991). A greater frequency of amino acid substitutions would be expected to occur within these domains than elsewhere in the protein. We observed one nucleotide variation within VDs that (Genotype F) resulted in amino acid changes in VD1, which may have been advantageous for the pathogen to escape immune pressure directed at *omp1*. It has been speculated that such *omp1* genovariants occur as a result of point mutations and recombination events selected by immune pressure (Hayes *et al.*, 1994). One variation in CD3 resulted in the amino acid replacement tryptophan to arginine, which probably caused little antigenic, functional, or conformational change to MOMP, as CD3 is not predicted to be surface exposed (Rodriguez-Maranon *et al.*, 2002). However, nucleotide substitutions that we detected in Genotypes E and G were synonymous (silent), which suggests that they were evolutionarily neutral.

The presence of a high specificity between the host and chlamydiae has been recognized and this relation has been considered as an adaptation mechanism. However, some studies have indicated that chlamydiae can also grow in laboratory animals, yolk sacs of embryonated eggs and *in vitro* cell cultures. Similarly Ozbek *et al.*

(2008), in a study on bovine aborted fetuses, found 5 (21.7%) out of 23 samples positive for *C. trachomatis*. The nested plasmid PCR is a sensitive and specific method for *C. trachomatis* detection. This technology has provided a valuable and sensitive means for molecular epidemiological analysis to identify novel routes of transmission of *C. trachomatis*.

In conclusion, the present study is the first description of *C. trachomatis* serovar distribution in Shiraz, Iran. The predominating serovars were F and E, as has been reported from other countries. Sequencing the *omp1* gene enables analysis of sequence variations within each genotype and provides discrimination between *C. trachomatis* strains, which is helpful in tracing the sexual transmission pathway, which ranges from individual contact to international transportation.

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