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Prevalence of *Chlamydia trachomatis* Infection in Cervical Samples from Infertile Women and Related Genotypes

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ABSTRACT

Aims *Chlamydia trachomatis* infection is a sexually transmitted disease that its majority infections are asymptomatic and can cause infertility. So, determining its frequency and prevalent genotypes in each zone is necessary to provide clues for clinicians and also to prevent or minimize its complications. The aims of this study were to investigate the prevalence of C. trachomatis infection in infertile women in Isfahan, Iran and its association with some clinical findings and determining the involved genotype in the understudy population.

Materials & Methods This experimental study was conducted among infertile women referring to two infertility clinics in Isfahan, Iran in 2018. 180 endocervical samples were selected using cross sectional Sampling method based on the defined clinical criteria for infertility and confirmed by gynecologist. Genotyping of positive samples was done based on PCR-RFLP of *omp1* gene and then DNA digestion with *Hpa*II, *Hinf*I and *Alu*I restriction enzymes. The relations between genotypes, clinical sings, age, primary and secondary infertility, and duration of infertility and abortion history were analyzed using chi-square test.

Findings The frequency of *C. trachomatis* infection in 180 samples was 10.5% in infertile women. E, F, and D genotypes were prevalent in this population. There was a significant association between infection and abortion among patients with primary and secondary infertility.

Conclusion The frequency of *C. trachomatis* infection in 180 endocervical samples is 10.5% in infertile women in Isfahan, Iran. E, F and D genotypes are prevalent in this population. *C. trachomatis* infection is prevalent in infertile women especially in secondary infertility.

Keywords *Chlamydia trachomatis*; Genotype; Infertility; Omp1 Gene

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Introduction

Chlamydia trachomatis is an obligate intracellular pathogen in columnar and transitional epithelial cells [1] and according to the estimation of World Health Organization, 92 million new cases of its genital infection occur worldwide annually and it is the most common bacterial cause of sexually transmitted infections (STI) [2, 3]. This pathogen has been highly adapted and dependent to human cells for nutrition and energy [4]. Different parts of the human body can be affected by infection due to this agent, so different diseases, and syndromes, including lymphogranuloma venereum, trachoma and urogenital infection may occur which is dependent to the involved serovar [5, 6]. Serovars A to C is mostly associated with trachoma, serovars D to K are mostly associated with urogenital infections and serovars L1 to L3 are commonly associated with lymphogranuloma venereum [7-9]. The majority of urogenital *C. trachomatis* infections in women, nearly up to 70 -80%, are asymptomatic [10].

As a result of untreated infections, pelvic inflammatory disease (PID) can occur that scares the inside of the reproductive organs and consequently can lead to serious complications including chronic pelvic pain, ectopic pregnancy, and infertility [11]. One of the most important aspects of this infection is its chronic status in the course of infection and as a result of the persistence of this bacterium in the host cells the risk of tubal factor subfertility will be increased [12]. Long-term persistence of *C. trachomatis* in genital tissues can cause unapparent and progressive damages to genital tubes. Therefore, all sexually active women are at risk of this infection [13]. However, comparing to other sexually transmitted diseases (STDs), *C. trachomatis* infection has fewer signs and this cause delay in its diagnosis which lead to ascending infections in the genital system [14]. In addition, untreated *C. trachomatis* infections can lead to abortion, stillbirth, preterm delivery and most importantly, infertility [15].

Hence the rapid and accurate diagnosis of this infection can limit its prevalence and prevent its undesired complications. Furthermore, with regard to the importance of involved serotype in the outcome of infection, finding the involved genotype in clinical cases is of great importance. Different diagnostic approaches are exist for *C. trachomatis,* but among them, PCR reaction is taken into consideration as the most sensitive, rapid and low cost method than others [16]. So, molecular techniques for typing the involved serotype in the *C. trachomatis* infection like as PCR-RFLP have been used for this purpose. As sequencing of the *omp1* gene in *C. trachomatis* showed a significant difference among genotypes [9], restriction fragment length polymorphism (RFLP) analysis of *omp1* gene has been used to differentiate genotypes of *C. trachomatis* by using different enzymes [17-19].

Several studies in Iran have been focused on determining the prevalence of *C. trachomatis* infection in women especially those with genital infection. Till now, no study has reported the prevalence of this pathogen in infertile women in Iran.

The aims of this study were to investigate the prevalence of *C. trachomatis* infection in infertile women in Isfahan, Iran and its association with some clinical findings and to determine the involved genotype in the understudy population.

Materials and Methods

This experimental study was conducted in Isfahan, Iran in 2018. The understudy population was infertile women referring to two infertility clinics in Isfahan. These cases were selected using cross-sectional Sampling method based on the defined clinical criteria for infertility and confirmed by a gynecologist. 180 endocervical samples from infertile women were assessed for the presence of *C. trachomatis.*

Endocervical samples were collected by cotton swab from infertile women, and were carried on ice in 1ml of PBS buffer (1×) to the microbiology laboratory. Some data including age, primary or secondary infertility, abortion history, medical treatment and duration of infertility were also recorded.

DNA extraction: Each microtube was vortexed and the obtained solution was centrifuged (13000 rpm, 30min). The deposit was resolved in 70µl of 10mM Tris-HCl (pH=8) and stored at -70°C for 48h. The thawed samples were boiled for 10min and the supernatant (10000 rpm, 2min) was harvested and stored at -20°C [17].

Primary screening: In case of *C. trachomatis,* amplification of plasmid sequence increases the sensitivity of PCR than the chromosomal DNA. So, CTP1 (5'-TAGTAACTGCCACTTCATCA-3') and CTP2 (5'-TTCCCCTTGTAATTCGTTGC-3') primers were used for this purpose [19]. The PCR reaction was preformed according to the method described by Taheri Beni *et al.* in 2010 [19].

Genotyping: The *omp1* gene was selected for genotyping. In order to increase the sensitivity, nested or semi-nested PCR reactions for this target must do followed with RFLP [18]. The positive samples in primary screening were subjected to *omp1* gene amplification as same as primary screening using two primers: CT1 (5'-GCCGCTTTGAGTTCTGCTTCCTC-3') and CT5 (5'-ATTTACGTGAGCAGCTCTCTCAT-3').

Then Semi-nested PCR was carried out on 2µl of PCR product using PCTM3 (5'-

TCCTTGCAAGCTCTGCCTGTGGGGAATCCT-3') as a forward primer and CT5 as a reverse primer and the product was electrophoresed in 1% agarose gel. In order to obtaining the RFLP pattern of positive samples, single digestion with *Alu*I (which differentiates 10 genotypes, A, C, E, F, G, I, J, K, L1 and L2, while can't differentiate B, Ba and D, and H and L3) was performed in a reaction containing semi-nested PCR product (10µl), nuclease free water (18µl), 10x Tango Buffer (2µl) and 1µl *Alu*I (Fermentas; USA) with incubation at 37°C for 4h and then at 65°C for 20min to deactivate the enzyme. Then triple digestion with *Hin*fI, *Eco*RI and *Hpa*II was performed to differentiate 11 genotypes including D, E, F, G, H, I, J, K, L1, L2 and L3, but genotypes A and C, and B and Ba, have similar patterns [19]. Firstly, a digestion was done by *Hpa*II (semi-nested PCR product (10µl), $MgCl_2$ (10mM), Tris-HCl (10mM; pH 7.6; 20µl) and 5U of *Hpa*II (Fermantas; USA), at 37°C for 4h, then the enzyme was inactivated at 80°C for 20min. Consequently, 2ml of the Tris-HCl (200mM), NaCl (75mM; pH 8) solution was added with 4U from each of *Eco*RI and *Hin*fI (Fermentas; USA) enzymes to the previously digested solution and incubated overnight at 37°C. Finally, these enzymes were inactivated at 65°C for 20min. The digested products were electrophoresed in 6% polyacrylamide gel (PAGE) at 100V for 120min and stained with ethidium bromide solution (0.5µg/ml) for 45min. Digested PBR322 with *Hae*III was used as weight marker.

The relations between genotypes, clinical signs, age, primary and secondary infertility, and duration of infertility and abortion history were analyzed using chi-square test.

Findings

From these 180 endocervical samples, 25, 119 and 36 samples were obtained from <25, 26-35 and 36-45 years old, respectively. 19 positive cases (10.5%) were found in primary screening based on PCR of plasmid gene (Figure 1).

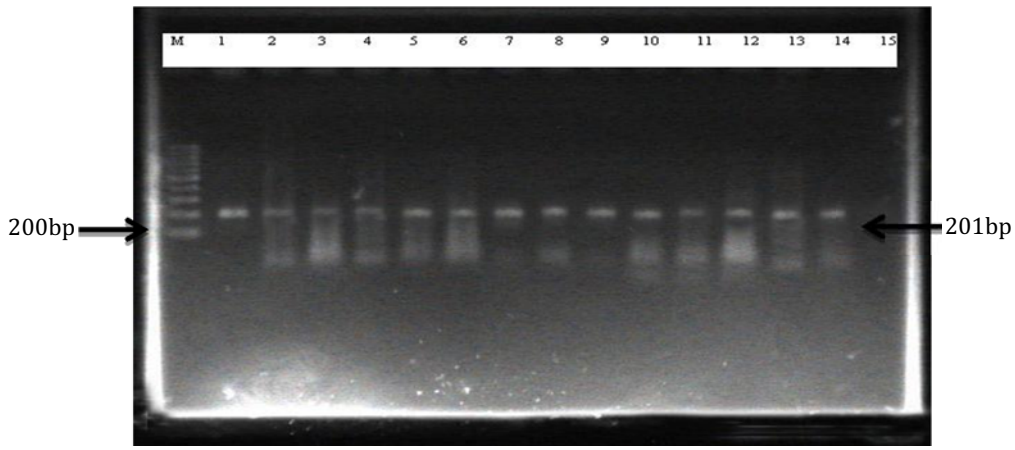

Figure 1) Positive clinical specimen for *C. trachomatis*; M: Marker (100bp); 1: Positive control; 15: Negative control; 2 to 14: PCR products of positive clinical specimen (201bp)

There wasn't any case infected with *C. trachomatis* in ≤25 years old group while, 13 positive cases (10.9%) were found in total samples (119 samples) of 26-35 years old group and 6 infected samples (16.6%) were found in the total samples (36 samples) of 36-45 years old groups. Three clinical signs including pelvic pain, bleeding and abnormal discharges are most frequently found in *C. trachomatis* infections. 84.2% of positive cases had clinical signs and 15.7% of them were asymptomatic, although no significant relation was found between clinical signs and *C. trachomatis* infections ($p<0.05$).

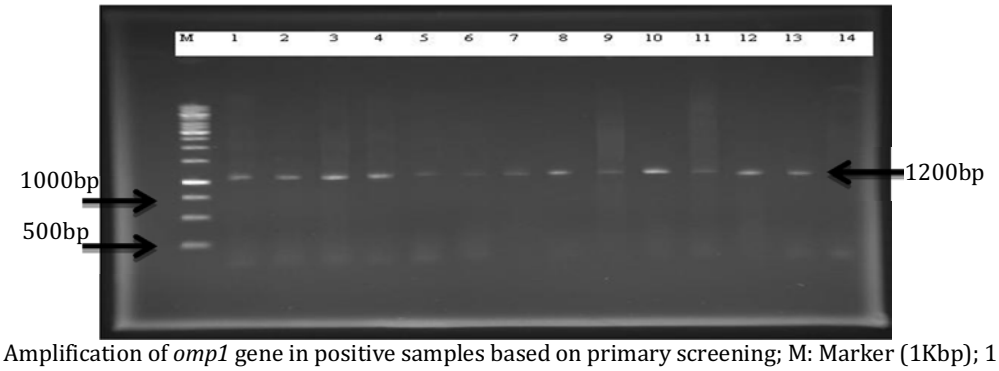

Figure 2) Amplification of *omp1* gene in positive samples based on primary screening; M: Marker (1Kbp); 1: Positive control; 14: Negative control; 2 to 13: Positive samples (1200bp)

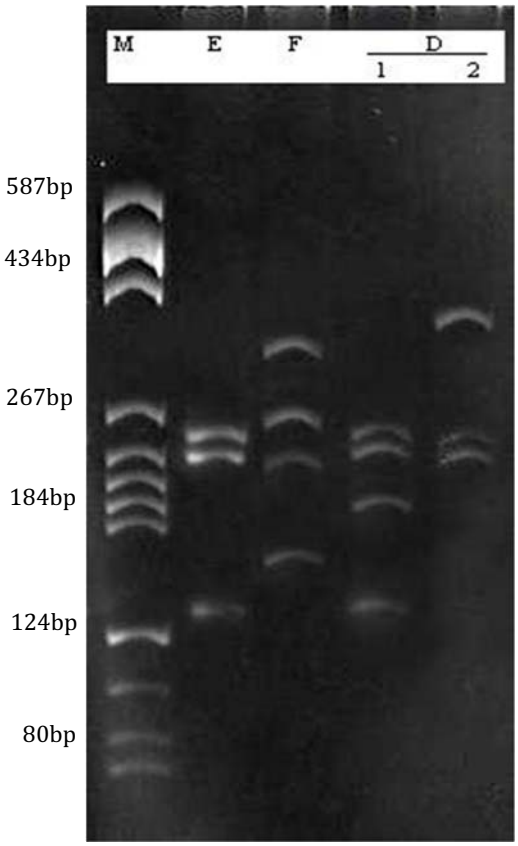

Figure 3) RFLP patterns of the *omp1* gene of three clinical genital *C. trachomatis* strains (E, F and D/Da) obtained by 6% polyacrylamide gel electrophoresis.
M: digested pBR322 with *Hae*III as molecular size marker
E and F indicate *Al*uI digestion of genotype E and F, respectively
D1: *Al*uI digestion of genotype D/Da
D2: *Hp*aII-*Eco*RI-*Hin*fI digestion of genotype D/Da

5.1% and 20.6% of cases were affected with primary and secondary infertility, respectively. So, there is significant relation between secondary infertility and *C. trachomatis* (p=0.03). Furthermore, it has found that 31.2% of women with abortion history were significantly infected with *C. trachomatis* (p<0.05). The duration of infertility was in the range of 1 to more than 16 years; 4.7% of cases from 1 to 5-year infertility group, 15.6% from 6 to 10-year infertility group, 25.0% from 11 to 15year infertility group and 27.0% of more than 16 years infertility history were positive for *C. trachomatis.*

Amplification of *omp1* gene in positive samples from primary screening was successful only in 18 samples (Figure 2) which were subjected to single digestion with *Al*uI and triple digestion with *Eco*RI, *Hin*fI and *Hp*aII (Figure 3).

Nine (50.0%) of positive samples were from genotype E, six (33.3%) were from genotype F and three were from (16.7%) genotype D. Other genotypes didn't find; 66.6% (6 cases) of E genotype were fall into 26 to 35 years old group and 33.3% (3 cases) of them were in 36 to 45 years old group. In case of genotype F also, 66.6% were in 26 to 35 years old group and 33.4% in 36 to 45 years old group. 100% D genotypes were in 26 to 35 years old group.

The majority of affected cases by genotype E, had abnormal discharges while, in genotype F, 50.0% of cases had pelvic pain. All infected cases with genotype F had clinical signs while; there was one asymptomatic case in each of E and D genotypes (Table 1).

Table 1) Relation between serovar and clinical signs (the numbers in parentheses are percent)

Clinical Signs	Serovar E	Serovar F	Serovar D
Pelvic pain	2 (22.2%)	3 (50.0%)	1 (33.3%)
Bleeding	1 (11.1%)	2 (33.3%)	0
Abnormal discharge	5 (55.5%)	1 (16.6%)	1 (33.3%)
Asymptomatic	1 (11.1%)	0	1 (33.3%)
Total	9 (100%)	6 (100%)	3 (100%)

All strains of genotype D and 66.6% of each of genotype E and F were found in secondary infertility cases. However, there was no significant relation between them (Table 2; p<0.05).

Table 2) Relation between serovars and type of infertility (the numbers in parentheses are percent)

Type of Infertility	Serovar E	Serovar F	Serovar D	Total
Primary Infertility	3 (33.4%)	2 (33.4%)	0	5
Secondary Infertility	6 (66.6%)	4 (66.6%)	3 (100%)	13

Abortion history was present in 55.5% of infected cases with genotype E, 33.4% of genotype F and 100% of genotype D. Finally, 44.5% of affected cases with different genotypes (50.0% genotype E, 37.5% genotype F and 12.5% genotype D) had an infertility history between 6 to 10 years. However, there was no significant relation between them (p<0.05).

Discussion

The aims of this study were to investigate the prevalence of *C. trachomatis* infection in infertile women in Isfahan, Iran and its association with some clinical findings and determining the involved genotype in the understudy population.

Chronic infections of *C. trachomatis* may be followed by infertility and abortion and different serovars of this pathogen are correlated with various clinical appearance. Since there is not STDs clinics for screening STDs in Iran, infertility due to this agent can be widespread while undiagnosed [19]. So, this study was conducted to survey the prevalence of *C. trachomatis* in infertile women.

The results of Carroli *et al.* [20] and Shafer *et al.* [21] revealed that endocervical swab samples are preferable than urine samples which have PCR inhibitors. Furthermore, the results of different

studies confirmed that boiling method is an effective and useful method for this purpose [18, 22]. So these methods were used for sampling and also DNA extraction.

The plasmid sequence due to more sensitivity was selected for primary screening. The results of Jenab *et al*. [22], Siala *et al*. [23] and Taheri beni *et al*. [19] also confirm the superiority of this target. The frequency of this infection in different area is variable and depends on cultural, social and economic status of understudy population. Therefore, the reported frequencies varied even in different parts of a city. No other similar study on infertile women has reported in Iran yet, but several studies have reported this infection in women referred to gynecological clinics including 18.5% frequency by Taheri Beni *et al*. in 2010 in Ahvaz [19], 21.37% by Zeighami *et al*. in women with abortion [24], 12.6% by Chamani–Tabriz *et al*. in Tehran [25] and 14.9% by Fallah *et al*. in Tehran [26]. The differences that found between these reports even in one region are due to differences in hygiene level, residence area, economy and culture. Furthermore, 2.9% frequency by Flipp *et al*. [27], 6% and 25% by Domeika *et al*. respectively in eastern European countries [28, 29] and 28.5% by Sturm-Ramirez *et al*. in Senegal have been reported [8].

The mean age of understudy population in this study was 31 years old. Although there was no significant relation between age and *C. trachomatis* infection, from the total infected women (19 cases) the majority (68.42%) of infected women were in 26-35 years old group which is in agreement with Fallah *et al*. [26] and Levidiotous *et al*. [30]. While in the study of Chen *et al*. [31] and Mascellino *et al*. [32] most of the infected women had less than 25 years old. These differences can be the result of cultural and religious believes, women do not have any sexual relationship before marriage in Iran. Furthermore, increase in marriage age has led to the lower frequency of this infection in women under 25 years old in Iran than other countries.

Absence of significant relation between infection and clinical signs is in agreement with Chen *et al.* [31] while is in contrast with the results of Mascellino *et al*. [32] and Van Bergen *et al*. [33]. Interestingly, a significant relation was found between history of abortion and infection ($p<0.05$) that is in agreement with Patel *et al*. [34].

A prominent finding was the significant relation between secondary infertility and infection ($p<0.05$) which is in agreement with Malik *et al*. [35]. These women due to longtime sexual activity are at higher risk of this infection.

The results of RFLP on amplified products revealed that E, F and D genotypes are prevalent in infertile women in Isfahan which is in agreement with Taheri Beni *et al.* [19]. In other studies, also these serovars comprised 60 to 80% of all genotypes e.g, in Barnes *et al*. [36] genotypes D (18%), E (32%), F (18%), G (3%), H (2%), I (5%) and K (4%) and in Persson and Osser in Sweden genotypes D (13%), E (40%), F (25%), G (5%), H (4%), I (7%), J (6%) and K (6%) were reported [36]. There was no significant relationship between genotypes and age groups. This is in agreement with Morre *et al*. [37] and Duynhoven *et al*. [38]. Most of infected women with genotype E had sign of abnormal discharges (55.5%) and 50% of those affected with genotype F had pelvic pain, but there was no significant relationship between the genotypes and clinical signs.

66.6% of infected women with genotype E, 66.6% of affected with genotype F and 100% of those with genotype D suffered from secondary infertility, but there wasn't significant relationship between these two parameters.

55.5% of infected women with genotype E, 33.3% of affected with genotype F and 100% of those have genotype D, showed a history of abortion but there wasn't significant relationship between abortion and serovars.

According to result of this study *C. trachomatis* infection may be involved in secondary infertility. Therefore, in the treatment of such cases, infection discovery and, subsequently, its treatment and eradication are necessary. It can be useful in the treatment of infertility and preventing the high cost of treatment due to misdiagnosis.

The limitations of this research were sampling and lack of history of patients included in the study. It is suggested that screening for this pathogen be regarded in gynecological clinics and the prevalent genotype is determined for further use in treatment and control of this infection.

Conclusion

The frequency of *C. trachomatis* infection in 180 endocervical samples is 10.5 % in infertile women in Esfahan, Iran. E, F, and, D genotypes are prevalent in this population. *C. trachomatis* infection is prevalent in infertile women, especially in secondary infertility. *C. trachomatis* infection may be involved in secondary infertility.

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Ethical Permission: The patients were informed about the study and their information was collected with their permission.

Conflict of Interests: The authors declare that there is no conflict of interest.

Authors' Contribution: Motamedi H. (First author), Methodologist/Discussion author (30%); Rasoolizadeh A. (Second author), Introduction author/Original researcher/Statistical analyst/

(40%); Shahshahan Z. (Third author), Assistant researcher (15%); Razavi V. (Fourth author), Assistant researcher (15%)

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