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Prevalence of *Chlamydia trachomatis* Sequence Types 4 and 80 in Infertile Couples in North Khorasan, Iran

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ABSTRACT

Background: Chlamydial infections could lead to ectopic pregnancy and infertility. Considering the high prevalence of infertility in Iran and little information about the role of urogenital bacterial infections in this disease, this study aimed to evaluate the prevalence and sequence types of *Chlamydia trachomatis* in the urogenital tract of infertile couples in North Khorasan.
Materials & Methods: Cervical or urethral swabs collected from infertile patients referring to two private clinics and the infertility center of Bent Al-Hoda hospital in Bojnurd during 2017-2021 were tested for *C. trachomatis*. These specimens were evaluated using PCR for *C. trachomatis orf8* gene. Multi-locus sequence typing (MLST) was performed on positive samples using PCR amplification of seven housekeeping genes (*GlyA*, *leuS*, *lysS*, *mdhC*, *pdhA*, *pykF,* and *yhbG*) following a previously described protocol.
Findings. Out of 268 samples tested, 44 (16.4%) samples were positive for *C. trachomatis*. Among which, 35 cases were obtained from women, and nine samples were from men. Of the 44 positive samples, 10 cases were not typable. Only two sequence types were detected among 34 typeable isolates: 25 (73.5%) isolates belonged to ST80, and nine (26.5%) samples belonged to ST4.
Conclusion: The high prevalence of ST4 and ST80 in most symptomatic infertile patients may be attributed to the higher pathogenicity of these types in the urogenital tract. However, our sample size was insufficient to draw such a conclusion., Further research on the prevention and treatment of Chlamydial infections could potentially help to reduce infertility in Iran.

Keywords: *Chlamydia trachomatis*, Infertility, Molecular typing.

CITATION LINKS

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Introduction

Chlamydia is a Gram-negative obligate intracellular pathogen characterized by its lack of motility. Among bacterial sexually transmitted infections (STIs), *Chlamydia trachomatis* is the predominant causative agent, accounting for approximately 127 million annually reported cases worldwide [2, 3]. *C. trachomatis* is widely recognized as a common and curable culprit of sexually transmitted infections in adult men and women [4]. It is considered a significant global public health concern by the World Health Organization (WHO) for several reasons. Firstly, it has a high prevalence and incidence rate, with millions of new cases reported annually [5, 6]. Secondly, if left untreated, *Chlamydia* could lead to severe complications in the reproductive system, causing infertility in both men and women. Thirdly, it increases the risk of transmission and acquisition of HIV [5, 6]. Fourthly, the infection often progresses with mild or no symptoms, contributing to its widespread transmission [7, 8]. Lastly, *Chlamydia* primarily affects young individuals, typically the most sexually active population. *C. trachomatis* exhibits a repertoire of 15 distinct serovars, which could be distinguished using monoclonal antibodies and polyvalent antisera. Serovars A, B, Ba, and C primarily contribute to the development of trachoma, whereas serovars D-K are associated with urogenital complications. Serovars L1, L2, and L3, on the other hand, are responsible for the development of lymphogranuloma venereum (LGV) [9]. Notably, *C. trachomatis* infections often remain asymptomatic and could persist for extended periods of time, spanning several years. In females, complications arose from *C. trachomatis* infections include urethritis, cervicitis, endometritis, and pelvic inflammatory disease (PID) [9, 10]. *C. trachomatis* plays a significant role as a causative agent in pelvic inflammatory disease, and its associated complications could lead to ectopic pregnancy and tubal factor infertility. Infertility is a complex medical condition that refers to the inability to conceive a child after trying for a long period of time, typically a year of regular unprotected intercourse. Infertility rates attributed to *C. trachomatis* infections vary, ranging from 12.8% following the first episode to 75% after three or four episodes [9]. Infertility caused by *C. trachomatis* infections is frequently observed, mainly due to asymptomatic infections, persistence of a carrier state, reactivation of latent infections, and challenges in complete eradication of *C. trachomatis* infections [11]. Tubal defects play a significant role in causing infertility, and recent findings have revealed that *C. trachomatis* infection is the leading cause of tubal peritoneal damage [12]. If left untreated, approximately 40% of infected women may experience pelvic inflammatory disease, and among these individuals, 20% are likely to develop infertility [9]. To avoid the potential detrimental consequences of *C. trachomatis* infections on male and female fertility, in many parts of the world, including Iran, routine tests are performed to detect *C. trachomatis* and other sexually transmitted bacteria among infertile couples. As outlined in the global health sector strategy on sexually transmitted infections (STIs) formulated by the World Health Assembly in 2016, it is of utmost importance to enhance data-gathering initiatives at the national level [13]. It is necessary to evaluate the prevalence and incidence rate and substantially diminish the global burden of *Chlamydia* [14]. Multi-locus sequence typing (MLST) is a promising molecular typing approach in the realm of global epidemiology for bacterial pathogens, specifically *C. trachomatis* isolates [7, 8, 15]. Implementation of MLST enables rapid and precise differentiation of *C. trachomatis* strains. This approach facilitates prompt and accurate identification of both newly discovered and previously documented sequence types (STs) of the pathogen. Such discrimination is crucial for effective epidemiological surveillance, therapy monitoring, and outbreak control of *C. trachomatis*-associ-

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ated infections [15-19]. A comprehensive assessment of various typing methodologies used for *C. trachomatis* isolates, including ompA, variable-number tandem-repeat (VNTR) genome loci, multiple-locus variable-number tandem repeats (MLVA), multi-locus typing (MLT) DNA microarray, and spatial laser speckle contrast analysis (s-LASCA) of virtual gene-based speckles (GB-speckles), demonstrated that MLST is the preferred approach for worldwide epidemiological applications [15]. This is particularly true for discriminating between different *C. trachomatis* strains at the intraspecific level [20-22]. Recent studies have shown that MLST-based phylogenetic trees of *C. trachomatis* share similarities with trees constructed using whole-genome sequencing, and both types of trees demonstrate comparable degrees of incongruence in the phylogenetic relationships of this bacterial species [16, 19, 23]. Studies have indicated that MLST offers superior accuracy, ease of implementation, lower costs, and significantly faster results than contemporary *C. trachomatis* genome-based methods for detecting sequence types (STs) [15, 19].

Several MLST schemes have been developed for *C. trachomatis*, employing PCR amplification and DNA sequencing of five to seven specific genomic loci linked to the chlamydial genome. Among these schemes, the scheme presented by Pannekoek et al. (2008) [7], which employs seven molecular targets, has gained recognition as the preferred option for worldwide epidemiological studies and tracking the molecular evolution of *C. trachomatis* [24]. This MLST approach encompasses asymptomatic individuals and couples infected with either the wild type of *C. trachomatis* or the novel variant [8, 25]. Significantly, implementing this MLST approach leads to the classification of *C. trachomatis* (CT) into three distinct sequence type (ST) groups: Groups I, II, and III [24]. Notably, in Pannekoek et al.'s (2008) study, urogenital CT strains were assigned to Groups I and III, while LGV strains formed Group II [7].

Objectives: The objective of this investigation was to determine the prevalence and sequence types (STs) of *C. trachomatis* in the urogenital tract of infertile couples in North Khorasan, Iran, in order to understand the epidemiology of this bacterium among Iranian patients.

Materials and Methods

In this study, 134 infertile couples (a total of 268 samples) were tested for sexually transmitted diseases, including *C. trachomatis*. Cervical or urethral swab samples were collected from patients in the age range of 22 to 45 years, visiting gynecology and infertility specialists in North Khorasan, Iran, during 2017-2021. Clinical specimens were transported to the diagnostic laboratory of Imam Reza hospital in Bojnurd, North Khorasan. The purpose of laboratory analysis was to detect the presence of *C. trachomatis* DNA using PCR following the methods described in a previous study [4]. This analysis was conducted to confirm the presence of an ongoing STD (sexually transmitted disease) as a cause of infertility or as one of the tests performed before IVF (*in-vitro* fertilization) or IUI (intrauterine insemination) in infertile couples. Among the female patients in the study, some were asymptomatic, while others had clinical symptoms related to cervicitis and pelvic inflammatory disease. These symptoms included prominent vaginal discharge, inter-menstrual and post-coital bleeding, lower abdominal pain, dysuria, and other manifestations observed during their annual routine clinical examinations. As for the male patients, they primarily presented symptoms of urethritis or epididymitis. Specifically, these symptoms included dysuria, urethral discharge, palpable swelling of the epididymis, and in one instance, fever.

DNA extraction: For DNA extraction, total DNA was isolated from clinical specimens obtained from patients using a genomic DNA

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isolation kit (KR-2000, GENET BIO, Korea). The DNA concentration of each specimen was quantified using a spectrophotometer from BioRad Laboratories (Redmond, WA, USA).

PCR detection of *C. trachomatis*: To detect *C. trachomatis* DNA, the *orf8* gene-specific primers were used. The forward primer sequence was 5-CTAGGCGTTTGTACTCCGTCA-3, and the reverse primer sequence was 5-TCCTCAGGAGTTTATGCACT-3, producing 200 bp products [26]. PCR reactions were performed with 25 µL reaction mixtures using the following thermal cycling program: initial denaturation: 94 °C for 5 minutes, cycling includes 40X: 94 °C for 45 seconds, 58 °C for 45 seconds, and 72 °C for 1 minute, and final extension: 72 °C for 3 minutes. For PCR product detection, 1.5% agarose gel electrophoresis was used.

Multi-locus sequence typing: All DNA specimens that tested positive in PCR were subjected to PCR amplification of seven housekeeping genes (*GlyA*, *leuS*, *lysS*, *mdhC*, *pdhA*, *pykF,* and *yhbG*) for subsequent MLST analysis, following the protocol outlined by Pannekoek et al. (2008) [7] (https://pubmlst.org). To construct the evolutionary tree using multi-locus sequence typing (MLST) data, the goeburst tool was used based on one to three variations between sequence types.

Statistical analysis: The results were transferred to a Microsoft Excel spreadsheet for analysis. Statistical analysis was performed using SPSS software Version 16.0**.** Similarities or differences were evaluated using ANOVA test. *P*-values of ≤ .05 were considered as statistically significant.

Findings

This study was conducted on cervical or urethral swabs and seminal fluid samples of infertile couples who visited gynecology and infertility specialists during 2017 to 2021 in North Khorasan, Iran. Indeed, out of the 268 samples tested, 44 (16.4%) samples were found to be positive for *C. trachomatis* based on PCR analysis (Fig. 1).

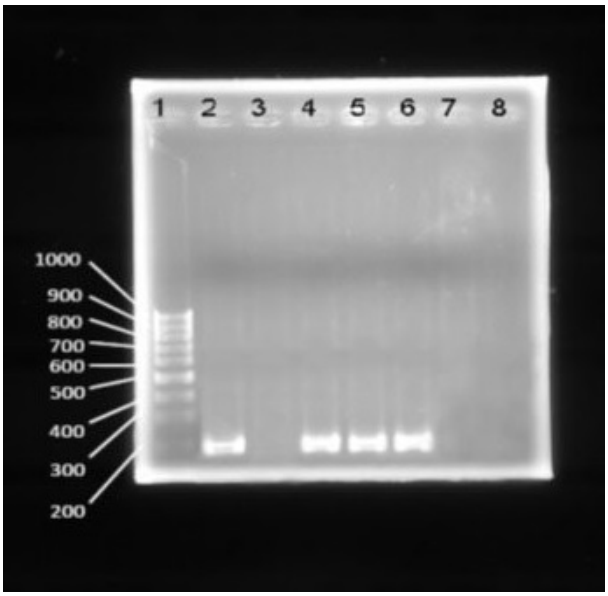

Figure 1) Lane 1: 100 bp ladder, lane 2: 200 bp PCR product of positive control (ATCC_VR_901BD), lane 3: negative control, lanes 4 to 6: 200 bp PCR product of positive clinical samples, and lanes 7-8: negative clinical samples of this study.

Of these 44 positive samples, 35 samples were obtained from women, and only nine samples were from men. Among the 44 positive cases, 15 (34.1%) cases were asymptomatic, while the remaining 29 (65.9%) exhibited typical symptoms associated with genital chlamydial infection, including cervicitis, urethritis, pelvic inflammatory disease, perihepatitis, or proctitis in women; urethritis, epididymitis, prostatitis, proctitis, or reactive arthritis in men; and finally, conjunctivitis, pharyngitis, and lymphogranuloma venereum in both [27] ($p \leq .05$). Among these symptomatic patients, only one case was a man with urethritis, and the rest were women (Table 1). It should be noted that some patients showed more than one clinical sign. In this study, the MLST scheme relying on seven housekeeping genes was used (Fig. 2) based on PCR amplification and sequencing of *GlyA*, *leuS*, *lysS*, *mdhC*, *pdhA*, *pykF,* and *yhbG* genes following the protocol previously outlined by Dean et al. (2009) [1].

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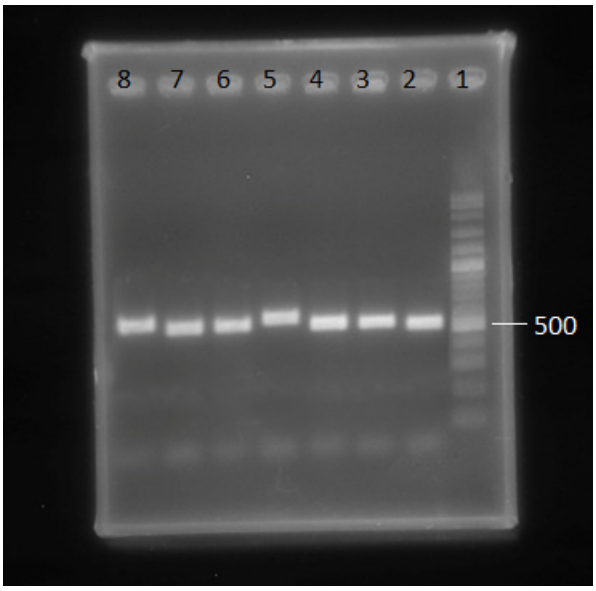

Figure 2) Lane 1: 100 bp ladder, lane 2: 519 bp *leuS* gene PCR product, lane 3: 525 bp *pykF* gene PCR product, lane 4: 522 bp *glyA* gene PCR product, lane 5: 576 bp *lysS* gene PCR product, lane 6: 519 bp *mdhC* gene PCR product, lane 7: 504 bp *yhbG* gene PCR product, and lane 8: 549 bp *pdhA* gene PCR product of *C. trachomatis* positive samples of this study.

Of the 44 *C. trachomatis*-positive samples, 10 cases were not typable with MLST, which may be due to low quality or insufficient quantity of the extracted DNA. Interestingly, only two sequence types were detected among 34 typeable isolates. Of the 34 typable samples, 25 (73.5%) isolates belonged to ST80, and the remaining nine (26.5%) samples belonged to ST4. ST80 is related to Group 3 based on locus variation evaluated by eburst [1], and ST4 belongs to singletons (Fig. 3).

Discussion

The present study results showed the low genetic diversity of *C. trachomatis* strains circulating among infertile couples in North Khorasan. Of the 268 urogenital samples tested, 44 (16.4%) samples were positive for *C. trachomatis*, while recent studies have reported a relatively lower prevalence of 8-12.3% for *C. trachomatis* in Iran [28-31]. In

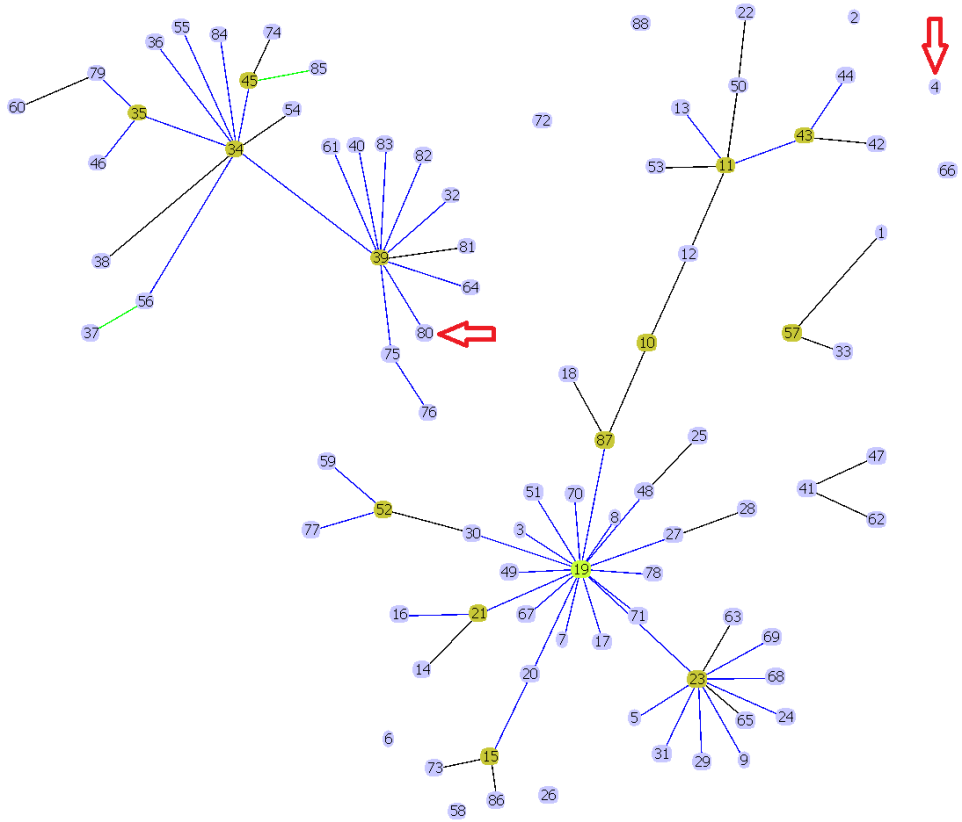

Figure 3) Neighbor-joining tree including SLV, DLV, and TLV of all *C. trachomatis* sequence types Known to this day including ST4 and ST80, found in this study (red arrows).

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Table 1) Prevalence of clinical signs in women participants in this study

	Cervicitis	Urethritis	PID[1]	Perihepatitis	Proctitis	Conjunctivitis	Pharyngitis	LGV[2]
N (%)	19 (67.9%)	4 (14.3%)	10 (35.7%)	0	5 (17.9%)	0	0	0

pelvic inflammatory disease, 2. lymphogranuloma venereum

studies conducted in other countries, the prevalence of *C. trachomatis* has been reported to be 12.4% in China [32],14.3% in the Netherlands [33], and 23% in India [34]. In the last case, the higher prevalence rate in India indicates the higher burden of *C. trachomatis* infection in this country compared to other countries. This elevated prevalence rate could be attributed to India's substantial population and variations in economic, cultural, and health care factors compared to other countries. The difference in prevalence rates could be attributed to variations in sample type, patients' conditions, sample size, diagnostic methods used, and specific settings in each study. In the present study, the prevalence of *C. trachomatis* was 16.4%, which appears to be high for two crucial reasons. Firstly, the sample type consisted of married couples with limited sexual activity. Secondly, due to cultural and religious factors in Iran, heterosexuality is incredibly restricted. Another finding of the present study is also in line with these factors: all of the *C. trachomatis* isolates in this study belonged to only two sequence types (ST4 and ST80), whereas most studies have reported highly variable sequence types. Feodorova et al. (2022) in their study on random samples of heterosexual males and females in Belarus found 12 sequence types, of which seven were common, and five were new. Most of their participants were asymptomatic, whereas in the present study, aside from infertility, 65.9% of patients exhibited other symptoms, especially in the urogenital tract [24]. Also, in another study, Thapa et al. (2020) reported a wide variety of sequence types in *C. trachomatis* isolates detected in endocervical samples of young women in Japan. In their study, most patients referred to specialists for initial screening, and some of them complained of symptoms related to bacterial vaginosis. They found 13 sequence types, consisting of nine common types and four new types. The most prevalent ST in their study was ST39, while ST80 was the most common ST in the present study, and ST4 was the most prevalent ST in the Belarus study [13]. So far, few studies have been done on *C. trachomatis* in Asia. In addition to Japan, there are some reports from Taiwan, Nepal, and Saudi Arabia [1, 35]. Compared to our isolates that mostly belonged to ST80 and ST4 and were detected in urogenital samples of men and women, in Taiwan, the most prevalent STs were ST6, ST11, and ST37, isolated from trachoma and conjunctivitis samples. In Nepal, the most prevalent STs were ST13, ST43, and ST44, isolated from trachoma samples, and in Saudi Arabia, the most prevalent ST was ST22 isolated from conjunctivitis samples. Based on these reports, the common types in each region are unique and different from other regions. Most Asian STs belong to Group 1 and are genetically close together, which may be due to trips between these countries. Of the detected STs in these studies, just ST22 belongs to Group 2, and ST6 is a singleton. Based on Fig. 3, ST11 and ST43 are the cores of Group 1 STs and are closely related. ST13, ST22, and ST44 also belong to Group 1. In this study, ST80 belonged to Group 2, and ST4 was a singleton, and it could be concluded that the STs detected in this study are not close to those of other Asian countries and are closer to those of Belarus.

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Compared with other reports on the distribution of STs in different diseases or asymptomatic patients, the present study findings regarding the prevalence of only ST4 and ST80 in the majority of symptomatic infertile patients may be attributed to the higher pathogenicity of these types in the urogenital tract. However, it should be noted that our sample size was insufficient to draw such a conclusion. North Khorasan is one of the smallest provinces of the country with limited medical facilities and centers compared to other parts of the world and even other cities of Iran, and many routine regular medical screenings that are performed in other parts of the world are not carried out in this province due to financial constraints of the population. On the other hand, it is near to Mashhad, the second largest city in Iran with more experienced doctors and significantly greater facilities, so that many patients who have financial capability visit that city for medical procedures. Thus, the sample size in the present study was small, which may be one of the reasons for obtaining different sequence typing results compared to those of other studies conducted in other parts of the world. To prove this, further extensive investigations in different geographical areas are required.

Conclusion

As to the best of our knowledge, this is the first study on sequence types of *C. trachomatis* isolates in Iran, and conducting similar studies in other parts of Iran is needed to better understand the dispersion of *C. trachomatis* genotypes in Iran. According to the findings, more appropriate preventive and therapeutic strategies should be adopted. Further research and interventions aimed at preventing and treating Chlamydial infections could potentially help reduce the burden of infertility in Iran.

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Ethical approval: Ethical clearance was granted by North Khorasan University of Medical Sciences ethical board (IR.NKUMS.REC.1401.006).

Authors' contributions: AM: carried out analyses and wrote the initial draft of the manuscript, KG: conceptualized and designed the study, AA: conceptualized and designed the study and carried out analyses, SA: conceptualized and designed the study, conducted the final revision of the manuscript, and carried out analyses.

Consent to participate: The authors declare that the participants were aware of the participation in this study and gave their consent.

Conflicts of interests: The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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