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Original Article

Molecular Genotyping of *Chlamydia trachomatis* in Iraqi Married Pregnant and Non-Pregnant Women

Shamkhi, G. J[1 *], Alkhuzai, R. A. H[2, 3], Al-Shukr, N. M. K[2]

1. Department of Biology, College of Science, University of Wasit, Wasit, Iraq
2. Department of Medical Laboratory Technique, the Islamic University, Diwaniya, Iraq
3. Department of Research and Studies, the Islamic University, Najaf, Iraq

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Corresponding Author: gaqeeli@uowasit.edu.iq

Abstract

It has been approved that the infection caused by *Chlamydia trachomatis* (*C. trachomatis*) is one of the major causes of infertility and adverse birth outcomes in populations. The *C. trachomatis* epidemiology among childbearing-age women in Iraq has not been recognized yet. This study aimed to detect the prevalence of *C. trachomatis* infection among pregnant and non-pregnant women using the polymerase chain reaction (PCR) assay and phylogenetic analysis of local isolates. In total, 200 endocervical swabs were collected from adult married pregnant (n=100) and non-pregnant women (n=100) from June to July 2021. Targeting the *omp1* gene, 9% of the total samples were positive for *C. trachomatis*, and significant increases were reported among non-pregnant compared to pregnant women. The PCR products of five positive local isolates were selected randomly, sequenced, and documented in the National Centre for Biotechnology Information (NCBI) with the accession numbers OK094104.1, OK094105.1, OK094106.1, OK094107.1, and OK094108.1. Analysis of the homology sequence of the local and NCBI-BLAST isolates revealed a significant association with the Russian (MF288585.1) isolate. Statistical analysis of reproductive data revealed a higher prevalence, odds ratio (OD), and risk in asymptomatic, compared to symptomatic cases. Although no significant variation was detected in prevalence rate among single and multiple symptomatic women, increases were observed in OD values and risk of multiple symptomatic women. Reportedly, chronic pelvic pain was more prevalent than pelvic inflammatory diseases, ectopic pregnancy, and infertility in single symptomatic women. Regarding the demographic characteristics (i.e., age, the place of residence, and occupation), prevalence and risk of infection were higher in women who were <30 years, lived in urban areas, and had a job, compared to women who were ≥30 years, lived in suburban and rural areas, and had a free job. In conclusion, the course of chlamydial infections is usually unpredictable, diverse, and asymptomatic and has remained almost unrecognized. Therefore, PCR-based methods can apply successfully to detect *C. trachomatis* in both pregnant and non-pregnant women.

Keywords: Chlamydiasis, Demographic factor, Iraq, Phylogenetic analysis, Polymerase chain reaction, Reproductive data

1. Introduction

Chlamydia trachomatis is a Gram-negative obligate intracellular bacterium, which belongs to *Chlamydiaceae* family of *Chlamydiae* phylum (1). Humans are the exclusive natural host of this bacterium, and those with asymptomatic chlamydial infections often do not seek treatment. Therefore, several complications can occur due to the ability of *C. trachomatis* to avoid destruction by the host's innate and adaptive immune systems through autophagy (2, 3). Prolonged exposure of the genital tract to *C. trachomatis* may lead to established pelvic

inflammatory diseases, tubal factor infertility, premature pregnancy termination, and increased susceptibility to ectopic pregnancy in some cases. The unrecognized infected cases act as a reservoir and transmit infections to their sexual partners, resulting occasionally in epididymitis and proctitis (4-6).

Diagnostic methods for detection of chlamydial infection vary according to factors, such as culture, antigen tests, and molecular assays other than indirect methods used for detection of specific antibodies generated against *C. trachomatis* (7). Regarding the culture factor, the sensitivity might be impaired by inadequate sample collection, storage, transport, toxic materials in the clinically collected specimen, as well as overgrowth of cell culture due to commensal microbial agents (8). Difficulties in standardization, labor intensity, and prolonged turn-around time are additional disadvantages of the traditional diagnostic methods (9). Therefore, molecular techniques based on nucleic acid amplification, such as polymerase chain reaction (PCR) have greatly improved the ability to detect *C. trachomatis* infections, and recently, endocervical or urethral swab tests were applied successfully (10). According to the World Health Organization, the annual occurrence of chlamydial infections was estimated to be about 100 million cases worldwide (11). From 2009 to 2015, the Centers for Disease Control and Prevention (Georgia, U.S), reported approximately 1.2-1.5 million *C. trachomatis* cases, which has made it one of the most prevalent sexually transmitted diseases (11). In Iraq, only a few reports have been recently performed to detect the prevalence of *C. trachomatis* infertile (12) and non-fertile women (12, 13).

Therefore, the present study aimed to detect the prevalence of *C. trachomatis* infections in pregnant and non-pregnant women using the conventional PCR assay and determine the association of PCR results with reproductive data and some demographic risk factors (i.e., age, residence, and occupation of the study population). Phylogenetic analysis of some documented local positive isolates in the NCBI was targeted, as well.

2. Materials and Methods

2.1. Sample and Data Collection

A total of 200 adult married women, including 100 pregnant and 100 non-pregnant women, attended the private clinic of gynecologists in Al-Qadisiyah province of Iraq and were subjected to the endocervical swab sampling from June to July 2021. Each cotton swab was cut from the stick by scissor and placed into an Eppendorf tube containing 1.5 ml of transport media (Vircell, Spain), and transported to the laboratory. Data related to the presence/absence of urogenital problems, such as age, residence, and occupation status, were collected as demographic risk factors.

2.2. Molecular Assay

According to the instructions provided by G-Spin™ Total DNA Extraction Kit (Intron, Korea) manufacturer, DNAs were extracted from the swab samples, following the protocol G, and examined using the Nanodrop system (Thermo-scientific, UK) to estimate the values of concentration (ng/µl) and purity (A260/A280). Targeting the region of the *C. trachomatis omp1* gene, one set of primers [(F:5′-ATGAAAAAACTCCTTGAAATCG-3′) and (F: 5′-CTCAACTGTAACTGCGTATTT-3′)] was designed as previously described (4) to prepare the master mix tubes at a final volume of 20 µl by a ready to use AccupPower® PCR PreMix Kit (Bioneer, Korea). Subsequently, the Thermal Cycler system (Bio-Rad, USA) was used for PCR amplification following one cycle of initial denaturation (95 ºC/2 min), 30 cycles of denaturation (95 ºC/30 sec), annealing (53 ºC/30 sec) and extension (72 ºC/30 sec), and one cycle final extension (72 ºC/5 min). Analysis of PCR products was performed using agarose gel (1.5%) stained with Ethidium bromide and electrophoresis at 100 volts, 80 mam for 90 min. The samples were considered positive at a product size of ≈1071 bp.

PCR products of five positive local *C. trachomatis* isolates were subjected to genetic sequencing by the Macrogen Company (Korea) using the Sanger dideoxynucleotide method, and the received data were analyzed using MSAA software (USA) and MEGA-X

software (USA) for phylogenetic tree analysis and homology sequence identity detection.

2.3. Statistical Analysis

All obtained data were documented using Microsoft Office Excel (Version 2016, Microsoft Inc., USA) and analyzed statistically in the GraphPad Prism (Version 6.0.1, GraphPad Software Inc., USA) using chi-square (x^2), one-way ANOVA, and odds ratio (OR). Data were represented as mean ± standard deviation (SD). A *P*-value less than 0.05 ($P<0.05$) was considered statistically significant.

3. Results

Targeting the *omp1* gene, the findings of the PCR assay showed that 8.5% of women were positive for *C. trachomatis*. Significantly, a higher prevalence of positivity was reported among the non-pregnant (13%), compared to pregnant (5%) women (Table 1, Figure 1).

Table 1. Positive results for testing 200 DNA samples by PCR

Reproductive status	No.	Positive	Negative
Pregnant	100	5 (5%)	95
Non-pregnant	100	13 (13%)*	87
Total No.	200	18 (9%)	182

*$P<0.05$

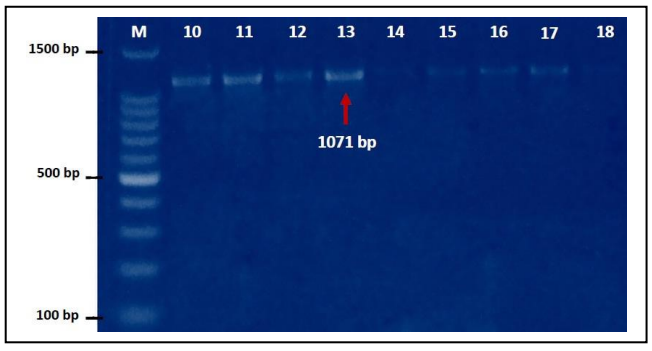

Figure 1. Electrophoresis of stained garose-gel with Ethidium bromide for PCR products at 100 volt, 80 mam for 90 min.
Lane (M): Ladder marker (1500-100 bp)
Lane (P): Positive control
Lanes (1-18): Positive samples for *C. trachomatis* at product size ≈ 1071 bp (lanes 1-5 for pregnant and lanes 6-18 for non-pregnant women).

The data from the reproductive system showed that the prevalence rate, OD, and risk of infection, respectively, were increased significantly among the asymptomatic cases (17.07% [7/41], 2.78, and 2.46), compared to symptomatic cases (6.92%, [11/159], 0.36, and 0.41). In symptomatic cases, although insignificant variation ($P\leq0.05$) was observed in the prevalence rate of single (4.76%, 3/63) and multiple (8.33%, 8/96) symptoms, significant increases were detected in values of OD and risk for cases of multiple symptoms (1.8 and 1.73, respectively) in comparison with the cases of single symptom (0.56 and 0.58, respectively). In single symptom cases, significant higher values were observed for prevalence, OD, and risk in patients with a chronic pelvic pain (9.09% [2/22], 4, and 3.73, respectively), compared to those reported in pelvic inflammatory diseases (2.86% [1/35], 0.38, and 0.4, respectively), ectopic pregnancy (0% [0/1], 0, and 0, respectively), and infertility (0% [0/5], 0 and 0, respectively) groups (Figure 2).

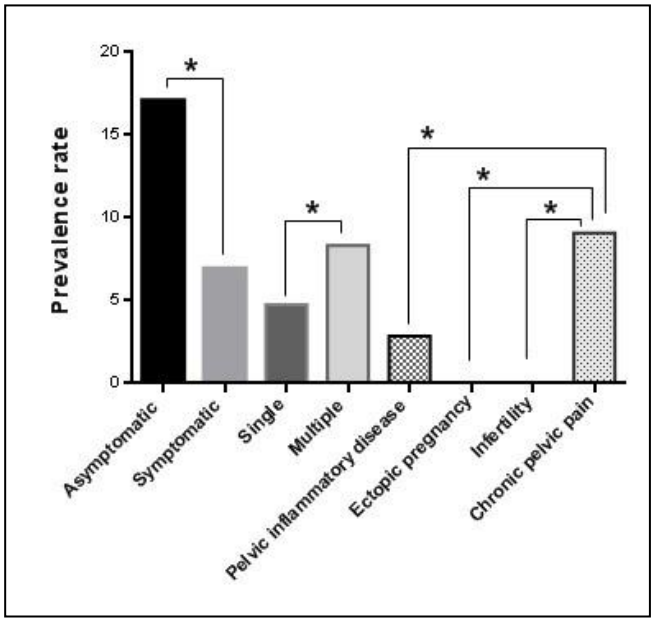

Figure 2. Prevalence rates of reproductive data among study population *$P<0.05$

Regarding demographic risk factors, significant differences were observed in the values of study groups (Table 2). Regarding the age, the prevalence of

infection (positivity) and risk was significantly higher in individuals <30 years (11.76% and 3.24, respectively), compared to those ≥30 years (3.7% and 0.31, respectively). The prevalence of positive infections and higher risks were detected in individuals residing in urban areas (11.56% and 0.63, respectively), compared to those residing in suburban (2.7% and 0.26, respectively) and rural areas (0% and 0, respectively). Concerning the occupation factor, a significant prevalence of infection (positivity) and higher risk were observed in cases having a job (13.03% and 2.32, respectively), compared to cases with a free job (5.56% and 0.43, respectively).

Based on of *omp1* gene, the locally sequenced *C. trachomatis* isolates were named *Chlamydia trachomatis* H. No. 1, 2, 3, 4, and 5 and documented subsequently in the NCBI under the accession numbers OK094104.1, OK094105.1, OK094106.1, OK094107.1, and OK094108.1. Phylogenetic analysis of the local *C. trachomatis* isolates revealed a significant association with the NCBI-BLAST Russian isolate (MF288585.1) at 99.61-99.81% range of identity, and 0.0015-0.0005% range of genetic changes/mutation (Figure 3, Table 3).

Table 2. Distribution of positive results according to demographic risk factors

Factor	Total No.	Positive	Odd ratio	Risk
Age (Year)				
< 30	119	14 (11.76%) *	3.5	3.24
≥ 30	81	3 (3.7%)	0.29	0.31
Residence (Area)				
Urban	147	17 (11.56%) *	6.89	0.63
Suburban	37	1 (2.7%)	2.39	0.26
Rural	16	0 (0%)	0	0
Occupation				
Having-job (Employee, student)	92	12 (13.03%) *	0.26	2.32
Free-job (Housewife)	108	6 (5.56%)	3.92	0.43

Significance level * (P<0.05)

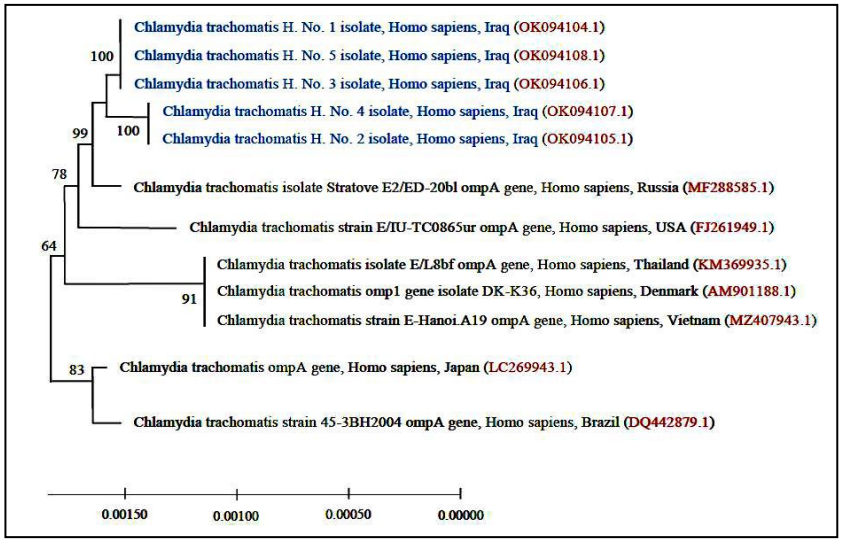

Figure 3. Phylogenetic tree analysis based on *omp*1 gene and partial sequence of local and NCBI-BLAST *C. trachomatis* isolates

Table 3. Homology sequence identity between the local and NCBI-BLAST *C. trachomatis* isolates

Local isolate		NCBI-BLAST isolate		
Name	**Accession No.**	**Country**	**Accession No.**	**Identity (%)**
Chlamydia trachomatis Human No. 1	OK094104.1	Russia	MF288585.1	99.71
Chlamydia trachomatis Human No. 2	OK094105.1	Russia	MF288585.1	99.61
Chlamydia trachomatis Human No. 3	OK094106.1	Russia	MF288585.1	99.81
Chlamydia trachomatis Human No. 4	OK094107.1	Russia	MF288585.1	99.81
Chlamydia trachomatis Human No. 5	OK094108.1	Russia	MF288585.1	99.81

4. Discussion

Sexually transmitted diseases represent a global burden caused by a dramatic increase in the number of new infections, particularly *C. trachomatis* cases, as one of the most prevalent bacterium worldwide. Therefore, screening programs were performed in the developed countries to detect the prevalence of these infections and prevent the respective complication (14, 15). In this study, the total prevalence of *C. trachomatis* infections determined by PCR was 9%. A review of the literature showed that the molecular prevalence of *C. trachomatis* in women was 22% in Iraq (16), 20.2% in Palestine (17), 11.1-13.8% in Iran (18), 5.2% in Italy (19), 13.5% in India (4), 10.4% in the United Arab Emirates (20), and 1.85-12.7% in Turkey (15, 21). This disparity in the prevalence of infection might be attributed to environmental and socio-economic factors, sampling method, the number of tested samples, diagnostic-based method, and targeted gene (14, 22). Other information surveys throughout many countries, including the USA, suggested that the rate of *Chlamydia* was not decreased, despite ongoing efforts to control the disease (23, 24). Bianchi, Frati (25) recorded that data on the occurrence and molecular epidemiology of chlamydial genital infection, in particular among the younger asymptomatic women, has remained unpretentious. In general, variation in the prevalence of genitally chlamydial infections is related to education, the number of sexual partners, marital status, income, race/ethnicity, and age (26).

In terms of reproduction, asymptomatic cases showed a high prevalence and risk, compared to symptomatic cases. Many studies have demonstrated that most acute infections are asymptomatic, resulting in late diagnosis with continuous pathogen transmission (25, 27, 28). Zimmerman, Potterat (29) mentioned that about 80% of genital chlamydial infections occur without apparent clinical symptoms or evidence of complication and often resolve spontaneously (29). However, little information is available regarding the clinical factors influencing untreated and uncomplicated genital illness. According to Morré, Van Den Brule (30), *C. trachomatis* infections can be both asymptomatic and symptomatic with frequent late complications that vary widely from 2 to 20%. Ascending infections, also known as pelvic inflammatory diseases, are long-term consequences that tend to be chronic and recur with scarring complications possibly related to hypersensitivity mechanisms (31). Haggerty, Gottlieb (32) reported that the number of complications is associated with the severity and the number of disease episodes. Bachmann, Richey (33) detected the existence of pelvic pain during the time between examination and therapy and assumed that 18% of women developed pain in three months, and more than 50% of women developed pelvic pain within 12 months. Zarb, Coignard (34) observed that the random examination of asymptomatic infection, tracing of contacts, and mandatory notification can help explain a high rate of notification. Mehrabani, Behzadi (20) showed that none of the women were aware of their infections before the survey; however, some clinical signs were apparent.

Our findings confirmed those obtained by Ali and Shia (16) who showed that women aged <35 years had a higher prevalence of infection, compared to women

in the age range of 40-55 years. However, this finding was inconsistent with those reported by Tosun, Cihanyurdu (21) who showed that there was no significant association between age and prevalence of infection. In this regard, Rabiah, Mutahar (35) reported that the infection was significantly more prevalent among old-aged women. Globally, *C. trachomatis* infection is most prevalent in young women (14-25 years), which can be explained by asymptomatic infection, inadequate partner treatment, and delayed development of protective immunity (36). In older age groups, a significant decrease in the rate of chlamydial infection presumably results from acquired immunity that can play a role in shortening disease duration as indicated by Bailey, Duong (37). However, there are obvious difficulties in estimating the duration of genital *C. trachomatis* infection. In the study performed by McCormack, Alpert (38), a reexamination of seven women with untreated cervical infection 15 months after the date of infection detection showed that four women were found to be infected on the second occasion, suggesting that some infected women could suffer from repeated episodes of infection. However, the existence of an inexpensive treatment against all infections and the fact that the presence of new or specific symptoms, if untreated, can lead to a rise in reproductive tract morbidity among women has made *Chlamydia* screening necessary for all sexually active women aged 25 years or younger (39-41).

A positive correlation was observed in this study between factors of occupation and place of residence and the prevalence of chlamydial infection. The results obtained by West, Lawlor (42) indicated that despite the increased equality of opportunities, the family background played a great role in the inheritance of income as well as education and social class. The increase in chlamydial infections in urban areas may be attributed to the fact that these areas are often highly contaminated. Neiderud (43) recorded that although urbanization has many advantages, it creates potential risks and challenges in terms of an increase in the prevalence of infectious diseases due to different factors, such as the high density of inhabitants (overcrowding) which facilitate the transmission of infection, and environmental contamination that increase the chance of infection. Patterson-Lomba, Goldstein (44) found that even with similar socio-economic indicators, the incidence of *Chlamydia* increased in larger areas, suggesting that doubling the size of the city population could result in an exceptional increase (more than two folds) in the incidence of sexually transmitted diseases.

Based on the results of this study, a significantly higher rate of infection was observed among working women and students. We highly propose that exposure to physical, psychological, or physiological stress can result in high competition, increases the pressure of life in society, and impacts public health significantly. Different stresses may change the immune response of a host, including the level of immunologic markers associated with activities of the neuroendocrine system affected by catecholamines and corticosteroids (45). Rabiah, Mutahar (35) reported that chlamydial infection was very much influenced by one's behavior and lifestyle.

According to Sun, Pal (46), the major outer membrane protein of *Chlamydia*, which encodes gene (*ompA*) and contains four symmetrically spaced variable domains (VDs-IV), maintains the structural rigidity of the outer membrane and facilities porin formation, permits diffusion of solutes through the intracellular reticulate body membrane, and is believed to play a role in pathogenesis and possibly adhesion. In this study, phylogenetic analysis of the *omp1* gene of the local isolates revealed their significant associations with the NCBI BLAST Russian isolate (MF288585.1), which was detected to be the cause of chronic asymptomatic genital infection and infertility in a couple undergoing *in vitro* fertilization (47).

Our data indicated that *C. trachomatis* and the negative impacts of infections in married women might be more widespread than expected. This illustrates the significance of efficient investigation techniques in the detection of infertility cases. In addition, the

characterization of *C. trachomatis* strains can provide valuable information concerning the variants circulating in the community. For more knowledge about the epidemiology of chlamydial infections, effective efforts should be made to prevent the spread of infection.

Authors' Contribution

G. J. S. and R. A. H. A., contributed equally to molecular examination, data collection, statistical analysis, and manuscript writing.

Ethics

The study protocol was approved by the Ethical committees of the Department of Biology, College of Science, University of Wasit, Wasit, Iraq; the Medical Laboratory Technique Department, The Islamic University, Diwaniya, Iraq; and the Research and Studies Department, The Islamic University, Najaf, Iraq. The swabs were collected by all participants following an unwritten agreement.

Conflict of Interest

The authors declare that they have no conflict of interest.

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