

Detection of Genital *Chlamydia trachomatis* Infection using PCR and Immunofluorescence Methods within Women Infertility in Iran

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For long time infertility was one of the most sequels in medical science. Microbial agents are one of its causes. Possible etiologic role of *Chlamydia trachomatis* in infertility has been suggested for years ago but it has not been approved completely yet. This study was a case-control type of investigation done over 150 infertile women without physiological deficiency for infertility and control group was 200 fertile safe and impregnated women. The presences of *Chlamydia trachomatis* in two groups examined by direct, indirect immunofluorescence tests and PCR. *Chlamydia trachomatis* was detected by direct immunofluorescence method in 23(15.3%) of infertile women compare with 7(3.5%) of control population. Using indirect immunofluorescence tests a positive test titer of 1:16 and above was detected in 34(22.6%) of infertile cases and in 9(4.5%) of control group. *Chlamydia trachomatis* was detected by PCR method in 48(32%) of infertile women compare with 13(8.7%) of control population. Thus, the results of our study suggest that there is a significant association between *C. trachomatis* infections and female infertility.

Key words: *Chlamydia trachomatis*, Infertility, Women, Iran.

Urogenital infections are caused by *Chlamydia trachomatis* worldwide¹. This bacterium is the most prevalent cause of bacterial sexually transmitted diseases (STD). The prevalence of these infections can be deferent depending on the country and population type. The prevalence of *C. trachomatis* infections among the sexually active women in developing countries is higher than developed countries^{2, 3} Annually

about one million *C. trachomatis* infections is reported among sexually active young women in the united states According to the Center for Disease Control and Prevention (CDC)⁴. Most of the infected people are asymptomatic so the majority of these infections are not detected and treated which can lead to pelvic inflammatory disease reaching of bacterium to the upper genital tract of infected women⁵. The etiological relation of pelvic inflammatory disease (PID) with microorganisms is not completely determined but presence of a variety of microorganisms including *C. trachomatis*, *Mycoplasma genitalium*, *Neisseria gonorrhoeae* and some other microorganisms have been shown in lower and upper genital tracts of women with PID⁶⁻¹¹. Nevertheless *C. trachomatis* is more suspected

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than other sexually transmitted diseases to causes symptomatic PID^{9, 12, 13}. PID can induce some disorders such as infertility and ectopic pregnancy. These disorders may be the results of the destruction of cilia covering of the fallopian and closure of fallopian tube¹⁴. Thus, detection and control of *C. trachomatis* infections prevalence is necessary to prevent of its related sequels. The period time of tubal inflammation and damages resulted from infection are two important factors which affect the efficiency of *Chlamydia* control programs. Therefore, it is important to detect and treat the infections before its development to short-term sequels like pelvic inflammatory diseases which in return can be developed to long-term sequels such as tubal factor infertility and ectopic pregnancy¹⁵. In the country of Iran, there is low epidemiological data regarding the prevalence of *C. trachomatis* infections and their sequels and it is clear that having more epidemiological knowledge about genital *C. trachomatis* infections prevalence could be very effective in choosing of efficient strategies for screening and treatment of infections. The purpose of this research was to determine the prevalence of *C. trachomatis* in healthy women and women with the disorder of infertility to evaluate the association of *C. trachomatis* infections on infertility in Iran.

MATERIALS AND METHODS

Population

This research was a case control study enrolling 350 women. The case group was constituted from 150 infertile women with an average age of 24.3 years who did not have any recognized physiological deficiency for infertility. The control group was comprised of 200 impregnated women with an average age of 25.2 years who had one or more successful childbirths and didn't have any history of infertility (Table 1).

Specimen collection

Two types of specimen from both the case and control groups were used in this study including endocervical mucosa and serums. Sampling from endocervical mucosa was performed using speculum and sterile swab.

Laboratory methods

Direct and indirect immunofluorescence methods were used for determining of presence or

absence of *C. trachomatis* infection in both of case and control groups. For direct test endocervical swabs were transferred on the clean slides and the slides were incubated in the room temperature for drying and fixation. One drop of anti-chlamydial monoclonal antibody was added on the slides and they were incubated at 37°C for 10 minutes. After that the slides were washed with PBS buffer and distilled water for 10 and 5 minutes respectively. Finally the slides were examined for *C. trachomatis* by fluorescence microscopy. In this technique the elementary bodies are observed as fluorescence green spots. For indirect test *C. trachomatis* serotypes D-K, L₁-L₃ and IOL-207(TWAR) were used as standard antigens. The serotypes were grown in egg individually and then they were placed in the determined places on the slides. A place on the slide was also specified to the mixture of egg yolk in PBS as negative control. The serums in dilutions from 1:16 to 1:256 were prepared and added to slides individually. After 30 minutes incubation at 37°C, the slides were washed and then were stained with fluorescence anti-human globulin. Finally the slides were examined by fluorescence microscopy and the results were recorded base on the color and fluorescence intensity of the slides (table 2).

PCR mixture and DNA amplification

The endocervical swab samples were obtained from the patients. Each swab was put in the sample collection vial containing the buffer. Several methods are available for DNA extraction. In the present study, we used boiling method. We were selected one pair of oligonucleotide primers specific for a region of the *C. trachomatis* gene (Accession No: AB695165.1) coding for the major outer membrane protein (MOMP). The sequences from 5' to 3' of these oligonucleotide primers are as follows:

Forward: 5'-CCT GTG GGA ATC CTG CTG AA-3'
Revers: 5'-GTC GAA AAC AAA GTC ACC ATA GTA-3'

A master mixture of these reagents was made for the samples along with the positive and negative controls. The final reaction mixture of 50µl for each sample contained 0.5µM each primer; 100µM each of dATP, dCTP, dGTP and dTTP; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl₂ and 1.25 units of Taq DNA polymerase enzyme and 9µl of sample DNA. Each microfuge tube

containing the PCR mix of 50µl was mixed and subjected to 40 cycles of amplification. Each cycle composed of sequential incubations of 94°C for 1 minute for DNA denaturation, 52°C for 1 minute for annealing primer to these templates, and 72°C for 30 seconds for DNA chain extension. At the end of 40 cycles, samples were kept for another 7 minutes at 72°C for completion of extension of DNA chain. The PCR product samples were immediately frozen for further analysis. The visualization of amplified product was carried out by agarose gel electrophoresis. A 10µl of post-PCR mixture was subjected to electrophoresis on 2% agarose gel in the presence of ethidium bromide. A DNA ladder was also run simultaneously to confirm size of the amplified product (103 bp). The DNAs were extracted from the bands on the gel using gel extraction kit (Qiagen, GmbH, Germany) and then were sequenced by Macrogen Inc, Seoul, Korea.

RESULTS

The results of direct and indirect immunofluorescence tests have been shown in table 3. As the results have show, the prevalence of *C. trachomatis* in case and control groups was achieved 15.3% and 3.5% respectively using direct immunofluorescence method. In indirect immunofluorescence method the prevalence of *C. trachomatis* was achieved 22.6% and 4.5% in case and control groups respectively. Too, in our study, prevalence of *C. trachomatis* in case and control groups was achieved 32% and 8.7% respectively using PCR method. The amount of P_{value} for the direct, indirect immunofluorescence and PCR results was calculated by Fisher test which was obtained $P_{value} < 0.001$ for direct tests results and $P_{value} < 0.004$ for indirect and PCR tests results (Table 3).

Table 1. The age average of case and control groups

Age groups	Case group (n=150)	Control group (n=200)
20	31	31
20-24	23	55
25-29	80	93
30-34	10	12
35-39	4	5
40>	2	4
Age average	24.3	25.2

Table 2. Determining of the positivity or the negativity of the tests base of fluorescence intensity and color of slides

Fluorescence intensity and color	Positivity or negativity
very shiny green	+++
Shiny green	++
green	+
yellowish green	±
orange	-

Table 3. Results of direct and indirect immunofluorescence tests for diagnosis *C. trachomatis* in case and control groups

Anti-chlamydial antibody		Positive in Case group n=150	Positive in Control group n=200	Fisher test results
Titer				
Direct immunofluorescence		23 (%15.3)	7 (3.5%)	$P_{value} < 0.001$
Indirect immunoFluorescence	1:16	10 (%6.6)	(2.5%)5	$P_{value} < 0.004$
	1:32	18 (%12)	2(1%)	
	1:64	6 (4%)	2(1%)	
	1:128	0	0	
	PCR	(%32)48	13(8.7%)	

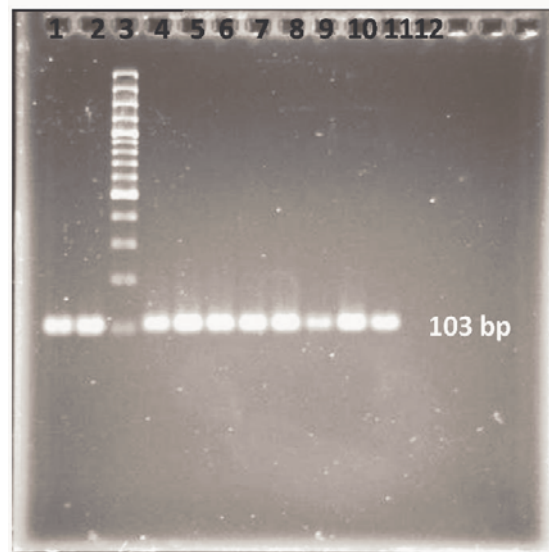


Fig. 1. Uniplex PCR for detection of MOMP gene in clinical strains of *C. trachomatis*.
Lane 1, 2, 4-10 clinical samples, lane 11, 12 positive and negative controls strains respectively,
Lane 3 molecular weight marker 100-bp DNA.

DISCUSSION

C. trachomatis is one of the most prevalent causes of sexually transmitted diseases. It is the major cause of urethritis and cervicitis, and their sequels such as PID and tubal factor infertility¹⁶. Generally *Chlamydial* infections are more destructive for reproductive health in women than men¹⁷.

Svenstrup *et al.* reported that 23% of women suffering tubal factor infertility (TFI) had antibodies against *C. trachomatis* compared with 15% of women in control group which had normal tubes¹⁸. In another research, Siemer *et al.* showed considerably higher prevalence of IgG and IgA antibodies (39% and 14%, respectively) among women with infertility compared with members of the control group (19% and 3% respectively)¹⁹. In a study which was carried out by Malik *et al.* the presence of *C. trachomatis* in infertile women was determined 28.1% which was significantly higher than that was detected (3.3%) in healthy women ($P_{value} < 0.01$)²⁰. Gaudoin *et al.* reported that 91.2% of woman with tubal occlusion have shown IgG anti-chlamydial antibodies in their sera²¹.

Our results are also compatible with the above studies. In the present study the presence of *C. trachomatis* infection in the 150 infertile women with an average age of 24.3 years as case group and 200 healthy women with an average age of 25.2 years as control group were examined using the direct, indirect immunofluorescence and PCR methods. The test for 15.3% of case group and 3.5% of control group was positive when the direct immunofluorescence method was performed. Indirect immunofluorescence test also determined that 22.6% of case group and 4.5% of control group members had an equivalent or higher titer of 1:16. The obtained amount of P_{value} from the results analysis of direct and indirect immunofluorescence experiments by Fisher test were $P_{value} < 0.001$ and $P_{value} < 0.004$ respectively which confirmed the significant association between urogenital *Chlamydial* infections and infertility. The difference between the results of direct and indirect immunofluorescence tests could be due to old *Chlamydial* infections or infections in the other places of the body.

CONCLUSION

Our results suggested that there is a significant association between *C. trachomatis* infections and female infertility. Therefore *C. trachomatis* can be one of the main ethological factors for female infertility. Consequently it is recommended that infertile women without any physiological deficiency should be examined for contamination with *Chlamydia trachomatis*. Finally, for detection genital *C. trachomatis*, PCR results is reliable than immunofluorescence tests.

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