Original Article

Diagnostic Value of PCR and ELISA for *Chlamydia trachomatis* in a Group of Asymptomatic and Symptomatic Women in Isfahan, Iran

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Abstract

Background: *Chlamydia trachomatis* infections are the most prevalent sexually transmitted bacterial infections (STI) in the world that lead to a cause of tubal factor infertility in women. The aim of this study is to determine the presence of *C.trachomatis* by polymerase chain reaction (PCR) and ELISA.

Materials and Methods: Endocervical swabs were collected from 80 women; 22 of them were asymptomatic and 58 symptomatic. Samples were examined by PCR designed to detect Chlamydial plasmid using specific KL1 and KL2 primers. Serum IgG and IgA antibodies to *C.trachomatis* were detected by ELISA. Since elevated CRP levels are a marker for inflammation, the presence of C- Reactive protein (CRP) has also been evaluated in all samples.

Results: The rate of *C.trachomatis* infection by PCR was revealed to be 27.2% and 18.9% in asymptomatic and symptomatic women, respectively The χ^2 test shows no significant difference (p value= 0.22). Serological screening was done on all samples. The high level of IgG and IgA to *C.trachomatis* infection was 29.4% and 17.6%, respectively. The presence of high levels of CRP, as a serological marker of persistence infection, was 31.8% and 34.4% in asymptomatic and symptomatic women, respectively. The high rate of CRP level in the samples indicates acute infections in both groups.

Conclusion: Genital *C.trachomatis* infection is the leading cause of tubal factor infertility. The present study shows that *C.trachomatis* infection could be present in symptomatic as well as asymptomatic women. Therefore, a screening test for *C.trachomatis* infection is recommended for all women who refer to gynecologic outpatient departments in Isfahan and other parts of Iran.

Keywords: *Chlamydia trachomatis, Chlamydia trachomatis* Infection, Sexually Transmitted Diseases, PCR, ELISA

Introduction

Chlamydia trachomatis is subdivided into 18 serotypes: (A-K), L1, L2, L3, Ba, Da, Ia, and L2a (1). *C.trachomatis* is a bacterial infection of the genital tract affecting both men and women; although up to 80% of infected women and 50% of infected men may be asymptomatic (2). When symptoms do occur, usually 1 to 3 weeks following exposure, they are followed by dysuria, abdominal pain and abnormal vaginal discharge (2-4). *C.trachomatis* (serotypes D-K, Da, Ia) is the most important cause of sexually transmitted diseases (STD) such as cervicitis, salpingitis, urethritis, urethral syndrome and pelvic inflammatory disease. During pregnancy, chlamydial genital infection increases the risk of spontaneous abortion, premature delivery and ectopic pregnancy. Infertility is a complication of these infections (3-6). Infants born to pregnant mothers with genital chlamydial infection are also often at risk of developing conjunctivitis and pneumonia (4, 6). There are several methods for detection of Chlamydia infection such as: cell culture, ELISA, micro immuno fluorescence(MIF), direct fluorescence antibody (DFA) and molecular methods such as PCR (7, 8). *C.trachomatis* infection is easily treated with antibiotics, so

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detection could be very helpful in controlling *Chlamydia* infections (9). The aim of this study is to evaluate the incidence of *C.trachomatis* infection in a cohort of asymptomatic and symptomatic women in Isfahan.

Materials and Methods

Study groups

This project was approved by the ethic committee of Shahid Beheshti Hoispital. Samples were collected after obtaining written informed consent from 80 patients who attended the Gynecology Outpatient Department of Shahid Beheshti Hospital in Isfahan, Iran. Endocervical swabs were collected from 58 symptomatic women ranging from 20 to 60 years old (mean 36.3 ± 8.8 years) and 22 asymptomatic women ranging from 19 to 56 years old (40 ± 10.5 years). All women were subjected to a thorough clinical examination and the case history was recorded individually. The median ages of women in both groups were 37.5 ± 9.4. Those who had received antibiotics in the last 4 weeks were excluded from the study.

Specimen collection

From each patient, an endocervical swab was transferred to a 15ml plastic vial containing 5 ml of sterile phosphate buffered saline (PBS) and stored at -70º C until the DNA isolation stage (10, 11). In addition, 5 ml of peripheral blood was collected from each patient for serological investigation (10).

Serological tests

Blood samples were taken from 80 women and sera were used to determine the level of IgG and IgA antibodies against *C.trachomatis* by p-ELISA (Medac, Germany) kits. All assays and calculations were performed according to the manufacture's instructions.

The p-ELISA is based on "a synthetic peptide" from the immunodominant region of the major outer membrane protein. The ELISA kit used in this study was very specific for detecting *C.trachomatis* and there was not any cross-reactivity with the other species of *Chlamydia*. Hence, it enabled us to detect only anti *C.trachomatis* antibodies in the samples, i.e. IgG and IgA.

This microtiter assay uses peroxidase-conjugated antihuman IgG and IgA antibodies to bind to *C.trachomatis* IgG and IgA antibodies. After incubation with tetramethyl-benzidine (TMB) substrate, the reaction is stopped by the addition of sulfuric acid. The absorption is read photometrically at 450 nm by an ELISA-reader. The intensity of the color is proportional to the concentration of the specific antibody in the sample. Cut-off values have been calculated according to the manufacturer's instructions. Results in the gray zone have been considered negative in the calculation (12, 13). The level of CRP was determined by using the CRP kit (Omega, United Kingdom). The assay was done according to the manufacturerr's instructions (14,15).

DNA extraction

The swab sample was removed from the vial and centrifuged at 2000 rpm for 15 min. The supernatant was discarded; the remainder was vortexed and transferred to a 1.5ml micro tube. This step was followed by centrifugation at 2000 rpm at room temperature for 15 min. The supernatant was removed and added to Tris-Base-EDTA (TE) solution before dividing it equally into 2 micro tubes. In order to find the best extraction, two different DNA extraction methods were used as followes:

1. Non-organic method with Proteinase K
2. Boiling for 10 minutes

The above mentioned methods were assessed as explained below.

Non-organic

Endocervical smears were collected in 400 µl 10mM Tris-HCl, pH 8.0 and 1mM EDTA. Each sample was supplemented with 4 µl proteinase K (10 µg/ml) and 4ml triton 10% (v/v), followed by incubation at 55º C for 90 minutes and then at 95º C for 30 minutes. The samples were maintained at -20ºC until used (11).

Boiling

Endocervical smears were collected in 400 µl 10mM Tris-HCl, pH 8.0 and 1mM EDTA and heated at 100º C using a water bath for 10 minutes. The samples were maintained at
-20ºC (1).

The quality of DNA extraction was confirmed by β globin PCR (10).

Detection of Chlamydia trachomatis by PCR

To detect the presence of *C.trachomatis* in the cervical DNA samples, a 241 bp fragment of the bacterial endogenous plasmid was amplified. The primers used for the C.trachomatis plasmid PCR were KL1 (5`-TCCGGAGCGAGTACGAAGA -3`) and KL2 (5`-AATCAATGCCCGGGATT-GGT -3`).

PCR was performed on 5µl of the extracted DNA sample in a final reaction mixture of 25 µl. The final reaction mix contained 3 mM $Mgcl_2$, 0.28 µM deoxynucleotide triphosphate, 16 pM of

each primers and 1 U of Taq polymerase. The PCR cycle was done as follows:
(94ºC /1 min, 55 ºC /1 min, 72 ºC / 1 min)×40, 72 ºC 8min (11). The PCR products were analyzed by 1.5 agarose gel electrophoresis (Fig 1). In this study, *C. trachomatis* serovar A was used as positive control and the sample that contained only distilled water was used as a negative control.

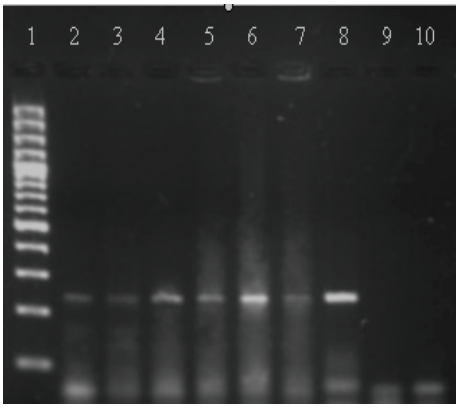

Fig 1: Analysis by agarose gel (1.5%) electrophoresis of the PCR products. The 241bp DNA corresponds to the specific Chlamydial amplified plasmid DNA sequence. Lane 1: 100 bp DNA ladder. Lanes 2, 4, 6,:-PCR products by boiling for 10 minutes method. Lanes: 3, 5, 7: PCR products by proteinase K method. Lane 8: Positive control. Lane 9,10: Negative control.

Statistical analyses

Data analysis was carried out by using the SPSS version 15 for Windows. For normally distributed data, means and standard deviations were calculated and compared by using Student`s t test, Chi-square, Fisher exact test and Mc-Nemar test. The x^2 test was applied to compare Chlamydia infection and history of STI. Statistical significance was the 5% level.

Results

The studied group of women ranged from 19-60 years old (mean 37.5 ± 9.4). There were 12.5% under 25; 32.5% between 25-35, 36.3% between 35-45, 15 % between 45-55 and 3.7% were more than 55 years old. The highest frequency of *C.trachomatis* cervical infection was found in the women aged 35 to 45 years (Table 1).
Successful amplification of a 241bp fragment of *C.trachomatis* plasmid genome was considered as a positive result by PCR. Out of 80 endocervical samples tested, 17 (21.25%) were positive for *C.trachomatis* (Fig 1). The prevalence of chlamydia infection by PCR was 27.2% and 19% in asymptomatic and symptomatic women respectively (Table 2).

Table 1: Effect of age and the prevalence of Chlamydia trachomatis infection detected by PCR

Age group (yr)	Positive No.(%)
<25	1/80 (1.25%)
25-35	5/80 (6.25%)
35-45	8/80 (10%)
45-55	1/80 (1.25%)
>55	2/80 (2.5%)
Total	17/80 (21.25%)

yr= years old; Positive No= Number of positive cases, percentage of cases represented in parenthesis

Out of 80 patients, 6.2% (5/80) were IgG seropositive and 3.7% (3/80) were IgA seropositive. Results are shown in detail in Table 2. In all the samples, two patients (2.5%) that were IgG seropositive also showed specific *C.trachomatis* IgA; both of which were in the symptomatic cohort of women. The sensitivity and specificity for IgG-ELISA were 29.4% and 100% and for IgA-ELISA were 17.6% and 100%, respectively.
Out of 80 samples that were tested for CRP, 27 showed a high level of CRP. Among these 27 patients who had a high level of CRP, 6 (22.2%) showed positivity for PCR. In addition, 2 samples (7.4%) were seropositive in ELISA. Out of these 2 samples, one patient showed IgG seropositivity and the other IgA seropositivity. The IgA seropositive sample belonged to the subfertile group (Table 2).
Based on PCR results, there was infection in 19.2 % (10/52) of the women with vaginal discharge and 25 % (7/28) of the women without vaginal discharge. Of those women with abdominal pain (33 of the patients), 6 were positive.

Table 2: The results of three different tests for detection of Chlamydia trachomatis

Test		Symptomatic Group No (%)	Asymptomatic Group No (%)	Total
PCR		11/58(19%)	6/22(27.2%)	17/80(21.25%)
ELISA	**IgG**	2/58(3.4%)	3/22(13.6%)	5/80(6.2%)
	IgA	3/58(5.1%)	0	3/80(3.7%)
CRP		20/58(34.5%)	7/58(31.8%)	27/80(33.7%)

Table 3.The Prevalence of Chlamydia trachomatis infection in women

Clinical manifestation	Result of clinical manifestation		Positive PCR	Negative PCR
Vaginal discharge	Positive	52	10(19.2%)	42
	Negative	28	7(25%)	21
Abdominal pain	Positive	33	6(18.2%)	27
	Negative	47	11(23.4%)	36
Past history of premature rupture of fetal membrane	Positive	0	0	0
	Negative	80	17(21.25%)	63
Past history of ecto- pregnancy	Positive	0	0	0
	Negative	80	17(21.25%)	63
Past history of abortion	Positive	15	5(33.3%)	10
	Negative	65	12(18.5%)	53
Past history of low birth weight child	Positive	10	2(20%)	8
	Negative	70	15(21.4%)	55
Past history of infertility	Positive	13	2(15.4%)	11
	Negative	67	15(22.3%)	52
Contraceptive methods	OCP	5	0	5
	IUD	8	4	4
	Condom	17	1	16
	Vasectomy	10	3	7
	TL	12	1	11
	Withdrawal	19	6	13
	None	9	2	7

OCP= Oral contraceptive pills; IUD = Intrauterine device; TL =Tubal ligation

Of women without abdominal pain (47 of the samples), 11 were positive. All women who took part in this study had no history of premature fetal membrane rupture and ecto pregnancy. In women with a past history of abortion, the prevalence was 33.3% (5/15) and in women without this clinical manifestation it was 18.5% (12/65). Those women with a past history of low birthdate of low birth weight child, showed 20% PCR positivity and the positivity among those women without this manifestation was 21.4%. In women with past history of infertility, the prevalence was 15.4% (2/13) and in women without having a history of infertility, it was 22.3% (Table 3).

Those women who previously used a contraceptive method (71 of the studied patients), showed 21.1% positivity for PCR. On the other hand, those who had not used any means of contraception showed 22.2% PCR positivity. Detailed results are shown in Table 3.

Discussion

C.trachomatis is one of the most frequent causes of sexually transmitted diseases and could seriously affect public health. Therefore, effective epidemiological control starting with an adequate method for correct and sensitive diagnosis is required (11, 16). The data presented in this study demonstrate, by PCR, up to 21.25% (17/80) of the women who attended the Gynecology Outpatient Department of Shahid Beheshti Hospital in Isfahan were infected with *C.trachomatis*. Comparing the two methods of DNA extraction (proteinase K and boiling), it was revealed that the boiling method is more sensitive. Moreover, it is very rapid and inexpensive so, the boiling method is recommended for DNA extraction. PCR is considered to be the most sensitive and specific diagnostic method for detecting infectious agents, including *C.trachomatis*.

All cervical samples have been tested by both the plasmid-PCR and MOMP-PCR methods. The plasmid-PCR showed 10 times higher sensitivity when compared to the MOMP-PCR. The higher sensitivity of plasmid PCR was due to having 10 copies of plasmid in the elementary body as compared to the MOMP gene that has only one (17).

In studies, Rose et al. reported that the prevalence of *C.trachomatis* infection in pregnant women was 7.7% (18). Gabriel et al. reported that in women in the UK, it was 13.2% (19). Patel et al. reported the prevalence of *C.trachomatis* infection was 11.4% among women seeking first-trimester pregnancy termination in a same day abortion clinic that

showed a relation between Chlamydial infection and abortion (20). Hashemi et al. reported the prevalence of *C.trachomatis* infection at 17% (21). Flipp et al. reported that the prevalence of *C.trachomatis* genital infection in sexually active 16-19 year old girls was 2.9%, many of them did not use any contraception (22). Chamani et al. reported the prevalence of *C.trachomatis* infection in urine samples of women in Tehran at 12.5%. Comparing to our results, the low frequency of positive patients could be due to the low accuracy of urine samples, especially in women, and using primers that were amplified 517 bp bonds that might cause false negative results (23). In another study done by Zaeimi Yazdi et al., the prevalence of *C.trachomatis* infection in cervical samples was 14.1% by using a DNA extraction kit (16). In comparison with our results, the low frequency might be due to the low specificity and sensitivity of the kit. Fallah et al. reported the prevalence of *C.trachomatis* in urine samples of women with MOMP-PCR and plasmid-PCR methods at 14.9%. The low prevalence could be the result of using urine samples that are usually less sensitive than endocervical samples and a low sensitivity DNA extraction method. In Fallah et al, the highest frequency of *C.trachomatis* cervical infection was shown in women aged 28 to 38 years old (3); but in our study, the highest *C.trachomatis* cervical infection frequency was found in women aged 35 to 45. Santos et al. reported the prevalence of *C.trachomatis* infection was 20.7% with the proteinase K DNA extraction method (11) and EI Qouqa et al., in 2008, reported a 20.2 % prevalence rate of *C.trachomatis* in Gaza done in endocervical samples with plasmid based PCR (24) which are in accordance with the results gained shown in this study (21.25%).

Education, job, vaginal discharge, abdominal pain, past history of infertility, past history of ecto pregnancy, past history of low birthdate, past history of low birth weight child, past history of premature fetal membrane rupture and past history of infertility were not significantly related to the rate of *C.trachomatis* infection; but a past history of abortion and contraceptive methods were statistically significant ($p<0.05$). The highest *C.trachomatis* cervical infection frequency was found in women ranging from 35 to 45 years old (Table 1) and in the users of withdrawal and IUD for contraception, respectively (Table 3).

By ELISA, increased titers of IgG and IgA against *C.trachomatis* infection were 6.2% (5.80) and 3.7% (3.80), respectively. Two patients amongst symptomatic women had elevated levels of antibodies for both IgG and IgA. The sensitivity and specificity for IgG-ELISA were 29.4% and 100%; and for IgA-ELISA it was 17.6% and 100%. Seropositivity for IgG was significantly related to symptomatic women ($p<0.05$), but for IgA it was not statistically significant. In a number of studies, Nazer et al. reported that the prevalence of *C.trachomatis* infection in asymptomatic patients by ELISA was 3.3%.The low prevalence of *C.trachomatis* infection in this study could be due to low sensitivity and specificity of the ELISA assay, particularly for IgA, and only selecting asymptomatic women (13).

On the contrary, in a survey done by Kalantar et al, it was shown that no positivity by ELISA was found in a cohort of 91 women. Also there was no positive PCR result (25).

Morré et al., reported that the prevalence of *C.trachomatis* in endocervical specimens by PCR was 28.8% and seroprevalence rates detected by *C.trachomatis* p-ELISA was 35% for IgG and 3% for IgA. The specificity is in accordance with our results, but the sensitivity is higher than our data (26).

Persistent *C.trachomatis* infections are assumed to increase the risk of tubal pathology. High levels of CRP and IgA against *C.trachomatis* are serological markers for persistent infections.

The serum Chlamydia IgG antibody testing (CAT) has been introduced as a screening test for tubal pathology in fertility work-up. IgA antibodies and C-reactive protein (CRP) are assumed to reflect chronic inflammation, so the combination of CAT and CRP appears to be a promising valuable set of serological tests to identify women at highest risk of tubal pathology (14, 15).

Hartog et al. reported that the level of CRP, IgG, IgA and antibodies to heat shock protein 60 (HSP60) were higher in the women with distal tubal pathology than women without distal pathology. In this study, two of the samples were positive for CRP, IgG and IgA antibody (2.5%) out of 80 women (Table 4). One of them had infertility. Barlow et al., in 2001, reported that chlamydial DNA was detected in 71% of patients with tubal factor infertility (TFI) (27).

Conclusion

This study showed that the rate of genital chlamydial infection was high among asymptomatic and symptomatic women in Isfahan, Iran. The high prevalence of *C.trachomatis* and its severe impact on public health, such as infertility and subfertility, indicates the necessity of implementing better diagnostic methods for its detection in diagnostic labs throughout Iran. It also suggests that patients diagnosed with genital Chlamydial infection should be referred to a gynecological clinic

for further investigation. Moreover, PCR could be an adequate alternative method, as a gold standard, for diagnostic purposes.

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