Arch. Immunol. Ther. Exp. (2010) 58:227–234
DOI 10.1007/s00005-010-0076-z

ORIGINAL ARTICLE

Comparison of Three Methods of DNA Extraction in Endocervical Specimens for *Chlamydia trachomatis* Infection by Spectrophotometry, Agarose Gel, and PCR

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Received: 9 April 2009 / Accepted: 4 February 2010 / Published online: 17 April 2010

Abstract *Chlamydia trachomatis* is the major cause of sexually transmitted disease in the world. The aim of this study was to determine the best method of DNA extraction for detecting *C. trachomatis* by polymerase chain reaction (PCR) in sexually active women ($n = 80$) attending Shahid Beheshti Hospital in Isfahan, Iran. Endocervical swabs were collected from 80 women, 22 of whom were asymptomatic and 58 symptomatic. Three different DNA extraction methods were used in this study (phenol-chlorophorm, proteinase K, and boiling). DNA yield was evaluated by spectrophotometry, agarose gel, and PCR. The internal control was assayed by β-globin primers (PCO4, GH20). The DNA cryptic plasmid was selected as the target for *C. trachomatis* and samples were examined by PCR using specific KL1 and KL2 primers. It was shown that DNA extraction by boiling was the most sensitive with the highest yield of DNA. Of the 80 samples, 17 (21.25%) showed positivity for *C. trachomatis* by PCR. The highest rate of *C. trachomatis* infection was found in the group aged between 35 and 45 years old and those who used withdrawal or an intrauterine device as methods of contraception. It was demonstrated that DNA extraction by boiling was the least expensive and a very rapid method that gave the highest DNA yield. The infection rate in the sexually active women, including symptomatic and asymptomatic, was 21.25%, with a presumably high prevalence compared with other studies done in this field.

Keywords *Chlamydia trachomatis* · Endocervical smears · DNA extraction · PCR · Agarose gel · Spectrophotometry

Abbreviations
IUD Intrauterine device
PID Pelvic inflammatory disease

Introduction

Chlamydia trachomatis (serotypes D-K, Da, Ia) is the most common cause of bacterial sexually transmitted infections, although up to 80% of infected women and 50% of infected men may be asymptomatic (Nisyrios 2006). When symptoms do occur, usually 1–3 weeks following exposure, they are followed by dysuria, abdominal pain, and abnormal vaginal discharge (Fallah et al. 2005; Manavi 2006; Nisyrios 2006). In women, untreated infection or chronic (persistent) infection of *C. trachomatis* can spread into the uterus or fallopian tubes and cause pelvic inflammatory disease. Pelvic inflammatory disease can cause permanent damage to the fallopian tubes, uterus, and surrounding tissues. The damage can lead to chronic pelvic pain, infertility, potentially fatal ectopic pregnancy, and premature delivery. Infants born to pregnant mothers with genital *Chlamydia* infection are also often at risk of developing conjunctivitis and pneumonia (Lehmann et al. 1999; Manavi 2006). Since most *C. trachomatis* infections

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are asymptomatic, early diagnosis is essential for the timely treatment of *C. trachomatis*-infected women to prevent the development of sequelae and the transmission of *C. trachomatis* infection to susceptible individuals. Polymerase chain reaction (PCR) has been established as the standard assay for the rapid diagnosis of genital *C. trachomatis* infection (Crotchfelt et al.1998; Hashemi et al. 2007). It was recently shown in national screening programs that utilizing sensitive and cost-effective PCR-based methods to identify *C. trachomatis*-infected women (Hashemi et al. 2007; Miller et al. 2004) reduces the high morbidity associated with this infection. There is not much information concerning the prevalence of *C. trachomatis* among Iranian women. Regional data regarding the rate of infection of *C. trachomatis* is essential to control its spread and help assess the impact of *C. trachomatis* on the reproductive health of the women. The aim of this study was to evaluate the best method for DNA extraction and to determine the prevalence of *C. trachomatis* infection in a cohort of women in Isfahan, Iran.

Materials and Methods

Study Groups

One hundred twenty women attending the gynecology outpatient department of Shahid Beheshti Hospital, Isfahan, were chosen to participate in this study. Forty women declined because of antibiotic therapy 4 weeks before performing the test (Fallah et al. 2005; Gopalkrishna et al. 2000). Written consent was obtained from the patients individually prior to sample collection. Endocervical swabs were collected from 58 symptomatic women aged between 20 and 60 (median: 36.5 ± 8.9) years and 22 asymptomatic women aged between 19 and 56 (40 ± 10.5) years. All the women were subjected to thorough clinical examination and a case history was recorded.

Specimen Collection

Sample collection was done during 2008. An endocervical swab was collected from each patient and transferred to a 15 ml plastic vial containing 5 ml of sterile phosphate-buffered saline (PBS). The swabs were stored at −70°C until the DNA extraction stage (Gopalkrishna et al. 2000; Santos et al. 2003). Individual smears of the patients were examined for pathological investigations.

DNA Extraction

The endocervical swab sample was removed from the vial and the PBS collection tube was centrifuged at 2,000 rpm for 15 min at room temperature. The supernatant was discarded and the deposit was vortexed and transferred to a 1.5 ml microtube. This step was followed by centrifugation at 2,000 rpm at room temperature for 15 min. The supernatant was removed and Tris-Base-EDTA (TE) solution (600 µl) was added before dividing it equally into three microtubes. For selecting the best method of DNA extraction, three different routine methods, i.e. the non-organic method using proteinase K, the organic method using phenol–chloroform, and boiling for 10 min, were used in this study and the yields of the extracted DNA were compared. These methods were conducted as explained below.

Organic Method Using Phenol–Chloroform

Briefly, 500 µl of a solution consisting of 1% SDS and 0.1 M NaOH was added to each microtube and heated at 80°C using a water-bath for 20 min. Following incubation, 500 µl of at 25:24:1 was added, vortexed, and centrifuged at room temperature (12,000 rpm, 10 min). The supernatant was removed to an autoclaved microtube and 1 volume of chloroform was added, vortexed, and centrifuged (12,000 rpm, 5 min). The upper aqueous layer was carefully removed to another clean microtube. Then 0.1 volume (30 µl) of 3 M sodium acetate was added and again mixed by vortexing. This step was followed by the addition of 1 volume isopropanol and incubated at −20°C overnight to allow precipitation of the DNA. Following centrifugation at 12,000 rpm at 4°C, the supernatant was discarded and the DNA was washed once with 75% ethanol. The pelleted DNA was dissolved in 50 µl of distilled water after drying completely in a fume hood. The extracted DNA was stored at −20°C till required (Shi et al. 2004).

Non-organic Method Using Proteinase K

Endocervical smears were collected in 400 µl of 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA. 4 µl proteinase K (10 µg/ml; Fermantase, Iran) and 4 µl triton 10%(v/v) were added and incubated at 55°C for 90 min followed by 95°C for 30 min. The samples were maintained at −20°C until used (Santos et al. 2003).

Boiling

Endocervical smears were collected in 400 µl of 10 mM Tris–HCl, pH 8.0 (Merck) and 1 mM EDTA (Sigma, Germany) and heated at 100°C in a water bath for 10 min. This was followed by mixing by vortexing and centrifugation at room temperature (10,000 rpm, 10 min). The

Fig. 1 Analysis of the extracted DNA products by agarose gel electrophoresis. *Lane 1* DNA extraction products by the proteinase K method; *lane 2* DNA extraction products by the phenol chloroform method; *lane 3* DNA extraction products by the 10 min boiling method

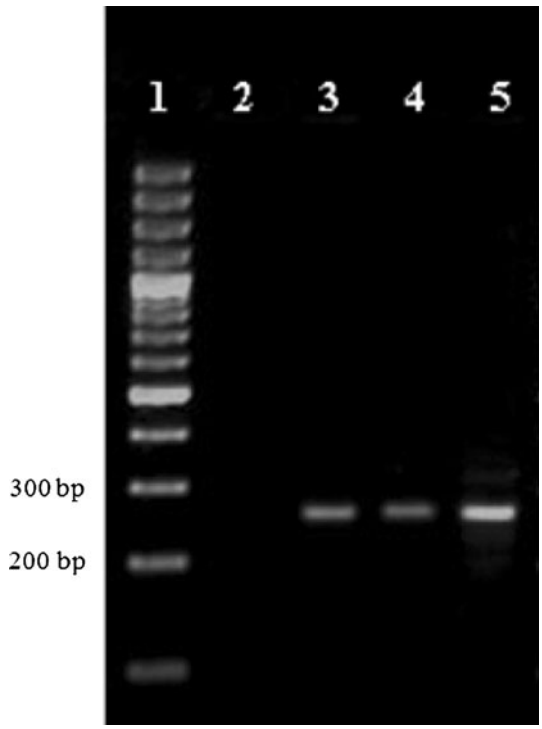

Fig. 2 Analysis of the PCR products by agarose gel (1.5%) electrophoresis. The 268 bp DNA corresponds to the specific β-globin DNA sequence. *Lane 1* 100 bp DNA ladder, *lane 2* negative control, *lane 3* PCR products by boiling for 10 min, *lane 4* PCR products by the proteinase K method, *lane 5* positive control

supernatant was removed to an autoclaved microtube and the extracted DNA stored at −20°C (Lan et al. 1993).

Analysis of DNA Extraction Products

The quality and quantity of DNA extraction by the different methods applied in this study were assessed by using spectrophotometry, agarose gel, and PCR amplification using β-globin primers followed by agarose gel electrophoresis to detect the human genomic DNA.

Analysis of DNA Extraction Products by Spectrophotometry and Agarose Gel

A spectrophotometer (Bio Photometer, Eppendorph, Germany) was used to measure the DNA yield according to the standard protocol recommended by the manufacturer. Briefly, 500 µl of the blank solution (TE) was added to the cuvette and adjusted to zero. Then 5 µl of the extracted DNA was added to 495 µl of the blank solution (TE) and the optical density (OD) was read at 260/280 nm. The extracted DNA products were analyzed by electrophoresis on 1.5% agarose gels in TBE buffer at room temperature using a voltage 80 V (Fig. 1). For any given gel analysis, the same volume of PCR products or extracted DNA products was loaded in each of the gel slots.

Application of β-Globin

Considering that the β-globin gene is part of the human DNA genome, β-globin primers were used as the internal control. Thus, β-globin PCR is supposed to be positive in all the samples in which DNA extraction was successful. In this study, the quality of the extracted DNA by different methods was confirmed by amplification of the β-globin gene in all the samples. The β-globin sequences were GH20: 5′-GAAGAGCCAAGGACAGGTAC-3′ and PCO4: 5′-CAACTTCATCCACGTTCACC-3′ (Metabion, Germany). The amplified size was 268 bp (Gopalkrishna et al. 2000). PCR was performed on 2 µl of the extracted DNA sample in a final reaction mixture of 25 µl. The final reaction mixture contained 2.5 mM $MgCl_2$ (Cinnagen), 0.3 mM deoxinucleotide triphosphate (Cinnagen), 20 pM of each primer GH20, PCO4 and 1 U of Taq polymerase (Cinnagen).

The amplification protocol was 10 min of denaturation at 94°C followed by 30 cycles of amplification. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. The final extension cycle at 72°C was prolonged for 8 min (Gopalkrishna et al. 2000). The PCR products were analyzed on a 1.5% agarose gel (Fig. 2). In this study, a

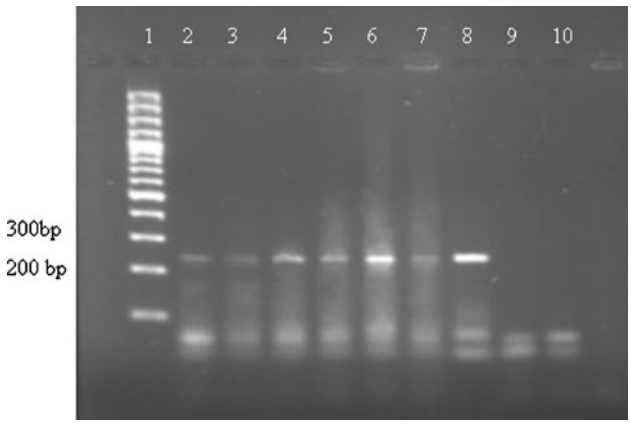

Fig. 3 Analysis of the PCR products by agarose gel (1.5%) electrophoresis. The 241 bp DNA corresponds to the specific *Chlamydia* amplified plasmid DNA sequence. *Lane 1* 100 bp DNA ladder; *lanes 2, 4, 6* PCR products by the boiling in 10 min method; *lanes 3, 5, 7* PCR products by the proteinase K method; *lane 8* positive control; *lanes 9, 10* negative control

sample containing distilled water instead of DNA was used as a negative control.

Detection of *C. 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 for the *C. trachomatis* plasmid PCR were KL1: 5′-TCCGGAGC GAGTACGAAGA-3′ and KL2 5′-AATCAATGCCCGGG ATTGGT-3′ (Metabion, Germany) (Santos et al. 2003). PCR was performed on 5 µl of the extracted DNA sample in a final reaction mixture of 25 µl. The final reaction mixture contained 3 mM $MgCl_2$ (Cinnagen), 0.3 mM deoxinucleotide triphosphate (Cinnagen), 16 pM of each primer KL1, KL2 and 1 U of Taq polymerase (Cinnagen).

The amplification protocol was 10 min of denaturation at 94°C followed by 40 cycles of amplification. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final extension cycle at 72°C was prolonged for 8 min (Santos et al. 2003). The PCR products were analyzed by 1.5% agarose gel electrophoresis (Fig. 3). In this study, *C. trachomatis* serovar A was used as a positive control and a sample containing distilled water instead of DNA was used as a negative control.

Statistical Analyses

Data analysis was carried out using the Statistical Package for Social Science (SPSS) version 15 for Windows. For normally distributed data, means and standard deviations were calculated and compared using the Student's *t*, chi-squared, and Fisher's exact tests. Confidence intervals (95%) were reported where appropriate. The χ^2 test was applied to compare the sensitivities of the DNA extraction methods (boiling with proteinase K) and contraceptive methods with *C. trachomatis* infection. Student's *t* test was applied to compare *Chlamydia* infection and average age. Fisher's exact test was applied to compare *Chlamydia* infection and vaginal discharge, abdominal pain, history of infertility, and history of abortion. Statistical significance was the 5% level ($P < 0.05$).

Results

DNA Extraction Products by Agarose Gel and Spectrophotometry

The quality of DNA extraction was analyzed by agarose gel. The best smears on agarose gel were obtained, respectively, by the boiling, proteinase K, and phenol-chlorophorm methods. The quantity of DNA extraction was examined by spectrophotometry. The OD 260/280 ratios acquired by the boiling, proteinase K, and phenol-chlorophorm methods were, respectively, 1.75, 1.67, and 1.65. Comparing the results revealed that the best results were from the boiling and proteinase K methods (Fig. 1). Due to the high sensitivities of boiling and the proteinase K assay, they were used for all the samples. The phenol-chlorophorm method was omitted because of its low sensitivity. By applying β-globin PCR, it was shown that the boiling method had the highest yield of DNA extraction (Fig. 2).

PCR Analysis

The successful amplification of a 241 bp fragment of the *C. trachomatis* plasmid genome was considered a positive result by PCR (Fig. 3). Out of 17 (21.25%) positive samples, 1.25% (1/80) of the women were under 25 years old, 6.25% (5/80) were between 25 and 35, 10% (8/80) were between 35 and 45, 1.25% (1/80) were between 45 and 55, and 2.5% (2/80) were over 55 years old (Fig. 4). Of the 80 endocervical samples tested, 17 (21.25%) were positive for *C. trachomatis* (Fig. 3). It is necessary to mention that all the infected patients were treated with doxycycline (twice daily for 2 weeks). Of the 22 samples of the asymptomatic group, 27.3% (6/22) were positive and of the 58 samples of the symptomatic group, 19% (11/58) were positive. The highest *C. trachomatis* cervical infection frequency was found in the women in the 35–45 age group and those who used withdrawal as a contraception method. The women who used a contraceptive method (71 of the studied patients) showed 21.1% (15/71) positivity for PCR. On the other hand, those who did not use any means of

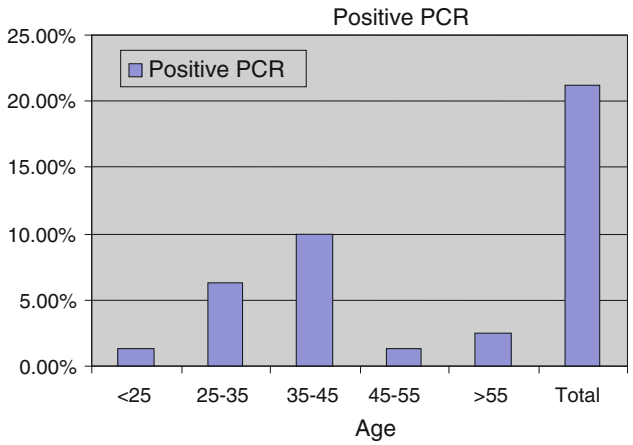

Fig. 4 Effect of age and the prevalence of *C. trachomatis* infection diagnosed by PCR

contraception showed 22.2% (2/9) PCR positivity. The relationship between *C. trachomatis* infection and some factors, for example vaginal discharge, abdominal pain, history of infertility, history of abortion, and using contraceptive methods, were examined. Details are shown in Table 1.

Pathological Results

Only one sample was reported to have an inclusion body of *C. trachomatis* by Gimsa staining in the pathology laboratory. The result was confirmed by PCR and included in the symptomatic group. This result indicated that Gimsa staining showed very low sensitivity for the detection of *C. trachomatis* infection.

Discussion

C. trachomatis is a common sexually transmitted infection with significant impact on public health. Therefore, effective epidemiological control as well as a correct and sensitive diagnostic method for *C. trachomatis* are required (Santos et al. 2003; Zaeimi Yazdi et al. 2006). Regarding different methods for the detection of *C. trachomatis* infection, based on different studies and our data, PCR is a sensitive and specific assay. Strand displacement amplification and transcription-mediated amplification are as good if not better in the diagnosis of *Chlamydia* infection. In the past, all specimens were tested by both the plasmid-PCR and MOMP-PCR methods (Mahony et al. 1993), but in this study we used plasmid PCR because it shows a 10-times higher sensitivity than MOMP-PCR (Mahony et al. 1993; Santos et al. 2003) due to its having ten copies of plasmid in the elementary body compared with the MOMP gene, which has only one. Obtaining high-quality DNA depends on the extraction technique used. The aim of this study was to evaluate various methods of DNA extraction in terms of DNA yield and amplification quality. Our data showed that up to 21.25% (17/80) of the women attending the gynecological outpatient clinic of Shahid Beheshti Hospital in Isfahan were infected with *C. trachomatis*. Comparing the three methods of DNA extraction, i.e. proteinase K, phenol–chloroform, and boiling, indicated that the boiling

Table 1 The prevalence of *Chlamydia trachomatis* infection in women

Clinical manifestation	Result of clinical manifestation	Positive PCR	Negative PCR	*P* value	
Vaginal discharge	Positive 52	10 (19.2%)	42	0.49 (Fisher's exact)	
	Negative 28	7 (25%)	21		
Abdominal pain	Positive 33	6 (18.2%)	27	0.55 (Fisher's exact)	
	Negative 47	11 (23.4%)	36		
History of abortion	Positive 15	5 (33.3%)	10	0.03 (Fisher's exact)	
	Negative 65	12 (18.5%)	53		
History of infertility	Positive 13	2 (15.4%)	11	0.45 (Fisher's exact)	
	Negative 67	15 (22.4%)	52		
Contraceptive methods	OCP 5	0	5	Using contraceptive method	0.04 (χ^2 test)
	IUD 8	4	4		
	Condom 17	1	16		
	Vasectomy 10	3	7		
	TL 12	1	11		
	Withdrawal 19	6	13		
	None 9	2	7	Not using contraceptive method	

OCP oral contraceptive pills, *IUD* intrauterine device, *TL* tubal ligation

Table 2 The sensitivity and specificity of PCR by boiling DNA extraction

	PCR total result		Total
	Negative	Positive	
Negative	63	2	65
	100% specificity	11.80%	
Positive	0	15	15
		88.2% sensitivity	
Total	63	17	80
	78.80%	21.20%	

Table 3 The sensitivity and specificity of PCR by proteinase K DNA extraction

	PCR total result		Total
	Negative	Positive	
Negative	63	8	71
	100% specificity	47.10%	
Positive	0	9	9
		52.9% sensitivity	
Total	63	17	80
	78.80%	21.20%	

Table 4 Prevalence of *C. trachomatis* in different studies done in Iran

Reference	Percentage	Methods	Number of subjects	City	Hospital	Year
Fallah et al. 2005	14.9	PCR	94	Tehran	Obstetrics and Gynecology clinic of Shoosh	2002
Chamani-Tabriz et al. 2007	12.6	PCR	1,052	Tehran	Obsterics and Gynecology clinics	2003
Zaeimi Yazdi et al. 2006	14.1	PCR	142	Tehran	Mirzakoochakkhan Women's hospital	2003–2004
Hashemi et al. 2007	17	PCR-EIA	123	Tehran	Mirzakoochakkhan Women's hospital	2004–2005

method had the highest sensitivity. Of the 17 positive samples, 7 (41.17%) were amplified by both the boiling and poroteinase K methods, eight were analyzed by boiling only, and 2 by proteinase K. The sensitivities and specificities of PCR done with the extracted DNA by the boiling and proteinase K methods were 88.2 and 100% and 52.9 and 100%, respectively (Tables 2 and 3). The χ^2 test showed that PCR is more sensitive when the DNA is extracted by boiling than by proteinase K ($P < 0.01$).

Our findings are in accordance with previous data (Lan et al. 1993). In separate studies in Iran done by Zaeimi Yazdi et al. (2006) and Hashemi et al. (2007), the prevalence of *C. trachomatis* infection in cervical samples in Tehran were, respectively, 14.1 and 17% by using the Diatom DNA extraction kit (IsoGene, Moscow, Russia) (Table 4). Compared with our results, the low frequency might be due to the low specificity and sensitivity of the kit. The prevalence of *C. trachomatis* infection in urine samples of women in Tehran were 12.5 and 14.9% (Table 4), respectively, by the Sambrook & Russel and phenol–chloroform methods for DNA extraction (Chamani-Tabriz et al. 2007; Fallah et al. 2005). The low prevalence of the infection in these studies compared with our data could be due to the lower sensitivity of urine samples compared with cervical samples and the low sensitivity and specificity of their DNA extraction methods. Literature reviews show that most of the researchers used kits instead of the methods that we used in this study. Thus, based on our findings, the boiling method is recommended for routine DNA extraction due to its rapidity and cost effectiveness.

Age, vaginal discharge, abdominal pain, and history of infertility were not significantly related to the rate of *C. trachomatis* infection, but a history of abortion and using contraceptive methods were statistically significant ($P < 0.05$). The average of age of the women infected with *C. trachomatis* (17 samples) was 40 ± 9.4 and of the uninfected women (63 samples) 36.8 ± 9.3 years (Table 5); therefore the independent *t* test did not show any significant relationship between age and infection ($P = 0.1$). On the other hand, the highest frequency of *C. trachomatis* cervical infection was found in women aged between 35 and 45 years old. Our finding is in accordance with those of Fallah et al.

Table 5 Comparison of average of age regarding to PCR results

Factor	Positive PCR		Negative PCR		*P*-value
	Mean	SD	Mean	SD	0.1
Age	40.1	9.4	36.8	9.4	0.34

(2005) and Hashemi et al. 2007) in Iran, but Zaeimi Yazdi et al. (2006) reported that prevalence was highest among women aged 25–29 and 35–39 years. Chamani-Tabriz et al. reported no statistically significant differences between patient ages and reproductive history (Chamani-Tabriz et al. 2007). Data gained in other countries are contradictive to our studies. For example, Mascellino et al. (2008) and Chen et al. 2006) reported separately that the rate of infection is higher in young women, with the highest frequency in women aged 25 years old. The discrepancy between the results could be due to there not being outpatient clinical centers for teenagers in Iran.

In our survey, it was demonstrated that there was no significant relationship between abdominal pain and vaginal discharge to *C. trachomatis* infection (Fisher's exact test). Fallah et al. (2005) found that a common sign in their patients was mucopurulent discharge and they did not find any significant relationship between pain in the lower abdomen and positive PCR of *C. trachomatis*. Zaeimi Yazdi et al. (2006) showed that the rate of *Chlamydia* infection was high among women who had a history of STI, but they did not mention abdominal pain and vaginal discharge, which is in contrast with the results of Hashemi et al. (2007) that showed that although 20- to 30-year-old women reported the highest frequency of STI history, they had the lowest relative frequency of *C. trachomatis* infection. The data gained in this study are in accordance with those reported by Chen et al. (2006), but they are in contrast with those of Van Bergen et al. (2005) showing that there was a relationship between a history of abortion and *C. trachomatis* infection. Patel et al. (2008) reported that the prevalence of *C. trachomatis* in women with a history of abortion was 11.4%, which demonstrated of the possibility of abortion caused by *C. trachomatis* infection. This factor was not considered in Iran by the researchers. In our study, the highest frequency of *C. trachomatis* cervical infection was found in the women who used the withdrawal method and intrauterine device (IUD) as a means of contraception, respectively (Table 1). Fallah et al. (2005) reported that the highest frequency of *C. trachomatis* infection were in users of IUDs and oral contraceptive pills for contraception.

The high prevalence of *C. trachomatis* and its severe impact on public health suggests the necessity of implementing better diagnostic methods for its detection in diagnostic labs. Moreover, patients with genital *Chlamydia* infection should be referred to an outpatient gynecological clinic for further screening to detect the acute and chronic phases of *C. trachomatis* infection. In chronic or persistent infection, treatment is more difficult because of the changed structure of *C. trachomatis*. Further screening, such as a combination of CRP and ELISA for the detection of IgG and IgA, could be useful in discriminating the chronic and acute phases of infection (den Hartog et al. 2006). The number of specimens used in this study was not enough for a precise evaluation of DNA extraction as well as the prevalence of *C. trachomatis*, so it is recommended the more samples be tested for a precise evaluation of the occurrence of the infection in the community.

Acknowledgments This study was supported by University of Isfahan, Isfahan, Iran.

Conflict of interest statement There is no conflict of interest in this article.

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