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Comparison of plasmid and chromosomal *omp1* gene-based PCR and two DNA extraction methods for diagnosing *Chlamydia trachomatis* in endocervical swab samples

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ABSTRACT

Objective: To evaluate the effectiveness of boiling and proteolytic DNA extraction methods and also to compare the sensitivity of plasmid polymerase chain reaction (PCR) and chromosomal *omp1* gene PCR for genital *Chlamydia trachomatis* swab samples in women. **Methods:** 710 cervical swab samples were obtained from women with symptomatic genital infection at 11 gynecology and obstetric clinics located in Ahvaz, Iran. DNA extraction was performed using proteolysis and boiling manners for all samples. Plasmid PCR and chromosomal *omp1* gene primary- and seminested-PCR were then performed separately on extracted DNA in boiling and proteolytic methods. **Results:** The prevalence of this infection was 17.6% as determined by plasmid-PCR, 13.2% by *omp1*-primary PCR and 15.8% by *omp1*-nested PCR. Sensitivities of boiling and proteolytic extraction-directed PCR were 93.6%, and 68.8%, respectively, which are significantly different (P=0.02). The sign of swab-induced bleeding was significantly found to be the most frequent among women infected with this bacterium (P=0.001) and had a sensitivity of 33.6% and a specificity of 80.5%. **Conclusions:** In order to obtain confident statistical results about sensitivity of each manner, in present study these evaluations were carried out for high numbers of samples (710 samples); high number of samples is statistical advantage of this study in comparing with other studies which were performed with low numbers of samples. Using boiling-DNA extraction manner and targeting plasmid sequence for PCR can increase the sensitivity of *C. trachomatis* diagnosis.

1. Introduction

Chlamydia trachomatis (*C. trachomatis*) causes several diseases and syndromes including trachoma, urogenital infections, conjunctivitis, infant pneumonia and lymphogranuloma venereum (LGV)[1]. This bacterium is one of the most prevalent causes of sexually transmitted diseases with approximately 92 million new cases of infections throughout the world annually[2]. Up to 70%-80% of urogenital *C. trachomatis* infections in women are asymptomatic[3]. Untreated infections in women may cause pelvic inflammatory disease (PID) that scares the inside of the reproductive organs and consequently can lead to serious complications including chronic pelvic pain, ectopic pregnancy, and infertility[4]. One of the important risk factors for genital *C. trachomatis* infection is young age because of high sexual activity[5]. Among the laboratory diagnostic methods, PCR that can be used to test cervical swabs as well as urine is becoming available. On cervical swabs the corresponding sensitivities of PCR, gene probe and EIA are 88.6%, 84% and 65%, respectively[6]. For achieving precise detection of this bacterium, increasing sensitivity of DNA amplification test is necessary that can depends on two important parts of DNA amplification, including appropriate selection of DNA extraction method and also DNA template for PCR. In order to early diagnose and treatment of genital *C. trachomatis* infection in women and to prevent the subsequent complications, finding guidelines for presumable diagnosis of this infection is useful and applicable; because of limitation in access to precise diagnostic tests in developing countries such as Iran, it is especially important in these countries. Our aims were to compare the sensitivity of boiling and proteolytic DNA extraction-directed PCR and to compare the sensitivity of

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plasmid-based PCR, *omp1* gene-based primary and nested PCR, and also to find visual guidelines from among three important clinical manifestations including two symptoms of abnormal vaginal discharge (AVD) and lower abdominal pain (LAP) and one sign of swab-induced bleeding (SIB) for presumable diagnosis of genital *C. trachomatis* infection in women in Ahvaz, Iran.

2. Materials and methods

2.1. Specimens

710 cervical samples were obtained from women with symptomatic genital infection at 11gynecology and obstetric clinics located at Ahvaz, Iran. 156 out 710 were unmarried which from them 121 (of 212) were in less than 25 years age group, 33 (of 328) were in 25-34 and 2 (of 170) were in over 34 years age group. For sampling, symptomatically infected women were defined as those presenting with one or more genitourinary clinical symptoms including abnormal vaginal discharge, lower abdominal pain, posticoital bleeding, dysuria, spotting, dysmenorrhoea and dyspareuria[7]. From these symptoms two symptoms of abnormal vaginal discharge and lower abdominal pain and one sign of swab-induced bleeding which more frequently reported by obstetricians and gynecologists were analyzed. Samples were collected with sterile cotton swabs from endocervix in a way that swabs could have the most possible contact with the epithelium so that swab-induced bleeding would occur in patients prone to bleeding. The swabs then were placed into tubes containing 1ml of 1X phosphate buffered saline (PBS) and transported on ice to the laboratory and stored at -20 ℃ prior to processing.

2.2. DNA extraction

For comparing two DNA extraction methods with each other, DNA extraction was performed using proteolysis and boiling for all samples. The sample tube was vortexed vigorously then swab was discarded. The pellet was used for extraction.

2.3. Proteolytic DNA extraction

400 μL of the clinical specimen was centrifuged at 13000 rpm for 30 min, the supernatant was removed and the pellet was resuspended in 100 μL of lysis buffer containing 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.1% Triton and 100 μg/mL of proteinase K. The tube was incubated at 55 ℃ for 90 min and then at 95 ℃ for 30 min to inactivate the proteinase K; The Cell debris was pelleted by centrifugation at 10 000 rpm for 2 min and the supernatant containing DNA was harvested and stored at -20 ℃ until PCR[8].

Boiling DNA extraction:

400 μL of the specimen was centrifuged at 13 000 rpm for 30 min, the pellet was resuspended in 100 μL of 10 mM Tris-HCl (pH 7.5) and stored at -70 ℃ for 48 h and then thawed; This freeze-thawed suspension was boiled for 10 min, centrifuged at 10 000 rpm for 2 min and the supernatant was stored at -20 ℃ until PCR[9].

2.4. Primary screening by β-Globin gene PCR

For ensuring that DNA remained intact during transporting and storing, and for examining presence of PCR inhibitors in samples, all samples were screened by PCR using primers GH20: 5′-GAAGAGCCAAGGACAGGTAC-3′ and PCO4: 5′-CAACTTCATCCACGTTCACC-3′ according to previous study[10].

2.5. Plasmid based PCR

Plasmid PCR was separately performed by DNA extracted from two extraction methods. The primers used for amplifying a 201 bp fragment of *C. trachomatis* endogenous plasmid were CTP1 (forward strand: 5′-TAGTAACTGCCACTTCATCA-3′) and CTP2 (reverse strand: 5′-TTCCCCTTGTAATTCGTTGC-3′). 10 μL of extracted DNA was used as template in PCR reaction. The final volume of PCR mixture was 50 μL and concentrations of ingredients were 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3), 200 μM from each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dCTP), 25 ρmol of each primer and 1U of Taq DNA polymerase. Thermal program of PCR was consisted of DNA denaturation at 95 ℃ for 4 min followed by 40 repeats of amplification, each repeat consisting of a denaturation step at 95 ℃ for 1 min, an annealing step at 55 ℃ for 1 min, and a chain elongation step at 72 ℃ for 1.5 min and a final elongation step at 72 ℃ for 4 min. The amplified products were visualized after electrophoresis through a 1.5% agarose gel containing ethidium bromide[11].

2.6. Omp1 gene based-PCR

2.6.1. Primary PCR

Primary PCR were carried out for all samples. An approximately 1200 bp fragment of the *omp1* gene was amplified using primers CT1 (forward strand: 5′-GCCGCTTTGAGTTCTGCTTCCTC- 3′) and CT5 (reverse strand: 5′- ATTTACGTGAGCAGCTCTCTCAT- 3′). PCR was carried out in a final volume of 50 μL. 10 μL of extracted DNA was used as template in PCR reaction. Final concentrations of ingredients were 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3), 200 μM from each deoxynucleoside triphosphate (dATP, dTTP, dGTP and dCTP), 25 ρmol of each primer (CT1 and CT5), and 1 U of Taq DNA polymerase. Thermal program of the PCR reaction was consisted of DNA denaturation at 95 ℃ for 5 min followed by 40 repeats of amplification, each repeat consisting of

a denaturation step at 95 ℃ for 1 min, annealing at 55 ℃ for 1 min, and elongation at 72 ℃ for 1.5 min and a final elongation step at 72 ℃ for 4 min[11].

2.6.2. Nested PCR

The nested PCR was carried out using primers PCTM3 (forward strand: 5′- TCCTTGCAAGCTCTGCCTGTGGGGAATC CT-3′) located 22 bp downstream of CT1 and previous primer CT5 as following manner, 1 μL of the primary PCR product as DNA template was added to a prepared PCR mixture. The amplification conditions of nested PCR were as same as the primary PCR. The amplification products were visualized after electrophoresis through a 1 % agarose gel containing ethidium bromide[11].

2.7. Statistical analysis

All evaluations in this study were analyzed by chi-square test. Data analysis was performed with SPSS statistical software version 15.0. A P-value less than 0.05 were considered significant.

3. Results

β-Globin gene PCR was positive in all samples; it performed only by DNA templates from boiling extraction. Since the aim of β-Globin gene PCR was ensuring that DNA has remained intact during transporting and storing steps, not for comparing two DNA extraction methods, this PCR was not carried out with DNA extracted by proteolytic method. A 201 bp fragment was amplified in 125 out of 710 (17.6%) samples by plasmid-PCR (Figure 1) and an approximately 1200bp was amplified in 94 out of 710(13.2%) by *omp1*-primary PCR and 112 out of 710(15.8%) by *omp1*-nested PCR (Fig. 2). Table 1 shows the prevalence of *C. trachomatis* infection in relation to age as determined by plasmid PCR. Table 2 shows two symptoms (abnormal vaginal discharge and lower abdominal pain) and one sign (swab-induced bleeding) in relation to the presence of *C. trachomatis* in absolute numbers and in percentages. From these symptoms/signs, the swab-induced bleeding was found more frequent among patients infected with *C. trachomatis* (P=0.001). In comparison of two DNA extraction methods, 78 of the 125 positive samples were amplified by both boiling and proteolytic methods-directed PCR, 39 by boiling only, and 8 by proteolytic method only.

Table 1

Prevalence of C. trachomatis infection among patients age groups.

Age group	PCR positive	PCR negative	Total
(Yr)	No (%)	No (%)	No (%)
<25	36 (17)	176 (83)	212(100)
25-34	68 (20.7)	260 (79.3)	328 (100)
>34	21 (12.3)	149 (87.3)	170 (100)

Table 2

Three clinical manifestations in relation to presence of C. trachomatis in absolute numbers and in percentage

Clinical Symptoms/signs	PCR positive No (%)	PCR negative No (%)	*P*-value
AVD[1]	70/125 (56%)	364/585 (62)	0.19
LAP[2]	26/125 (20.5%)	95/585 (16)	0.22
SIB[3]	42/125 (33%)	114/585 (19.5)	0.001

[1] Abnormal Vaginal Discharge, [2] Lower Abdominal Pain, [3] Swab Induced Bleeding.

Table 3

Comparison of boiling- and proteolytic-directed PCR with total resolved samples

PCR	No. of resolved samples		total	Sensitivity (%)
	positive	negative		
Boiling-directed PCR				
Positive	117	0	117	93.6
Negative	8	585	593	
Proteolytic-directed PCR				
Positive	86	0	86	68.8
Negative	39	585	624	

Table 4

Comparison of omp1 based-primary PCR and omp1 based-nested PCR with total resolved samples by plasmid based PCR.

PCR	No. of resolved samples		total	Sensitivity (%)
	positive	negative		
omp1 based-primary PCR				
Positive	94	0	94	75.2
Negative	31	585	616	
omp1 based-nested PCR				
Positive	112	0	112	89.6
Negative	13	585	598	

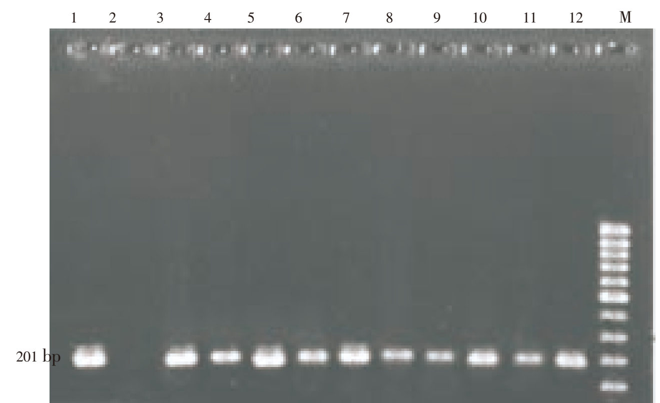

Figure 1. Agarose gel (1.5%) electrophoresis of 201bp amplified fragment of the specific Chlamydia plasmid DNA sequence from a number of PCR-positive specimens. Lane 1 and 2: positive and negative control, respectively; Lane 3 to 12: a number of positive clinical specimens; lane M:100bp ladder.

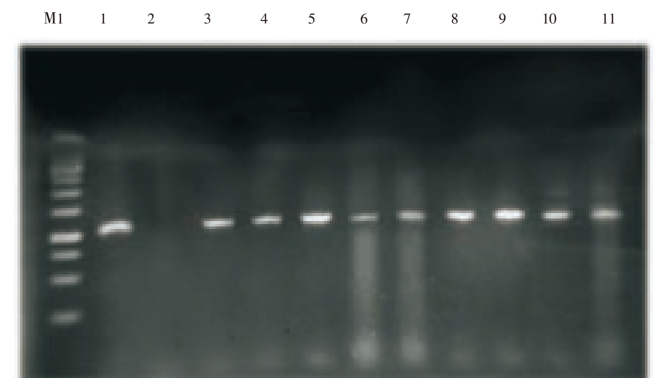

Figure 2. Agarose gel (1%) electrophoresis of approximately 1200 bp Amplified fragment of omp1 gene from a number of Chlmydia positive specimens. Lane M: 1kb ladder, lane1: positive control for primary PCR, lane 2 negative control, lane 3 to 6: a number of primary PCR-positive specimens, lane 7 to 10: a number of nested PCR-positive specimencs, lane 11: positive control for nested PCR.

4. Discussion

Determining the prevalence of genital *C. trachomatis* infection is critical for carrying out appropriate

epidemiological programming in order to control this infection, therefore selection of careful methods in diagnostic techniques, is very important. For precise statistical evaluation and comparison of different methods in diagnosis of this bacterium, the numbers of samples must be enough; for this reason, relatively high number of patients were investigated in present study (710 samples) in compared with other study (80 samples)[10]. The overall prevalence rate of *C. trachomatis* infection among women in Ahvaz was 17.6% as determined by plasmid-PCR in this study. Since there are not sexually transmitted diseases (STD) clinics in Iran and high risk women attend sporadically in gynecology, obstetric and other clinics, probably, if there were STD clinics in Iran and cervical samples collected from these clinics were analyzed, the prevalence of this infection would has been evaluated more than the current evaluated prevalence. For comparing our results with other studies in Iran, a number of those were presented here. A research was performed to determine the prevalence of *C. trachomatis* infection in women attending obstetrics and gynecology clinics in Tehran, Iran, 12.6% (133/1052) of samples were positive for this bacterium by PCR [12]. Other research in Ahvaz was carried out for determining the prevalence of *C. trachomatis* infection among females with vaginal discharge by plasmid PCR. In this study, out of 202 patients, 33 (16.3%) were positive for this bacterium[13].

Overall prevalence rate of *C. trachomatis* infection as determined by plasmid PCR were 17% in group I (under 25 years), 20.7% in group II (25-34 years) and 12.3% in group III (over 34 years). The highest prevalence of *C. trachomatis* infection (20.7%) was seen in group II (25-34 years). The prevalence rates of this infection between unmarried (7.7%, 12 /156) and married (20.4%, 113/554) patients were very different, this difference was significant (P<0.001). The prevalence in group I, II, and III without calculation of unmarried patients were 29.6% (27/91), 22% (65/295), and 12.5% (21/168), respectively. Interestingly in unmarried population, highest prevalence was seen in group II. In contrast to our results the highest prevalence rate of this infection in other countries, for example in UK, was found in young women aged under 25 years due to increased sexual behavior risk[14,15], but in this study with calculation of unmarried population, it was not seen. In several studies in Iran the highest prevalence was not also seen in young woman (under 25 years old)[10,16,17]. Based on mentioned points, the reason of this difference might be due to the difference in traditional and religious beliefs. One of these beliefs in Iran is virginity of young women till marriage, that is, one girl should be virgin until she gets married. Since genital *C. trachomatis* is a sex transmitted pathogen and virginity is a limiting factor for sex intercourse among young women, therefore probably virginity of young women is one of the reasons that highest prevalence of this infection is not seen among the young women in this study and other studies in Iran. Of course there are some other factors which influence this difference but the mentioned one is likely the most outstanding.

Comparing boiling and proteolytic DNA extraction indicated that the boiling had the higher sensitivity. Of the 125 positive samples, 78 were amplified by both boiling and proteolytic methods, 39 by boiling only, and 8 by proteolytic method only. The sensitivities of plasmid PCR performed with the extracted DNA by the boiling and proteolytic methods were 93.6% and 68.8 %, respectively, compared with the total resolved samples (Tables 3 and 4). This difference was significant (P=0.02). Results of present study are in accordance with findings of previous study in which sensitivity of boiling and proteolytic methods were 88.2% and 52.9%[10]. In a study it was not seen difference between two mentioned methods[9]. Based on our results, simple, low time-consuming, and low-cost boiling method was suggested for epidemiological researches.

In order to determine the sensitivity of *omp1*- primary and -nested PCR in comparison with plasmid-PCR, these techniques were done for all samples. Our finding showed that the *omp1*-primary and -nested PCR were less sensitive than plasmid-PCR. Sensitivities of primary- and nested-PCR were 75.2% and 89.6%, respectively (Table 4). This discrepancy could be due to the different copy number of the target DNA or different lengths of the amplified DNA fragments. Other study confirmed our findings[18]. Since most researches especially in Iran are performed on crude cell suspensions in which copy number of Chlamydia is low, our results for selecting appropriate DNA extraction method and target DNA could be advantageous for increasing sensitivity of detection.

The sign of swab-induced bleeding was found more frequently among patients infected with *C. trachomatis* (P=0.001); this finding is in agreement with other study[19]. This sign was seen in 33.6% and 19.5% of patients with *C. trachomatis* PCR-positive and negative infection, respectively. This sign could be used as an guideline for obstetricians and gynecologists for presumable diagnosis of genital *C. trachomatis* infections in women, especially this guideline is useful in developing countries such as Iran that have limitation in access to diagnostic techniques. Since specificity and sensitivity of this sign are 80.5% and 33.6%, respectively, the swab induced bleeding positive or negative infections can not indicate definite presence or absence of *C. trachomatis* but the presence of the sign as an alarm notifies clinicians for more accurate diagnosis and treatment of genital *C. trachomatis* infection in women.

Conflict of interest statement

We declare that we have no interest conflict.

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