Detection of *Chlamydia trachomatis* in endocervical specimens by an enzyme-linked polymerase chain reaction assay

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ABSTRACT

Chlamydia trachomatis (CT) is the most common cause of sexually transmitted infections (STI) worldwide and its early detection and treatment can reduces the high morbidity associated with this infection. In this study a sensitive diagnostic polymerase chain reaction (PCR)-based enzyme immunoassay (PCR-EIA) method was developed which detects CT in women with cervicitis. Endocervical swabs collected from 123 women (20-55 years) with cervicitis were tested by both conventional PCR, and PCR-EIA assays, using identical sets of primers to amplify a CT-specific plasmid. For the conventional PCR, amplicons were detected by agarose gel electrophoretic analysis and the PCR-EIA assay used biotin-labeled primers, strepavidin-coated plates, a digoxigenin-labeled probe, and a final enzyme-linked colorometric analysis (405 nm) was used to measure the CT amplicon. The frequency of positive CT infection by conventional PCR and PCR-EIA assay was 7% and 17%, respectively. The highest frequencies of CT infection were among women of 31-40 years old group (25%). The PCR-EIA limit of detection, calculated by linear regression analysis, was10 pg of CT DNA (r^2=0.9642). The degree of agreement (Kappa) between the conventional PCR and PCR-EIA method was 0.556 (p<0.0001).

Keywords: *Chlamydia trachomatis*, PCR-EIA, Cervicitis, Sexually Transmitted Infections

INTRODUCTION

Chlamydia trachomatis (CT) is the most prevalent cause of bacterial sexually transmitted infections (STI), with an estimated 100 million cases reported annually throughout the world (1). Diagnosis of genital CT infections in women is important because CT **c**ervicitis commonly leads to pelvic infections, which may results in sequelae complications such as pelvic inflammatory disease (PID), an increased risk of ectopic pregnancy and infertility (2-3). Since most CT infections are asymptomatic (4-5), early diagnosis is essential for the timely treatment of CT-infected women to prevent development of sequelaes and transmission of CT infection to susceptible individuals**.** Polymerase chain reaction (PCR) has been established as the standard assay for rapid diagnosis of genital CT infection (6-8). Recently, by national screening programs it has been shown that utilizing sensitive and cost-effective PCR-based methods to identify CT-infected women (9-10), reduce the high morbidity associated with this infection.

At the present time, screening programs to identify women with CT infections do not exist in Iran, mostly because conventional PCR assays are costly, laborious and unsuitable for high volume CT testing. However, various PCR-based enzyme immunoassays (PCR-EIA) have been devised as rapid and convenient methods for CT diagnosis which are not costly and are able to handle large number of specimens (11-14). In this report, development and utility of a cost-effective PCR-EIA test for CT detection which facilitates the successful launch of CT screening program among women in Iran is described.

MATERIALS AND METHODS

Patients and Specimens

The participants in this study included 131 women with cervicitis who visited the outpatient obstetrics and gynecology section of a women's health center in Tehran, Iran complaining of pelvic pain and/or vaginal discharge. Consenting participants completed a questionnaire addressing demographic data prior to their cervical examination, which included evaluation of the presence of mucopurulent endocervical discharge, friability, and ectropin. After removal of cervical mucus, samples of the endocervical canal secretions were collected on two cotton swabs for laboratory diagnosis of *C. trachomatis*.

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Specimen processing and DNA extraction

Eight of 131 endocervical specimens, were eliminated from the study due to incomplete clinical data, or inadequate specimen quality. Endocervical swabs were washed in 500µL of phosphate buffered saline and the fluid was stored at -20°C until further processing. DNA was extracted from 100µL of the endocervical sample fluid using the DIAtom Prep100 kit (IsoGene Inc., Moscow, Russia) and was stored at -20°C until used.

PCR Amplification

Briefly, a 377bp fragment of a cryptic plasmid (specific to *C. trachomatis*) was amplified with primer BP1 (sense) 5'- AACCGTTTTTAATAG-TGGCA-3' and BP2 (antisense) 5'- TTCTGG-CCAAGAATTATCC- 3' through 30 cycles (pre-denaturation step at 94 °C for 3.5 min., denaturation at 94 °C for 30 sec., primer annealing at 52°C, and 30 sec. primer extension at 72°C, and a post-extension step at 72°C for 3 min.) in 25µL PCR reaction (buffer consisting of 10 mmol/L TRIS, pH 8.3, 50 mmol/L KC1, 2.5 mmol/L $MgCl_2$, and 0.01% gelatin); 0.2 mmol/L each of dATP, dGTP, dCTP, and dTTP; 2.5U *Taq* DNA polymerase; and 0.5 µmol/L primers for conserved plasmid nucleotide sequences of *C. trachomatis.*

PCR-EIA

Digoxigenin-labeled *C. trachomatis* amplicons were measured by a microtiter-based enzyme immunoassay (PCR-EIA). Briefly, 10 µL of amplicon was denatured with 20µL of sodium hydroxide and incubated at room temperature (RT). After 10 min, 220µL of hybridization buffer containing 10 pmol of the 5′ end biotin-labeled internal oligonucleotide BP3 probe 5'-AGCAGCTGCGAAAAAGAGAC
(complementary to primers internal sequences) specific for *C. trachomatis,* was added. The amplicon was hybridized in solution for 30 min at 54°C, and the nucleic acid mixture was added to a strepavidin-coated 96-well microtiter plate.

Using a solution hybridization-based enzyme immmunoassay (Roche Diagnostics, Mannheim, Germany), 200µL of 1:100 diluted peroxidase-conjugated anti-digoxigenin IgG was added to each well and plates were incubated for 30 min. at 37°C with occasional mixing. After five washings, 200µL of the ABTS substrate solution was added, and the plate was incubated for 30 min. at 37°C. The optical density (OD) of the color reaction was measured by spectrophotometry at 405nm absorbance (reference filter: 492 nm). The PCR-EIA run was considered valid if all negative control OD values were <0.1, and the PCR positive control value was >1.0 OD units. The OD value of background "cut off" was 0.055 OD units, calculated as the mean of all EIA negative-control OD values plus 3 standard deviations (SD). The average OD value of 102 negative PCR-EIA samples was 0.155 OD units and a sample was considered positive if the specimen's OD value was greater than the background cut-off value by three fold (i.e. 0.170 OD units). Mean OD value of 3 separate positive control CT samples was 1.81 ± 0.789.

Statistical Analysis

All statistical analysis of data, including determination of standard deviation, and degree of agreement (i.e. *Kappa* statistic), was carried out by the SPSS v.10 statistical software.

RESULTS

Figure 1 shows the electrophoretic analysis of amplified chlamydial DNA from endocervical specimens demonstrating the CT specific 377bp amplicon in some of the endocervical specimens. The PCR amplification was specific for *C. trachomatis,* since the negative control samples did not show any CT amplicon (Fig.1). All nine specimens that were CT positive by the conventional PCR were also positive by the PCR-EIA assay as well. However, the PCR-EIA assay identified *C. trachomatis* DNA from 12 endocervical specimens, which did not display the CT specific amplicon by the conventional PCR.

Overall, 17% (21/123) of the specimens were positive for chlamydial DNA by the PCR-EIA assay as compared to only 9 (7%) of specimens which were positive by conventional PCR (Table1). There was no difference in overall relative frequency of CT infection among women by either PCR-EIA or the conventional PCR results. Figure 2 shows PCR-EIA assay's chlamydial DNA range of detection by using various concentrations of standard *C. trachomatis* DNA. Regression analysis of the PCR-EIA standard curve, covering the range of 60ng to 25pg of *C. trachomatis* plasmid DNA, demonstrated a remarkably linear (r^2=0.9642) curve with an approximate *C. trachomatis* DNA detection limit of 10pg (Fig. 2). In contrast, the sensitivity of the conventional PCR, determined by visualization of various dilutions of amplified CT DNA on agarose gel was 950pg of *C. trachomatis* DNA (data not shown). The PCR-EIA analysis of PCR negative *C. trachomatis* DNA samples as well as distilled water samples showed OD values of 0.004 and 0.075, respectively. The range of corresponding OD values were 1.615- 0.265 OD units.

Table 1. Frequency of *C. trachomatis* infection in women with cervicitis as detected by conventional PCR and PCR-EIA methods.

Patient Age Group	No. (%)	(+) Chlamydia Test (% of total)	
		PCR	PCR-EIA
20-30	57 (47)	3 (2.4)	7 (5.6)
31-40	43 (35)	5 (4.1)	10 (8.1)
41-55	23 (18)	1 (0.8)	4 (3.2)
Total	123	9 (7.3)	21 (17.1

Figure 1. A representative gel electrophoresis analysis of amplified *C. trachomatis* plasmid DNA from endocervical specimens showing the CT specific target amplicon. Lane 1, molecular weight markers (MW 100bp units); lane 2, positive control *C. trachomatis* DNA showing the 377bp amplicon; lane 3, negative control (distilled water); and lanes 4-11, endocervical specimens from women with cervicitis.

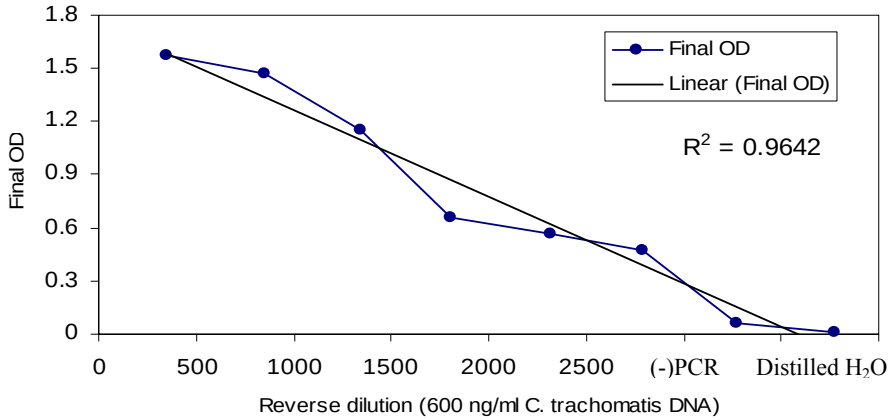

Figure 2. Standard titration curve of *C. trachomatis* amplicon by PCR-EIA assay. Serial dilutions of the 377bp CT amplified product (1:100 to 1:2,500) were hybridized, and quantified in the immunoassay read at 405nm absorbance.

DISCUSSION

Early detection of genital Chlamydial infection is extremely important in reducing the morbidity due to chlamydial STI among women, and decreasing the high cost of reproductive health care**.** In order to detect CT in endocervical samples, a sensitive PCR-EIA assay was developed which rapidly detects CT among women with cervicitis. The employed *C. trachomatis* PCR-EIA method has the following features; 1) The nucleotide sequences of "Primer and Probe set" have not been previously used, and 2) this is the first PCR-EIA developed in Iran for detection of *C. trachomatis*.

Similar to the gel-based conventional PCR, our PCR-EIA is a rapid method, but has the advantage of being cost-effective and amenable to quantitative measurement, as well as the ability of measurement of chlamydial DNA in large number of specimens. To our knowledge, this is the first report of a devised PCR-EIA assay for detection of *C. trachomatis* in Iran.

Initial studies were aimed at setting up a microtiter-plate based PCR-EIA assay, which has similar sensitivity as the conventional CT-PCR, as well as the convenience of large sample through-put and quantitative measurement capability. A cost effective chlamydial PCR-EIA can finds

widespread use for diagnosis of CT infection in Iran and can facilitates implementation of nationwide screening programs. Unlike the conventional PCR, the newly developed PCR-EIA method is neither laborious, nor time consuming, and allows for standardization. Our results demonstrate a small scale development of a CT PCR-EIA in Iran. However, before its widespread use in any country, this method should be tested by additional validation studies, including rigorous inter- and intra assay variation measurements using a larger number of specimens from various body sites. Moreover, the results should be compared to the results of other chlamydia detection assays.

The fact that developed PCR -EIA detects CT DNA in specimens negative by conventional PCR, suggests that PCR-EIA is a highly sensitive method for diagnosis of genital chlamydial infections. Furthermore, PCR-EIA detection of chlamydial DNA over a wide range of concentrations indicates that PCR-EIA can detects even low concentrations of CT in endocervical samples. Therefore this PCR-EIA may be helpful in diagnosis of early genital chlamydial infections, which is essential for the appropriate and timely treatment of CT-infected women and preventing transmission of *C. trachomatis* to susceptible individuals.

Moreover, the PCR-EIA for *C. trachomatis* has all the advantages of the conventional PCR, and is a valuable method to be utilized by CT screening programs where large number of samples, low cost and rapid detection is desired. Our findings lend support to the implementation of a nationwide screening program to identify and treat Iranian women with CT infections using sensitive molecular techniques such as PCR-EIA. Similar studies in other countries have shown that such programs are cost-effective and leads to reduced morbidity associated with chlamydial infections (16).

The higher frequency of *C. trachomatis* genital infections among women with cervicitis as compared to a recent report from Tehran (17) may be related to the high sensitivity of PCR-EIA used in this study, as well as the difference in the type specimens analyzed in the previous report. Since most women with genital chlamydial infection are asymptomatic, it is believed that incidences of CT infection is probably higher than the 17% rate observed in this study and such CT screening programs will makes a significant impact on improving the reproductive health of women by prevention of long-term sequelae associated with PID (3, 18).

The higher frequency of CT genital infections among women in Iran in comparison to 4-9% prevalence rates reported from Holland, Colombia, Canada, and the United States (19-22), warrant a more comprehensive study utilizing the PCR-EIA assay to screen a large number of women who suffer from chlamydial infection, thus allowing for better estimates of the real magnitude of the reservoir of asymptomatic CT infections among Iranian women. In light of epidemiological studies that have shown chlamydial genital infections can serve as biological cofactors for transmission of the human immunodeficiency virus (HIV) (23-25), and a co-factor in human papillomavirus (HPV) infection (26), screening programs for genital chlamydial infections not only reduce the morbidity associated with other sexual transmitted pathogens in women, but also decrease the general cost of health care in developing countries, such as Iran.

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