ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Molecular Diagnosis of Genital *Chlamydia trachomatis* in Women with Pelvic Inflammatory Disease

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ABSTRACT

Frequency of STI infections has a low rate in Muslim and Arabic societies due to injunction and ethics to prevent non-marital sex and sexual promiscuity. In Iraq, there has been very little surveillance program to screen *C. trachomatis* infection, thus this research aimed to detect frequency of genital chlamydia in female with PID via genetic diagnosis of *C. trachomatis* by the use of *16S rDNA* and *16S-23S rDNA* spacer and comparison between them. This study included 232 endocervical swab samples collected from female patients suffering from signs and/or symptoms of PID; and were diagnosed as having PID by the gynecologist and had risk factors for this infection. These swabs were subjected to DNA extraction, and further processing by polymerase chain reaction. Using 16S rRNA and 16S-23S rRNA spacer genes as genetic markers via conventional PCR; both of them gave approximate results 7/232 (3.02%) versus 8/232 (3.45%) respectively. The highest frequency distribution 4/8 (50%) of *C. trachomatis* among the age group of 20-40 years, while 2/8 (25%) from both age group less than 20 years, and more than 40 years separately. While association with the use of different types of contraceptives; 50% of chlamydial positive patients used oral contraceptive pills during their life. While those using intrauterine contraceptive devices representing 25%, and remaining cases represented 12.5% used barrier methods and 12.5% did not use any type. The study concluded that *16S rRNA* and *16S-23S rRNA* spacer genes are considered as sensitive and specific methods for the identification of *C. trachomatis* in women.

Key words: *Chlamydia trachomatis*, 16SrRNA, 16S-23S rRNA spacer, PCR

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ISSN: 0975-8585

INTRODUCTION

Pelvic inflammatory disease (PID) is an infection of the female upper genital tract that involves any combination of the uterus, endometrium, ovaries, fallopian tubes, pelvic peritoneum and adjacent tissues. PID consists of ascending of different infectious agents from the lower to upper genital tract [1].

Chlamydia trachomatis is one of the leading pathogens of sexually transmitted diseases. It is an obligate intracellular bacterium, with unique developmental cycle. Its infection mostly is asymptomatic, without antibiotic intervention, long-term *C. trachomatis* infection of the female genital tract may result in chronic inflammatory pathologies, including pelvic inflammatory diseases, ectopic pregnancy, and infertility [2]. Variable risk factors are related to increase incidence, whether are linked with the host or the bacterium [3].

Number of epidemiologic studies have revealed that the cumulative risk of PID and long-term reproductive outcomes rise according to the number of repeat *C. trachomatis* infections. Conversely, it remains unclear whether the increased risk is due to longer exposure time or repeated infections induce more immunopathology [4].

Diagnosis of chlamydial infection is based on isolation of bacteria in tissue culture; the gold standard for diagnosis of genital *C. trachomatis* infection. But difficulties associated with this method include, technical complexity, time consuming (requires at least 48-72 hours), laborious with strict requirements related to collection, transport and storage of specimens; are limited factors for its use [5, 6].

The use of molecular biological methods in the detection of *C. trachomatis* in clinical specimens is rapidly increasing, because these are sensitive and specific methods for detection of small quantity of bacterial DNA in clinical samples [7]. This is done by nucleotide sequence analysis of genes in the rRNA operon, as the *16S* and *23S rRNA* genes, by the use of genus- and species-specific regions of rRNA [8]. The vitality and widespread of *rRNA* genes makes them a best target for bacterial identification.

Very limited reports are published on the frequency of *C. trachomatis* in developing countries and most of them depended on seropositivity by detecting antichlamydial antibodies via ELISA and other immunoassay; thus the aim of this study involved the detection of *C. trachomatis* genetically in patients with PID by the use of *16S rDNA* and *16S-23S rDNA* spacer and comparison between these two molecular detection techniques.

MATERIALS AND METHODS

This study approved by the ethical committee of College of medicine -University of Babylon, Iraq.

Subjects of the Study

This was a retrospective (Case-control) study. Clinical samples were collected from patients admitted to the out-patient clinics of Gynecology and Obstetrics, in two hospitals of Babylon Province, during the period from March to October 2015.

The study involved 232 female patients subjected for endocervical swab sampling. The age of patients ranged from 18 to 48 years. These females were diagnosed by the gynecologist as having PID, according to the characteristic criteria of national guidelines for pelvic inflammatory disease [9] and according to the signs and symptoms, abdominal and pelvic ultrasound, in addition to be having risk factors.

Samples collection

After obtaining the permission from each female subject for examination and sampling, she was rested in lithotomy-dorsal position. A methodical inspection was done for lesions and vaginal/cervical discharge. Sterile vaginal speculum was introduced and fixed by the clinicians, using proper lighting and environment. After cleaning the exocervix with cotton swab, cervical mucus or inflammatory exudates was removed before the introduction of swab into the endocervical canal.

Later on, two to three cotton swabs were used for each patient; as the specimens were collected by inserting swab about 1 cm into the endocervical canal and were rubbed by rotating it against the wall of endocervical canal vigorously or scraping to get more cells from the endocervix. Then swabs were removed carefully to avoid any contact with vaginal secretions and immersed in plain tube-containing 1 ml of phosphate buffered saline (PBS) transport medium or normal saline. The speculum was withdrawn. The sample was labeled with the patient's data or number. The specimen was transported to the laboratory using iced-box. This method is rapid, inexpensive and fairly specific, but its disadvantage is the subjectivity [10, 11].

DNA extraction and PCR protocol

The collected endocervical swabs from patients were subjected to DNA extraction procedure. It was performed according to protocols recommended by manufacturer (Geneaid/USA). The achieved DNA was stored at 2-8°C for further applications and processing for molecular identification of *C. trachomatis* using two pairs of primers to increase the probability of detection. First one is the *16SrRNA* gene, using conventional PCR, while the second targets the more conserved regions of the *16S-23S rRNA* spacer gene, by the usage of touch down protocol PCR which used to increase specificity and sensitivity; sequences of both genes and their conditions are listed down in **Table-1.**

Table-1: Primer sequences and PCR conditions with their amplicon size

Gene's Name	Primer Sequence (5´- 3´)	Size (BP)	Conditions	Reference
Identification Genes				
16S rDNA	F-5'CGAGTCGGCATCTAATACTAT3' R-5'AAAACGACATTTCTGCCGC3'	402	94°c 5min 1x 94°c 1min / 58°c 30s / 72°c 1min — 30x 72°c 5min 1x	[12]
16S-23S spacer *rRNA*	F-5'GGCGTATTTGGGCATCCGAGT AACG3' R-5'TCAAATCCAGCGGGTATTAACC GCCT3'	315	95°c 75s 1x 94°c 45s / 62-52°c 30s (-1/4cycles) / 72°c 1min — 60x 72°c 5min 1x	[13]

PCR amplification was confirmed by agarose gel electrophoresis by visualization against UV light [14]. The electric current was allowed to pass at 70 volt for 50 min. UV trans-illuminator was used 280 nm for the observation of DNA bands, and the gel was photographed using digital camera [15].

Statistical analysis

Frequency distribution of *C. trachomatis* was introduced in the form of percentage and histograms.

RESULTS

Out of the total 232 females, only eight (3.45%) were diagnosed genetically to be *Chlamydia* positive, as shown in **Figure-1**, while the remaining (224/232) were negative for this bacterium. This result was only 7/232 (3.02%) produced the specific 402bp DNA fragment compared with allelic ladder by the use of 16SrRNA; as shown in **Figure-2**. While, the use of genus- and species-specific *16S-23S spacer rRNA* with touchdown PCR protocol increases the result to eight (8/232), represent 3.45%, as shown in **Figure-3.** Comparison between two tests was presented in **Figure-4.**

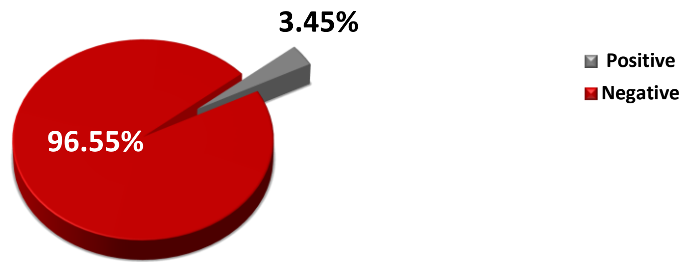

Figure-1: Frequency (%) of *C. trachomatis* in endocervical swab from women with PID.

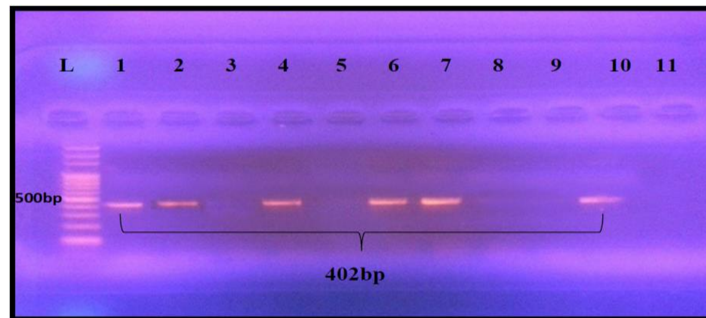

Figure-2: 1.5% Agarose gel electrophoresis at 70 volt for 50 min for *16SrRNA* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L: 1500 bp ladder; lane (1, 2, 4, 6, 7 and 10) were positive for *Chlamydia trachomatis* in endocervical swabs among patients with pelvic inflammatory disease. The size of product is 402 bp.

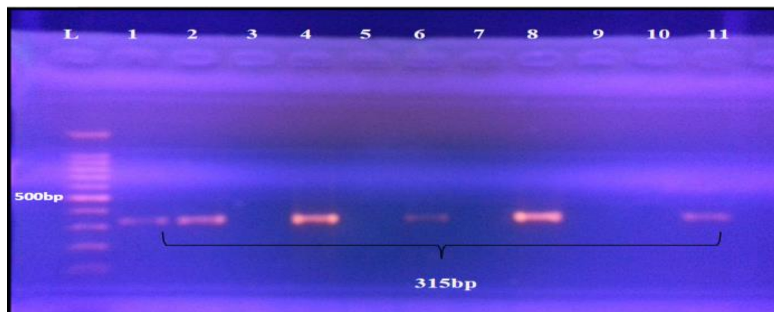

Figure-3: 1.5% Agarose gel electrophoresis 70 volt for 50min for *16S-23SrRNA* PCR products visualized under U.V light 280 nm after staining with ethidium bromide. L: 1500 bp ladder; lane (1, 2, 4, 6, 8 and 11) were positive for *Chlamydia trachomatis* in endocervical swabs among patients with pelvic inflammatory disease. The size of product is 315 bp.

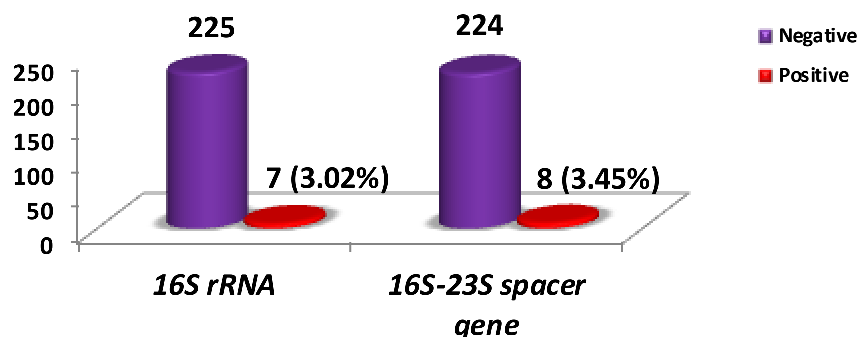

Figure-4: Comparison between positive chlamydial results using *16S rRNA* and *16S-23S rRNA* spacer genes.

Also, analysis of frequency distribution of *C. trachomatis* among age groups of patients with PID revealed that the highest frequency among the age group of 20-40 years was 4/8 (50%), while 2/8 (25%) from the age group less than 20 years, and 2/8 (25%) from the age group more than 40 years. This mean the frequency was about (75%) above age 20 years, as compared with (25%) at the age 20 years and below, as in **Table-2.**

Table-2: Frequency of C. trachomatis in endocervical swabs of patients with PID according to age groups.

Age groups (years)	Frequency (No. (%))
< 20	2 (25%)
20-40	4 (50%)
> 40	2 (25%)

While, the analysis of chlamydial risk factors, particularly the use of different types of contraceptives; showing that 50% of chlamydial positive patients used oral contraceptive pills during their life. While those using intrauterine contraceptive devices (IUCD) representing 25% of chlamydial cases. The remaining cases represented 12.5% who used barrier methods and 12.5% of them did not use any type of IUCD, as in **Table-3.**

Table-3: Distribution of C. trachomatis among patients with PID according to the use of variable types of contraceptives.

Contraceptive type	Chlamydial results
Oral Pills	4(50%)
Intra-Uterine Contraceptive Devices	2(25%)
Barrier Methods	1(12.5%)
Not using any type	1(12.5%)
Total	8(100%)

DISCUSSION

The diagnosis of PID is based principally on clinical evaluation which is ambiguous, as no single historical, physical, or laboratory test finding is both sensitive and specific enough for the diagnosis of PID. So, combinations of both diagnostic and clinical findings can improve sensitivity and specificity [16].

The occurrence of chlamydial genital infection in women fluctuates in different groups and different communities, according to the exposure to variable risk factors. It was establish that *C. trachomatis* considered to be a leading cause of PID and female infertility worldwide and responsible for approximately 10-30% of all PID cases [17], with numerous studies have tried to assess the percentage of untreated *C. trachomatis* infections leading to PID, a study estimated that 8-10% of women with chlamydial infection developed into PID [1].

Very limited reports were published on the frequency of *C. trachomatis* in Iraq and developing countries and most of them depend on detecting chlamydial IgG and IgM antibodies via immunoassay [18-20]. However, these immune studies ought not to be used for screening, for the reason that previous chlamydial infections habitually produces long-lived antibodies that cannot be easily distinguished from the antibodies produced in a recent infection [2]. Beside, that screening tests needed to be simple and cost effective diagnostic tests, and the intracellular localization of the pathogen generates an additional challenge for routine diagnosis. Diagnosis of chlamydial infection is even more difficult in asymptomatic and in chronic or persistent infections where pathogen load would be low [21].

Several studies done in various Iraqi governorates, showed wide range of frequency variability; ranging from (0%) in Kerkuk [22] to 26.5% and 39.7% in Baghdad and Mosul respectively [23, 20].

The lower frequency (3.45%) in this study is agreed with different studies conducted in the Arabic World; in the United Arab Emirates (2.6%) [24], Jordan (3.9-5%) [25, 26] and Qatar (5.3%) [27], while in Saudi Arabia (15%) [28]. Additionally, in Iran two separated studies reported that frequency of *C. trachomatis* was (8.3%) and (22%) respectively [29, 30].

This distinction in the frequency is related to sample size, age of the participants, population studied with variable socioeconomic factors, along with different practices used.

In comparison to this lower rate, that is expected to be lower than that in the Western countries, as UK (16%) [31], USA (15%) [32], Brazil (10.9%) [33].

This restriction in the chlamydial positivity among study sample could be due to the fact that the Arabic people (including the Iraqi population) are culturally and socially conservative in their attitudes towards free social life.

Since *C. trachomatis* is a non-cultivable, thus molecular techniques are useful for the identification of this microorganism. As PCR is rapid and has a superior sensitivity in comparison with tissue-culture, ELISA or DFA staining [23]. Due to the cost and problematic, stringent requirements; in addition to that, these serological tests based on the use of specific antibodies, but the problem with these antibodies is that the specific antichlamydial antibodies may indicate current or past chlamydial infection in sites other than the genitourinary tract [34].

Many PCR-based studies targeted variable chlamydial genes, as cryptic plasmid, outer membrane proteins and others; in this study we used 16SrRNA and 16S-23S rRNA spacer genes, where rRNA as 16S, 23S and their spacers genes were used to study bacterial identification, taxonomy and phylogeny because they are the most communal housekeeping genetic markers. Moreover, no variation in the 16S-23S rRNA spacer regions between human isolates of *C. trachomatis*. So, it is likely that a species or subspecies group with a certain housekeeping gene complex 16S-23S rRNA has host specificity and is adapted to a certain host [12].

rDNA gene is particularly advantageous in identifying unusual bacteria that are difficult to identify by conventional methods, and the ultimate solution for identification of etiological agents of infectious diseases caused by rarely encountered bacteria. This is providing identification in >90% of cases [35].

Results of age analysis among study patients could be agreed with results in variable studies were approved in some developing countries, as the topmost age period for *C. trachomatis* infection was between 20-30 years old about (65%) [36, 37].

In the developed countries, STI including chlamydial disease were more common among patients in the age group less than 25 years old, this is due to different cultural setting and the screening programs focus mainly on women at this age group [31, 33].

The most common demographic associate of infection with chlamydial infection in women is young age (<20 years). This could be elucidated by the anatomic differences in the cervix of the younger women, wherein the squamo-columnar junction, a primary host target for *C. trachomatis*, is everted and thus more exposed. While, the less common spread of infection in the older age group has been ascribed to low exposure to *C. trachomatis* and by physiological changes which lessen sensitivity to the gaining [38].

A number of studies were carried out had implicated oral contraceptives as a co-factor for chlamydial infection and other STDs. Also, suggested that these STDs may contribute to the spread of HIV infection. Thus the relationship between oral contraceptives and chlamydial and other STD is an important public health problem [39].

The use of these hormonal contraceptives associated with an increased risk of acquisition and transmission of STD as chlamydia, due to many causes; where they exhibit momentous immune regulatory effects that may impact the host's susceptibility to pathogens; this is due to their ability to impede the secretion of key regulators of cellular and humoral immunity by activated T cells and dendritic cells [39, 40].

CONCLUSIONS

The uses of a sensitive and specific method for the identification of *C. trachomatis* in women by using the *16S rRNA* and *16S-23S rRNA* spacer genes. Low frequency of chlamydial infection in association with PID among Iraqi female patients might be due to Islamic and Arabic traditions and ethics.

ACKNOWLEDGEMENTS

The authors are thankful to Department of Microbiology, College of Medicine, Babylon University, Iraq, for the facilities provided in the completion of the work. They also thankful to all patients for their cooperation.

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