Original Research Article

Molecular Detection of *Chlamydia Trachomatis* In Women with Bad Obstetric History

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Abstract

Bad obstetric history is multifactorial common female genital disorders. A significant percentage of cases are attributed to infectious agents, of which *Chlamydia trachomatis* attracted less attention. This study aimed to assess the role of *Chlamydia trachomatis* in a sample of Iraqi women with bad obstetric history using molecular methods. This case-control study recruited 100 women with bad obstetric history (referred as cases onward) and 40 age-matched apparently healthy women as controls. Bacterial DNA was isolated from homogenized placental tissues obtained from each participant post-delivery. The 16S ribosomal gene of the *Chlamydia trachomatis* was amplified with specific set of primers using conventional PCR. Twenty-Three placental samples (23%) gave positive results for gene amplification from cases, while none of women in control groups gave such a result. The infection rates were significantly higher in 31-39 age group, aborted women in the first trimester and in women with more than 4 pregnancies compared to other age groups, women aborted in the trimester, and women with 4 or less pregnancies respectively. These data highly suggested the association of *C. trachomatis* with bad obstetric history. Screening program for detection of these bacteria in pregnant women should be considered.

Key Words: *Chlamydia trachomatis*, Bad obstetric history, PCR

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استخدام الطرائق الجزيئية للكشف عن بكتريا الكلاميديا تراكوماتس في النساء اللاتي لديهن تاريخ ولادة سيء

الخلاصة

تاريخ الولادة السيء عبارة عن اضطرابات شائعة متعددة الاسباب تصيب القناة التناسلية الانثوية . تعزى العديد من هذه الحالات الى العوامل الخمجية ومن بينها بكتريا كلاميديا ترامكوماتس التي لم تحظ بالاهتمام المناسب هدفت هذه الدراسة الى تقييم دور بكتريا كلاميديا ترامكوماتس في حصول حالات تاريخ الولادة السيء في عينة من النساء العراقيات ممن لديهن تاريخ ولادة سئ. تم في هذه الدراسة تطويع ١٠٠ امراة ممن لديهن تاريخ ولادة سيء (مجموعة الحالات) و ٤٠ امراة سليمة ظاهريا كمجموعة سيطرة . استخلص الـ DNA من انسجة المشيمة بعد جمعها ومجانستها من كل امراة وتمت مضاعفة جين 16S الرايبوسومي للبكتريا باستخدام بادئات خاصة وبطريقة PCR التقليدية. أعطت ٢٣ عينة من مجموعة الحالات (٢٣%) نتائج موجبة لتضاعف الجين في حين لم تعط اي عينة من مجموعة السيطرة مثل هذه النتيجة. كانت نسبة الاصابة أعلى معنويا في الفئة العمرية ٣١–٣٩ سنة و النساء المجهضات في الثلث الاول من الحمل وفي النساء اللاتي لديهن اكثر من اربع ولادات مقارنة بالفئات العمرية الاخرى و النساء المجهضات في الثلث الثاني من الحمل والنساء اللاتي لديهن اربع ولادات او اقل على التوالي.تشير هذه البيانات الى اهمية بكتريا كلاميديا ترامكوماتس في حالات تاريخ الولادة السيء ويجب الاخذ بالاعتبار تبني برامج الكشف عن البكتريا في النساء الحوامل .

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Introduction

Classically, bad obstetric history (BOH) was defined as the occurrence of three consecutive pregnancy losses. However, recently this definition was extended to involve any previous unfavorable outcome such as two or more consecutive abortions, intrauterine fetal growth retardation or death, stillbirth and congenital anomalies [1,2]. Several causes are implicated in BOH, may be the most prominent of which are chromosomal aberrations and anti-phospholipid antibody syndrome [3,4].

Although less attributed, infectious agents do have an important role in BOH. About 15% to 66% of cases are attributed to different infections [5]. Of those agents, *Chlamydia trachomatis* is so far considered as the most sexually transmitted bacterial disease worldwide [6]. It is an obligate intracellular bacterium with an estimated 92 million new annual infections [7]. Researchers frequently attributed different gynecological illnesses such as salpingitis, ectopic pregnancy, pelvic inflammatory diseases, stillbirth, miscarriage and infertility in women to these bacteria [9,10]. There are two great challenges associated with difficulty in controlling of such infections.

The first one is that as many as 90% of infections in both men and women are asymptomatic. Those subjects act as reservoirs and are capable to transmit the microorganism to their sexual partners[10]. The other challenge is related to technical difficulty for culturing because of intracellular nature of the bacteria [9]. With the advent of molecular methods, it becomes more easily to accurately diagnose a wide range of infectious diseases including infection with C. trachomatis. In Iraq, there is paucity in the report addressing the role of *C. trachomatis* in female reproductive disorder. So, this study aimed to use polymerase chain reaction (PCR) to investigate the prevalence of *C. trachomatis* in women with BOH.

Materials and Methods

Placental tissue samples were collected from 100 pregnant women with BOH (referred as cases onward) and from 40women healthy with normal delivery who were attending the Gynecology outpatient clinics, wards and emergency unit in Al Imamain Al Kadhimain Medical City, and Baghdad Teaching Hospital during the period from December 2015 to May 2016.Informed consent was obtained from all the women who participated in this study. This study approved by Research Ethical Committee (REC) in the College of Medicine /AL-Nahrain University. Inclusion criteria included married women at sexually-active ages and negative for TORCH (toxoplasmosis, other (syphilis, varicella-zoster, parvovirus B19), rubella, cytomegalovirus, and Herpes infections). Exclusion criterion was women who were positive for anti-phospholipid antibodies.

Preparation of Tissue Homogenate

Twenty five gram of the placental tissue was homogenized with 10ml of PBS by using tissue homogenizer [11] for about 1 min. at 4°C. The resulting suspension was subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenate centrifuged for about 15 min. at a speed of 5000 rpm and temperature of (2-8) °C. The supernatant was then collected carefully and stored at (-80°C) for the subsequent measurement.

DNA Extraction and Gene Amplification

DNA was extracted from placental homogenate using DNA isolation kit (DNA-sorb-B (Sacace/Italy) Kit) according to the manufacturer's instruction. The concentration and purity of the purified DNA was quantified by the use of nanodrop instrument following

the instruction of the manufacturer, The specific pair of primer was used in conventional PCR to detect the presence of16Sribosomal gene *of C. trachomatis* [12]. Forward:5'-TGG CGG CGT GGA TGA GGC AT-3' and Reverse: 5'-CTC AGT CCC AGT GTT GGC GG-3' with a fragment length of 300 bp. The master mix contents were thawed at room temperature before use. For each reaction within each single pre-mixed PCR reaction tube, 2μL from each forward primer and reverse primers, 5μL of DNA template, 12.5 μL of GoTaq® Green Master Mix were added. The volume was completed to 25μl with deionized nuclease–free, and tubes were then spun down with a minicentrifuge to ensure adequate mixing of the reaction components. Non-template negative control was used to validate the reaction. The tubes were placed on the thermo-cycler (Cleaver Scientific Thermal Cycler TC32/80) previously programmed with the following PCR conditions: 94°C for 5 min followed by 40 cycles of 94°C for 20 sec, 65°C for 20 sec, and 72°C for 20 sec, terminating in 72°C for 5 min. Ten μL of each PCR product was subjected to 1%

(wt/vol) agarose gel electrophoresis with ethidium bromide (0.5 μg /ml; Sigma) Five microliters of the 100bp DNA ladder (KAPA Biosystems) were mixed with one microliter of blue/orange 6X loading dye and subjected to electrophoresis in a single lane. Amplicon visualization was performed using an UV light transilluminator and then photographed using digital camera (Sony-Japan).

Statistical Analysis
Statistical Analysis system (SAS) software was used for all statistical analysis continuous variables were expressed in mean ± standard deviation (SD). The Pearson's Chi-square test or Fisher exact test was used for comparing the categorical variable. A two-sided significant level of 0.05 was considered to indicate a statistically significant difference.

Results
Demographic and Clinical Data of the Study Population:
Base line characteristics of the study population are shown in table 1.

Table 1: demographic and clinical characteristics of the study population

Variables	Cases(100)	Controls (40)	*P*-value
Age (mean ± SD)	27±6.6	29.4±8.14	0.823
Co-morbidity			
Diabetes Mellitus	15(15%)	9(22.5%)	0.205
Hypertension	17(17%)	11(27.5%)	0.122
Asthma	1(1%)	2(5%)	0.196
Epilepsy	1(1%)	0(0%)	…….

Regarding the last delivery outcome (at which the samples were collected), 25 (25%) of cases had normal deliveries, whereas 75(75%) had abnormal deliveries with stillbirth (18, 24%), abortion (40,53.3%), congenital abnormalities (16, 21.33%), and neonatal death (1, 1.33%). All women in control group had normal delivery outcomes.

Molecular Detection:
The results of the amplification of *C. trachomatis* gene by conventional PCR showed that gene was present in 23 (23%) out of 100 placental tissues, and the PCR product of this gene was 300 bp (Figure-1). All control groups were negative for this gene.
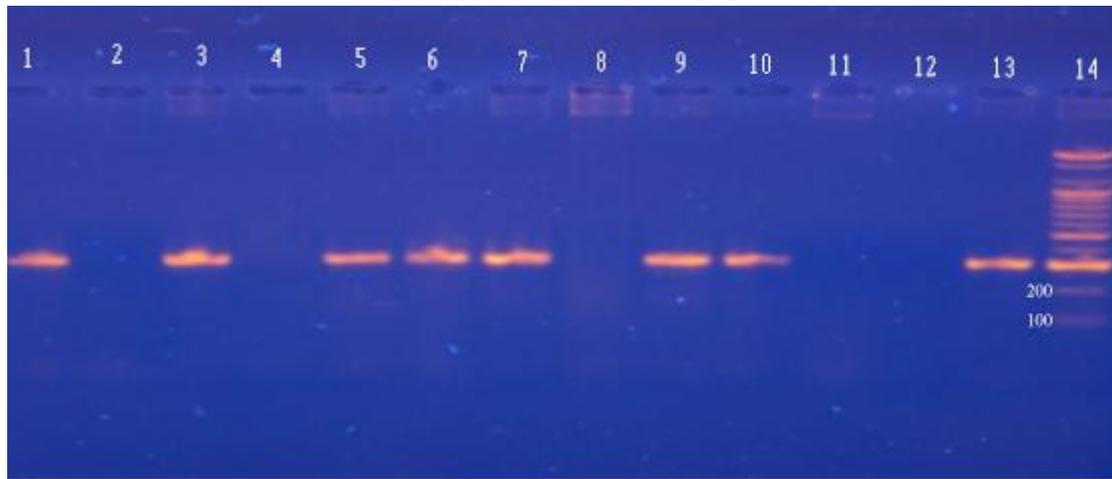
Figure 1: Gel electrophoresis of PCR of *Chlamydia trachomatis* S ribosomal gene. Lanes 1,3,5,6,7,9,10,13: positive results, lanes 2,4,11,12: negative results, lane 8: negative control, lane 14: 100 bp DNA marker.

The rate of *C. trachomatis* among different age classes is presented in table 2. The highest rate was observed in age group (30-39) years which was 11 (26.8 %), which differed significantly from other age classes.

Table 2: Prevalence of *Chlamydia trachomatis* among age groups

Age class	Positive cases		Negative cases		
	No.	%	No.	%	
<20 years (13 cases)	1	7.6	12	92.3	P< 0.001
20-29 years (37 cases)	5	13.5	32	86.4	
30-39 years (41 cases)	11	26.8	30	63.8	
≥40 years (9 cases)	5	55.5	4	44.4	

Table 4 shows the association of *C. trachomatis* infection with abortion time and with the number of pregnancies. Fifteen women (65.21%) had an abortion during the first trimester of pregnancy, while 8 women (34.79) during the second trimester with significant difference. On the other hand, more than half of infected women (56.52%) had more than 4 pregnancies compared to 4 women (17.39%) with 1-2 pregnancies and 6 women (26.05%) with 3-4 pregnancies with significant differences.

Table 4: Association of *C. trachomatis* infection with abortion time and number of pregnancies.

Variables	No	%	P- values
Abortion			
In the first trimester	15	65.21	0.038
In the second trimester	8	34.79	
Number of pregnancies			
1-2	4	17.39	0.006
2-4	6	26.08	
<4	13	56.52	

Discussion

The study revealed relatively high prevalence (23%) of *C. trachomatis* in women with BOH. Comparing with global studies, this percentage seems to take an intermediate rank. The prevalence in the whole Europe was recoded to be ranged from 4.1% to 25% among young women with BOH [13]. In Iran, 14.99% and 22.9% rates were recorded by two authors among women with spontaneous abortion, although both of them used PCR technique for detection [12,14]. Using enzyme linked immune-sorbent assay (ELISA), Baud *et al*. [9] reported higher prevalence of anti- *C. trachomatis* IgG antibodies in Switzerland women with miscarriage than in healthy women (15.2% vs 7.2%). In Serbia, Arsovic *et al.* [15] detected anti- *C. trachomatis* IgA antibodies in 21.3% of women with miscarriage. Finally, Wilkowska-Trojniel *et al*. [16] in Poland, reported an association between positive IgG specific antibodies for *C. trachomatis* and the number of miscarriages the women had faced. About 21% of women with one miscarriage were positive, while this percentage rises to 36.9 in those with two miscarriage compared to 4.4% in healthy women.

Several mechanisms by which *C. trachomatis* imposes its notorious effect in female genital tract have been suggested. The most plausible hypothesis accuses chlamydial heat shock protein 60 (HSP60) to induce this effect. Firstly, there is a high analogy between this protein and human HSP [17]. Accordingly, antibodies will be formed against self-protein in infected woman, especially in follicular fluid. These antibodies negatively influence the embryonic growth and increase the chance for adverse pregnancy outcome [18]. Secondly, chlamydial HSP could act as antigen and stimulate toll-like receptor 4 (TLR-4), and it thereby causes trophoblast apoptosis [19]. Finally, there is some evidence that this protein has a cross-reactivity with embryonic protein (the early pregnancy factor), and, thus, the host antibodies react with embryonic components leading to miscarriage [20].

The study revealed that the age group 30-39 years had significantly higher frequency of *C. trachomatis* compared to other age groups. This result is not in line with that recorded by Christian *et al*. [21] who found that young adults are the main affected age group. This discrepancy may be due to the fact that young group is more sexually active and they have frequent partner change in the population where the last study has done. By contrast, in Iraqi society, where Muslim religious is the majority, this behavior is banned which reduce the chance for getting sexually transmitted diseases including *C. trachomatis.*

The bacteria were more frequently detected in aborted women in the first trimester than those aborted in second trimester. One study in India did not find significant difference in the prevalence of *C. trachomatis* infection in mid-pregnancy and at labor [22]. Generally, time of pregnancy was not documented as risk factor for chlamydial infection. Rather, the pregnancy itself may increase the opportunity for colonization of these bacteria due to the accompanied changes in immune status of the pregnant women [7]. As there were more aborted women in first trimester who had *C. trachomatis*, this suggests that ability of the bacteria to induce abortion in early stages. However, the role of other causes of abortion should be excluded.

Finally, the study showed that women with over 4 pregnancies are more prone to *C. trachomatis* infection. This may reflect the immunosuppressive status of recurrent pregnancy and hence the high opportunity of the bacteria to establish the infection.

All in all, these data indicate the significant role *C. trachomatis* in BOH. Thus more attention should be paid to these bacteria and through regular investigation of pregnant women. Otherwise, miscarriage, stillbirth, ectopic

pregnancy, fetal growth retardation or adverse pregnancy outcomes may be inevitable. In this regard, Center for Disease Control (CDC) recommends screening for *C. trachomatis* for pregnant women in her first prenatal visit.

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