Immunological and molecular study of *Chlamydia trachomatis* as causative agent of abortion in Al-Muthanna province

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Summary

Chlamydiasis during pregnancy should be considered a significant risk factor for adverse pregnancy outcomes in humans. 120 women who had a single or repeated abortion were selected for this study, and they were referred with a physician report for TORCH tests to determine the final diagnosis of pregnancy loss. The control were (40) healthy pregnant women with a history of a normal pregnancy. The innate immunity in abortive women was higher than normal pregnancies, that was estimated by Nitroblue Tetrazolium test done to estimate the phagocytic activity, there was a significant increase (P=0.009) in phagocytic activity in the leukocytes of abortive women which was (22%) higher than that in control. Also, the cellular immune response was higher in abortive women than that in control. Methyl Thiazolyl Tetrazolium assay was performed to estimate lymphocyte transformation index of peripheral blood leukocytes in abortive women. The results of Methyl Thiazolyl Tetrazolium assay showed a significant increase (P=0.001) in the lymphocyte transformation index in the lymphocytes of abortive women which was (27%) higher than control. The serological detection by ELISA showed that anti-*C. trachomatis* IgG was (14.2%), and the molecular detection by Quantitive Real Time-Polymerase Chain Reaction showed positive results (17.5%) of total abortive women. The present study demonstrated a high level of relationship between *C. trachomatis* and abortion among women in the study samples. The age group (20-25years) was the most susceptible to chlamydial infection and the infection was higher in recurrent miscarriages than in single miscarriage.

Keywords: *Chlamydia trachomatis*, Miscarriages, Recurrent miscarriage, Quantitive Real Time-Polymerase Chain Reaction.

Introduction

Miscarriage can be defined as spontaneous loss of a pregnancy during the first twenty four weeks of gestation. Early miscarriage occurs during the first trimester of pregnancy (less than: 12 weeks of gestation) and occurs in up to one in five pregnancies. Late miscarriage occurs during the second trimester (12 to 24 weeks of gestation) and is less common (1). *C. trachomatis* infection, which most frequently is asymptomatic, is the most common bacterial sexually transmitted infection and a major public health concern globally. In 2012, the World Health Organization (WHO) estimated 130.9 million urogenital cases among adults worldwide (8.9 million in the WHO European region) (2). In the United States prevalence is highest in persons aged ≤ 24 years (CDC, 2014). Despite significant advances in chlamydial research, a prophylactic vaccine has yet to be developed (3). This Gram-negative obligate intracellular bacterium, which often causes asymptomatic infection, do not exhibit any symptoms, patients with *C. trachomatis* urogenital infections often remain undiagnosed and untreated, so may cause pelvic inflammatory disease (PID), ectopic pregnancies, miscarriage, and infertility. *Chlamydia* exists in two developmental forms: the elementary body (EB), which is infectious, nonreplicating, and extracellular; and the reticulate body (RB), which is noninfectious, replicating, and intracellular. Infection begins when the small EB is internalized by the cell. In the genital tissues, *C. trachomatis* normally infects the cervical (women) or urethral (men) epithelium layer, in the epithelial cells it requires Th1 immunity for optimal clearance. Both T cell subsets have been shown to recognize *C. trachomatis* antigens presented by APC, such as outer membrane protein 2 (Omp2), polymorphic outer membrane protein D (POMP-D), MOMP, heat shock protein 60

(hsp60) and chlamydial protease activating factor (CPAF) (3).

Materials and Methods

The samples were collected during the period from November 2016 to February 2017 in Children and delivery's Hospital at Al-Muthanna Province, 120 women who had single or repeated abortion were selected for this study, and they were referred with a physician report for TORCH tests to determine the final diagnosis of pregnancy loss (Table, 1) showed the distribution of demographic characteristics of the abortive women. The 40 control were healthy pregnant women with a history of a normal pregnancy. Blood samples were taken from each subject for measuring the phagocytic activity and lymphocyte transformation index, and to proceed ELISA test. At the same time an endo-cervical swab was taken from each woman in this study, the collected swabs were sent directly to the laboratory to conduct the DNA extraction process. The DNA then used in quantitive real-time Polymerase Chain Reaction.

Table, 1: The distribution of demographic characteristics of the abortive women.

Item		Frequency	Percentage
Age groups	≤ 20	13	10.8%
	21-25	34	28.3%
	26-30	42	35.0%
	31-35	23	19.2%
	≥36	8	6.7%
	Total	120	100%
Region	Rural	75	62.5%
	Urban	45	37.5%
	Total	120	100%
Gestational age	1st	60	50%
	2nd	44	36.7%
	3rd	16	13.3%
	Total	120	100%
Number of miscarriages	Single	58	48.3%
	Recurrent	62	51.7%
	Total	120	100%

Nitro blue Tetrazolium: The assay was carried out in relation to a method offered by (4) for all abortive women and control. NBT was used for evaluation of phagocytic activity percentage. The results were read by ELISA reader at 490-650 nm and the phagocytic activity percentage calculated according to the following equation (5):

{(OD test sample – OD control)/OD control}× 100%.

Preparation of lymphocytes: According to (6) the lymphocytes were separated from abortive woman blood. Peripheral blood was collected from all the subjects in heparin tubes and diluted in 1:1 ratio with 1M phosphate buffered saline (PBS). Lymphoprep (density gradient separating medium) was used to separate the lymphocytes, diluted blood was carefully overlaid on lymphoprep solution gradient without allowing the solution to become mixed. After cooled centrifugation (4˚C), the mononuclear cells were visible as thin gray zone in the interface between the plasma and RBC layers. By using a fine Pasteur pipette, the zone containing lymphocytes was taken quickly into a new 15 ml conical tube. Lymphocyte ring was washed thrice by adding RPMI 1640 media to the lymphocyte suspension to obtain a final volume of 10 ml and a cooling centrifuge (4˚C). Cell viability was checked by trypan blue staining and cells were counted using haemocytometer as per standard procedure. Cells were finally suspended in RPMI-1640.

Methyl Thiazolyl Tetrazolium: The procedure of MTT assay (3- (4, 5- dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) was done according the steps of the procedure of (4). The optical density was read by ELISA reader at 490 nm and the lymphocyte transformation percentage was calculated according to the following equation (5):

{(OD test sample – OD control)/OD control}× 100%.

ELISA assay: This performed according to the procedure of *C. trachomatis* IgG ELISA kits, CAL biotech, USA. Depending on the principle that: Diluted patient and control serum is added to wells coated with purified antigen. IgG specific antibody, if present, binds to the antigen. All unbound materials are washed away and the enzyme conjugate is added to bind to the antibody-antigen complex, if present. Excess enzyme conjugate is washed off and substrate is added. The plate is incubated to allow the hydrolysis of the substrate by the enzyme. The intensity of the color generated is proportional to the amount of IgG specific antibody in the test specimen.

DNA extraction was performed according to the manufacture instructions of (QIAamp® DNA Mini kit, Germany), the protocol of DNA purification from the swabs. Ensuring the presence of DNA by gel electrophoresis and freezing the collection tube which containing DNA.

Quantitative Real-time- Polymerase chain reaction (qPCR) primers used in our study are F′5-CCTGTGGGGAATCCTGCTGAA-3′ and R′5-GTCGAAAACAAAGTCACCATAGTA-3′ yielding a 240 bp fragments of the MOMP gene in *C. trachomatis* (7). Quantitive real time polymerase chain reaction was performed according to manufacture instructions of (2x SYPER® Green qPCR Mix kit, Dongsheng Biotech, China) which contains (KCL, MgCl2, dNTPs, hot start Taq DNA polymerase, 1X SYPER® Green and other optimized buffer components). SYBER green dye binds to double-stranded (ds) DNA, thus providing a fluorescent signal that reflects the amount of dsDNA product generated during PCR.

Results and Discussion

The Nitro Blue Tetrazolium test is used for evaluating the phagocytic activity percentage of leukocytes. P. value ≤ 0.05 was considered statistically significant, our study showed a significant increase (P=0.009) in phagocytic activity in abortive women which was (22%) higher than healthy women (Table, 2), depending on the equation which mentioned in materials and methods section. NBT is a basic test to assess neutrophilic capacities in bacterial and non-bacterial irresistible sicknesses. The extent of a total number of NBT test positive neutrophils serves as a measure of the sufficiency of the host reaction to infection (8). Increasing the intercellular reduction of NBT dye to formazen (deep blue compound) by the neutrophils, confirming the intracellular killing property of phagocytosing neutrophils (9).

Researchers (10) augmented oxidative stress in infertile women with persistent chlamydial infection, it found that parameters of oxidative stress, superoxide anion and index of oxidative stress, were significantly elevated in infertile patients with persistent chlamydial infection compared to seropositive and seronegative patients. His findings point to the possible impact of *C. trachomatis* infection on prooxidative-antioxidative balance that can influence fertility potential in women with persistent chlamydial infection (10). The MTT assay is used for evaluating the lymphocyte transformation index % of leukocytes in blood. Our results showed a significant increase (P=0.001) in lymphocyte transformation index in the lymphocytes of abortive women which was (27%) higher than healthy women (Table, 3), depending on the equation which mentioned in materials and methods section.

Table, 2: Nitro Blue Tetrazolium (NBT) index results of abortive women.

Groups	Mean ±SD	Phagocytic activity percentage of abortive women	P. value
Control group	1.12±0.10	22%	0.009
Patients group	1.37±0.29		

Table, 3: Lymphocyte transformation index for abortive women

Group	Mean ±SD	Lymphocyte transformation index of abortive women	P. Value
Control group	0.077±0.008	27%	0.001
Study group	0.098±0.018		

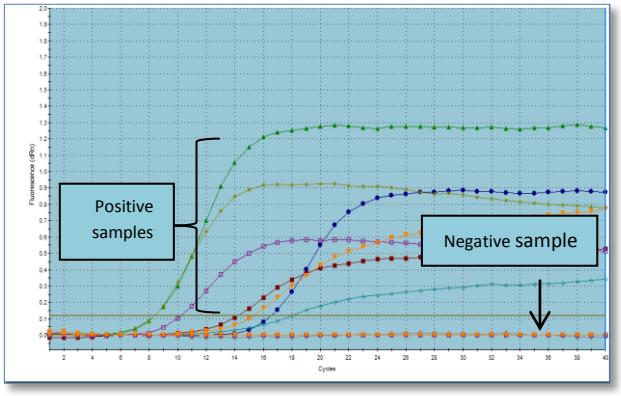
Determination by ELISA showerd that *Chlamydia trachomatis* IgG was found in (14.2%) of abortive women, and showed no significant differences between the age groups. This indicates that *C. trachomatis* may infect all ages, but the age group 21-25 years (Table, 4) was the highest infected with *C. trachomatis* similar to (11), infection rates are highest among younger women, partly because their immature cervical cells are more vulnerable to infection, but older age wasn't protected. For sexually active women who are not pregnant, screening test is recommended in those under 25 and others at risk of infection. Risk factors include a history of chlamydial or other sexually transmitted infection, new or multiple sexual partners, and inconsistent condom use.

Guidelines recommend that all women who attend for emergency contraceptive be offered Chlamydia testing, with studies showing up to 9% of women aged <25 years had Chlamydia (12).

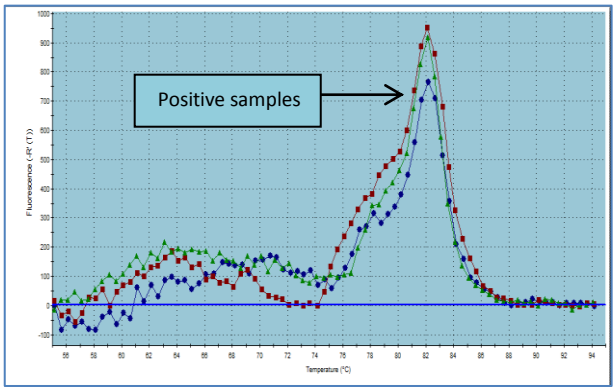
Table, 4: The ratio of *Chlamydia trachomatis* IgG according to age groups.

Age groups	C.trachomatis IgG		Total
	+	-	
≤ 20	1	12	13
21-25	6	28	34
26-30	4	38	42
31-35	5	18	23
>36	1	7	8
Total	17	103	120
P. value	0.621		

Molecular detection: The ratio of positive results in qPCR were (17.5%) (Fig. 1 and 2), higher than in ELISA that may return to the intracellular nature of these pathogens. Thus, humoral immunity is not so important like cellular immunity in protection and elimination of these microbes. The prozone phenomena may be another cause, as the titer of Abs is higher than Ags so the Ab-Ag binding will not occur, or due to the presence of incomplete IgG. Also the low titer of Abs in Immunocompromised mothers may show a false negative result. The cases without antibodies indicate that the women may test early in the course of the disease before their body had a chance to produce antibodies. If such women acquire primary infection during gestation they are at risk of transmitting the infection to their fetuses, so they will need to be tested in 2-3 weeks (13).



Figure, 1: Real-Time PCR Amplification Plots for MOMP Gene in *C. trachomatis* that display a positive and negative DNA samples by using syber green 1 RT-PCR amplification.



Figure, 2: Dissociation curve for MOMP gene in *C. trachomatis*, that display a positive DNA samples.

This study showed highly significant differences between single and recurrent miscarriages in abortive women with anti-*C. trachomatis* IgG (P =0.000) (Table, 5). The highly presence of *C. trachomatis* IgG in recurrent miscarriages in agreement with (14), who mention that there is an association between *C.trchomatis* and abortion recurrence.

Miscarriage could be induced by asymptomatic *C. trachomatis* infection spreading to the fetal tissue or endometrium. Few miscarriages occur during *C. trachomatis* primary infection, and this explains the absence of association with IgA. Several patients exhibited *C. trachomatis*–positive serologic results without *C. trachomatis* DNA suggests that miscarriage might also occasionally be induced by damage from a past chlamydial infection or persistent *C. trachomatis* antibodies that might interfere with embryonic antigens (15). The study (16) inspected 120 ladies with a specific end goal to evaluate the recurrence of *C. trachomatis* diseases in patients with a past filled with premature delivery. In a gathering of ladies with one unconstrained premature delivery, higher IgG particular antibodies were identified in serum by ELISA in 21.1% of the women, in women with at least two unconstrained unsuccessful labor 36.4%.

Table, 5: The Association between IgG and the number of miscarriages

Number of miscarriages	*C. trachomatis* IgG		Total
	+	-	
Single	1	57	58
Recurrent	16	46	62
Total	17	103	120
P.value	0.000		

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دراسة مناعية وجزيئية للتحري عن *Chlamydia trachomatis* كعامل مسبب للإجهاض في محافظة المثنى

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الخلاصة

ان الإصابة الأولية للأم بداء الكلاميديات أثناء الحمل يعد عامل خطر لنتائج الحمل السلبية. أختير 120 امرأة مجهضة اجهاض مفرد أو متكرر بعد احالتهن للمختبر بتقرير طبي للتحري عن نوع المسبب لتحديد التشخيص النهائي لفقدان الحمل، وأختير 40 عينة من الحوامل الطبيعية مجاميع سيطرة. وقد تبين من خلال التجارب ان المناعة الطبيعية في النساء المجهضات أعلى من المناعة الطبيعية للنساء الحوامل، والتي قيست بإختبار قياس الفعالية البلعمية، حيث أظهر التحليل الإحصائي فرق معنوي (0.009) في معدل البلعمة لكريات الدم البيضاء للنساء المجهضات بنسبة أعلى من الحوامل الطبيعية بمقدار (22%). وقيست الإستجابة المناعية الخلوية باختبار تحول الخلايا اللمفاوية، وقد أظهرت النتائج زيادة معنوية (0.001) في التحول اللمفاوي للخلايا اللمفاوية في دم النساء المجهضات بنسبة أعلى من الحوامل الطبيعية بمقدار (27%). وقد استعملت الطرق المصلية والجزيئية للتحري عن *Chlamydia trchomatis*، حيث اجري اختبا الألايزا غير المباشر للجسم المضاد IgG وسجلت نتائج الاختبار نسبة (14.2%) حالات موجبة. كما أجري تفاعل البلمرة المتسلسل الكمي، وقد سجلت النتائج الموجبة بواقع (17.5%). أظهرت الدراسة وجود علاقة وطيدة بين الإصابة بداء الكلاميديات وحدوث الاجهاض، وكانت الفئة العمرية (20-25) سنة هي الأكثر إصابة بداء الكلاميديات، وأن داء الكلاميديات كان أكثر حدوثاً في حالات الاجهاض المتكرر.

الكلمات المفتاحية: داء الكلاميديات، الإجهاض، الإجهاض المتكرر، تفاعل البلمرة المتسلسل الكمي.