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Molecular detection of *Chlamydia trachomatis* infection among males with abnormal semen

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Abstract

Many studies revealed that *Chlamydia trachomatis* is the most prevalent bacteria that cause sexually transmitted infections in the world. Most of these infections are asymptomatic and there is a remarkable relationship between CT infections and male infertility. Therefore, the present study is established to determine the effect of *Chlamydia trachomatis* infection on seminal fluid quality in males suffering from infertility compared with fertile males. Semen samples were collected from 63 infertile male and 13 fertile male as control group, attending the clinical laboratory for routine seminal fluid analysis. Seminal fluid was analyzed according to World Health Organization guidelines, the whole genomic DNA was extracted for molecular study. Real time PCR technique was used to specifically detect the presence of CT DNA in semen samples.

C. trachomatis was demonstrated in the semen samples of 11 (17.4%) infertile male and all control samples were negative. Infertile, infected males had semen samples that showed statistically significant differences in the mean of total sperm count, motility and morphology as compared with fertile uninfected control samples. These differences indicate that chlamydial infection of genital tract could negatively influence the quality of seminal fluid.

Keywords: male infertility, semen analysis, *chlamydia trachomatis*, genital pathogens.

التحري الجزيئي عن الأصابة ببكتريا المتدثرة الحثرية *Chlamydia trachomatis* في المني غير الطبيعي عند الذكور

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الخلاصة

اشارت العديد من الدراسات أن بكتريا *Chlamydia trachomatis* هي أكثر الانواع انتشارًا في العالم التي تسبب العدوى المنقولة جنسيا، ومعظم هذه العدوى لا تسبب أعراض ولوحظ ان هناك علاقة بين هذه العدوى والعقم عند الذكور ، ولذلك جائت هذه الدراسة لتحديد تأثيرعدوى المتدثرة الحثرية على نوعية السائل المنوى لدى الذكور الذين يعانون من العقم مقارنة بالذكور الخصيبين ، جمعت عينات السائل المنوي من 63 ذكر عقيم و 13 من الذكور الخصبة كمجموعة سيطرة، يراجعون المختبر السريري لتحليل السوائل المنوية الروتينية ، تم تحليل السائل المنوي وفقا ل المبادئ التوجيهية لمنظمة الصحة العالمية ، استخلص الحمض

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النووي الدنا Genomic DNA للدراسة الجزيئية ، استخدمت تقنية تفاعل البلمرة المتسلسل اللحظي Real (time PCR) للكشف بصورة محددة عن وجود الحمض النووي للبكتريا في عينات السائل المنوي.
اظهرت النتائج تواجد بكتريا *Chlamydia trachomatis* في(11)(17.4٪)عينة من السائل المنوي للذكور المصابين بالعقم وكانت جميع نتائج مجموعة التحكم سالبة.اظهرت عينات السائل المنوي للذكور العقيمين المصابين فروق ذات دلالة إحصائية في المتوسط الإجمالي لعدد الحيوانات المنوية والحركة والشكل مقارنة مع عينات التحكم غير المصابة. يشير هذا الاختلاف إلى أن هذه العدوى في الجهاز التناسلي قد تؤثر سلبًا على جودة السائل المنوي.

Introduction

Infertility is the failure of a couple on their reproductive age to conceive a child after one year of unprotected intercourse, it is considered as a public health concern according to the World Health Organization (WHO) , since it approximately influences between (8-12%) of couples around the world and it is rated to affect as many as 70 million couples[1]. Infertility reasons are equally divided between male and female factors, both accounting for 40% of infertility incidence, and 20% is considered to be combined or unexplained factor[2] .

Subfertility is defined as the reduced fertility that is treatable, it also describes couples who take a long time to conceive, male subfertility is widespread, and it causes a great worry to the couples [3], male infertility is considered as a multifactorial disease with an incidence rate of 50% of infertile couples [4] .

Many males suffer from pathological problems that is the primary cause of infertility, but unfortunately, they are not properly diagnosed and treated, so that the female partner would be in charge of infertility problem, therefore it is crucial to understand the causes of male infertility to be diagnosed and treated properly[5] , many sides of male infertility are poorly understood and the medical therapy for these males is unsuccessful, about 90% of male factor infertility is due to, either low sperm number in semen or to the production of poor quality sperms (reduced progressive motility, morphological defects) [6].

98*-/Infections of the reproductive tract by viruses, bacteria, fungi and parasitic microorganisms which are transmitted through sexual activity and cause sexually transmitted diseases (STDs), could be a major concern since many infections are asymptomatic or show few symptoms and cause about 15% of male infertility, it affects male by changing semen parameters such as reduced sperm count and motility , also causes obstruction of reproductive system, epididymitis and testicular damage [7,8]،genital tract infections are an important factor of infertility for both males and female [1,9]

Among many etiological factors that affect male infertility, infections of the genital tract are constitute an important, hidden and disruptive factor, and it causes approximately about 8-35% of male infertility[10]. Different types of microorganisms have been associated with male infertility. The degree of association depends on the type of the infection caused. Each microorganism has a specific mechanism to affect infertility, either directly by reducing sperms motility, or indirectly by causing an obstruction in the seminal tract [11,12]

Chlamydia trachomatis, other sexually transmitted bacteria and many viruses such as Herpes simplex type1, type2). Human Papillomavirus have been detected in semen of asymptomatic males. These pathogens cause acute and chronic infections, the resultant inflammations in the male reproductive system, cause a damage in the function of spermatozoa that lead to a reduced sperm quality, decreased sperm count and motility [11,13] . *Chlamydia trachomatis* is the most common etiological agent of sexually transmitted diseases after Herpes simplex Virus and Human Papillomavirus [14].

The aim of this study is to investigate the effect of *Chlamydia trachomatis* infection on semen quality, as a risk factor for male infertility using specific kits for real time PCR to detect the presence of chlamydial DNA in the semen of infertile males.

Materials and methods

Subjects

The samples required in this study were seminal fluid from males suffering low fertility and difficulty to conceive, attending clinical laboratories for routine seminal fluid analysis.

Patient samples have been selected according to their questioner information's and their seminal fluid analysis. The patient group consists of males who have no children and a problem in conceiving with a poor seminal fluid quality. The value of sperm count, motility and morphology were below the lower reference limits determined by WHO standard manual of 2010. Their age ranged from (20) to (52) years old, while males who have one child at least and a normal seminal fluid analysis were considered as a control group and their age ranged from (21) to (45) years old.

Semen collection and analysis

The seminal fluid was collected after (3-4) days of sexual abstinence by masturbation into a sterilized specific container in the laboratory from all patients and control males.

Sperm count, motility, and morphology were assessed according to the World Health Organization standard manual,[15]the semen samples were allowed to liquefy for 30 minutes at (37℃) before analysis, after seminal fluid analysis, an amount of the fluid mixed in 1.5ml Eppendorf tubes with a mucolytic transport media to be transported to the lab and preserved in freezer (-20℃) until the time of DNA extraction.

DNA extraction

DNA-sorb-AM nucleic acid extraction kit (AmpliSens, Russia) was used in this study to extract whole genomic DNA from seminal fluid, this kit designed to extract and purify DNA manually from clinical materials such as (seminal fluid, urine, discharges of urogenital tract, throat, etc.), following the manufacturer's instructions, then DNA concentration and purity was determined using Nanodrop (Nas-99, China), gel electrophoresis also was used to check DNA integrity, and the extracted DNA was tested for human beta globin gene as a reference gene, to check that there were no PCR inhibitors in the samples, human β-globin gene primers were designed for this study and the primers sequence is listed in Table-1, cycling conditions of the amplification reaction of β-globin gene are listed in Table - 2.

Table 1-Sequences of primers used to amplify Beta-globin gene

Gene name	Primer sequence	Fragment size
Hemoglobin subunit beta(*HBB*) Chr.11:5226940-5227097	**F:**AGTCAGGGCAGAGCCATCTA	158bp
	R:CCTCACCACCAACTTCATCC	

Table 2-Cycling conditions for PCR amplification of β-globin gene

Steps	Temperature	Time	No. of cycles
Denaturation 1	95℃	15 min.	1
Denaturation 2	95℃	15 sec.	40
Annealing	58℃	25 sec.	
Extension	72℃	20 sec.	
Final extension	72℃	5 min.	1

Detection of *C. trachomatis*

AmpliSens® PCR kit (AmpliSens, Russia) is used for in vitro nucleic acid amplification test to detect the presence of *Chlamydia trachomatis* DNA in any clinical material, using real time hybridization fluorescence detection of amplified products. The principle of the detection is based on the amplification of the selected pathogens genome specific regions using specific primers, and the amplified product is detected by fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermos-cycling (AmpliSens, Russia). All steps for PCR reaction preparation were performed according to the manufacturer's instructions. The amplification program is listed in Table-3.

Table 3-Amplification program

Step	Temperature (℃)	Time	Cycles
Hold	95	15 min.	1
Cycling 1	95	5 sec.	5
	60	20 sec.	
	72	15 sec.	
Cycling 2	95	5 sec.	40
	60	20 sec.	
	72	15 sec.	

Statistical Analysis

The Statistical Analysis System- SAS (2012) [16] program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage. Least significant difference –LSD test (ANOVA) was used to significant compare between means in this study.

Results and Discussion

Seminal fluid analysis

Seminal fluid samples were collected after 3-4 days of sexual abstinence by masturbation and examined according to WHO laboratory manual for the examination and processing of human semen. The samples with low sperm concentration (≤ 15 million/ml). Sperm progressive motility (≤ 32%) and abnormal sperm morphology (≤ 30%) were considered as a patients group, males with normal sperm concentration, motility and morphology, having one child at least were considered as a control group, semen parameters for all patients. The control group are listed in Table-4, the age mean ± standard deviation of the patients group was (32.2 ±6.8) years and for control group was (34.0 ±6.5) years.

Table 4-Patients and control in variables of seminal parameters

Variable	Mean ± SD		T-Test
	Patients (n=63)	Control (n=13)	
Age (years)	32.28 ± 6.88	34.07 ± 6.52	4.144 NS
Sperm count (million/ml)	9.42 ± 8.70	38.46 ± 9.43	5.356 **
Total motility (%)	22.62 ± 15.52	72.69 ±14.08	9.287 **
PR motility (%)	2.89 ± 5.45	52.3 ±13.63	4.506 **
Morphology (%)	27.06 ± 16.50	56.1 ±17.57	10.125 **
** (P<0.01).			

NS: Not statistically significant
PR motility: Progressive motility

DNA extraction

Genomic DNA extraction was achieved by using DNA-sorb-AM nucleic acid extraction kit to all seminal fluid samples (patients and control). DNA concentration and purity were determined by using Nanodrop (NAS-99, China). All samples showed a concentration range of (50-250) ng/µl and a purity range from (1.5-2.00). DNA integrity was detected by agarose gel electrophoresis subsequent the Nanodrop estimation. DNA with good integrity must be shown as a single clear band with no smears when visualized under UV light after ethidium bromide staining as showed in the Figure-1.

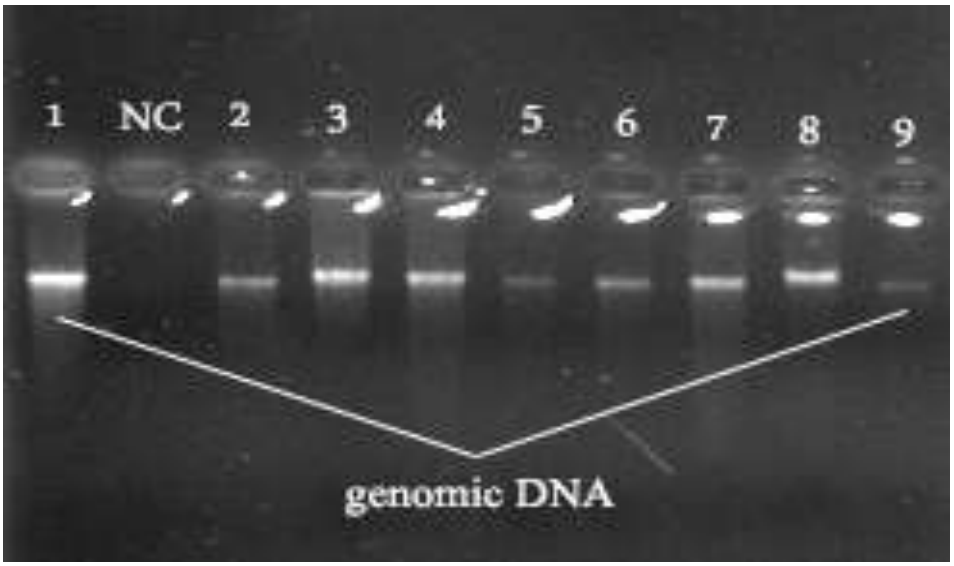

Figure 1-Electrophoresis of the extracted genomic DNA from the samples of seminal fluid on 1% of agarose for an hour at 90 voltages followed by ethidium bromide staining for 20 minutes and UV light visualization with the aid of gel documentation system.

Line (1-9): DNA bands
NC: Negative control for extraction.

Molecular detection of Beta globin gene by PCR

Conventional PCR technique was used to amplify the desired β-globin gene region for all samples as internal control for DNA extraction. It was an important step to check DNA integrity, and only positive samples for β-globin gene detection were involved in the following step of this study, after PCR amplification. Gel electrophoresis was performed to detect the presence of the amplified region in a band of molecular size 158bp as shown in Figure-2.

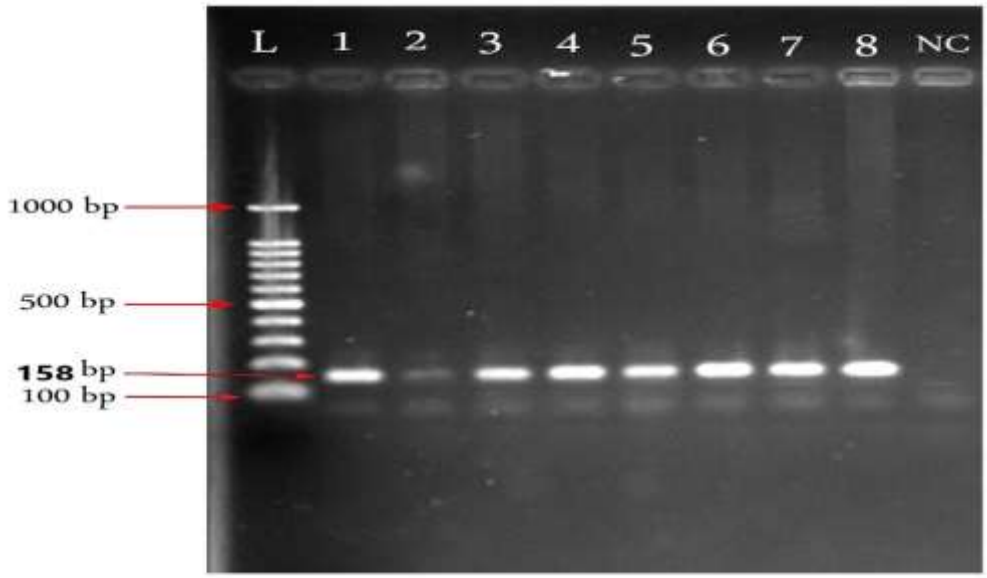

Figure 2-β-globin gene PCR product in different DNA samples.

L: DNA Ladder (100-1000bp).
Line (1-8): 158bp bands of β-globin gene amplified region.
NC: Negative control for amplification.

Real time PCR for the qualitative detection of *Chlamydia trachomatis*

Amplisens® PCR kit used in this study to detect the presence of chlamydial DNA in semen samples of both patients and control group.

Analysis of the results was performed by the software of real time PCR instrument by measuring the fluorescent signals accumulated in channels. The signal of *C. trachomatis* DNA amplification product was detected in channel of the JOE fluorophore and the signal of internal control (IC). DNA amplification product was detected in channel of the Cy5 fluorophore. The amplification was recorded as a Ct value (cycle threshold), as shown in Figures -(3, 4). The results of RT-PCR detection revealed that out of the 63 samples detected, 11 (17.4%) samples showed to be infected with *C. trachomatis*, Table-5 shows the difference of semen parameters values between infected, uninfected and control groups, males positive for the infections showed lower values as compared with others. These results are compatible with the findings of [7,8,17], which confirm the negative influence of C. trachomatis infection on semen parameters.

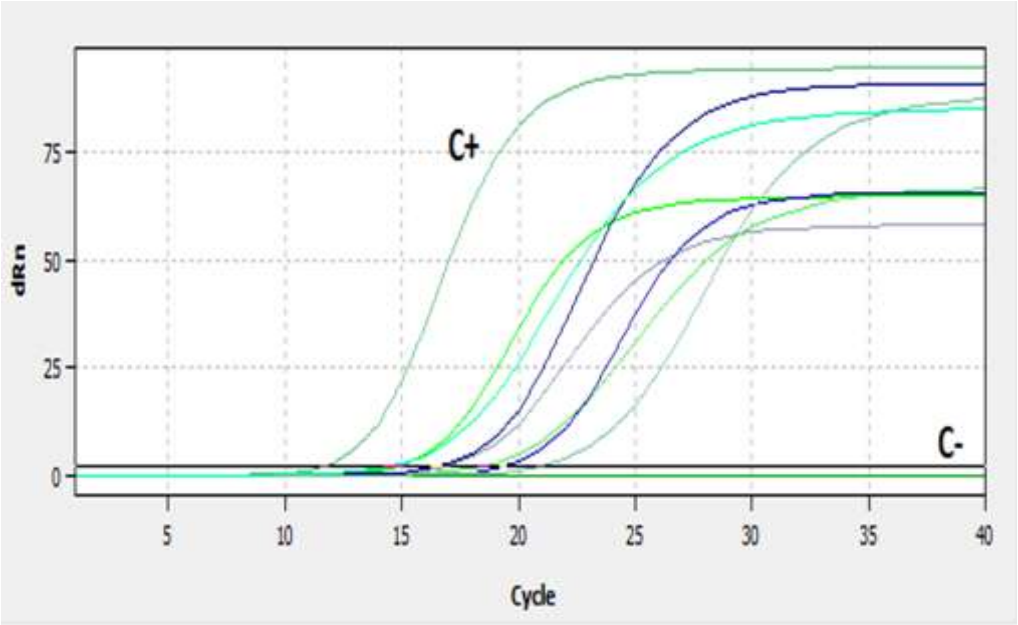

Figure 3-the channel for JOE fluorophore that detects *C. trachomatis* DNA amplification.

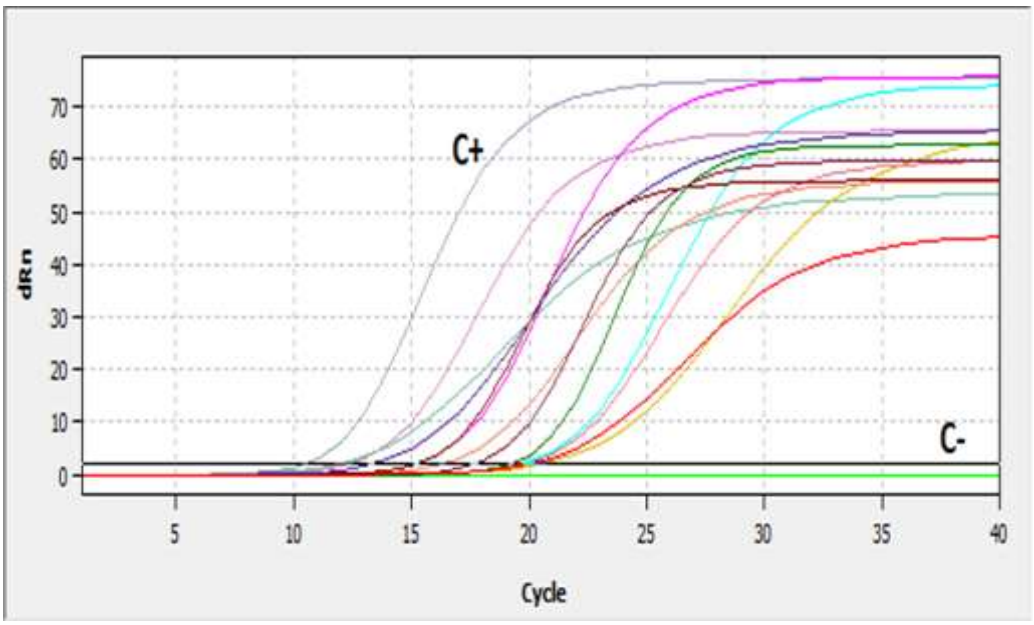

Figure 4-the channel for Cy5 fluorophore that detects IC DNA amplification.

Conclusion

In conclusion, using Real time PCR for the detection of *C. trachomatis* provides a sensitive, quick and accurate technique to detect the presence of these bacteria in the semen of asymptomatic males, which is useful for more future studied to detect other types of infections. The results of the present study demonstrate that *C. trachomatis* infection seems to be spread in infertile males and could lead to a decrease in sperm concentration, motility and morphology,

Therefore, this result supports the possibility of *C. trachomatis* playing a crucial role in the etiology of male infertility.

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