ISSN 2515-8260 Volume 08, Issue 03, 2021

Molecular Analysis of *Chlamydia trachomatis* in Infertile Women in Basrah

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ABSTRACT-

Background Chlamydia trachomatis is a gram negative bacteria, which involve in sexually transmitted disease. This study was aimed to detect C. trachomatis by molecular methods and to evaluate Chlamydial infections in women suffering from primary and secondary infertility with special emphasis.

Method 200 endocervical cytobrush were obtained from 200 infertile women having primary and secondary infertility. The work has been carried out at Basra Maternity

and Child Hospital for molecular analysis of C. trachomatis . The primer CT1& CT4 w used to investigate the 144bp of MOMP of C. trachomatis in the endocervical brush samples.

Results Out of 200 infertile women 96(48%) were positive for C. trachomatis by PCR.

Conclusion The percentage of C.trachomatis in primary infertile women with blocked tubes was higher than women with patent tubes and also higher than C. trachomatis in secondary infertile women with both blocked and patent tubes.

. Key words: Chlamydia trachomatis, Infertile, women, PCR.

ISSN 2515-8260 Volume 08, Issue 03, 2021

Introduction

Chlamydia is a gram negative, non motile obligate intracellular pathogen [1.] *Chlamydia trachomatis* is the common treatable cause of bacterial sexually transmitted infections in both men and women [2] . *C. trachomatis* has 15 serovars characterized by monoclonal antibodies and polyvalent antisera. Serovars A, B, Ba and C causes trachoma, serovars D-K lead to urogenital problems, while serovars L1,L2 and L3 cause lymphogranuloma venereum [2]**.** *Chlamydia trachomatis* infections are asymptomatic and can be present for many years [3.] Complications in females occur such as urethritis, cervicitis, endometritis and pelvic inflammatory disease (PID) [4]**.** *Chlamydia trachomatis* is an important causative agent of pelvic inflammatory disease and its complications cause ectopic pregnancy and tubal factor infertility. Infertility rates range from 12.8% after first episode to 75% after three or four episodes of Chlamydial infections. Infertility by *Chlamydia trachomatis* is common and this is due to asymptomatic infections ,a persistant carrier state ,reactivation of latency and difficulty in eradicating Chlamydial infections [5.]

Tubal defect is an important reason of infertility, and is now discovered that *C. trachomatis* infection is the most reason for tubal peritoneal damage [6] Infertility by *Chlamydia trachomatis* is common and this is due to asymptomatic infections, a persistent carrier state, reactivation of latency and difficulty in eradicating Chlamydial infections [5.] If left uncured, up to 40% of infected women will suffer from pelvic inflammatory disease and 20% of these ladies will develop infertility [7.]

Nucleic Acid Amplification Test (NAAT) this test is the more specific and sensitive than culture and serological methods they are widely used now for the diagnosis of *Chlamydia trachomatis* [8], because they don't depend on viable Chlamydiae and due to the amplification process. This test can be applied in first – void urine and vaginal swabs with almost the same specifity and sensitivity to cervical and uretheral samples .NAATs amplify either the the target nucleic acid ,DNA or ribosomal RNA (r RNA) or the probe after it has annealed to the target nucleic acid [4.] The most common method that gives a positive result after 5-7 days of *Chlamydia trachomatis* transmission is polymerase chain reaction(PCR) [9.]NAAT are now the common assays used for the diagnosis of *Chlamydia trachomatis* ,its specifity is about 100% but its sensitivity is 90-96 % depending on specimen and amplification reaction [10]**.**

Materials and Methods:

The study population is consisted of 200 infertile women who were attending the infertility center at their reproductive age with primary and secondary infertility. The Ethical Committee of the College of Medicine, University of Basrah, Iraq has ethically proved this work. 200 Endocervical swabs were collected from the patients for DNA extraction and PCR. During the gynecological examination that was done by a gynecologist , a sterile speculum was inserted into the vagina [11.] Before obtaining

ISSN 2515-8260 Volume 08, Issue 03, 2021

a specimen for *Chlamydia trachomatis* test, a sterile guaze was used to clean the vagin. Cervical cytobrush was inserted 1-2 cm into the endocervical canal (after the squamocolomnur junction) and rotated the swab was rotated for 20-30 seconds and then kept in a suitable medium [12] . The samples were kept on ice pack till reaching the laboratory where immediate processing was held according to the manufacture instruction . DNA extraction from the samples was done by using DNA –Sorb A extraction kit by applying the manufacture instruction. Lysis solution and washing solution were warmed up to 65 °C. (1.5) ml Eppendrof tubes were prepared and labeled ,(300)µl lysis solution was added to each tube and 100µl of each sample was added to the appropriate tube,the tubes were incubated at 65 °C for 5 minutes after their vortexing all the tubes were centrifuged for 5 minutes at a full speed (1400 rpm) then the supernatant was transferred into new tubes (20)µl of the sorbent was added to each tube after it has been vortexed vigorously .The tubes were then incubated at room temperature for 3 minutes after being vortexed , and this step was repeated twice all the tubes were centrifuged at 5000 rpm for 30 second, and the supernatant of each tube was carefully removed and discarded (500) µl of washing solution was added to each tube followed by vortexing vigorously and centrifugation for 30 second at 1000 rpm the supernatant of each tube was removed carefully and discarded .Step 10 was repeated then all tubes with open caps were incubated with open caps for 5-10 minutes at 65 °C, the pellet of each tube was resuspended in 100µl of DNA-eluent then incubated for 5 minutes at 65°C with periodic vortexing .Finally the tubes were centrifuged for 1 minute at 1200 rpm and the supernatant that contain the DNA was transferred into new tubes and stored at -20°C the newly extracted DNA was visualized using agarose gel electrophoresis [13] . Agarose gel was prepared by adding 25 ml of 0.5X TBE buffer to 0.2 gm of agarose in a beaker if the mini tray was used (or 100 ml of TBE buffer with 0.8 gm agarose if the maxi tray was used). The solution was heated on the hot plate until all the gel particles are dissolved and the solution is clear ,the dissolved agarose are left to cool down and a drop of red stain was added .

The comb was placed at one end of the tray and the agarose was poured into the casting tray, agarose was allowed to become a gel at room temperature for about 20 -30 minutes .The gel must be 3mm to 5mm thick and must not contain bubbles, then the comb was gently removed ,lifted out of the gel.The tray was filled with TBE buffer until covered all the gel surface ,it is important to use the same buffer in both the gel and the electrophoresis tank . DNA samples were subjected for loading and running in agarose gel, 8µl of DNA samples was mixed with 3µl of b romophenol blue, the mixing was dispensed carefully into the wells of agarose gel, the entire gel was subjected to equal electric current ,the cathode was connected to the wells side of the tray and the anode to the other side . The gel was run at 60 V until the bromophenol blue dye migrate to the end of the gel and DNA bands was detected and examined under UV transilluminator .

ISSN 2515-8260 Volume 08, Issue 03, 2021

Molecular analysis of *Chlamydia trachomatis* was done by performing polymerase chain reaction (PCR) .The primers sequence were supplied by Biolabs (England). The primers for the present study was designed by [14] . Molecular analysis of *C.trachomatis* among patients attending Khartoum Teaching Hospital .

The primer sequence was taken and manufactured by Biolabs (England) .

Primer	Primer sequence	Length(bp)
CT1	(CCT/GTG/GGG/AAT GCT/GCT/GAA	144bp
CT4	(GTC/GAA/AAC/AAA/GTCATCCAGTA/GTA	144bp

The primers was dilluted in DNase free water for the reverse primer we used 729µl ,and for the forward primer 865µl was used as mentioned in the sheet .

The reaction set up for PCR

Compenent	25 µl reaction	50 µl reaction	Final concentration
One Taq Quick – Load 2X Master Mix with Standared Buffer	12.5 µl	25 µl	1X
10 µM Forward primer	0.5 µl	1µl	0.2µM
10 µMReverse primer	0.5 µM	1µl	0.2 µM
Template DNA	Variable	Variable	<1,000 ng
Nuclease –free water	To 25 µl	To 50 µl	

Thermocycling conditions for amplification

STEP	TEMP	TIME
Initial denaturation	94C°	30 sec.
30 cycles	94C° 57C° 68C°	15-30 sec. 15-60 sec. 1 min/kb
Final Extension	68 C°	5 min
Hold	4-10 C°	

For the analysis of the result the aliquots were subjected to electrophoresis in 2 % agarose gel with the DNA ladder to determine the size of the amplified gene .

ISSN 2515-8260 Volume 08, Issue 03, 2021

Results

According to **Table 1** the age groups that were enrolled in the population study were (≤ 20, 21-30, 31-40 and >40). The percentage of primary infertile women was 82% and secondary infertile women were 18%. The percentage of infertile women with blocked tubes was 28.5% and with patent tubes was 71.5%.

Table 1 : General characteristics of the data contributed to this study

Age	Infertility type*				Tubal blockage**				Total
	Primary		Secondary		Yes		No		
	N	(%)	N	(%)	N	(%)	N	(%)	
≤20	9	(100)	0	(0)	0	0	9	(100)	9
21-30	81	(86.2)	13	(13.8)	18	19.1	76	(80.9)	94
31-40	50	(78.1)	14	(21.9)	24	37.5	40	(62.5)	64
>40	24	(72.7)	9	(27.3)	15	45.5	18	(54.5)	33
Total	164	(82)	36	(18)	57	(28.5)	143	(71.5)	200

X^{2}*=0.130, p>0.05 X^{2}=0.002, p<0.05**

. Out of 200 patients 164(82%) had primary infertility while 36(18%) revealed with secondary infertility. This table shows that 67% of women with primary infertility had ovarian defect, 29.3% with tubal blockage and 3.7% appeared with unexplained infertility. Secondary infertile women revealed also ovarian dysfunction, tubal blockage and unexplained infertility in percentage (72.3%, 25% and 2.7%), respectively **(Table 2)**.

Table 2 Frequency of types of infertility according to etiological factors

Disease	Primary infertility		Secondary infertility		Total
	N	(%)	N	(%)	
Ovarian defect	110	67%	26	72.3%	136
Tubal blockage	48	29.3%	9	25%	57
Unexplained	6	3.7%	1	2.7%	7
Total	164	(82)	36	(18)	200

ISSN 2515-8260 Volume 08, Issue 03, 2021

P>0.05

Out of 200 infertile women that were enrolled in this work to identify *C. trachomatis*, 96(48%) were positive for *C. trachomatis* by PCR.

The primers (CT1&CT4) were used to identify *C. trachomatis*. Both CT1&CT4 (forward and reverse) encode major protein of outer membrane (MOMP) of *C. trachomatisomp1*gene **(Figure 1).**

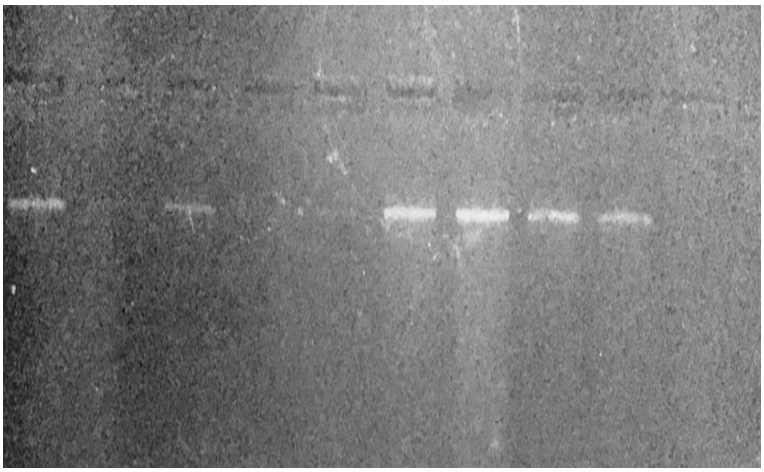

Figure 1 *C. trachomatis* total DNA.

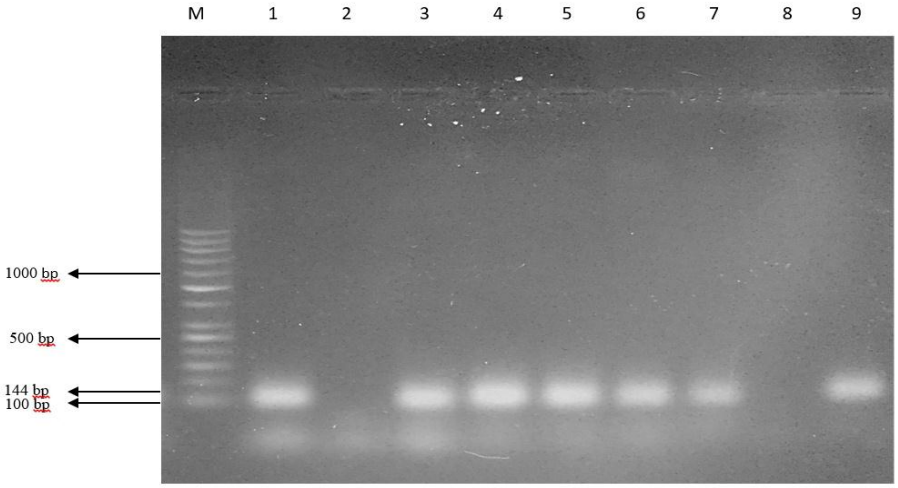

Figure 2 PCR amplification of MOMP primers (CT1&CT4) of *C.trachomatis* from endocervical specimens L: DNA Ladder (10000bp), Lane 1,3,4,5,6,7,9 positive specimens for *C.trachomatis* and Lane 2,8 negative specimens.

ISSN 2515-8260 Volume 08, Issue 03, 2021

Out of 200 enrolled women 96(48%) had *C. trachomatis* gene detected by PCR while 104 (52%) were negative. (**Table 3).** The results show no statistical differences.

Table -3 Detection of *C. trachomatis* by PCR assay

Infertility types	*C.trachomatis*				Total
	Positive		Negative		
	N	(%)	N	(%)	
Primary	**82**	**(50)**	**82**	**(50)**	**164**
Secondary	**14**	**(38.9)**	**22**	**(61.1)**	**36**
Total	**96**	**(48)**	**104**	**(52)**	**200**

X^2=0.227, p>0.05

The results showed that *C. trachomatis* was detected in all age groups **(Table .4)** without any significant differences between age groups (P>0.05).

Table -4 Detection of *C. trachomatis* among infertile women according to age groups

Age groups	*C. trachomatis*				Total
	Positive		**Negative**		
	N	**(%)**	**N**	**(%)**	
≤ 20	**4**	**(44.4)**	**5**	**(55.6)**	**9**
21-30	**44**	**(46.8)**	**50**	**(53.2)**	**94**
31-40	**31**	**(48.4)**	**33**	**(51.6)**	**64**
>40	**17**	**(51.5)**	**16**	**(48.5)**	**33**
Total	**96**	**(48)**	**104**	**(52)**	**200**

X^2=0.966,p>0.05

ISSN 2515-8260 Volume 08, Issue 03, 2021

The percentage of *C. trachomatis* in primary infertile ladies with blocked tubes **(Table 5)** was (56.2%) and in secondary infertile women with blocked tubes was (66.6%) without any significant differences between primary and secondary infertility and blocked tubes ($p>0.05$). It also shows the percentage of primary infertile ladies with patent tubes was (47.4%) and in secondary infertile ladies with patent tubes was (29.6%), with significant differences between type of infertility and patent tubes ($p<0.05$).

Table -5 Detection of *C. trachomatis* by PCR among infertile women according to type of infertility and tubal blockage

Tubal blockage	Type of infertility	*Chlamydia trachomatis*				Total
		Positive		Negative		
		N	(%)	N	(%)	
Blocked*	Primary	27	(56.2)	21	(43.8)	48
	Secondary	6	(66.6)	3	(33.3)	9
Patent**	Primary	55	(47.4)	61	(52.6)	116
	Secondary	8	(29.6)	19	(70.4)	27
Total		96	(48)	104	(52)	200

$X^2=0.561$, $p>0.05$*

$X^2=0.094$, $p<0.05$**

Discussion

The present work has proved the use of molecular and serological techniques in the diagnosis of *C. trachomatis* in infertile women. The prevalence of current and past infections using the sensitive and specific techniques would give a better understanding about the situation of the disease in any community. It is thus important to determine its prevalence in infertile women with different clinical conditions. Best to the knowledge this is the first study using PCR assay for the detection of *C.trachomatis* in both endocervical brush and plasma in Basrah. In this study the primer pairs identified the 144bp sequence of *omp1*gene (Figure 2) of *C. trachomatis* infection present in the patient endocervical swab sample. The PCR has been reported as a major method for detecting the principle outer protein *omp* gene from the 15 *C.trachomatis* serovars [2]**.** In present study the highest percentage of *C. trachomatis* infection was at the age group 21-30 years (45.8%) followed by other age groups.

ISSN 2515-8260 Volume 08, Issue 03, 2021

This result is in agreement with that [15, 16] reported that younger age group are associated with high rates of *C.trachomatis*.

The high percent of *C.trachomatis* is detected in young age group is because young groups are at the reproductive age and are more sexually active than elders which have the role to evaluate the chance of spread of bacteria. This infection is asymptomatic, so women only came to the clinics when symptoms of the lower genital tract infection appears, pregnancy loss and had infertility. Also in recent study ladies with primary and secondary infertility were contributed. The highest percentage of *C.trachomatis* was found in primary infertile ladies. These results agree with that of Sambrook and Russell [12].

In conclusion the percentage of *C.trachomatis* in primary infertile women with blocked tubes was higher than women with patent tubes and also higher than *C.trachomatis* in secondary infertile ladies with both blocked as well as patent tubes. There is no conflicting between authors as far as this work is concerned.

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