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Research Article

Molecular determination of *Chlamydia trachomatis* of infertile woman by 16SrRNA in Mosul City

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Abstract

Chlamydia trachomatis (CT) is the most common negative gram bacteria obligate intracellular pathogen that causes sexually transmitted diseases, including ocular trachoma worldwide. the objective was to diagnose genital CT infection among reproductive-age women attending Al-Batool Hospital in Mosul-Iraq. including Immuno-chromatographic test and quantitative Polymerase chain reaction (qPCR). A total of 125 females were included in this research, out of which 100 were infertile and 25 were fertile. Endo-cervical swabs were collected from all participants during the study period (July 2022 to September 2022). The immune chromatographic for chlamydia (ICT) and qPCR method was utilized to amplify and quantify chlamydial 16srRNA, known for its high specificity and sensitivity. qPCR is considered an ultrasensitive marker for the detection of CT. and was used to confirm the positive cases identified by ICT. Among the 125 women tested, a total of 12 cases (12%) were found to be positive for CT infection, all positive cases identified through immunographic test showed increased amplicon copy numbers with variable concentrations when analyzed by qPCR, providing strong evidence for the accuracy and reliability of molecular test. Out of the 100 infertile women, 12 were positive for CT infection, while none of the 25 fertile women tested positive. These findings suggest a potential association between CT infection and female infertility. The research demonstrated the utility of quantitative Polymerase chain reaction (qPCR) in diagnosing genital Chlamydia trachomatis infection among reproductive-age women.

Keywords: *Chlamydiae trachomatis*, 16srRNA

Introduction

The most prevalent gram-negative bacterium that causes sexually transmitted illnesses is *Chlamydia trachomatis* (Ct). Every year there are over 4 million new cases of CT in the United States and an estimated 152 million cases worldwide (Feodorova et al., 2022; Grieshaber et al., 2022). Globally, the majority of sexually transmitted diseases (STDs) are caused by Chlamydia trachomatis (Feodorova et al., 2022). Dysuria and vaginal discharge are first brought on by CT's infection of the cervix as well as the urethra. If the infection is not identified and treated, it may spread to the fallopian tubes and result in pelvic inflammatory diseases like cervicitis, endometritis, and salpingitis (Fatholahzadeh et al., 2012). Infections of CT are 80–90% asymptomatic (Wilkowska-Trojniel et al., 2009)(Folger 2014). Young females have been found to have the greatest rates of CT infection, making them a reservoir for more transmission (Hasanabad et al., 2011). In the past few years, the Chlamydiaceae family has been expanding. Currently, this family contains thirteen species of the genus *Chlamydia* . Sequence analysis of the 16S ribosomal RNA (rRNA) gene has been widely used to identify bacterial species and perform taxonomic studies (Petti et al., 2005). Bacterial 16S rRNA genes generally contain nine “hypervariable regions” that demonstrate considerable sequence diversity among different bacterial species and can be used for species identification (Ranjan et al., 2016). Due to its sensitivity and specificity in detecting minute amounts of bacterial DNA in clinical samples, molecular biological techniques are being used more and more often to identify CT in clinical specimens (A. P. Liguori et al., 2011). The vitality and widespread of rRNA genes make them the best target for bacterial identification Widely 16S rRNA gene (Fu et al., 2022). 16SrRNA is often used as an ultrasensitive marker for CT detection because of its species specificity and high copy number in CT. Nine "hypervariable regions" (V1-V9) of the bacterial 16S (rRNA) gene show a significant amount of sequence variability among different bacteria Sequences that are distinctive to a certain species might serve as targets to diagnostic tests and with the other scientific inquiries. Since never one part able to distinguish between all bacteria, comprehensive investigations that contrast the relative benefits of each location for particular are necessary. Diagnostic objectives are required (Fang et al., 2022).

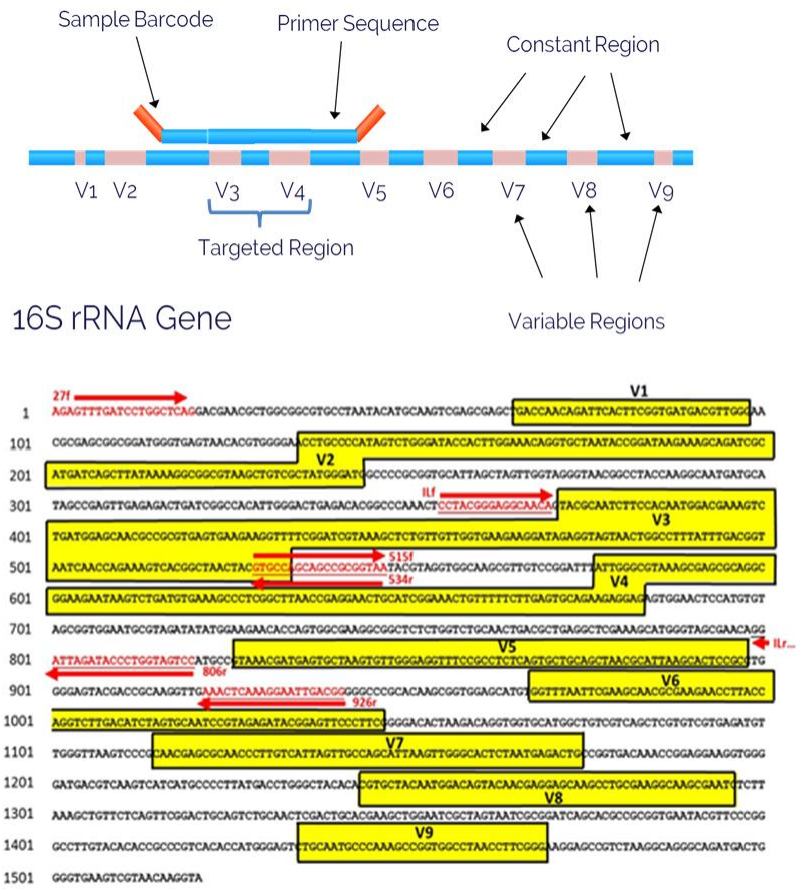


Figure 1 The variable areas were revealed by 16S rRNA gene and metagenomic sequencing (Fang et al., 2022)

The Aims of the Research

The difficulties in controlling infections with Chlamydia trachomatous is the delayed diagnosis, so this research aimed to find a simple molecular diagnostic method of genital CT infection and Highlight the importance of molecular detection of chlamydial 16srRNA by qPCR among reproductive-age woman in Mosul city and found if there is a correlation between chlamydia infection and women infertility.

Materials And Methods

Infertile Iraqi females (125) from Mosul were enrolled in this research. 100 of them were suffering from infertility and 25 were fertile women considered as control. These women attended the Private gynecology Clinic at Al-Batool Teaching Hospital at Mosul City. Cervical swaps The set of samples was carried out from June to August 2023.

Swab collection

cervical swabs had been taken and divided into two groups the first one placed in the 0.3 l phosphate buffered saline (PH 2-4) to extract DNA by extraction kit (Add bio INC Daejeon, Republic of Korea) DNA kept at -20° C until usage in accordance with the manufacturer's instructions

the second had been immunological tested for CT by (ECO test/UK). Black, C.M. 1997 kit

Immunological detection

Chlamydia trachomatis detection test by chromatography test

- The test devices, specimens and reagents were all allowed to reach room temperature prior to testing.
- In the extraction tubes, 5 full drops of reagent A were put. The swabs were inserted immediately, compressed to the bottom of the tubes rotated for 10 times and were let to stand for 2 minutes.
- Seven full drops of reagent B were added to the extraction tubes. The bottom of the tubes was compressed, the swabs were rotated until the colour of the solution was turned to a clear light blue or yellow if the swab was bloody and was let to stand for 1 minutes.
- The dropper tips were fitted to the tops of extraction tubes. Three full drops of the extracted solution were added to specimens well (S) of the test device.
- The results were read at 10 minutes. The appearance of 2 red lines, one line in the control region (C) and another line in test region (T) indicated positive results, while the appearance of single line in control region indicated negative result.

Moleculer Detection of CT

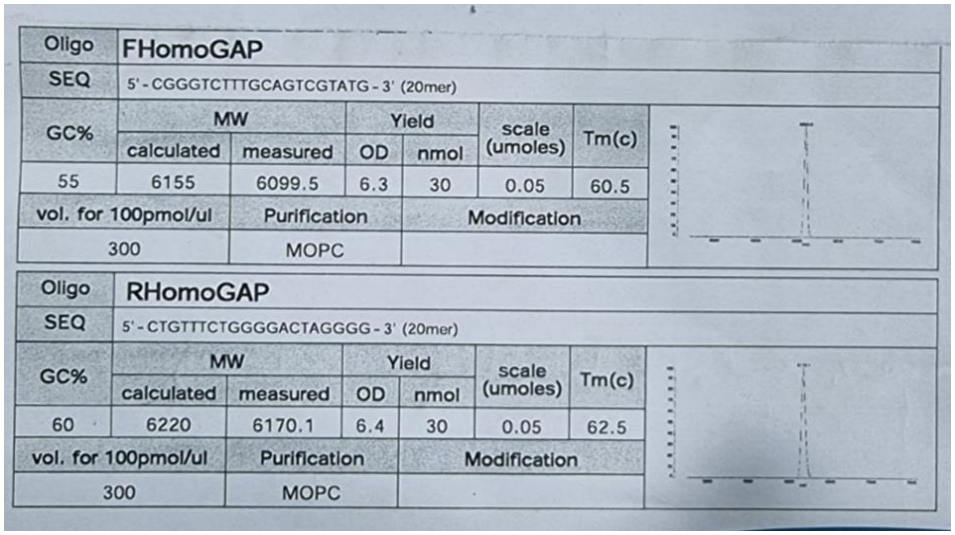
Extraction of cervical swab DNA

The AddPrep Genomic DNA Extraction Kit provides an easy, quick, and affordable way to isolate genomic DNA from blood, tissue, and plants. Centrifuging tubes containing cervical swab secretion at 13000 RPM for 20 minutes. Next, the sediment added to the 1.5 ml eppendroph tube after the supernatant was removed. Utilizing a DNA extraction kit, the sediments were utilized for

DNA extraction. (AddPrep Genomic DNA Extraction Kit ADD BIO INC Daejeon of Korea). DNA samples were divided into separate 0.2 ml microtubes with phosphate buffer and stored at - 20 °C until the time of the PCR test in order to prevent DNA deterioration DNA concentration and purity. The concentration and purity of extracted DNA were measured via nano-drop spectrophotometric analysis (Thermo Scientific).which read DNA or RNA, concentration while DNA purity had been obtained by the ratio of 260/280, show in the Figure 3.1 Map proceger and Figure 3.2 Nano drop device.

Detection of chlamydia 16srRNA via the qPCR technique

Primer were designed using NCBI (https://ww The house keeping gene utilized in qPCR to compared with test sample (w.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi) primer baste (Figure2). The bulk of the genome's active genes are housekeeping genes, and it goes without saying that survival depends on the expression of these genes. The housekeeping gene expression levels are adjusted to fit the needs of different tissues' metabolisms. Because of the poorly understood promoter patterns and transcription start mechanism, biochemical research on the housekeeping gene promoters have proven challenging.



Oligo	FHomoGAP					
SEQ	5'-CGGGTCTTTGCAGTCGTATG-3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
55	6155	6099.5	6.3	30	0.05	60.5
vol. for 100pmol/ul	Purification		Modification			
300	MOPC					

Oligo	RHomoGAP					
SEQ	5'-CTGTTTCTGGGGACTAGGGG-3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
60	6220	6170.1	6.4	30	0.05	62.5
vol. for 100pmol/ul	Purification		Modification			
300	MOPC					

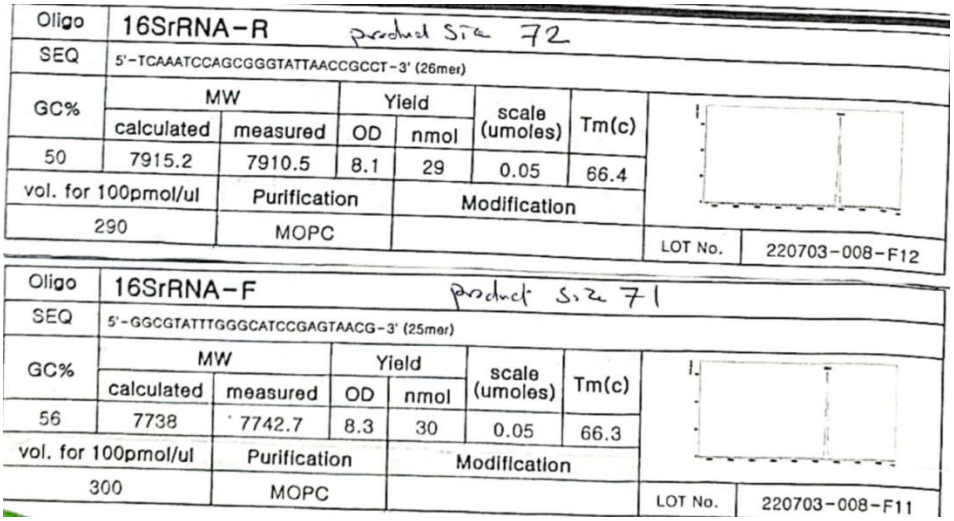
Figure 2. The house keeping gene utilized in qPCR to compared with test

For the House Keeping gene of Chlamydia trachomatis, two distinct primers were created:

Forward start: from ('5-CGGGTCTTTGCAGTCGTATG-3')

Reverse start: from ('5- CTGTTTCTGGGGACTAGGGG-3')

Primers for 16srRNA expressing gene were designed using NCBI (https://www.ncbi.nlm.nih.gov /tools/primer-blast/primertool.cgi) primer baste (Figure 3).



Oligo	16SrRNA-R product size 72						
SEQ	5'-TCAAATCCAGCGGGTATTAACCGCCT-3' (26mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
50	7915.2	7910.5	8.1	29	0.05	66.4	
vol. for 100pmol/ul	Purification		Modification				
290	MOPC					LOT No.	220703-008-F12

Oligo	16SrRNA-F product size 71						
SEQ	5'-GGCGTATTTGGGCATCCGAGTAACG-3' (25mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
56	7738	7742.7	8.3	30	0.05	66.3	
vol. for 100pmol/ul	Purification		Modification				
300	MOPC					LOT No.	220703-008-F11

Figure 3 16srRNA primer design from NCBI

For the Chlamydia trachomatis genome's 16 s ribosomal gene, two distinct primers were created.

Forward start: from (GGCGTATTTGGGCATCCGAGTAACG-3')

Reverse start: from ('5- TCAAATCCAGCGGGTATTAACCGCCT-3')

Real time PCR: The reaction were performed with real - time quantitative PCR machine using Gotaq® qpcr master mix (72050) from USA promega company for the detection of amplification product. The system contains a fluorescent DNA- binding dye, the BRYT green ® DYE, that exhibits greater fluorescence enhancement upon binding to double strand DNA than SYBR® green I. and GoTag® qPCR master mix. Thermal cycling was started with a two - minute hot start polymerase activation at 95 $_{o}$C, and this was followed by a first denaturation step of 15 - seconds at 95 $_{o}$C and an annealing and extension in one minute at 60 $_{o}$C Table 3.4 and Figure 3.5.

Table 2 PCR reaction components

Reagents	Initial Concentration
Master mix	150µl
Forward primer	30 µl
Reverse primer	30 µl
D.W	30 µl

Total	240 µl
Take from mixture 8 µl - DNA template	2 µl

20 µl added from each well

Housekeeping component Table 2.

Table 3 Maintenance and cleaning facets

Reagents	Initial Concentration
- Master mix - Forward primer - Reverse primer - Total	40µl 12µl 12µl 64 µl
Take from mixture 8 µl - DNA template	2 µl

10 µl added from each well

Thermal map (application) data, tabulated Table 3.6.

Table 4 Thermal map (program)

No.	Step	Tm (C)	Time	No.of cycle
1	Polymerase activation	95	2 min	1
2	Denaturation	95	15 sec	40
3	Annealing	60	1 min	
4	Extension	60	1min	

Results And Discussion

Using a cassette test for chlamydia known as the fast immunochromatographic card test from 100 infertile patients 12 was chlamydia positive test at the age of (25-38) years old (Figure 5). While all fertile group 25 sample (control) was chlamydia negative (Table 5) and (Figure 4). Therefore, the frequencies of chlamydia positive among the infertile cases were 12%.

Figure 4 Immunochromatographic card test for chlamydia detection; A. Positive result showing two red lines at T (test) and C (control) regions. B. Negative result showing single line at C region.

Table 5 Chlamydia positive infection in fertile and infertile cases

	No. (%)		Total number	*P*-Value
	Chlamydia positive	Chlamydia negative		
Infertile	12 (12%)	88	100	<0.0001
Fertile	0	25	25	**
Total	12 (9.7%)	113 (90.3%)	125	Significant

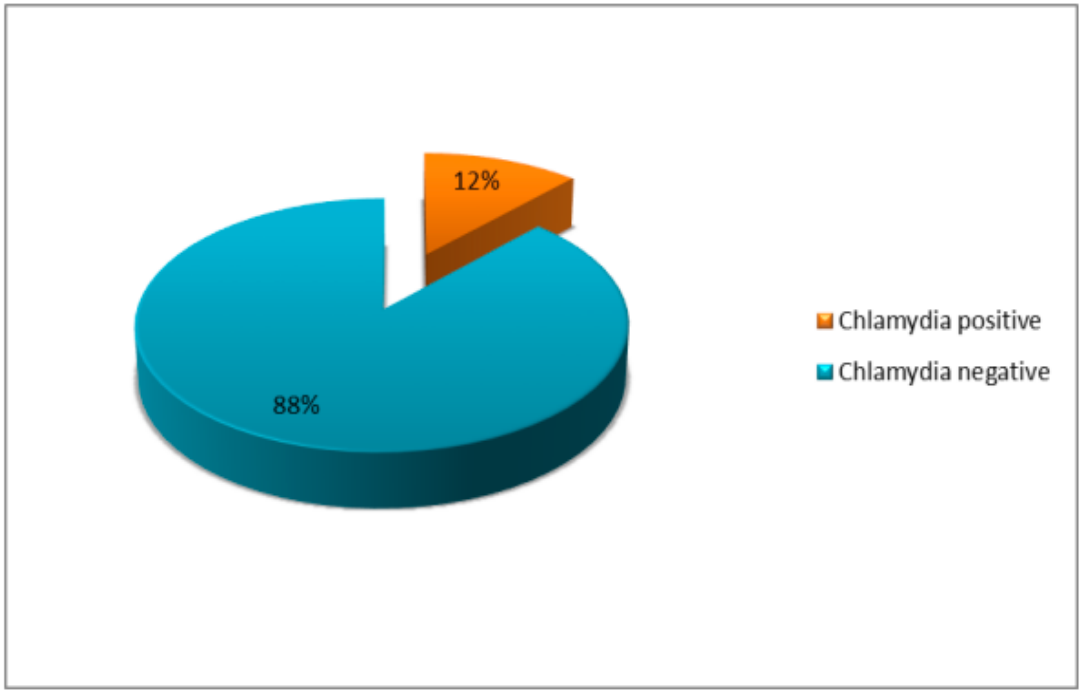


Figure 5. Chlamydia positive infection in fertile and infertile cases

In this research, the prevalence of chlamydia was 12% in instances of infertile and 0% in cases of fertile individuals. The frequency of chlamydia in both patient groups varied widely, according to prior investigations. These findings showed that 3.4 – 44 percent of infertile patients had chlamydia (Peng et al., 2020). According to (Gibney & Drexhage, 2013; Möller et al., 2010). While in the fertile groups it was ranging from 5- 14.5% (Mertz et al., 2010; Möller et al., 2010). These several in the spread of chlamydia between the several studies might be refer to geographical or to social variation and for the type of test that used in the detection of chlamydia. Therefore, chlamydia infection is expected in about 1/5 to 1/6 of adult married women irrespective of being infertile or not. Such a common prevalence should be looked after carefully to prevent serious sequelae as PID or infertility. It was reported previously that chlamydia could be an important cause of tubal infertility (Rethman et al., 2010).

Molecular Diagnosis of CT

The DNA had been extracted from infertile patients (12) from cervical swabswhich were reading by Nano Drop 9 and the concentration mean of the 12 positive sample were 1.34 ng /ul and the purity was 1.8–2.

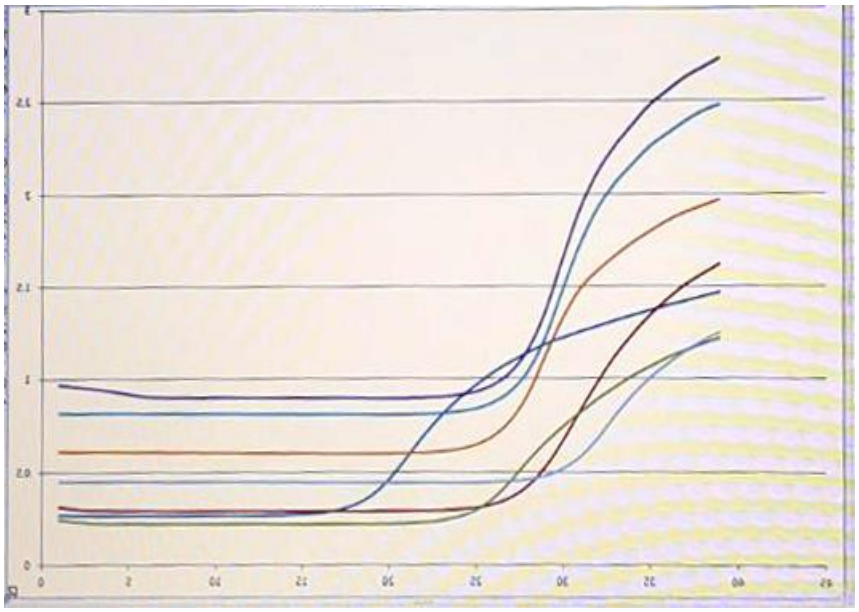


Figure 5. Results of 16sRNA by Qpcr

Diagnosis of chlamydia trachomatis 16srRNA by qPCR

From 100 infertile women 12 (12%) were positive, resulte by PCR(Figure 6) molecular detection for 16srRNA as shown in (Figure 4.16) that revealed increased amplicon copy number of 16srRNA in all cases of positive CT With variable extent.

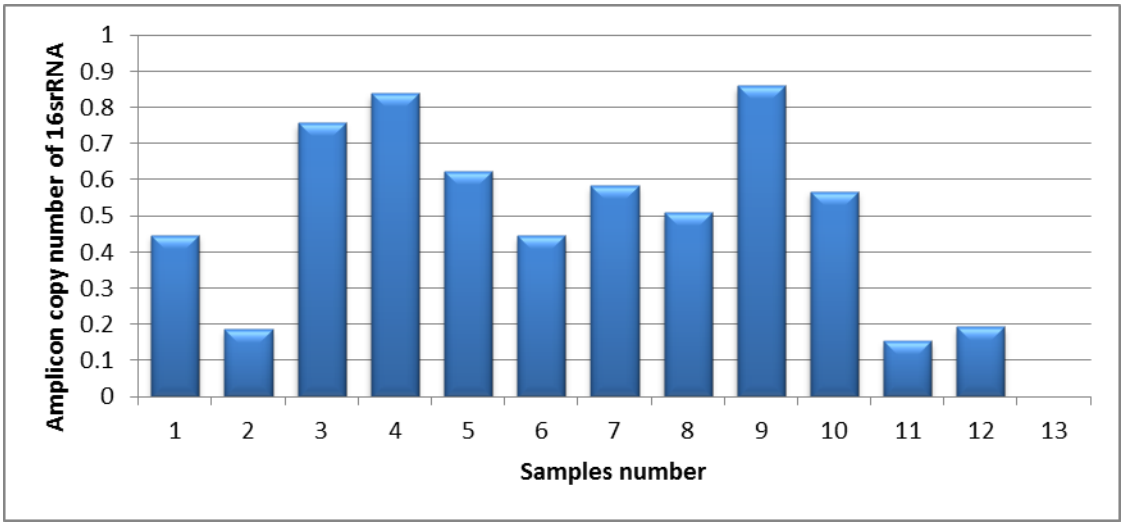


Figure 6. Amplicon copy number of 16srRNA gene in the positive cases of CT

The specificity of the q PCR utilized in the current research was better than that of our traditional gene PCR because it could identify extremely little amounts of DNA in each reaction. The result

of this study revealed that 12 of 100 swab samples of infertile women had been positive for CT - 16srRNA by qP.C.R technique with different amplicon copy numbers of 16SrRNA (Figure 7).

The specificity of the q PCR utilized in the current research was better than that of our traditional gene PCR because it could identify extremely little amounts of DNA in each reaction. These results were accepted with other researcher results such as (Ahmed, 2012). A study confirmed that the PCR technique is a useful method for detecting CT taken from endocervical area by utilization of Chlamydia plasmid genes (KL1 and KL2) that amplified by a specific PCR from Iraqi women.

(Mohammed et al., 2017; Sonmez et al., 2008) Additionally, the Real-time PCR results showed a substantial association with women who are infertile, beside (Rashidi et al., 2009), analysis revealed a 13.7% incidence of CT infection in Iranian women who were infertile, (Sachdeva et al., 2009) explained that Early diagnosis lowers the likelihood of illness progression and chronic infection transmission. Since it is simpler to do, provides quicker results, and is performed in a closed-tube, it is less likely to be contaminated, qPCR has become more and more popular in recent years for identifying genital chlamydia.

(C. Liguori et al., 2016) in their studies concluded that the application of molecular technologies that used for detection of CT in clinical samples is expanding quickly By using genus- and species-specific portions of r-RNA, this is accomplished by nucleotide sequence analysis of genes in the r-RNA operon, such as the 16S and 23S r-RNA genes.

In addition (Fadrosh et al., 2014) revealed that16S rRNA and 16S-23S rRNA spacer genes are recognized as sensitive and specific approaches for the detection of CT in females when used as genetic markers by conventional PCR. (Mohammed et al., 2017) Confirmed. In Iraqi laboratories, the qPCR has the potential to support CT detection in the first assessment of infertility comparison with the ELISA and Rapid Test.

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