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The role of host TLR receptor gene polymorphism aassociated with *Chlamydia trachomatis* infection in infertile women.

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Abstract

Background : Female infertility represents a growing public health concern worldwide and in Iraq, where infectious, genetic, and immunological factors in common contribute to defective reproductive outcomes. The most common bacterium spread through sexual activity is *Chlamydia trachomatis*, which can cause infertility and other confusion and infections without any symptoms. Thus, having an effective diagnostic method is essential to reducing the long-term effects of illness. **Aim:** this study aimed to probe the associated between the *TLR4 (+896)A>G* gene polymorphism and susceptibility to *C. trachomatis* contagion among infertile females in Iraq. **Method:** A case –control study was supervised on 100 infertile women and 100 fertile controls attending infertility clinics Al-Hilla city between March June 2025. endocervical swabs were collected for detection o *C.trachomatis* using real-time PCR, while blood sample were used for genotyping the *TLR4(+896) A>G* via Tetra ARMS-PCR technique**.** Result: Real-time PCR detected *C. trachmastis* infection in 42% of infertile woman, while all control subjects tested negative , indicating a statistically significant diffrence. Genotypic analysis demonstrated a significantly higher frequencyof GG among infertile woman both with and without *C. trahomatis* infection, compared with controls. The GG genotype was related with markedly increased risk of infertility (OR = 4.41 in infected women and OR= 5.91 in non infected women). Allelic analysis further revealed that the G allele was significantly more prevalent among infertile woman, coferring an elevated risk of disease. **Conclusion**: the finding suggest a strong association between the *TLR 4 (+896) A>G* polymorphism , especially the GG genotype and G allele, and female infertility in Iraq women.

Keyword: **Infertility, *C. trachomatis*, TLR4, Tetra-ARMS-PCR**

1-Introduction

Infertility is a significant global health issue, affecting millions of handful worldwide . it is define as the incapacity to assume after on year of unprofitable sexual intercourse, and it can have intense emotional, and psychological consequence for individual and couple [1]. About 60–80 million women worldwide suffer from infertility, and the prevalence rate augmented by 0.37 percent annually between 1990 and 2017. [2]. A recent meta-analysis reported a global pooled prevalence of infertility at 46.25% [3]. Iraq have skilled a continuous extent over 16 years has been attributed to various factor such as genetic, war, smoking, occupation, psychological, stress, lifestyle, and diet [4]. primary infertility was characterised as the capability to achieve pregnancy following uniform sexual intercource without planning for one year or more. on the other hand, secondary infertility was define as the incapacity to become after captivating in reguler sexual activity without contraception for six month or more despite having earlier experiecenced a victorious pregnancy [5].

Chlamydia trachomatis is a gram-negative obligate intracelluler bacterium that belongs to the chlamydiaceae family of chlamydia species and is responsible for one of the most widspread sexually transmitted infections(STIs) in the world and a significant contributer to infertility in wome .It can evade the hosts innate and adaptive immune system by using autophagy [6]. Chlamydial infection were the most frequent STIs that lead to abnormal vaginal discharge in 2021 [7]. The world Health organization (WHO) estimates that 129 million people worldwide have chlamydial infections in 2020 [8]. Screening to identify chlamydial infections is necessary because most genital chlamydial infections are typically asymptomatic and can be transmitted to sexual partners [9]. If these infections are not treated, they can cause infertility and increased susceptibility to ectopic pregnancy [10]. Infection with *C. trachomatis* is becoming more commonplace worldwide right now. Other than indirect approaches for detecting particular antibodies produced against *C. trachomatis*, diagnostic procedures for chlamydial infection detection differ depending on parameters, including culture, antigen testing, and molecular assays

[11]. PCR is the most used test that responds positively after 5-7 days of *C. trachomatis* transmission [12].

Additionally, TLR polymorphisms have been linked to heightened vulnerability to a variety of bacterial infections. [13]. Nevertheless, few studies have looked into how TLR single nucleotide polymorphisms (SNPs) affect C. complications related to a trachomie infection. The TLR4 polymorphism was linked to higher C among these. trachomatis. [14]. There have also been reports linking TLR4 SNPs to an increased risk of tubal pathology after C. contamination with trachomatis. [15]. TLR4 polymorphisms *Asp299Gly* and *Thr399Ile* stood related with a declined incidence *C. trachomatis* in tubal factor infertility patients [16]. Furthermore, intronic and synonymous polymorphisms are rarely valued for their function in genetic connotation studies, despite the fact that non-synonymous and promoter region SNPs provide a suitable relevance. Overall, given the significance of TLR polymorphisms linked to heightened vulnerability to different infections and the onset of disease, the current study was created to examine the function of specific prevalent TLR4(+896) SNPs to C. women's infertility and trachomatis infections.

MATERIALS AND METHODS

Subjects and sample Collection

The study population consisted of 100 infertile women attending the infertility clinics in AL-Hilla city throughout the period of the end of March to Jun 2025 at their reproductive age with primary and secondary infertility and 100 fertile women as control groups who had no complaints of infertility. The attachment principles for the control grouping were the ability to become pregnant and 30 days without antibiotic use.

The endocervical swabs stood composed as of all participants (infertile and control women). The swab was preserved in phosphate buffer saline and then centrifuged at 14000rpm for 20 min, and then the swab was removed after spinning. The sample was maintained in a freezer until DNA extraction and real-time PCR were performed. From each group, 3 ml of venous blood was drawn using a disposable syringe, dispensed into a EDTA tube. Blood were stored in a freezer to detect *TLR4 (+896) SNPs* polymorphism of both the study and control groups.

DNA Isolation from Endocervical Swabs

DNA was extracted from the 200 endocervical swabs using the SRC® G-Negative Bacteria DNA Extraction Kit. The DNA extractions were performed as per the directions supplied by the

manufacturer. The quantity and quality of DNA were determined using a NanoDrop® spectrophotometer.

DNA Isolation from whole blood

The gSYAN DNA extraction kit (Frozen Blood) Geneaid was used to extract DNA from the 200 whole blood samples. USA, and carried out in compliance with business directives. The Nanodrop spectrophotometer (THERMO) was used to examine the extracted blood's genomic DNA. USA), which measured the amount of DNA (ng/µL) and used the absorbance at 260/280 nm to check the purity of the DNA.

Tetra-ARMS-PCR Method

The T-ARMS-PCR was achieved on extracted DNA using the sequence of a primer supplied by Macrogen (Korea) for detection and genotyping of TLR4 gene polymorphism in 200 whole blood sample from patients and healthy control samples. The reaction was performed in a final volume of 25 µl containing 5µl of DNA template, Twelve points five milliliters of G2 Green Master Mix, one microliter of each of the following primers—Forward inner primer (wild allele), Reverse inner primer (mutant allele), Forward outer primer, and Reverse outer primer—and three points five milliliters of nuclease-free PCR water were used to adjust the volume. 35 cycles of 30 seconds each of denaturation at 95 °C, annealing at 62 °C, and extension at 72 °C were performed after the initial denaturation, which lasted 5 minutes at 95 °C. At 72 °C, the final extension phase was carried out for five minutes. The amplified products were run on 2% agarose gel, and the outcomes were recorded after being seen under a UV transilluminator.

Statistical analysis

Version 25.0 of the Statistical Package for the Social Sciences (SPSS) (IBM Corp.) stayed used to investigate the data. New York, USA, Armonk. A chi-squared test was employed to compare the proportional associations. It was determined that $P \leq 0.050$ was statistically significant. **Ethics Approval**

The Ethics Committee of the College of Medicine's Department of Microbiology gave its approval to this study, University of Al-Qadisiyah, as did the Iraqi Ministry of Health. before they were included in the study tests. All participants in the study completed a written informed consent form before enrollment into this study.

RESULTS

Detection of *C. trachomatis* by real-tim PCR

One hundred infertile women and One hundred healthy control women stood experienced for current *C. trachomatis* infection by real-tim PCR using specific target sequences primers. Our study found 42 (42.0%) infertile women have positive result for *C. trachomatis*, while all healthy control women give negative result for detection and quantification C. trachomatis by means of Real Time PCR method and the difference was significant.

Table (1): Identification of *C. trachomatis* among patients and healthy control.

Groups	Total *n* (%)	Real Time PCR technique		*p*-value
		Positive *n* (%)	Negative *n* (%)	
Infertile women	100	42 (42.0 %)	58 (58.0 %)	0.001 ¥ S
Healthy women	100	0	100 (100.0 %)	
X^2	53.165			

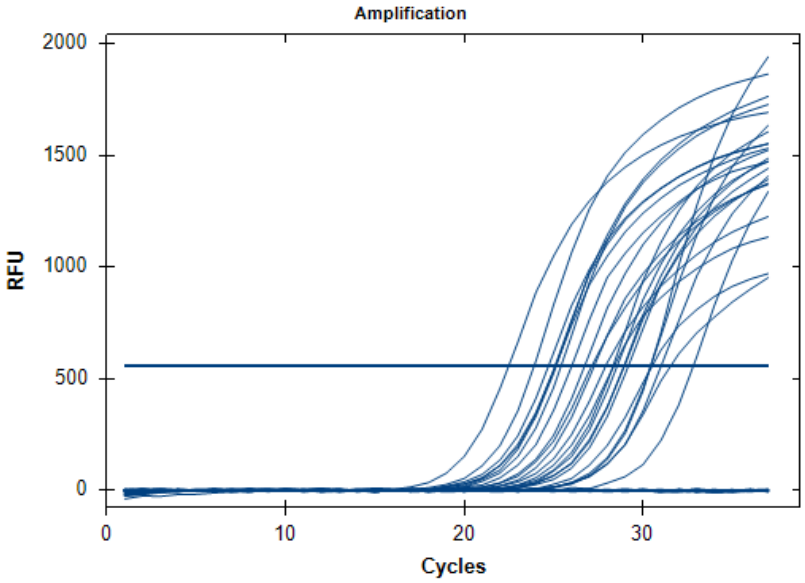

Figure (1): Real Time PCR amplification plots of 16S ribosomal RNA gene for detection *Chlamydia trachomatis* samples.

Participant Characteristics

Table (2) displays the demographic details of the sick and control issues. Participants in the population study ranged in age from 18 to 45. The mean age of infertile women with *C. trachomatis* contagion was 30.57 ± 5.76 years old, 31.81 ± 6.41 years old for infertile women without *C. trachomatis* infection, and that of control issues stayed 32.14 ± 6.84 years old and there was non-significant alteration among sick and control issues in mean age (P = 0.533). Concerning age group, both patients groups (infertile women with C. trachomatis contagion and infertile women without C. trachomatis contagion) show higher frequency with 20-29 years age groups, 17 (40.5%) and 25 (43.2%) respectively, but these result was non-significant when compared to healthy control women (P= 0.799).

Table (1) Assessment between sick and control groups in Age group.

Characteristic	**Infertile with *C. trachomatis* *n* = 42**	**Infertile without *C. trachomatis* *n* = 58**	**Healthy control *n* = 100**	***P***
Age (years)				
Mean ±SD	30.57 ± 5.76	31.81 ± 6.41	32.14 ± 6.84	0.533 † NS
Range	18–45 years	18– 45 years	18– 44 years	
< 20, *n* (%)	4 (9.5%)	2 (3.4%)	8 (8.0%)	0.799 ¥ NS
20-29, *n* (%)	17 (40.5%)	25 (43.2%)	34 (34.0%)	
30-39, *n* (%)	15 (38.7%)	21 (36.2%)	38 (38.0%)	
≥ 40, *n* (%)	6 (14.3%)	10 (17.2%)	20 (20.0%)	

Types of female infertility

Our study found that 16 (38.1%) of the infertile women with *C. trachomatis* infection were primary type of infertility and 26 (61.9%) were secondary types of infertility. The same results was found in infertile women without *C. trachomatis* infection, where show primary types of infertility lower than the secondary types 25 (43.1%) versus 33 (56.9%), and the differences was non-significant as shown in table (3).

Table (3): Frequency distribution of patients with infertility according to types of female infertility.

Study groups	**Types of female infertility**		**Total**	**p-value**
	Primary	**Secondary**		
Infertile with *C. trachomatis*	16 (38.1%)	26 (61.9%)	42	0.615

Infertile without *C. trachomatis*	25 (43.1%)	33 (56.9%)	58	¥ NS
Total	41 (41.0%)	59 (59.0%)	100	

Detection of *TLR4 (+896) A>G* Polymorphism

The appraisal of genotypes and allele occurrences concerning *TLR4 (+896) A>G* SNP between patients and apparently healthy control table (4). Concerning genotype frequency, The frequency distribution of genotypes among infertile women with C differed significantly. infection with trachomatis and a seemingly healthy control. In infertile women with C, risk analysis showed that the heterozygous A/G genotype was a non-significant risk factor with an OR of 1.27, while the homozygous GG genotype was a important danger factor (OR= 4.41). infection with trachomatis. This indicates that compared to patients with other genotypes, those with the homozygous GG genotype are roughly four times more possible to develop illness. Infertile women without C were the subject of the risk analysis. trachomatis infection, the current findings showed that infertile women without C had a significant risk factor for the homozygous GG genotype (OR= 5.91). infection with trachomatis. Therefore, compared to patients with other genotypes, those with the homozygous GG genotype are about six times more possible to develop illness.

Table (4): The frequency of the TLR4 (+896) A>G polymorphism in sick and controls.

TLR4 (+896)	**G1 *n* = 40**	**G2 *n* = 58**	**C *n* = 100**	**G1 vs C**		**G2 vs C**	
				P value	**OR**	**P value**	**OR**
Genotype frequency							
GG	14 (33.3%)	22 (37.9%)	11 (11.0%)	0.001*	4.41	0.001*	5.91
A/G	11 (26.2%)	16 (27.6%)	30 (30.0%)	0.589	1.27	0.260	1.57
AA	17 (40.5%)	20 (34.5%)	59 (59.0%)	**Reference**			
Alleles frequency							
G	39 (46.4%)	60 (51.7%)	52 (26.0%)				
A	45 (53.6%)	56 (48.3%)	148 (74.0%)				
	P	**OR**	**95%CI**				
G1 vs C	0.001*	2.47	1.44 -4.20				
G2 vs C	0.001*	3.05	1.88 -4.93				

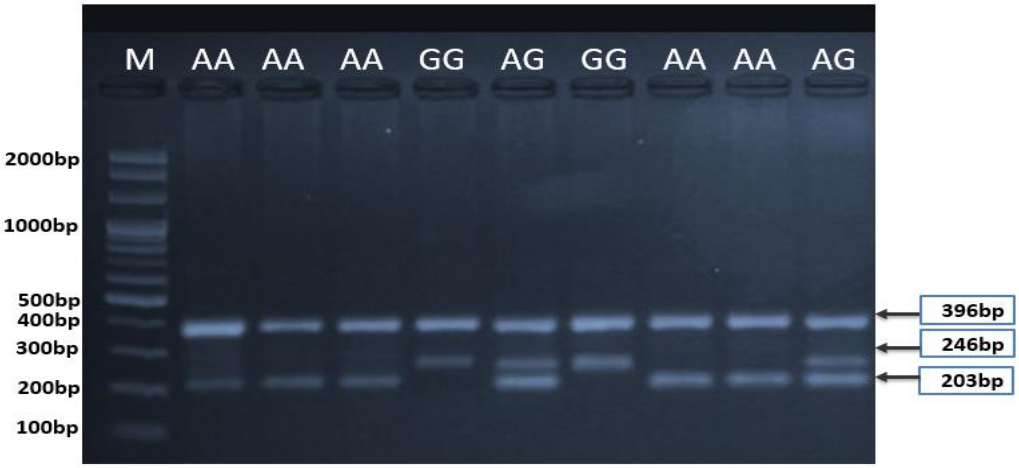

Figure (2): The ARMS-PCR product analysis of the TLR4 (+896) A>G gene polymorphism in patient blood samples was displayed in the agarose gel electrophoresis image.

Discussion

There is imperfect data on the incidence of chlamydial contagion, predominantly in underdeveloped nations, including Iraq, where standard laboratory testing for this disease is inaccessible [17]. Other than indirect approaches for detecting particular antibodies produced against *C. trachomatis*, diagnostic procedures for chlamydial infection detection differ depending on parameters, including culture, antigen testing, and molecular assays [11]. PCR is the most used test that responds positively after 5-7 days of C. trachomatis transmission [12].

It has been proposed that chlamydial infection may reduce fertility by causing an inflammatory reaction to produce anti-sperm antibodies (ASA) that might impede sperm movement and prevent sperm-ovum contact [18]. The present result show that 42 (42.0%) of infertile women provide positive result for *C. trachomatis*. social and cultural factor that hinder women from reporting sexual symptoms, limited access to testing facilities in numerous healthcare centers, and the predominantly symptomless nature of the infection can contribute to elevated levels of undiagnosed chlamydial infections. inaddition, social and cultured factors that impde women from reporting sexual symptoms. limited antrance to testing facilities in numerous healthcare centers, and the predominantly symptomless nature of the infection can contribute to elevated levels of undiagnosed chlamydial infections and antibodies. The present outcomes approve with trainings accompanied in Iraq; Sahi and Mohammed [19]. in Basra revealed that the prevalence of *C. trachomatis* infection in women with infertility stayed 43.2%. Salman *et al.,* [20]. in Kirkuk demonstrated that the prevalence of *C. trachomatis* infection in women with PID was 41.96%, While Sharief *et al.,* [12] in Basra they reported a slightly higher prevalence 48.0% for *C.*

trachomatis. Menon *et al.,* [21], which comprised 239 women, designate that up to half of infertile females could have *C. trachomatis* as a reason or causative factor. But the present result lower than the result of den Heijer *et al.,* [22] discovery that *C. trachomatis*-positive females had around 70% greater chance of suffering infertility.

the reduced incidence of C. trachomatis was discovered in Iraq. [23], found that 22 percent of infertile patients used the PCR technique, and Shamkhi et al. ", [24] in Baghdad was 17.07%. found that 22 percent of infertile patients used the PCR technique, and Shamkhi et al. ",". [25]. There is a significant difference in infection rates between the two groups. Our study's infection rates varied from previous research, which may be attributed to certain limitations, including the sample size, economic and environmental variables, specimen-collecting methods, diagnostic-based methods, and targeted genes. Consequently, PCR has been established as the preferred method for identifying infections, which is why it was selected as the gold standard for this study [26]. The PCR technique is widely recognized as the most reliable method for STI diagnosis [27].

The present results shows the mean age of infertile women with *C. trachomatis* infection was 30.57 ± 5.76 years old, 31.81 ± 6.41 years old for infertile women without *C. trachomatis* infection, and The mean age of the control subjects was 32.14 ± 6.84 years, and the alteration between the patients and control subjects stayed not statistically important (P = 0.533).), these results aligns with Ajani *et al.,* [26]; They show that the women were within the reproductive age range, with a mean age of 33.98 ± 3.85 and a extent of 24 to 40 years. The present results lower than the results of Piscopo *et al.,* [28], which showed the mean and standard deviation of age was 36.3±4.6 and their age ranged from 22 to 48 years. the starting age of sexual activity and C. A strong correlation exists between trachomatis infection. [29]. The age assortment of sick at diagnosis stayed wide (between 18 years and 45 years). There is a fluctuating tendency of infections among early adulthood and middle-aged women. The present study indicate most Infertile with *C. trachomatis* were diagnosed at the age between 20-29 years old, implying that C was more likely to affect young women. trachomatis infection, which could result from their sexual activity, poor vaginal hygiene, lack of proper sexual education, and low educational attainment. [30]. A study conducted on 498 women in Brazil revealed that young people (less than 30 years old) were the risk factors for Chlamydia trachomatis. [31]). Khalaf *et al.,* [32], showed the highest incidence rate of infertile women was for the age group of 20-29 years.

In our study, the results indicate high frequency of infertility in secondary type in compared to primary type of infertility in both group of patients, but differences was non-significant (p=0.615). Where, 16 (38.1%) of infertile with *C. trachomatis* have primary infertility and 26 (61.9%) with secondary infertility. The present finding is close to results of Omar and Darogha, [33] study in Iraq which revealed that 21 (33.87%) of infertile with *C. trachomatis* with primary infertility and 41 (66.12%) with secondary infertility. Also agree Sahi and Mohammed [19] who revealed 37.2% of infertile females with *C. trachomatis* contagion have primary infertility and 62.8% with secondary infertility.

One of the main health problems affecting women worldwide is infertility, which is caused by infections with a number of pathogens, including C. trachomatis; TLR4 is known to be essential for triggering an inflammatory response in response to these pathogens. [34]. As a particular cell receptor for bacterial constituents, TLR4 can act on the cell surface and endosomes to recognize the linker and start the innate immune response's activation of cytokine construction and the adaptive immune response's conduction. It is a crucial mediator in the identification of Gram-negative bacteria's lipopolysaccharide (LPS). [32]. One of the main causes of the systemic inflammatory response that can result in organ failure, sepsis, and septic shock is LPS. [35]. The goal of the current study, one of the first of its kind, was to look into how TLR4 SNPs relate to infertility and pathogen susceptibility in Iraq.

Three exons and two introns make up the human TLR4 gene, which is found on chromosome 9q33.1. SNP rs4986790, also referred to as 896A/G, is a non-synonymous mutation in this gene that affects the conserved residue of aspartic acid in amino acid 299 of the protein sequence (Asp299Gly) on, in, or at the domain of the extracellular structure of TLR4. It is described by substitution within the third exon of the TLR4 gene, from an adenine (A) to a guanin (G) at position 896 (896A>G). It is accountable for altering the encoded protein's function, reducing its functionality, i.e. e. missense. [32]. Analyzing host genetic factors, like SNPs, can help identify patients who are more likely to experience unfavorable disease outcomes, people who are more likely to contract a particular infection, and help develop more effective treatment strategies. [36]. *TLR4 (Thr399Ile) gene* polymorphism has been suggested to be strong association with diabetic neuropathy is more common in those with T2DM [37].

Our result found the homozygous *GG genotype of TLR4 (+896) A>G* was a important risk factor (OR= 4.41), for *C. trachomatis* infection in infertile women. This consisit with study of Taylor *et*

al., [38] who found the *genotype CC* was related with augmented risk of Chlamydia infection in an African American population. But disagree with our results Chauhan *et al.,*[13], did not invention *TLR4 rs4986790 (Asp299Gly) polymorphism* (AG, p=0.472; GG, p=0.821) to be associated with cervicitis.

On the other hand *TLR4 rs4986790 AG* genotype stayed non-significant hazard factor with an OR of 1.27 of *C. trachomatis* infection. A higher quantity of inflammatory cells in the sputum of patients with chronic obstructive pulmonary disease was linked to the same genotype. [39]. Instead, Chauhan *et al.,* [13], showed that the *TLR4 rs rs4986790 AG* genotype had a preventative effect on cervicitis. Further information on the impact of C may be obtained by examining how the aforementioned polymorphisms affect the pattern of TLR4 gene expression. cytokine construction and the host immune reaction in trachomia infections. Our findings indicate a strong correlation between TLR4 polymorphism and infertility, as well as C. contamination with trachomatis. However, there were numerous limitations to our study as well. For instance, because it was a case-control study conducted in a hospital, selection bias could not be eliminated.

CONCLUSION

Chlamydia trachomatis infection was highly prevalent among infertile women in the studied population, confirming its significant role as a contributing factor to female infertility with both type. Still, the type of infertility does not significantly affect the likelihood of *C. trachomatis* infection. These outcomes highpoint the significance of allowing for C. trachomatis as a possible feature in cases of infertility, regardless of the type. Also the *TLR4 (896) A>G* polymorphism displayed a strong association with infertility,with the GG genotype nascent as a major genetic risk factors. The influence of the *GG genotype* on infertility was noted in both infected and non-infected infertile women, showing that TLR4 genetic variation may modify reproductive health separately of active chlamydial infection. additional large-scale and multicenter studies are suggested to validate these result and to probe the functional effect of TLR4 polymorphism on immune directive in the female reproductive tract.

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