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Immunological And Genotyping Study of *Toxoplasma Gondi* and Its Relationship with Toll Like Receptor 4 "TLR4" Polymorphism in Aborted Women

Ihsan K. A. Alkardhi[1,2*], Hatem Masmoudi[3], Hayder A. Muhammed[4], and Hayet Sellami[5]

1. Department of Biology, College of Science, University of Sfax, Tunisia

2. Department of Basic Medical Science, College of Nursing, University of Al-Qadisiyah, Iraq, **ORCID: 0000-0002-4677-0428,**3. Laboratory of Immunology, Habib Bourguiba University Hospital, Faculty of Medicine, University of Sfax, Tunisia,**orcid=0000-0002-5161-2846,**4. Department of Microbiology, College of Veterinary Medicine, University of Kerbala, Iraq, **ORCID: 0000-0001-7594-8283**,5. Fungi and Parasitic Laboratory, Drosophila Research Unit, Faculty of Medicine, University of Sfax, Tunisia, **ORCID: 0000-0003-1655-8589**

***Corresponding author: Ihsan K. A. Alkardhi,Email: Ihsan.khudhair@qu.edu.iq,mobile: 009647806546535**

ABSTRACT

Background: Toxoplasma gondii (*T. gondii*) causes toxoplasmosis, a dangerous and prevalent disease. Toll-like receptors (TLRs) are the best-studied pattern recognition receptors in mammals.

Objective: The current study was conducted at Al-Diwaniya Maternity and Children Teaching Hospital, Diwaniyah city, Iraq, from December 2020 to August 2021. **Methods:** Blood samples and placenta tissue pieces were collected prospectively from 30 patients newly diagnosed with toxoplasmosis and 64 healthy controls. Women in both groups underwent a spontaneous abortion. A human Toll-Like Receptor 4 (TLR4) ELISA kit was used to measure TLR4 levels and blood DNA extraction. ARMS-PCR was adopted to analyze the polymorphism for TLR4 Asp299Gly, and SAG3 marker was used to analyze the genotype of *Toxoplasma* strains.

Results: The frequency distribution of the A allele and G allele was statistically non-significant between the patients and the control group. The polymorphism analysis of the Asp299Gly SNP in the TLR4 gene showed abortion had no significant association with toxoplasma infection (P> 0.05). Genotype II was more prevalent in aborted Iraqi women (22/30) than in control. The obtained result revealed that the concentration of TLR4 in serum women infected with type I *Toxoplasma* was significantly higher (P< 0.05) in the AA genotype of TLR4 Asp299Gly (mean 1328 pg/ml, SD: 266.1pg/ml), compared to AG genotype (mean; 923.6 pg/ml, SD: 27.2 pg/ml).

Discussion: These findings highlighted the significance of TLR molecules in *Toxoplasma gondii* infection and the need for close collaboration between practitioners and to lessen the illness burden of toxoplasmosis.

Keywords: *Toxoplasma gondii*, Sag3, TLR4, Asp299Gly Polymorphism, ARMS-PCR.

INTRODUCTION

Toxoplasma gondii (*T. gondii*) causes toxoplasmosis, a dangerous and prevalent disease. Toll-like receptors (TLRs) are the best-studied pattern recognition receptors in mammals(1).

They play critical roles in microbial and parasite Pathogen-Associated Molecular Pattern (PAMP) and Damage-Associated Molecular Patterns (DAMP) identification and immunological responses against *T. gondii*. TLR4 is a critical receptor for innate and adaptive immune responses against *T. gondii*(2). *Toxoplasma gondii* is an intracellular parasite that causes toxoplasmosis *T. gondii*, a widespread parasite in animals and humans, especially in immunocompromised individuals. Tachyzoites, bradyzoites, and sporozoites are *T. gondii's* infectious patterns. Cats are definitive hosts, while non-feline vertebrates are intermediate hosts(3).

Considering the importance of TLR molecules in *T. gondii* infection and TLR genetic alterations in pregnancy illnesses, the current study aimed to investigate the TLR4 Asp299Gly SNPs in infected aborted women

Parasite virulence is linked to SAG3, a source involved in the parasite invasion process (5). Surface Antigens (SAG's) of Toxoplasma isolates are being sequenced and exploited for diagnostic purposes. Sag3 gene in human and animal samples of *T. gondii* was used for genetic variation to determine toxoplasma typing (4).

Understanding the modes of transmission and epidemiology of *T. gondii* in human patients requires the genotyping of this parasite. Therefore, the present study determined the prevalence of *Toxoplasma* genotype in aborted Iraqi women.

MATERIALS AND METHODS

The data were collected prospectively from 30 patients newly diagnosed with toxoplasmosis and 64 healthy controls. Women in both groups underwent a spontaneous abortion in Al-Diwaniya Maternity and Children Teaching Hospital, Diwaniyah city, Iraq, from December 2020 to August 2021.

Blood samples and placenta tissue pieces were collected prospectively from 30 patients newly diagnosed with toxoplasmosis that underwent spontaneous abortion as well as 64 apparently healthy women as control. Five milliliters (ml) of venous blood were divided into two volumes; 3 ml were kept in a gel tube to be placed in a centrifuge set at 3000 rpm for about 5 minutes, and 2 ml were kept at the temperature of -20 °C in anticoagulants tubes to be used later in the

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molecular diagnostic study. The sera were centrifuged from gel tubes and stored in 1.5 microcentrifuge tubes at -20 °C for long-term storage to preparation for future immunological research.

TLR4 serum was measured using ELISA kits with anti-human TLR4 antibody (Sunlong®, China) in accordance with the directions provided by the manufacturer. The color intensities of the samples were measured with ELISA reader (Biotek®, USA). For the analysis of the genetic variation of TLR4 Asp299Gly "rs4986790", tetra-primer amplification refractory mutation system–polymerase chain (ARMS–PCR)" reaction was used following a study by Child *et al.* [5]. The primer sequences used are described in Table 1. For each sample, the following reagents were used in the preparation of the mix: 1 µL TLR4 Inner forward "A" (10 pmol / µL, Intron), 1 µL TLR4 Inner Reverse "G" (10 pmol/µL, Intron), 1 µL of TLR4 Outer Forward (10 pmol/µL, Intron), 1 µL of TLR4 Outer Reverse (10 pmol/µL, Intron), 12.5 µL GoTaq ® Green Master Mix, and 5.5 µL sterile MiliQ H_2O. The final volume of the mix was 22 µL, and 3 µL genomic DNA was added for mixture to become 25 µL.

The total volume at the end of the reaction was 25 µL. PCR was performed using Thermocycler (Biobase®, China). The cycles used were as follows: 95°C (5 minutes), 33 cycles of 94°C (45 seconds), 58°C (45 seconds), 72°C (45 seconds), 72°C (7 minutes), and 4°C (holding temperature). Fragment amplification was confirmed in gel electrophoretic, and was run in agarose (Intron) 2% using TBE 1X (Intron) at 60 V to check the presence of a band in the gel. A molecular weight marker was used. The PCR products were stained with safety red DNA staining (LGC Biotechnology®, China) and visualized under ultraviolet light through the Transilluminator (Biobase®, China).

Table (1): Sequences of the primers of TLR4 Asp299Gly used for ARMS-PCR:

Primer TLR4 Asp299Gly	Primer Direction	amplicon size (b p)
Outer F.	5′-CCTGAACCCTATGAACTTTATCC-3	385
Outer R.	5′-GTTAACTAATTCTAAATGTTGCCATC-3	
Inner F.	5′-GCATACTTAGACTACTACCTCGAaGA-3	147
Inner R.	5′-GTCAAACAATTAAATAAGTCAATAAaAC-3′	292

TLR4 Asp299Gly genotyping was determined using conventional PCR with four primers. At first, an A/G "rs4986790-containing 385-bp product was amplified by two outer primers. The two allele-specific primers (inner primers) used the segment of outer primers as a template to generate 147 base pairs for the A allele and 292 base pairs for the G allele.

Determination of *Toxoplasma* genotype:

To determine the genotype of *Toxoplasma* in the 30 women infected with toxoplasmosis, the sag3 primer was used in DNA sequencing analysis (Table 2). The methodology was carried out as stated by Sudan et al.[6] The DNA from the placenta of the 30 women infected with toxoplasmosis was extracted using Add Prep Genomic DNA extraction kit"(Addpio®, South Korea), according to the information provided by the manufacturer. Sag3 primers were used in DNA sequencing analysis. This approach was carried out according to the methods outlined by Grigg et al. [7]

Table (2): Nucleotide sequences primer of Sag3 gene

Sag3	Primer Direction	Product size (source by :5)
Sag3 Forward	5′-ATGCAGCTGTGGCGGCGCAG-3′	1158
Sag3 Reverse	5′-TTAGGCAGCCACATGCACAAG-3′	

The PCR master mix was prepared according to the instructions provided by the manufacturer, 12.5 L of GoTaq® Green Master Mix was utilized, including 5 µL DNA target, 1 µl sag3 forward primer (10 Pico mole), 1 µl sag3 Reverse primer (10 Pico mole), and 5.5 µl of sterile MiliQ H_2O. The PCR mix was amplified directly in the thermocycler (Biobase, China) according to the optimization protocol: 1 cycle consisted of Pre-denaturation 95 ºC for 5 minutes, 35 cycle consisted of denaturation 95 ºC for 35 seconds, annealing 06 ºC for 35 seconds, extension with 72 ºC for 55 seconds and final extension with 72 ºC for 5 minutes, 5 µL from PCR product was electrophoresis in agarose gel and the product was visualized using a UV Transilluminator. The remaining 20 µL from PCR products were subjected to DNA sequencing to identify genetic variation. The positive PCR SAG3 gene products were shipped in an ice bag to Macrogen Company in Korea for DNA sequencing on an AB DNA sequencing machine through DHL.

Molecular Evolutionary Genetics Analysis (version 6.0) was used to calculate evolutionary distances by employing the"Maximum Composite Likelihood technique using the Phylogenetic Tree (UPGMA) Method and the Multiple Sequence Alignment Analysis of the Incomplete SAG3 Gene Based on the Clustal W Alignment Analysis by Utilizing Software (Mega 6.0).

The collection of *T. gondii* sequences in Genbank of *T. gondii* sequences was performed using "NCBI (https://www.ncbi.nlm.nih.gov/)"to compare local SAG3 to other global sequences. The main

inclusion criterion was linear DNA sequences of the SAG3 marker with a wide geographical representation from different regions of the world with definitive or intermediate hosts of similar size. The size of the SAG3 marker was 1158 bp. Phylogenetic analysis using multiple sequence alignments, especially when the sequences are not highly conserved, necessitates the removal of poorly matched locations and divergent sections, as these regions are unlikely to be homologous and may be saturated by repeated replacements.

Ethical approval:

The study was approved by the Ethics Board of Al-Diwaniya Maternity and Children Teaching Hospital and an informed written consent was taken from each participant or their parents in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical analysis

Statistical analysis was performed on the data collected for this study using the Statistical Analysis System-SAS (2012) software. The mean and standard deviation were calculated for all data. The Chi-square act test was performed for categorical data and the student's t-test for continuous data, with a significance threshold of 0.05 for all tests.

RESULTS

Serum TLR4 concentrations in women infected with *Toxoplasma gondii* and control:

Serum TLR4 levels were greater in the patient group (mean 1388.7 pg/ml, SD 509.6 pg/ml, p=0.001), compared to the control group (mean 801.5 pg/ml, SD 261.3 pg/ml, p=0.001). The characteristics of the women infected with Toxoplasma and the control groups are provided in Figure 1.

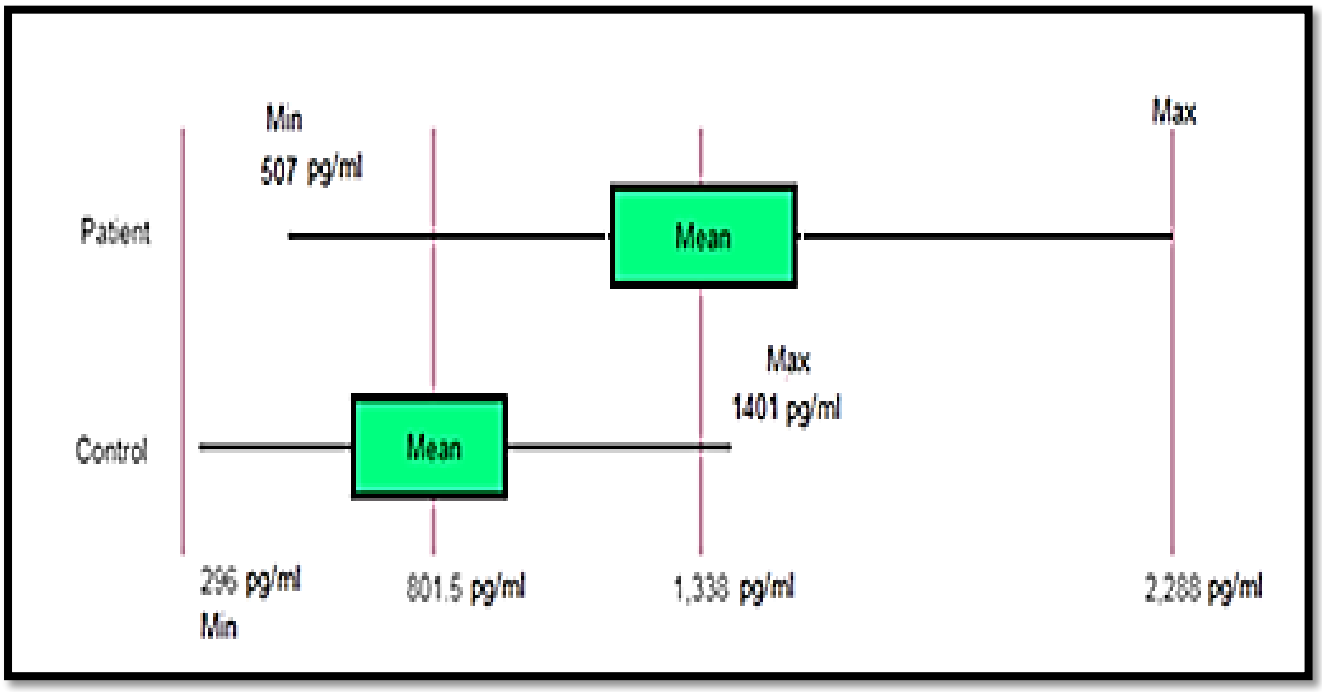

Figure 1: Box plot as graphic representation of numerical data that shows TLR4 concentration in patient and control. The box plot shows only the following statistics on a one-dimension chart, Min (Minimum), Mean, and Max (Maximum).

TLR4 Asp299Gly genotyping results:

Samples from 94 individuals were subjected to Tetra primer PCR to determine the TLR4 Asp299Gly A/G "rs4986790" polymorphism. Of these, 85 (90.4%) had the AA genotype, and 9 (6.9%) had the AG genotype. No individuals carrying the GG genotype were observed. Women infected with *Toxoplasma* and control groups were consistent in Hardy-Weinberg equilibrium (p =0.212 and p =0.93, respectively). In healthy control group, 60 (93.75%) had the AA genotype, and 4 (6.25%) had the AG genotype. In the patient group, 25 (83.3%) had the AA genotype, and 5 (16.7%) had the AG genotype. It was determined that there was not a statistically significant difference (P>0.05) between AA and AG genotype frequency (odd ratio [OR] = 0.33 95% confidence intervals [CI] : 0.08-1.345, Table 3). Within the control group, the occurrence probability of the A allele was calculated at 96.9% (62/64), and that of the G allele was 3.1% (2/64). As can be seen in Table 3 and Figure 2, A allele and G allele were 90% (27/30) and 10% (3/30), respectively, and the observed differences between A and G alleles were not statistically significant in the patient group (OR= 0.29, 95% CI: 0.04-1.83, P< 0.05).

Table 3: TLR4 polymorphism frequency by genotype and allele in case and control

Genotype frequency	Patient	Control	Odd ratio	95% CI	P value
AA	25	60	Reference	-	-
AG	5	4	0.33	0.08-1.345	0.122
GG	0	0	-	-	-
Total	30	64			
Allele frequency					
A	27	62	Reference		
G	3	2	0.29	0.04-1.83	0.189
Total	30	64			

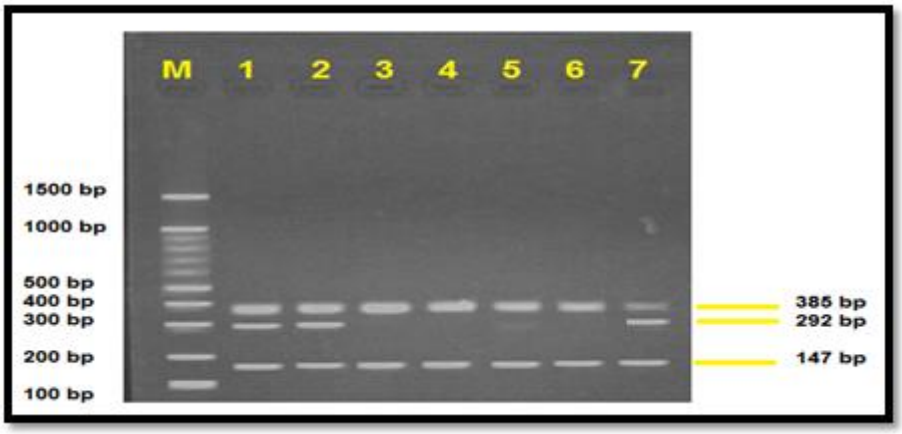

Figure 2: Agarose gel in 2% electrophoresis, under light ultraviolet, polymorphism for TLR4 Asp299Gly A/G. Sample of three individuals with the AG genotype represented in the 1, 2, and 7 wells, and samples of four individuals with the AA genotype represented in the 3, 4, 5, and 6 wells. M: represented molecular weight DNA markers 100 bp.

Typing of *Toxoplasma gondii* isolates:

The DNA amplification of the SAG3 gene was positive for the 30 samples (figure 3).

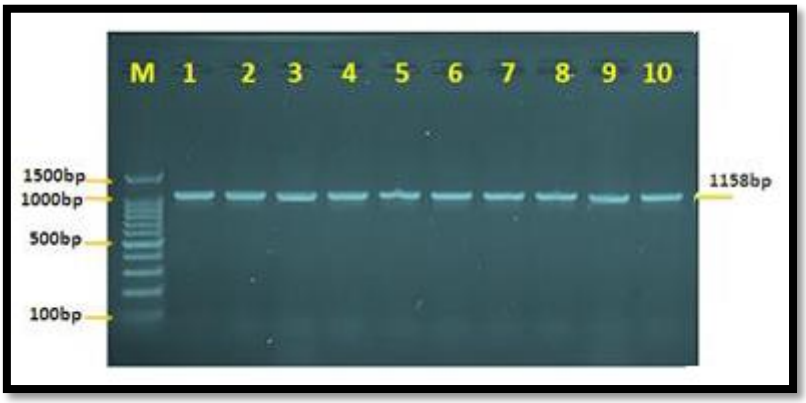

Figure 3: Analysis of TLR4 polymorphism frequency by genotype and allele in cases and controls.

Table 5 and Figure 3 display the distribution of the three *T. gondii* genotypes across the research population. Considering *T.gondii* typing, genotype II was the most common, followed by genotype I with 26.7%, and genotype III with 0%

Table 4: Number of *T.gondii* typing with a percentage

T. gondii typing	No.	Percentage
Type I	8	26.7%
Type II	22	73.3%
Type III	0	0
Total	30	100
Statistical analysis	X^2 (df 1) = 37.2, P< 0.05	

The Phylogenetic Tree Analysis based on a complete sequence of the SAG3 gene in local *T.gondii* Human isolates used for genotyping is represented in Figure 4 and Table 5. The evolutionary length was calculated by a phylogenetic UPGMA tree (MEGA X version) and the Maximum Composite Likelihood shape.

In the first analysis after the alignment, a matrix with a size of 420 bp was obtained. A phylogenetic tree with topology biased by differences in the size of sequences generating noise at the time of alignment and trees with a group of excluded sequences. In the second analysis, a size of 420 bp with a total of 8 sequences was obtained after alignment and debugging trees with topology showing the lineages of interest (Table 5).

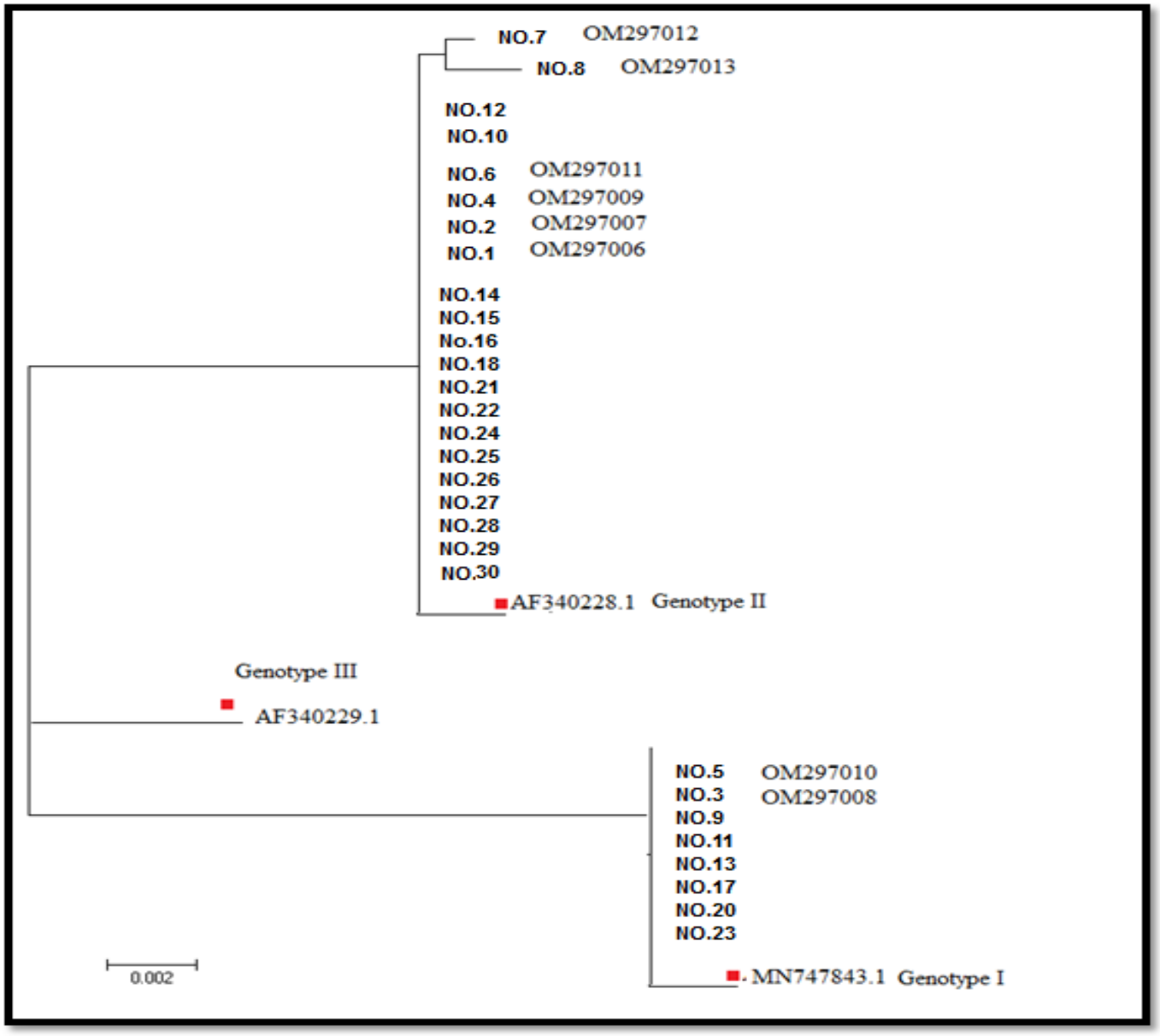

Figure 4: Phylogenetic tree analysis of *Toxoplasma gondii* human isolates based on SAG3 gene sequence. Phylogenetic UPGMA tree Maximum Composite Likelihood technique was used to calculate evolutionary distances (MEGA X version).

Table 5: *Toxoplasma gondii* surface antigen (SAG3) gene homology using NCBI BLAST *Toxoplasma gondii* genotypes isolates from humans

Toxoplasma gondii isolate No.	Genbank accession number	Homology sequence identity		
		Genotypes	Genbank accession number	Identity %
IQ.No.1	OM297006	Genotype II	AF340228.1	100%
IQ.No.2	OM297007	Genotype II	AF340228.1	100%
IQ.No.3	OM297008	Genotype: I	MN747843.1	100%
IQ. No.4	OM297009	Genotype II	AF340228.1	99.90%
IQ.No.5	OM297010	Genotype: I	MN747843.1	99.32%
IQ. No.6	OM297011	Genotype II	AF340228.1	99.90%
IQ. No.7	OM297012	Genotype II	AF340228.1	99.29%
IQ. No.8	OM297013	Genotype II	AF340228.1	100%

Relationship between *Toxoplasma* genotype and Concentration of TLR4 in serum of women and TLR4 Asp299Gly polymorphism:

The concentration of TLR4 in serum from women infected with *Toxoplasma* type I was significantly higher (P< 0.05) in AA genotype of TLR4 Asp299Gly (mean 1328.2 pg/ml, SD: 266.1pg/ml), compared to AG genotype (mean; 923.6 pg/ml, SD: 27.2 pg/ml). Regarding the concentration of TLR4 in women's serum infected with *Toxoplasma* type II, there was no significant difference (P> 0.05) between the AA genotype of TLR4 Asp299Gly (mean 1587.6 pg/ml, SD: 1518.2 pg/ml) and AG genotype (mean; 783.2 pg/ml, SD: 22.14 pg/ml, Table 6).

Table 6: Relationship between *Toxoplasma* genotype and concentration of TLR4 in serum of women and TLR4 Asp299Gly polymorphism

T. gondii typing	No. of genotype Frequency TLR4 Asp299Gly	Concentration of TLR4 Pg/ml Mean ±SD
Type I (N=8)	AA (N=6)	1328.2± 266.1
	AG (N=2)	923.6±27.2
	GG (N=0)	0
P value		0.001
Type II (N=22)	AA (N=19)	1587.6± 254.1
	AG (N=3)	1518.2 ± 22.14
	GG (N=0)	0
P value		0.074
Type III	0	-

DISCUSSION

To the best of the researchers' knowledge, this is one of the first studies in Iraq that investigated the role of immunological variables, human genotype, and parasite strain in the onset of disease. It is known that factors, such as dietary habits, climate, parasite strain, and host genetics may be related to disease development [8, 9]. *T. gondii* induces a potent Th1-type response that involves the production of cytokines, including IFN γ, TNF-α, IL-12, and the induction of NO. Studies related to the immune response of the host are important, as numerous questions have not yet been clarified [10, 11].

Toll-like receptors recognize PAMP functioning as central elements of innate immunity due to their direct involvement in controlling the adaptive immune response, and failure of this recognition may be important for the parasite survival [12, 13]. The data in the current study were collected from 30 aborted women with toxoplasmosis in the Al-Diwanyia Maternity and Teaching hospital" in Iraq and 64 control that underwent spontaneous abortion.

The patient group had significantly higher serum TLR4 levels. Given TLR4's role in inducing innate and adaptive immune responses against *T. gondii*, this result is not surprising. TLRs initiate pro-inflammatory cytokine production and the adaptive immune response during *T. gondii* infection [14].

Human TLR4 sequencing revealed that most variations in nonsynonymous substitution polymorphisms are located in the third exon coding for the TRR domain. The frequency of these nonsynonymous polymorphisms (which modify the amino acid) is less than 1% in the human population. The exception as in one nonsynonymous polymorphisms A/G transition was described in the population with a frequency greater than 5%, leading to the replacement of aspartic acid by glycine (Asp299Gly) in the fourth exon and alteration of the extracellular domain[15]. Polymorphisms at TLR loci can be used to establish the involvement of TLRs in humans. Polymorphism of the Asp299Gly SNP in the TLR4 gene in the current study had no association (P> 0.05) with abortion in women infected with *Toxoplasma* (OR = 0.33, 95% CI : 0.08-1.345). Polymorphisms at TLR loci can be used to establish the involvement of TLRs in humans. ***Wujcicka et al.*** [16] found that the GACG multiple-SNP variant, falling within the parameters of all four types of polymorphisms (TLR4 1196 C > T, TLR9 2848 G >A, TLR2 2258 G > A and TLR4896 A> G, was correlated with a decreased risk of the parasitic infection [15].

T. gondii has three major genetic categories of I, II, and III, with recombinant and atypical strains. PCR-based molecular genotyping has been used in clinical samples. In epidemiological research, PCR-RFLP, microsatellite analysis, and DNA sequencing are utilized to characterize *T. gondii* [17].

In the present study, SAG3 marker was used to genotype the isolates. The Genotype II was more prevalent in aborted Iraqi women (22/30). This result was in agreement with another study who found a prevalence of 80% for genotype II [18]. **Badr *et al.*** [19] discovered a larger percentage (44%) of Type I in Egyptian women who had abortions, compared to Type II (33%), and no case was recorded for Type III. The absence of genotype III in the present study, the non-virulent type, can be explained by the origin of samples which were from aborted women.

The *T. gondii* genotyping in the current study evaluated only one marker. Insufficient molecular markers may cause a considerable part of diversity to be ignored or genotypically different parasites to be misidentified. In Europe and the U.S., archetypical clonal lineages (Types I, II, and III) predominate, while the genotypical profile of *T. gondii* varies greatly in Brazil, with an increasing number of atypical strains. Atypical strains cause severe toxoplasmosis in immunocompetent patients and congenital toxoplasmosis newborns [20].

According to numerous research findings, the strain of *T. gondii* alone is insufficient to predict the results of toxoplasmosis in humans, and the severity of the disease appears to be multivariate[21]. The current study has shed light on the significance of TLR molecules in the progression of *T. gondii* infection. Additionally, it was discovered that the concentration of TLR4 in the serum of women who are infected with Toxoplasma type I was higher and significantly higher (P<0.05) in AA genotype of TLR4 Asp299Gly (mean 1328 pg/ml, SD: 266.1pg/ml), compared to AG genotype (mean; 923.6 pg/ml, SD: 27.2 pg/ml). Toxoplasmosis requires effective, realistic solutions. The One Health strategy should be used to lower the illness burden of toxoplasmosis.

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