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Original Article

Sequencing of TNFα and IFNϒ genes associated with *Toxoplasma gondii* infection among Erbil residents-Iraq

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Abstract

Toxoplasmosis is a serious disease that affects all age groups and may even threaten the lives of some people. Fifty serum samples were taken from patients has Toxoplasmosis who attended Rizgary Teaching Hospital in Erbil City, and 38 serum samples were taken from healthy individuals as a control group, with ages between 18-60 years old, from the period January 2024 to March 2025. The purpose of the research is to determine the gene sequence of TNFα and IFNϒ associated with Toxoplasmosis. The results showed that the infection prevalence rate in the age group (18-27) years was 17 (56.7%) in comparison to healthy individuals 13 (43.3%), and in the age group (28-37) years was 20 (62.5%) compared to healthy control 12 (37.5%), and in the age group (38-47) years was 10 (76.9%) in comparison to the controls 3 (23.1%), while in the age group (≥47) years was 3 (23.1%) compared to the controls 10 (76.9%) with significant variations (P=0.03). The prevalence rate of Toxoplasmosis in males was 17 (48.6%) in comparison to the healthy controls 18 (51.4%), while in females it was 33 (62.3%) compared to the healthy individuals 18 (51.4%), with no significant variations (P=0.29). The mean level of Toxoplasmosis IgM was (2.28±0.18) when compared to healthy group (0.12±0.03), and the mean level of Toxoplasmosis IgG was (2.12±0.18) when compared to healthy control (0.09±0.02). The mean level of TNF-α (pg/ml) was (10.34±0.39) in comparison to the control group (4.89±0.31), and the mean level of INF-γ (pg/ml) was (10.72±0.36) in comparison to the controls (4.80±0.29) with highly significant variations (P=0.001). Moreover, direct correlation between Toxoplasma IgM with IgG was (r=.568), with TNF-α was (r=.607), with INF-γ was (r=.528), with highly significant variations P= (.000, .000, .000) respectively. Also, there were direct correlations between Toxoplasma IgG with IgM (r=1), with TNF-α (r=.550), with INF-γ (r=.576), with highly significant variations P= (.000, .000, .000) respectively. The TNFα gene sequence ID 7132 of rs767455 in the position AA was changed to AG and GG, respectively, in samples 1-10 in comparison to the control group. Also, the INFϒ gene sequence ID 3458 of rs2430561 in the position TT was changed to TA and AA, respectively, in samples 1-10 in comparison to the control group.

Keywords: Sequence, TNFα, IFNϒ, Genes, *Toxoplasma gondii*, Erbil City.

1. Introduction

Toxoplasmosis is an important parasitic disease worldwide and is related to certain psychiatric disorders and sterility]1]. It was assumed that Toxoplasmosis exerts some negative impacts on reproductive capability in both men and women]2[. Congenital infection caused by trans placental transmission can lead to a wide variety of manifestations in the fetus and infant, including spontaneous abortion, still-birth, a new-born with classic signs of congenital Toxoplasmosis such as hydrocephalus or microcephalus, cerebral and calcifications [3]. *Toxoplasma* infection in infertile human couples was higher than that in fertile couples, perhaps associated with anti-sperm antibodies, which are higher in couples infected with *T. gondii*]4[. A study on *toxoplasmosis* in infertile men revealed that out of 100 cases of infertile men, 36% had positive serum *Toxoplasma*-IgG and IgM, which concluded that *toxoplasmosis* can be a cause of men's sterility]5[. It was known that latent toxoplasmosis influences infected person's morphology and increases the possibility of the birth of male offspring in both mice and humans; all these traits may be correlated with the detected variations in the testosterone concentration between *Toxoplasma*-free and *Toxoplasma*-infected individuals]2[. It is obvious that any hypothalamic–pituitary–gonadal (HPG) axis disorder resulting from direct or indirect reasons may lead to changes in normal male reproductive parameter functions. It is of interest to say that *toxoplasmosis* was recognized as a risk factor that influences male's sterility owing to spermatogenesis malfunction]6[. Recurrent toxoplasmosis in females results in fertility problems, and one of the causes why the endometrium does not allow the fertilized eggs to adhere is the chronic inflammation of different etiologies]7[. Chronic inflammations result in functional intrauterine disorders and reduce the endometrial receptivity, which negatively influences the embryo implantation process and its initial developments]8[. These chronic infections include toxoplasmosis, brucellosis, listeriosis, rubella, herpes and

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cytomegalovirus]9]. It was hypothesized that peripheral cytokine release in response to toxoplasmosis may reach to the hypothalamus and start a series of actions causing inhibition of the pulsatile release of the gonadotropin-releasing hormone and resulting in the consequent pituitary-ovarian axis function's impairments]10[. Although in many studies *T. gondii* was isolated from caprines, ovine, swine and human seminal fluid, there is virtually no risk of venereal transmissions]11[. *Toxoplasma gondii* can be effective on some indicators e.g., TNF-α [12]. IFNγ is required for stimulation of T-cell response and resisting *T. gondii*. IFNγ pathways cause activation of effector mechanisms in various cell types, resulting in control of toxoplasmosis [13].

2. Materials and Methods

2.1. Study population and sample collection

Fifty serum samples were taken from patients has Toxoplasmosis who attended Rizgary Teaching Hospital in Erbil City, and 38 serum samples were taken from healthy individuals as a control group, with ages between 18-60 years old, from the period January 2024 to March 2025.

2.2. Serological and cytokine analysis

Toxoplasma IgM and IgG antibodies were done by sandwich test ELISA technique. TNF-alpha and IFN gamma by enzyme-linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The kit was from Mybiosources.

2.3. Genetic analysis

TNFα and IFNϒ genes were detected by conventional PCR and were sequenced by Sanger sequencer with the specific primers (Table 1).

2.4. Statistical analyses

The SPSS-20 version, Chicago, IL, US, was used for data analysis. Data was studied using the Chi-square test. Correlation test was done to examine the relationship between markers.

3. Results

3.1. Prevalence rate of Toxoplasmosis

The results showed that the infection prevalence rate in the age group (18-27) years was 17 (56.7%) in comparison to healthy individuals 13 (43.3%), and in the age group (28-37) years was 20 (62.5%) compared to healthy control 12 (37.5%), and in the age group (38-47) years was 10 (76.9%) in comparison to the controls 3 (23.1%), while in the age group (≥47) years was 3 (23.1%) compared to the controls 10 (76.9%) with significant variations (P=0.03). The prevalence rate of Toxoplasmosis in males was 17 (48.6%) in comparison to the healthy controls 18 (51.4%), while in females it was 33 (62.3%) compared to the healthy individuals 18 (51.4%), with no significant variations (P=0.29). The distribution of Toxoplasmosis according to residence showed that the rate of patients who were residing in rural areas was 27 (61.4%) and in urban areas was 23 (52.3%), with no significant variations (P=0.51). In addition, the distribution of Toxoplasmosis according to smoking showed that 23 (53.5%) of patients were smokers and 27 (61%) were non-smokers with no significant variations (P=0.68). Also, the distribution of Toxoplasmosis according to the cat breeding showed that the study groups were equal with no significant variations (P=0.88), as shown in Table 2.

3.2. Immunologic parameters in the study groups

The mean level of Toxoplasmosis IgM was (2.28±0.18) when compared to healthy group (0.12±0.03), and the mean level of Toxoplasmosis IgG was (2.12±0.18) when compared to healthy control (0.09±0.02). The mean level of TNF-α (pg/ml) was (10.34±0.39) in comparison to the control group (4.89±0.31), and the mean level of INF-γ

Table 1. specific primers for TNFα and IFNϒ genes amplification.

Primer Name	Sequence 5`-3`	Annealing Temp. (˚C)	Product size (bp)
rs767455-F	GTAAAACGACGGCCAGTCCCTCCTCTCTGCTTTAATTT	55	664
rs767455-R	CAGGAAACAGCTATGACACTCCCACTCCCT TCTTT		
rs2430561-F	TGTAAAACGACGGCCAGTCGTTGCTCACTGGGATTT	55	1029
rs2430561-R	CAGGAAACAGCTATGACCATGTCTTCCTT GATGGTCTC		

Table 2. Demographic representation of the studied groups.

Properties		Case	control	*P-value*
Age range (years)	(18-27)	17 (56.7%)	13 (43.3%)	0.03
	(28-37)	20 (62.5%)	12 (37.5%)	
	(38-47)	10 (76.9%)	3 (23.1%)	
	(≥47)	3 (23.1%)	10 (76.9%)	
Gender	Male	17 (48.6%)	18 (51.4%)	0.29
	Female	33 (62.3%)	20 (37.7%)	
Residency	Rural	27 (56.3%)	21 (43.7%)	0.51
	Urban	23 (57.5%)	17 (42.5%)	
Smoking habit	Yes	23 (53.5%)	20 (46.5%)	0.68
	No	27 (60%)	18 (40%)	
Cat breeder	Yes	25 (58.1%)	18 (41.9%)	0.88
	No	25 (55.6%)	20 (44.4%)	

(pg/ml) was (10.72±0.36) in comparison to the controls (4.80±0.29) with highly significant variations (P=0.001), as illustrated in Table 3.

3.3. Correlation between immunologic parameters

Direct correlation between Toxoplasma IgM with IgG was (r=0.568), with TNF-α was (r=0.607), with INF-γ was (r=0.528), with highly significant variations P= (0.000, 0.000, 0.000) respectively. Also, there were direct correlations between Toxoplasma IgG with IgM (r=1), with NF-α (r=0.550), with INF-γ (r=0.576), with highly significant variations P=(.000, .000) respectively as shown in Table 4.

There was no statistically significant linear correlation between serum IL-5 and IgG-Toxoplasma antibodies (r=0.085, P=0.46). The mean values of IgM-Toxoplasma antibodies were 0.394 in women who had chronic toxoplasmosis, while in those who had no chronic toxoplasmosis, the mean value was 0.298, and there were statistically significant differences between the mean values of the two parameters (P=0.02). There was no statistically significant correlation between IgM-Toxoplasma antibodies and IgG-Toxoplasma antibodies (r =0.156, P=0.08). Results showed that there was statistically significant linear correlation between IgM Toxoplasma-antibodies and serum IL-5(r=0.441 [24].

2.4. The amplification of *rs767455* specific region and TNFα genes sequencing

The amplifications of rs767455 of specific regions of TNFα genes in the blood specimens were carried out. Species were fractionated on 2% agarose gel electrophoresis stained with Eth.Br. M:100 ladder, as shown in Figure 1.

Table 5 and Figure 2 show the TNFα gene sequence ID 7132 of rs767455. The position AA was changed to AG and GG, respectively, in samples 1-10 in comparison to the control group.

The results also showed that the altered IL-1β GA, AA genotype is highly significantly increased in women with miscarriage and toxoplasmosis (P=0.03), OR = 10 and 95% confidence interval (1.32-81.48); (P=0.0007), OR = 0.07 and 95% confidence interval (0.01-0.32).

The amplification of rs2430561 specific region of INFϒ gene in human samples was fractionated on 2% aga-

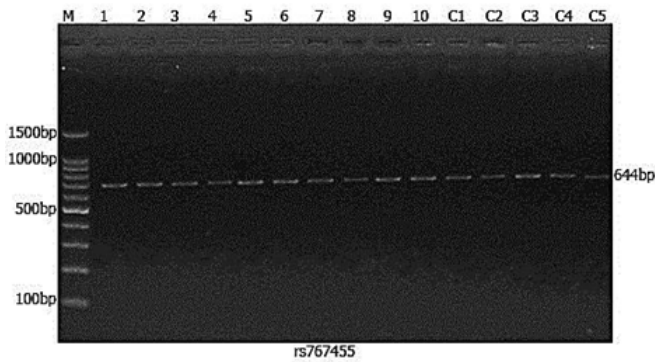

Fig. 1. The amplification of *rs767455* specific region. Lanes 1-C5 resemble 664bp PCR products.

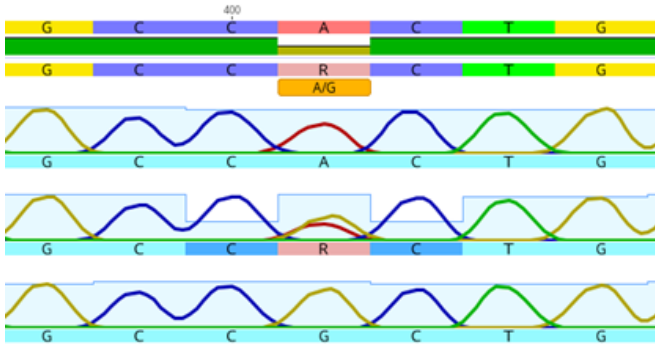

Fig. 2. Analysis of SNP *rs767455* of TNFRSF1A gene using Sanger sequencing. Single "A" peak indicates a "A" homozygous alleles. Single "G" peak indicates a "G" homozygous alleles. Presence of the "A" and "G" peaks indicates A/G heterozygous alleles.

Table 3. Mean levels of immunologic parameters in the study groups.

Parameter	Groups	Mean±SE	t-test	P-value
IgM (U/ml)	Case	2.28±0.18	11.27	0.001
	Control	0.12±0.03		
IgG (U/ml)	Case	2.12±0.18	10.95	0.001
	Control	0.09±0.02		
TNF-α (pg/ml)	Case	10.34±0.39	10.82	0.001
	Control	4.89±0.31		
INF-γ (pg/ml)	Case	10.72±0.36	12.59	0.001
	Control	4.80±0.29		

Table 4. Correlation analysis between IgM and IgG levels with TNF-α (pg/ml) and INF-γ (pg/ml).

		IgM (U/ml)	IgG (U/ml)	TNF-α (pg/ml)	INF-γ (pg/ml)
IgM	Pearson Correlation	1	.568**	.607**	.528**
	P-value		.000	.000	.000
	N	50	50	50	50
IgG	Pearson Correlation		1	.550**	.576**
	P-value			.000	.000
	N		50	50	50

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5. The sequence of TNFRSF1A Gene ID 7132.

TNFRSF1A Gene ID 7132	
SNPs	rs767455
Wild	AA
Variation	A>G
Samples	
1	AG
2	AG
3	GG
4	AG
5	GG
6	AA
7	AG
8	GG
9	AG
10	AG
C1	AA
C2	AA
C3	AG
C4	AA
C5	AG

rose gel electrophoresis stained with Eth. Br. M: ladder as shown in Figure 3.

Table 6 and Figure 4 showed that the INFϒ gene sequence ID 3458 of rs2430561, the position TT was changed to TA and AA, respectively, in samples 1-10 in comparison to the control group.

4. Discussion

The findings about infection prevalence disagreed with a study in Saudi Arabia by (Almalki et al., 2024), who revealed a highly significant disparity (p=0.02) in the portrayal of males and females, as females constituted (73.4%) while males constituted (26.6%). The mean age of positive cases was (23.27) years, while the mean age of negative cases was (15.54) years [14]. Also, in Al-Najaf Province/Iraq, a study by Jaber and Noori (2021) found that there was a significant increase(P<0.05) in the incidence of toxoplasmosis in the age group (21-30) years and in females compared to males. The high incidence of toxoplasmosis in Iraq, especially in Najaf province, was observed after taking random individuals who were asymptomatic from various areas [15]. On the other hand, the latex test performed by Alkubaisi and Al-Zubaidy (2024) revealed that females with (>40) years showed the highest infection rate (45.8%) in comparison with patients in the age group (31-40) years (33.8%) and the age group (21-30) years (13.0%) (P≤0.01). Females who lived in urban areas had a lower infection rate (29.7%) compared to rural resident females (55.4%) [16]. Also, Jaber and Noori (2021) found in Najaf province that the spread of toxoplasmosis in rural areas is greater than in urban areas [15]. Regarding raising cats, what was stated by Dunay and Zólyom (2024) is consistent with these results, which reported that a significantly higher proportion of cats living outdoors were seropositive (38.8%) compared to those living indoors (18.6%) (P = 0.022) [17].

Toxoplasma gondii IgM antibodies give a picture of the acute infection of the disease. Our findings matched

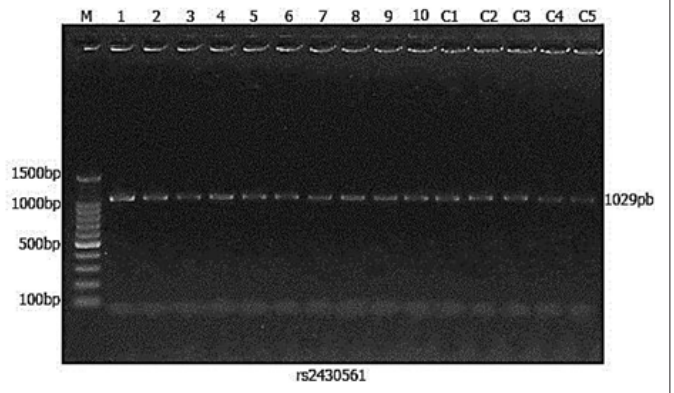

Fig. 3. Amplification of rs2430561 specific region of IFNϒ Human samples. Lanes C1-A34 resemble 1029bp PCR products.

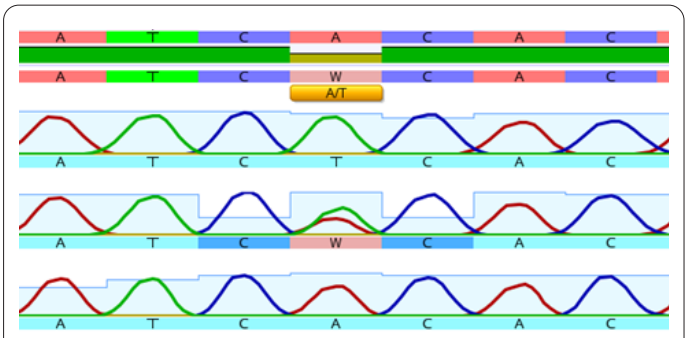

Fig. 4. Analysis of rs2430561 SNP of IFNG gene using Sanger sequencing. Single "T" peak indicates a T homozygous allele. Single "A" peak indicates a A homozygous allele. Presence of the "T" and "A" peaks indicates T/A heterozygous allele.

Table 6. The sequence IFNϒ GENE ID 3458.

IFNϒ GENE ID 3458	
SNPs	rs2430561
Wild	TT
Variation	T>A
Samples	
1	TA
2	AA
3	TA
4	TA
5	AA
6	AA
7	TA
8	TA
9	AA
10	TA
C1	AA
C2	AA
C3	AA
C4	TA
C5	AA

with (Vargas-Villavicencio et al, 2022) who showed that subjects who had acquired infections reported a duration of IgM antibodies for (40-50) weeks, and in pregnant women for (25-65) weeks and in others, even the existence of IgM antibodies was followed further than 70 weeks. A heterogenic kinetics in people with acquired infections

can be observed, partly depending upon the method applied; however, it can be suggested that the IgM persistence was observed in acquired and gestational infections [18]. Furthermore, Mohyuddin et al (2023) concluded that in T. gondii-seropositives, a significant positive associations were found between *T. gondii* IgG serointensity and frailty [19]. Grada et al (2024) found that IgG antibody was detected in 325 (70.04%) of toxoplasmosis patients. We have noticed a highly positive IgG antibody against *Toxoplasma gondii* in elderly people, females, as well as in rural area residents. We also observed an association between toxoplasmosis and some risk factors, such as activities which include contact with soils, low income level, as well as low educational achievement. Their results indicated a high prevalence in psychiatric individuals living in Western Romania [20]. In addition, for the increased TNFα level with Toxoplasmosis, Mohammed et al (2024) demonstrated that Toxoplasmosis biochemical marker values were the focus of their study. To evaluate the infection-related marker TNF alpha, TNF-α regulates inflammation, anti-tumor responses, and homeostasis via interacting with TNF-R1 and TNF-R2. TNF-α has a vital function in treating *T. gondii* by hematopoietic stem cell regulation and development of progenitor cells. Adverse impacts like lymphoproliferative diseases were known to take place, despite using of therapeutic anti-TNF-α treatments, which also indicates that inhibition of parasite's invasion is one main mechanism by which cytokines are operating. TNF-α can directly affect intracellular parasite proliferation to various extent [12]. Also, Montoro et al (2025) reported that the anti-TNF-α treatment dependably stimulates *T. gondii* latent cyst reactivation in mice model. In humans, *T. gondii* reactivation by TNF-a blockade is rare, despite the extensive use of TNF-a blockers [21]. The elevation of IFNϒ in Toxoplasmosis was in harmony with Ihara and Yamamoto (2024), who concluded that the innate immune system first recognizes toxoplasmosis and triggers chemokines and pro-inflammatory cytokines for acquired immunity promotion. The axis of IL-12/IFN-γ is of particular importance, and on inhibition of this pathway, infections become lethal and uncontrolled. In mice, receptors like (TLR11), (TLR12), and chemokine receptors contribute to recognizing *T. gondii* and to immune response modulation [22]. Song et al (2024) reported that IFN-I is able to inhibit *T. gondii* proliferation, although other studies revealed that it is useful for parasite's growth. *T. gondii* is also able to secrete protein which impacts IFN-I production pathway and downstreams the regulation of induced interferon-stimulated genes (ISG), thus, it avoids immune destruction by the hosts [23].

About direct correlation between Toxoplasma IgM with IgG, these results agreed with (Alvares et al, 2024) who showed that the mean values of IL-5 were the same, as it was 16 in the two groups of women who had/ and those who did not have toxoplasmosis.

About the TNFα gene sequence ID 7132 of rs767455, these results agreed with (Mousa and Jasim et al, 2024) who found that with TNFα-174 G/C and IL-1β +3954 G>A, the associations are established between genetic polymorphism and toxoplasmosis. Also, the genotypes GC at IL-6 (G/C) appeared to be highly correlated [25]. Also, these findings agreed with (Khudhur et al, 2022) who showed that the presence of SNPs in TNF receptor [TNFRSF1A (rs767455), TNFRSF1B (rs1061622)] encoding genes could influence patients' outcomes to etanercept in a specimen of Iraqi AS patients [26]. On the other hand, Mohammed et al (2025) targeted microRNA 155 of the tumor necrosis factor gene and found that there was high gene expression in those infected with toxoplasmosis in combination with the coronavirus [27].

About INFϒ gene sequence ID 3458 of rs2430561, Al-Baldawy et al (2023) results are similar to these findings, who reported that the heterozygous genotype (TC) frequency for SNPs TLR-5 genes (rs2072493) was significantly higher in Group1 than Group2 (P=0.004). At allelic levels, the mutant allele frequency (allele C) was significantly higher in Group1 than in Group2 [28]. On the other side, Areeshi et al, (2021) showed that the (IFN-γ) +874 A>T (rs2430561) gene polymorphism roles were assessed in variable ethnicities with pulmonary tuberculosis (PTB) infections, and reported inconsistent results. In the current study, a meta-analysis was done to determine the precise associations between IFN-γ +874 A>T gene polymorphisms and PTB susceptibility [29]. Similar findings were shown by Tachibana et al (2023), who demonstrated that interferon-γ (IFN-γ)-dependent virulence gene. The screening result suggests GRA72 role for normal GRA17/GRA23 localizations and IFN-γ-dependent roles for UF Mylation-related gene [30]. Also, Nast et al (2020) reported that IFN-γ upregulated histone mark H4ac, H3K9ac & H3K4me3, while downregulated H3S10p at promoters of primary and secondary responses. Histone modification was abolished by toxoplasmosis [31]. Also, Ihara et al (2024) showed that many mechanisms of IFN-γ-induced anti-*T. gondii* defense caused parasite growth inhibition. These involve production of nitric oxide (NO) and indoleamine 2,3-dioxygenase, and parasitophorous vacuole destruction by IFN-γ-inducible immunity-related GTPase group (IRGs and GBP) [22].

The study showed that the TNFα gene sequence ID 7132 of rs767455 at position AA was changed to AG and GG, respectively, in samples 1-10 than control group. In addition, the INFϒ gene sequence ID 3458 of rs2430561 at the position TT was changed to TA and AA, respectively, in samples 1-10 in comparison to the control group.

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