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Cytokine Gene Polymorphisms of Tumor Necrosis Factor-alpha (TNF-α-238) and its Role in Susceptibility or Resistance to Toxoplasmosis Infection in Samples of Women from Salahaddin Governorate

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A B S T R A C T

Toxoplasmosis, one of the most common parasitic infections in human, is caused by the intracellular protozoan *Toxoplasma gondii*, and cytokines have been demonstrated to have a role in its pathogenesis. Moreover, single nucleotide polymorphisms (SNPs) of cytokine genes have been suggested to have role in its etiologic. Therefore, the aim of the presented study to investigated SNPs of tumor necrosis factor gene (TNF-α-238) and its association with toxoplasmosis in 52 toxoplasmosis-aborted women were admitted to Tikrit Teaching Hospital during the period October 2013 - May 2014 to terminate their pregnancy, from Tikrit Governorate. These SNPs were ascertained by PCR-SSP (polymerase chain reaction-sequence specific priming) method. In addition, 66 apparently healthy women were included as controls. Both Rapid Test Cassette and Enzyme Immunoassay for IgG and IgM) revealed that cases 30.8% of cases were positive for IgG and IgM anti-Toxoplasma antibodies, which is may aid to understand the immunogenetic predisposition in the development of toxoplasmosis through investigating cytokine gene polymorphisms. This study showed that the single nucleotide polymorphisms (SNPs) of TNF-238 gene demonstrated significant positive and negative association with toxoplasmosis in the sample of aborted women, and the GA genotype frequency was significantly increased in patients compared to controls (50.0 vs. 30.3%; P = 0.037).

Introduction

T. gondii is highly prevalent intracellular protozoan pathogen that infects a broad range of warm-blood animals including human (Mckee *et al.*, 2004; Waree, 2008). It's an orally acquired apicomplexans protozoan parasite (Ju *et al.*, 2009). In

addition to a sexual cycle which is limited to members of the felidae (Cats), the parasite can infect a wide variety of warm-blood host species cell types by use of an asexual cycle (Eslamirad *et al.,* 2012; Halonen, 2013). The parasitic infection with a worldwide distribution is common among individual living under poor sanitary condition in developing countries (Darde, 2004; Bahia-Oliveiria *et al.,* 2009).

Toxoplasmosis is reserved to describe the clinical or pathological disease caused by *T. gondii* (Montoya, 2002). The population structure of *T. gondii* is complex and shows distinct geographic patterns, the prevalence of this infection range from 15 to 85% (Albuquerque *et al.,* 2009).

T. gondii infects at least one third of the world's population. The infection is usually asymptomatic in most immunocompetent individuals, but there is more severe in immunocompromised individuals and in the case of pregnant women, transmission frequency and disease severity are oppositely related. Early maternal toxoplasmosis (first and second trimester) may result in severe congenital infection and can result in death of fetus in *utero* and spontaneous abortion, while late maternal infection usually results in normal appearing newborns (Harker *et al.,* 2015). However, the pathogenesis of foetal *T. gondii* infection is incompletely understood, but it is has been proposed that immunological alterations at the maternal-foetal interface, such as an imbalance in the production of T helper Th_1 and Th_2 lymphocytes associated cytokines may increase the risk of foetal infection, and different cytokines and cell populations have been reported to be involved in the effecter and regulatory phases of immune response against the parasite.

Cytokines play an important role in pathogenesis of toxoplasmosis and pregnancy loss; however and equally important, variations in genes that encode cytokines have been shown to interfere with the expression of these molecules and may have an important role in gene regulation in inflammatory response and resistance or susceptibility to infections including toxoplasmosis (Ortiz-Alegría *et al.,* 2010). Single nucleotide polymorphisms (SNPs) in the promoter region of cytokine genes have been demonstrated to be associated with the development of toxoplasmosis, for instance, $IFNG_{+874}$ T/A SNP of IFN-γ gene has been indicated to have a role in clinical manifestations of toxoplasmosis (Neves Ede *et al.,* 2012). Further study suggested $IL6_{-174}$ SNP genotypes that were related with a lower production of IL-6 might be associated with the occurrence of toxoplasmic retinochoroiditis (Cordeiro *et al.,* 2013).

Materials and Methods

Collection and diagnosis the positive samples

A total of 118 Arab women were enrolled in the study, and they were distributed in two groups:-

1). 52 toxoplasmosis patients, The patients were admitted to Tikrit Teaching Hospital during the period October 2013 - May 2014 to terminate their pregnancy (aborted women). Their sera were tested for anti-*T. gondii* antibodies by two laboratory methods, the first was Rapid Test Cassette (CTK Biotech Inc., USA), while the second confirmatory test included testing the sera for IgG and IgM antibodies by two Enzyme Immunoassay kits (EIA-IgG and EIA-IgM; ACON Laboratories Inc., USA). Only patients with positive results in the two tests

were involved in the study, and they had an age range of 20–40 years.

2). 66 samples as control. The control sample included apparently healthy women (University staff and blood donors), and their sera were negative for anti-*T. gondii* antibodies by both diagnostic kits. They also matched patients for age range and mean**.**
From each participating subject, 5 ml of venous blood was drawn using disposable syringe, and distributed into two aliquots. The first aliquot (3 ml) was dispensed into a plain tube and left in the refrigerator (4°C) for 15 minutes to clot, and by then, it was centrifuged (15 minutes at 3000 rpm) in a temperature-controlled centrifuge (4°C). The separated serum was distributed into aliquots (0.5 ml) in Eppendorf tubes, which were frozen at -20°C. The second aliquot (2 ml) was transferred to EDTA tube and frozen at -20˚C until DNA extraction for genotyping of cytokine gene polymorphisms. The diagnosis of toxoplasmosis was carried out by using to test kits. The first (OnSite Toxo IgG/IgM rapid test-cassette) was screening test, while the second (*Toxoplasma* IgG and IgM EIA test kits) was a confirmatory test**.**

The OnSite Toxo IgG/IgM Rapid Test is a lateral flow chromatographic immunoassay for the simultaneous detection and differentiation of IgG and IgM anti-*T. gondii* antibodies in human serum or plasma. This kit is intended to be used as a screening test and as an aid in the diagnosis of infection with *T. gondii*.

The *Toxoplasma* IgG EIA test kit is a solid phase enzyme immunoassay based on indirect principles for the qualitative and quantitative detection of IgG antibodies to *Toxoplasma* in human serum or plasma. While the *Toxoplasma* IgM EIA test kit is a solid phase enzyme immunoassay based on immunocapture principle for the qualitative detection of IgM antibodies to *Toxoplasma* in human serum or plasma**.**

Genomic DNA Extraction

The genomic DNA was extracted from EDTA blood using the AccuPrep® Genomic DNA Extraction Kit (Bioneer Corporation, Korea), which is designed to provide a fast and simple technique for the preparation of purified and intact DNA from mammalian blood.

The DNA yield was spectrophotometrically assessed by using a NanoDrop (ND-2000 spectrophotometer; USA), in which the sample was read at two wavelengths that were 260 and 280nm. If the outcome was 1.6-1.9, the sample was considered as free of contamination and having a sufficient amount of DNA for a further analysis (Frank and Tait, 2012).

Genotyping of Cytokine Polymorphisms

The Cytokine CTS-PCR-SSP Tray Kit was used to detect cytokine gene polymorphisms. The kit was developed in the laboratories of Department of Transplantation Immunology, University Clinic Heidelberg (Heidelberg, Germany). It enables the user to detect some of the polymorphisms described in the promoter regions of *IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* genes, as well as some polymorphisms in the translated regions of *TGFB1* and *IL4R* genes**.** The method of detection is a PCR-SSP (polymerase chain reaction-sequence specific priming) assay, which allows the definition of the polymorphic variants of the genes that are present in the individual under test.

The first step included a preparation of PCR Reaction Mix, which consisted of 138 µl master mix (CYT), 2.8 µl Taq DNA

polymerase (5 Unit/µl), 329 µl deionized distilled water and 50 µl DNA sample (0.10-0.15 µg/µl). The mix was mixed by vortex thoroughly and kept on ice. This was followed by taking the PCR tray and placing it inside a sample holder, and the adhesive seal was carefully removed from the tray. To each well of the first 48 wells, 10 µl of the PCR Reaction Mix was added in the sequence given in (Figure 1).

The tray was placed in the thermal cycler and the thermal cycling profile was optimized and validated for the use with the CTS-PCR-SSP TRAY KITS, and as shown in (Table 1).

Agarose gel electrophoresis

After thermo-cycling, PCR products were visualized by submarine agarose gel electrophoresis.

Preparation of agarose gel: To prepare 2% agarose, mixing 7 grams of agarose, 7 ml of 50x TAE (Tris-acetate-EDTA) buffer and 350 ml of deionized distilled water in a 500ml conical flask. The mixture was boiled to dissolve the agarose, after that, it was cooled to 60°C, and by then 17 µl of ethidium bromide (10 mg/ml) was added and well-mixed. The gel was poured on an electrophoresis plate (25 x 25 cm) fixed on an even surface, and then four combs (each with 24 teeth) were placed and the gel was allowed to set for one hour at room temperature.

Running electrophoresis: When the thermo-cycling was terminated, the PCR tray was handled outside the thermo-cycler carefully and the strip caps that covered the wells were removed from the tray. This was followed by removing the combs from the gel, which was covered with running TAE buffer (approximately 2–3 mm above the gel surface). Then, 10 µl of each PCR product was loaded onto the respective gel wells in a sequence that was given in (Figure 1). The electrophoresis was run for 20 minutes at 170 volts.

Interpretation of electrophoresis patterns: After the end of electrophoresis, the band was visualize on UV light trans-illuminator (312 nm) after removed the gel. Finally a picture was taken, and the genotype of each cytokine was recorded according the chart provided by the supplier of the genotyping kit (Figure 2).

Statistical analysis

Genotypes of cytokines were presented as percentage frequencies, and significant differences between their distributions in toxoplasmosis patients and controls were assessed by two-tailed Fisher's exact probability (P)**.** Relative risk (RR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between a genotype with the disease. The RR value can range from less than one (negative association) to more than one (positive association). If the association was positive, the EF was calculated, while if it was negative, the PF was given (Ad'hiah, 1990).

Results and Discussion

Diagnostic test of toxoplasmosis

Both diagnostic tests revealed that 16 cases (30.8%) were positive for IgG and IgM anti-*Toxoplasma* antibodies; however, cases positive for one class of antibodies showed some differences. In the Rapid Test Cassette, 36.5% of cases were positive for IgG antibody, while it was 61.5% in the Enzyme Immunoassay. For IgM antibody, an opposite picture was drawn and the

corresponding percentages were 32.7 and 7.7%, respectively. Such differences were significant ($P \leq 0.01$), as analyzed by Pearson Chi-square test (Table 2).

Cytokine Gene Polymorphisms

The study dealt with SNPs of 13 cytokine and cytokine receptor genes (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*, *IL2*, *IL4*, *IL4R*, *IL6*, *IL10*, *IL12B*, *IFNG*, *TNF* and *TGFB1*) in order to define the type of association (positive, negative or no association) between these SNPs and toxoplasmosis in aborted women. These SNPs were ascertained by PCR-SSP (polymerase chain reaction-sequence specific priming) method, in which different PCR mixes were used to define these polymorphisms at different positions, which were revealed after agarose-gel electrophoresis of the PCR amplicons, and their patterns of migration in the gel were gave in (Figure 3).

Table.1 Optimization and validation of thermo-cycling for the use with the CTS- PCR-SSP TRAY KITS

Stage	Temp	Duration/sec	Cycles
Initial denaturation	94°C	120	10
Denaturation	94°C	15	
Annealing + Extension	65°C	60	
Followed by			
Denaturation	94°C	15	20
Annealing	61°C	50	
Extension	72°C	30	
Hold	4°C	900	

Table.2 Results of serum-testing in toxoplasmosis patients by rapid test cassette and enzyme immunoassay

Anti-*Toxoplasma* Antibody	Positive Cases			
	Rapid Test Cassette		Enzyme Immunoassay	
	No.	**%**	**No.**	**%**
IgG only	19	36.5	32	61.5
IgM only	17	32.7	4	7.7
IgG + IgM	16	30.8	16	30.8
Total	52	100.0	52	100.0

Pearson Chi-square = 11.361; D.F. = 2; $P \leq 0.01$

Table.3 Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of *TNF*$_{-238}$ genotypes and alleles in toxoplasmosis patients and controls

TNF$_{-238}$ Genotype or Allele	Patients (No. = 52)				Controls (No. = 66)			
	Observed		Expected		Observed		Expected	
	No.	%	No.	%	No.	%	No.	%
GG	4	7.7	5.6	10.7	8	12.1	4.9	7.4
GA	26	50.0	22.9	44.0	20	30.3	26.2	39.7
AA	22	42.3	23.6	45.3	38	57.6	34.9	52.9
G	34	32.7	Not Estimated		36	27.3	Not Estimated	
A	70	67.3			96	72.7		
H-W X^2 P ≤	Not Significant				Not Significant			

Table.4 Statistical analysis of associations between *TNF*$_{-238}$ genotypes or alleles and toxoplasmosis

TNF$_{-238}$ Genotype or Allele	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
GG	0.60	0.05	Not Significant	0.17 - 2.11
GA	2.30	0.28	**0.037**	1.09 - 4.86
AA	0.54	0.27	Not Significant	0.26 - 1.12
G	1.30	0.08	Not Significant	0.74 - 2.26
A	0.77	0.17	Not Significant	0.44 - 1.35

Figure.1 Cytokine genotyping PCR tray showing the sequence of PCR Reaction Mix adding. The word "Lane" refers to a well's corresponding gel lane

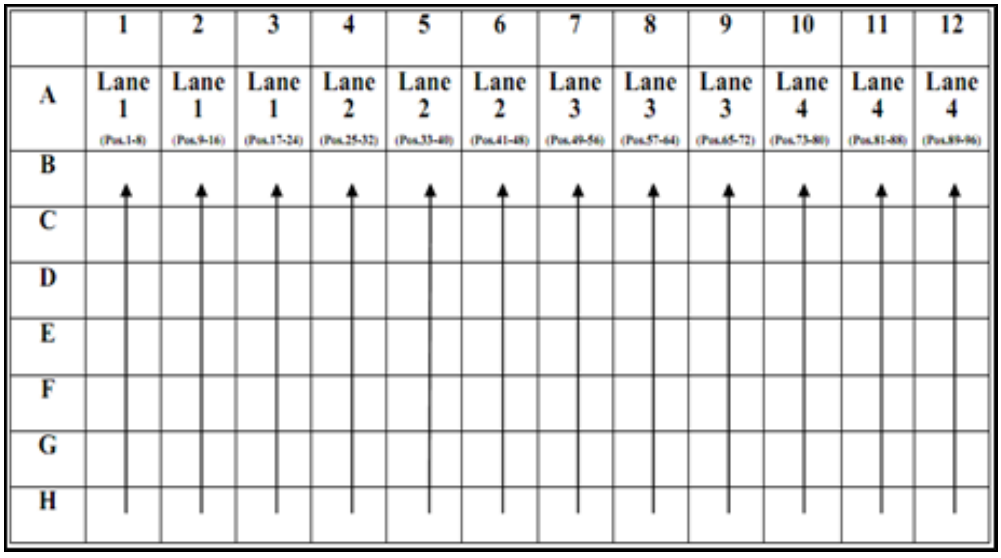


Figure.2 Patterns of visualized bands after agarose gel electrophoresis. Two internal positive controls were included: a fragment of the human β-globin gene (90 bp) or IL-2, IL-4, IL-6, and IL-10, or the human C- reactive protein gene (440 bp) for IL-1α, IL-1β, IL-1R, IL-1Ra, IL-4Rα, IL-12, IFN-γ, TGF-β and TNF-α

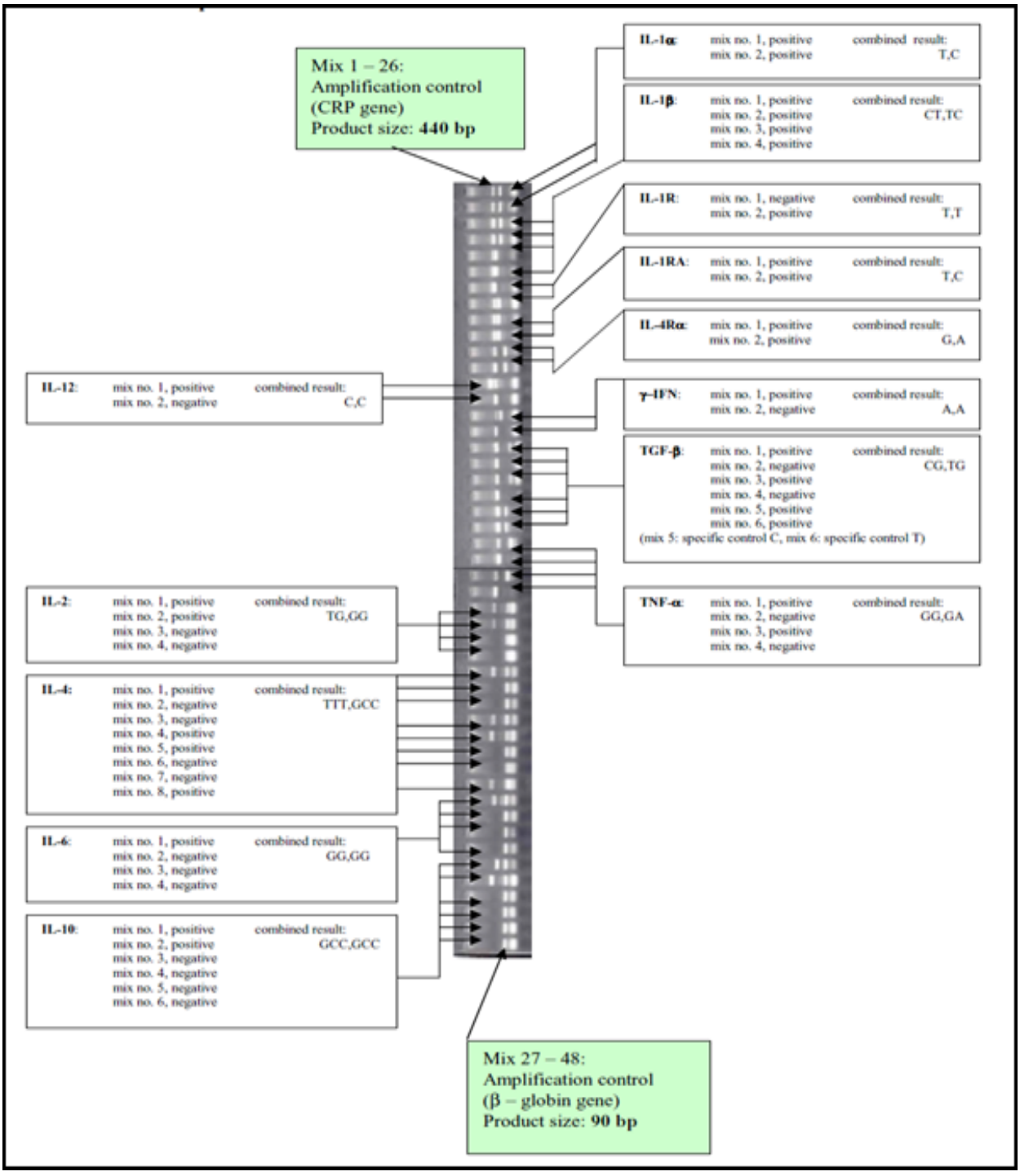
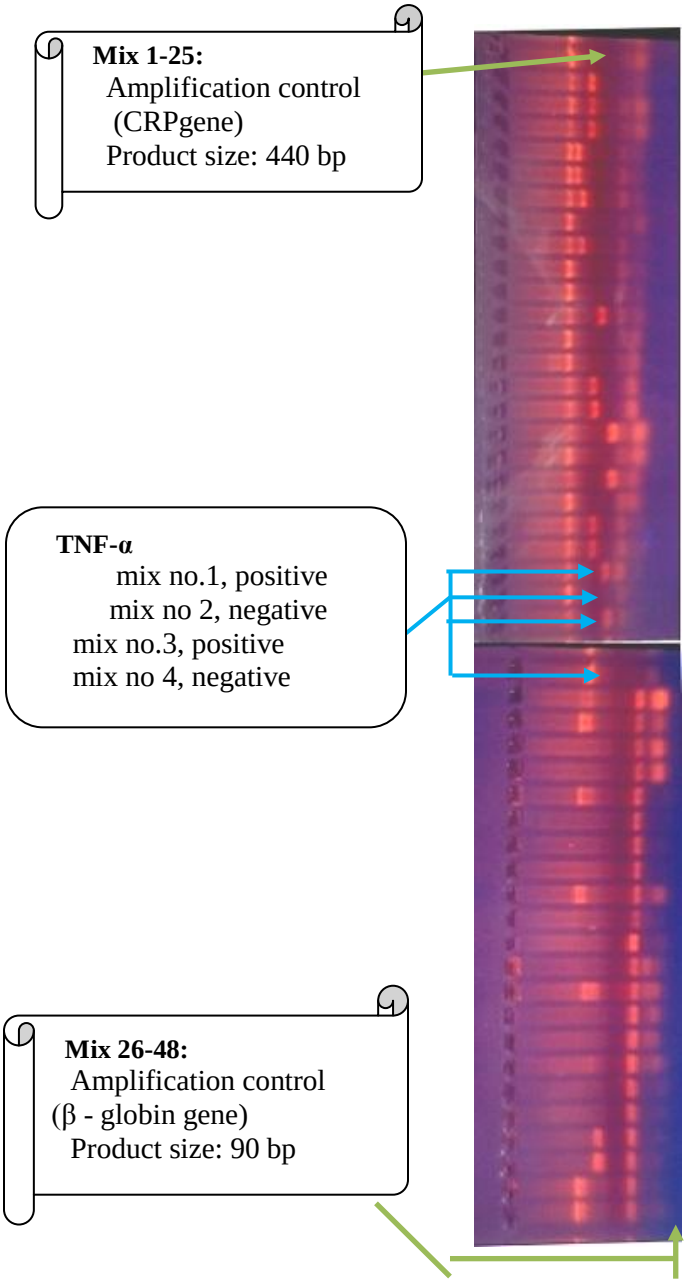


Figure.3 Agarose gel electrophoresis patterns of cytokine amplified gene regions visualized by UV trans-illumination (internal controls are also given)



Tumor Necrosis Factor Gene (*TNFα*$_{-238}$)

At the position -238 of *TNF* gene, there were no significant differences between the observed and expected frequencies of *TNF*$_{-238}$ genotypes in toxoplasmosis patients or controls; therefore an agreement with H-W equilibrium was established. However, it was noticed that GA genotype frequency was significantly increased in patients compared to controls (P = 0.037). Such positive association scored RR of 2.30 and the EF was 0.28 (Tables 3 and 4).

Tumor necrosis factor-α gene was further implicated in the etiopathogenesis of toxoplasmosis, *TNF*$_{-238}$ GA genotype was observed to have a significantly increased

frequency in patients compared to controls, and the RR of such positive association was 2.30, and therefore an increased risk to develop toxoplasmosis might be associated with such heterozygous genotypes. As early as 1992, it was recognized that resistance to toxoplasmic encephalitis in inbred mice might be associated with specific alleles in *TNF* gene, and susceptible mice expressed elevated levels of TNF-α mRNA in brain tissue as early as six weeks post-infection with *T. gondii*.

However, resistant mice and all mice that are uninfected showed no detectable TNF-α mRNA expression in brain tissue. Such differences in the *TNF* gene between susceptible and resistant mice were localized to the first intron, the promoter, and the 3' end of the gene. Their data suggested that differences in regulation of TNF-α production in brain tissue could contribute to differences in toxoplasmic encephalitis susceptibility (Freund *et al.*, 1992). In contrast to the latter study and the present study, (Cordeiro *et al.*, 2008) reported no significant variations between genotypes or alleles of TNF_{-308} SNP and toxoplasmic retinochoroiditis in Brazilian patients. Although, there is some inconsistency in these results, the role of TNF-α in pathogenesis of toxoplasmosis cannot be underestimated (Butler *et al.*, 2013), and certainly further investigations are required to reach a better profile of *TNF* SNPs in etiopathogenesis of toxoplasmosis.

Genetic polymorphisms of *TNF* genes have also been investigated in RSA, and variants in the *TNF* promoter region were implicated in the RSA pathogenesis. Four studies revealed that TNF_{-308} SNP was associated with RSA (Reid *et al.*, 2001; Daher *et al.*, 2003; Alkhuriji *et al.*, 2013; Parveen and Agrawal, 2013), while two further studies did not confirm the association with RSA (Lee *et al.*, 2013; Piosik *et al.*, 2013). This discrepancy was suggested that might be due to racial differences in genotype frequencies of this SNP.

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