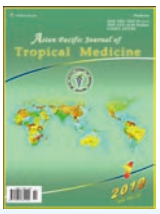

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Diagnosis of *Toxoplasma gondii* infection in pregnant women using automated chemiluminescence and quantitative real time PCR

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ABSTRACT

Objective: To identify serodiagnosis and quantification of *Toxoplasma gondii* (*T. gondii*) infection among pregnant women in Salmas, northwest of Iran. **Methods**: In this cross-sectional study, 276 blood samples were collected from pregnant women referred to the health care centers in Salmas city. The demographic variables were also recorded. Titers of anti-*Toxoplasma* IgM and IgG antibodies (Ab) were determined using the chemiluminescence immunoassay. Quantitative real-time PCR targeting the *T. gondii* repeated element gene was also performed on the blood sample. **Results**: Out of all, 19.92% (55/276) and 2.17% (6/276) patients were seropositive for anti-*Toxoplasma* IgG and IgM Ab, respectively. Moreover, the presence of *T. gondii* DNA was observed in 12.31% (34/276) blood samples. A significant relationship was observed between the IgG Ab seropositivity and contact with the cat as a risk factor (P=0.022). **Conclusions**: The seroprevalence rate of *T. gondii* infection in pregnant women is relatively low. Consequently, the seronegative pregnant women are at risk, and a considerable rate of positive blood samples for the presence of parasite's DNA should not be ignored. Besides, quantitative real-time PCR could be considered as an accurate method for diagnosis of acute toxoplasmosis especially when the precise results are of the most importance in pregnancy. Limiting contact with cats is also suggested for pregnant women.

1. Introduction

Toxoplasma gondii (*T. gondii*), an obligate intracellular parasite, infects almost all warm blooded animals and is of great importance when cause infections in humans. Toxoplasmosis is highly prevalent infection throughout the world and *T. gondii*

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infection is considered as a serious public health problem, with more than 30% of the human populations infected[1,2]. The parasite can infect humans by ingesting undercooked or raw meat containing tissue cysts of the parasite. Furthermore, it can be also transmitted by eating fruits, vegetables and other food sources contaminated by the parasite's oocysts. The soil is also reported to be a potential source of human infections[2,3].

T. gondii infection is mostly mild and asymptomatic, however, it can cause severe complications to the fetus or immunocompromised humans. *T. gondii* can transmit the infection during pregnancy to the fetus through placenta and cause congenital toxoplasmosis[2,4]. Therefore, pregnant women, as well as children with congenital infection, are important risk groups. In seronegative pregnant women, acquired primary *T. gondii* infection may affect pregnancy and leads to miscarriage or fetal disorders[5,6]. Acute toxoplasmosis in pregnant women like other healthy non-pregnant individuals is usually asymptomatic. One of the most seen clinical manifestations of acute infection is regional lymphadenopathy[2]. Chronic maternal infection usually does not affect the fetus. Although pregnant women with *T. gondii* infection generally have no symptoms, the acute infection during pregnancy can lead to potentially tragic outcomes for the fetus and newborn[2,6].

Fetus or neonate with congenital toxoplasmosis may suffer from visual defects, hearing loss, neurological complications, hematological disorders, seizures, and/or death. Encephalitis, hydrocephalus, hepatitis, microcephalus, lymphadenopathy and death are the other consequences of the congenital toxoplasmosis[7]. Generally the clinical manifestations in infected fetus depend on the maternal immune response, the parasite virulence, and the trimester of pregnancy. Hence, rapid diagnosis and treatment are essential for reducing the severity of the fetal disease[7,8].

Toxoplasma screening test for pregnant women is part of the TORCH panel (*T. gondii*, rubella, cytomegalovirus and herpes infections)[9]. The diagnosis of toxoplasmosis is complicated and challenging. Diagnosis of the infection is mostly based on the serological tests, determining concentrations of anti-*Toxoplasma* IgM and IgG antibodies (Ab) in the patients' sera. These antibodies can last for months or even years after acute infection, thus these methods cannot differentiate recent infection from past infection. In fact, the use of molecular techniques, that are sensitive and rapid, is crucial for the early diagnosis of congenital toxoplasmosis and active toxoplasmosis in pregnancy[4,8]. Therefore, the aim of the present study was to identify serodiagnosis, and quantification of *T. gondii* infection among pregnant women referred to the health care centers in Salmas city, northwest of Iran, in 2016.

2. Materials and methods

2.1. Sample collection

In this cross sectional study, 276 blood samples were collected from pregnant women referred to the health care centers in Salmas city, North West of Iran, from the 1st May 2016 to the 1st December 2016 (Figure 1). The study was approved by the Ethical Committee of Tabriz University of Medical Science (No. 94/2-5/17). All pregnant women signed informed consent for enrollment to the study. Demographic variables were collecting by interview: previous contact with cats; clinical symptoms; educational status; occupation; age; history of consuming raw and undercooked meat, unpasteurized milk or raw vegetable.

The taken blood samples were divided into two groups, one for serum isolation which was left sedentary to clot, and the other in sodium-heparin tubes for buffy coat isolation. Serum samples were isolated after centrifugation of the clotted blood at roughly 2 500 rpm for 10 min. The isolated sera were kept frozen at -20 ℃ until being examined. The buffy coat was also isolated by spinning the blood sample containing anti-coagulant at 1 500 rpm for 10 min and stored in 70% ethanol at -20 ℃ for DNA extraction.

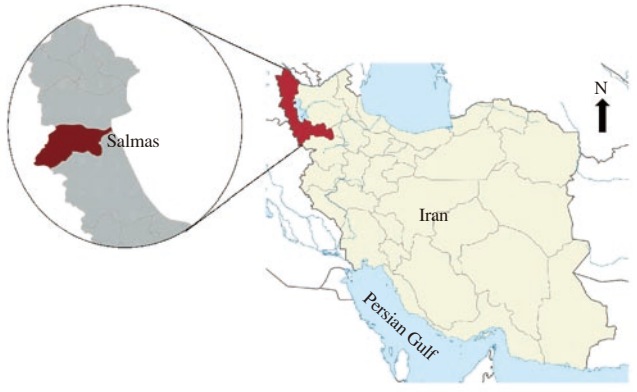

Figure 1. Geographical position of Salmas district, northwest of Iran.

2.2. Determination of anti-Toxoplasma IgM and IgG antibodies

Anti-*Toxoplasma* Abs were determined by Ab capture chemiluminescence immunoassay (CLIA) using anti-*Toxoplasma* IgG and IgM Abs CLIA kits (LIAISON® Toxo IgM and IgG, DiaSorin S.P.A, Italy) applied in LIAISON device (DiaSorin, Germany). The tests were performed by the device automatically according to the manufacturer's setting. Samples with IgM Ab titer <6 IU/mL were regarded as negative, 6 to 8 IU/mL as borderline and 8 IU/mL as positive results. Furthermore, samples with IgG Ab titer <7.2 IU/mL were graded as negative, 7.2 to 8.8 IU/mL as equivocal and 8.8 IU/mL as positive results[8]. All borderline results were twice repeated.

2.3. DNA extraction

The buffy coats were washed twice with phosphate-buffered saline to remove the ethanol, and DNA was extracted using blood genomic DNA extraction mini kit (YTA co., Iran). The extracted DNA samples were kept frozen at -20 ℃ for further use.

2.4. Quantitative-PCR

Quantitative PCR reactions (Q-PCR) were performed as previously described[10,11] in 20 μL of reaction volume with SYBR Green qPCR Master Mix 10 μL (YTA co, Iran) mixed with 1.4 μL template DNA, 7 μL distilled water and 0.8 μL of each primer at a concentration of 1 pmol/μL. The following primers were used for amplification of 164-bp fragment of repeated element (RE) gene from *T. gondii*; F 5'-AGG GAC AGA AGT CGA AGG GG-3' and R 5'-GCA GCC AAG CCG GAA ACA TC-3'. The amplification protocol was: 10 min at 95 ℃℃, 40 cycles at 94 ℃ for 30 s (denaturation), 55 ℃ for 30 s (annealing), and 72 ℃℃ for 30 s (amplification). Amplification was done by magnetic induction cycler real time PCR machine (Bimolecular system, Australia). Tubes and plastic materials were used as provided by Bimolecular system for magnetic induction cycler machine. All experiments were done in triplicate. Melting curve analysis was performed to verify the correct product size and to ensure the absence of side products or primer dimmers. Also, *Toxoplasma* DNA extracted from tachyzoites (RH strain) and water were used as the template for positive and negative control, respectively. In order to evaluate the number of parasites in the blood samples, Q-PCR threshold cycle value was used according to standard curve obtained with DNA samples from a range of serial dilutions ($1\times\times10^{7}$-$1\times\times10^{1}$ parasite/mL) of RH strain tachyzoites.

2.5. Data analysis

Data was recorded and analyzed with the SPSS v.18 software (SPSS Inc., Chicago, ILL, USA) using Mann-Whitney and *Chi*-square tests. The *P* value <0.05 was considered as significant.

3. Results

In total, 276 pregnant women, with the mean age±standard deviation of (33.1±7.5) years were enrolled in the study and screened for *Toxoplasma* infection. Out of all, 19.92% (55/276) and 2.17% (6/276) pregnant women were positive for anti-*Toxoplasma* IgG and IgM Abs, respectively. Also, 5/276 (1.81%) pregnant women were positive for both IgM/IgG Abs. Detailed information is summarized in Table 1.

Moreover, molecular analysis was performed using Q-PCR on the seropositive pregnant women (either IgG or Ig M positive) (*n*=56) for confirmation of their *Toxoplasma* infection status. *T. gondii* DNA was found in 34/276 (12.3%) blood samples. All of the six women with positive IgM Ab had parasite's DNA in their blood, detected by Q-PCR (*P*<0.001) (Table 1). Interestingly, 58.18% (32/55) of patients with positive anti-*Toxoplasma* IgG Ab had also positive result for *T. gondii* obtained by Q-PCR (*P*<0.001) (Table 2). The IgM positive (6/34) and IgG (32/34) positive individuals harbored an average (2 454.0±918.5) and (1 014.0±255.3) parasite/mL (Mean±SEM) , respectively. Although, IgM-positive individuals showed greater parasite loads; however, there was no statistically significant difference in parasite load between IgM-positive and IgG-positive women (*P*>0.05).

Table 1
Results of anti-*Toxoplasma* IgM and IgG antibodies in 276 studied pregnant women [*n* (%)].

Anti-*Toxoplasma* antibody	Pregnant women	
	Positive	Negative
IgM positive only	6 (2.17)	270 (97.83)
IgG positive only	55 (19.92)	221 (79.10)
Both of IgM and IgG	5 (1.81)	271 (98.19)
Either IgG or IgM	56 (20.30)	220 (79.70)
PCR	34 (12.30)	242 (87.70)

Table 2
Relationship between ELISA results and RT-PCR for *Toxoplasma* in 276 pregnant women.

Anti-*Toxoplasma* antibody		PCR		Total	*P*
		Positive (*n*=34)	Negative (*n*=242)	(*n*=276)	
Anti-*Toxoplasma* IgM	Positive	6	0	6	<0.001
	Negative	28	242	270	
Anti-*Toxoplasma* IgG	Positive	32	23	55	<0.001
	Negative	2	219	221	

Using the Kolmogorov-Smirnov test, none of the quantitative variables had abnormal distribution, thus for analysis of these variables, non-parametric Mann-Whitney test was used. The IgG and IgM Ab concentrations and age of the studied women were statistically significantly higher in women with positive results of *T. gondii* by Q-PCR (Table 3).

Table 3
Relationship ofmean age of studied pregnant women, anti-*Toxoplasma* IgM and IgG Abs titer and results of RT-PCR test for *T. gondii*.

Anti-*Toxoplasma* antibody	PCR	Mean rank	Mann-Whitney *U*	*Z*	*P*
Age	Positive	160.57	3 363.5	-1.726	0.084
	Negative	135.40			
IgG titer	Positive	237.53	611.0	-8.378	<0.001
	Negative	122.07			
IgM titer	Positive	168.97	2 908.0	-3.733	<0.001

Considering the demographic variables, the frequency of IgG Ab seropositivity was significantly higher among the women being in contact or having a cat as a pet (*P*=0.022). Furthermore, all the

Table 4

Relationship between different socio-demographic variables and results of anti-*Toxoplasma* IgM Ab, IgG Ab and RT-PCR.

Variable		IgG		*P*	IgM		*P*	PCR		*P*
		Positive	Negative		Positive	Negative		Positive	Negative	
Duration of pregnancy (month)	1	5	20	0.354	0	25	0.118	4	21	0.628
	2	11	58		1	68		7	62	
	3	14	58		1	71		8	64	
	4	3	15		3	15		4	14	
	5	7	33		0	40		4	36	
	6	12	23		1	34		6	29	
	7	1	11		0	12		0	12	
	8	2	3		0	5		1	4	
Occupation	Employed	23	106	0.451	0	129	0.031	15	114	0.436
	Housewife	32	114		6	140		19	127	
Education	Illiterate	1	4	0.077	0	5	0.563	0	5	0.533
	Guidance school degree	18	58		2	74		12	64	
	High school diploma	14	45		2	57		7	52	
	Associate degree	2	12		1	13		2	12	
	Bachelor's degree	11	84		0	95		8	87	
	Master's degree	9	14		1	22		5	18	
	Doctoral degree	0	2		0	2		0	2	
Consumption of undercooked meat	Yes	23	95	0.427	2	116	0.480	18	100	0.214
	No	32	124		4	152		16	140	
Consumption of raw vegetable	Yes	24	115	0.152	3	134	0.644	17	122	0.536
	No	31	104		3	132		17	118	
Consumption of unpasteurized milk	Yes	43	156	0.208	6	193	0.147	29	170	0.820
	No	12	62		0	74		5	69	
Cat contact	Yes	29	150	0.022	3	176	0.345	22	157	0.538
	No	26	69		3	92		12	83	
Symptom	Yes	4	12	0.175	1	15	0.461	4	12	0.150
	No	51	207		5	253		30	228	

six IgM Ab positive individuals were housewives (P=0.031). No statistically significant relationship was observed considering PCR result, IgG and IgM Ab seropositivity among the other studied demographic variables (Table 4).

4. Discussion

Toxoplasmosis is the ubiquitous parasitic infection. The importance of acquired toxoplasmosis is mainly in pregnancy, with the risk of severe congenital infection. Chronic maternal infection usually does not affect the fetus, however, the acute toxoplasmosis during pregnancy can lead to serious complications in fetus[5,6]. The current study investigated the prevalence of *T. gondii* infection among pregnant women referred to the health care center in Salmas city, northwest of Iran using serological and molecular methods. The results showed that 19.92% and 2.17% of the studied pregnant women were seropositive for anti-*Toxoplasma* IgG and IgM Abs, respectively. Remarkably, *Toxoplasma* DNA was detected in 58.18% seropositive pregnant women.

There are many studies on the *T. gondii* prevalence in the world[6,12]. The overall seroprevalence rate of *T. gondii* infection among the Iranian general population is estimated 39.3%[1]. In meta-analysis by Mizani *et al.*, the seroprevalence of *T. gondii* Abs using the random-effect model in the pregnant women and girls were 43% (95% *CI*=38%-48%) and 33% (95% *CI*=23%-43%), respectively[5].

Salmas is located in the northwest Iran. In the literature, the seroprevalence of *T. gondii* infection was reported as 47.00% and 45.12% in other parts of West Azerbaijan, Iran[13,14]. In the studies reported from neighboring province, East Azerbaijan, the IgG Ab seropositivity was from 35.1% to 38.66%[15,16]. In Turkey, a neighboring country of Iran, the *T. gondii* Absseroprevalence of in women of reproductive age was determined to be 58.3% for IgM Ab and 1% for IgG Ab[17]. The present study shows the low prevalence of *T. gondii* infection in pregnant women in Salmas compared to the other nearby cities.

The diagnosis of *T. gondii* infection is based on serological methods for demonstration of specific IgG, IgM, and IgA Abs[8,18]. There are several serology techniques that could be used. The enzyme-linked immunosorbent assay and indirect immunofluorescence assay are the most common methods in Iran, and recently the CLIA method has been used[15]. In the present study, screening of anti-*Toxoplasma* IgG and IgM Abs in the pregnant women was done by CLIA. This method is more sensitive, automated, reliable, and convenient technique. However,

the seropositivity for anti-*Toxoplasma* Abs is one of the most challenging situations in pregnant women[18].

Determination of active and acute toxoplasmosis in pregnant women and commencement of a sufficient anti-*Toxoplasma* treatment, if needed, can prevent the congenital toxoplasmosis and improve the prognosis. Molecular techniques, as alternative tools, have been used for detection of *T. gondii* DNA in clinical samples[4,19,20]. Currently, Q-PCR has been used for detection and quantification of *T. gondii* DNA in different clinical samples. The variation of the test performance is mostly associated with target genes and primer composition. Most investigators have used the B1 or RE gene for detection[21-23].

Hence, in the present study Q-PCR molecular technique was performed based on the RE gene of *T. gondii* for confirmation of active and acute toxoplasmosis. Using Q-PCR, the presence of *T. gondii* DNA was detected in 12.30% of patients' blood samples. All individuals with positive anti-*Toxoplasma* IgM Ab had also parasite's DNA in their peripheral blood. Interestingly, 58.18% of anti-*Toxoplasma* IgG Ab positive women had also positive results by PCR. Based on these results, Q-PCR technique could be used as a relevant confirmatory test in diagnosis of acute toxoplasmosis. Although laboratory diagnosis of toxoplasmosis is performed using serology, molecular method such as real time PCR is also important in confirmation. As a limitation in this study, the PCR was not performed on seronegative samples due to lack of funding. Hence, in future studies it is better to survey all samples using molecular method. Salehi *et al.* showed that 33.3% of the pregnant women were seropositive for *T. gondii* infection in Arak, Iran. They also found parasite's DNA in one cord blood sample after delivery, confirming congenital infection[24]. Our results showed that the parasite's DNA could be found in more than 50% of the *T. gondii* seropositive pregnant women. Furthermore, the high prevalence and parasite load are indicative as important markers for screening before and during pregnancy. Type Ⅱ strains are the most prevalent cause of human toxoplasmosis in North America and Europe. Conversely, the majority of strains isolated in Spain were type Ⅰ [25,26]. Fuentes *et al.* determined the types of *T. gondii* and its association with human toxoplasmosis using genetic analysis of the SAG2 locus[26].They showed that type Ⅰ strains were more commonly (75%) found in cases with congenital infection. Type Ⅰ strains are considered as the most virulent, fast growing and usually causing high level of parasitemia. Therefore *T. gondii* infection of pregnant women with genotype Ⅰ can lead to severe fetal abnormalities or abortion.

Further the association between *T. gondii* infection and potential risk factors were analyzed. Among the pregnant women who participated in the study, there were no significant differences considering age (P=0.084). On the other side, previous contact with cats has been shown as statistically significant risk factor for toxoplasmosis (P=0.022). These results are in agreement with previous meta-analysis data[1,5]. Surprisingly, all IgM Ab positive pregnant women were housewives (P=0.031). Our results indicate that housewives had higher risks for exposure to this parasite. Although the frequency of IgG Ab seropositivity was higher in housewives than employed women, there was no statistically significant difference between occupational groups.

In conclusion, the results of the present study show that, pregnant women in Salmas city have a low anti-*Toxoplasma* Abs seroprevalence compared to most parts of Iran. The seronegative pregnant women are considered susceptible to *T. gondii* infection and women of childbearing age are at the higher risk. Therefore, pregnant women require regular checks for seroconversion. On the other hand, considerable rate of positive blood samples for the presence of parasite's DNA should be considered. Based on the present findings, Q-PCR is an accurate method and it could be used for fast and precise diagnosis/confirmation of acute and active toxoplasmosis. Furthermore, defining of the population structure at risk for *T. gondii* infections could lead to more effective prevention strategies. Finally, limitation of the contacts with the cats is also highly suggestive to pregnant women.

Conflict of interest statement

Authors declare there is no any conflict of interest. The sponsor or funding organization had no role in the design or conduct of this research.

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