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

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**ABSTRACT**

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**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. Titters 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*...
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 °C 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 °C for DNA extraction.

#### 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 °C 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 °C, 40 cycles at 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C 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 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 °C, 40 cycles at 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 30 s (amplification). Amplification was done by magnetic induction cycler real time PCR machine (Bimolecular system, Australia).

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).

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 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 | | |
|--------------------------|-----------|---|---|
|                          | Positive (n=34) | Negative (n=242) |       |
| Anti-Toxoplasma IgM      | 6          | 0  | 6   | <0.001|
| Anti-Toxoplasma IgG      | 28         | 242| 270 |       |
| T. gondii PCR 89         | 32         | 23 | 55  | <0.001|

Table 3
Relationship of mean 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         | 332.27 | -1.726 | 0.084|
| IgG titer                | Positive      | 237.53         | 611.0 | -8.378 | <0.001|
|                          | Negative      | 122.07         | 981.9 | -8.378 | <0.001|
| IgM titer                | Positive      | 168.97         | 2908.0 | -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
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 IgG Abs seroprevalence 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,

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**Table 4**

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

| Variable                        | IgG Positive | IgG Negative | P     | IgM Positive | IgM Negative | P     | PCR Positive | PCR Negative | P     |
|---------------------------------|--------------|--------------|-------|--------------|--------------|-------|--------------|--------------|-------|
| 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  |              |              |       |
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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