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Seroprevalence and molecular detection of *Toxoplasma gondii* in young healthy blood donors in Northern Iran

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Abstract. *T. gondii* is a life-threatening infection in immunocompromised patients which may be transmitted through blood transfusion. The present study aimed to evaluate the seroprevalence and molecular detection of *T. gondii* infection and the associated risk factors among young healthy blood donors in the central part of Mazandaran province, northern Iran. Blood samples were taken from 500 participants and the serum was separated. All serum samples were tested for the presence of anti-*T. gondii* antibodies (IgG) and then all positive samples were evaluated for IgM antibodies using commercial ELISA kits. All IgM positive samples and 66 randomly selected IgG positive samples were further tested by PCR of the REP-529 gene. Anti-*Toxoplasma* antibodies (IgG) avidity test was performed for 142 IgG positive samples which were randomly selected. In the current study, anti-*T. gondii* antibodies (IgG) and (IgM) were found in 316 (63.2%) and 3 (0.95 %) participants, respectively. Seropositivity rate of *Toxoplasma* was higher among blood donors living in rural areas (P=0.000) and those with a history of soil and animal contact (P<0.05). PCR of the REP-529 gene showed *T. gondii* DNA in 21 out of 66 samples. The REP-529 gene was not detected in IgM positive samples. Low avidity antibodies (IgG) was found in 23.2% of the IgG positive samples. In conclusions, this study found that the prevalence of toxoplasmosis among young healthy blood donors in north of Iran was high. To reduce the risk of parasite transmission, leukofilteration method are recommended for donated blood used for immunosuppressed patients.

INTRODUCTION

Risk of infection through blood/ blood products transfusion is a challenge for health care systems around the world (citation). Global safety of blood components should be considered as a goal in any country, although the risk of transfusion transmitted infections have been reduced (Garraud *et al.*, 2016; Kiely *et al.*, 2017). However, screening tests to detect infective agents such as the Hepatitis B virus, Hepatitis C virus and human immunodeficiency virus (HIV) are implemented in all blood transfusion organizations throughout the world but several blood borne bacterial and parasitic infections, which can be transmitted through transfusion, remain undetected (Karimi *et al.*, 2016). Regarding parasitic infections, parasites which have an intracellular life cycle may be transmitted more frequently than other

parasites by blood transfusion (Singh & Sehgal, 2010).

T. gondii is an obligate intracellular parasite that is hosted in cats as definitive host and in all warm blooded animals as intermediate host (Tavakoli Kareshk *et al.*, 2017). It can be transmitted to human by several routes including ingestion of *T. gondii* oocysts, eating undercooked contaminated pork or beef containing tissue cysts, organ transplant, congenital and blood transfusion (Dalimi & Abdoli, 2012). *T. gondii* infection is asymptomatic in immunocompetent cases but it is life threatening in immunocompromised individuals such as acquired immunodeficiency syndrome patients and transplant recipients. It is also known that this infection can result in congenital toxoplasmosis which may lead to serious abnormalities in fetuses or newborns (Xiao & Yolken, 2015). Transmission of *T. gondii* infection to leukemic patients by packed cell was reported by a study performed more than 40 years ago by Siegel *et al.* (1971). Furthermore, recent studies demonstrated *T. gondii* DNA in the blood samples obtained from blood donors (Foroutan-Rad *et al.*, 2016). Hence, screening blood for acute *T. gondii* infection is an interesting subject for researchers because a significant proportion of the population experiencing transfusions stand at risk for toxoplasmosis.

Generally, the seroconversion of anti-*Toxoplasma* antibodies [IgG], rising antibody titre and presence of *Toxoplasma*-IgM antibodies, avidity of antibodies and molecular method are used to distinct recent and latent toxoplasmosis in clinical samples. The prevalence of anti-*T. gondii* antibodies [IgM] in healthy blood donors are reported as 2.9% in Sonora, Mexico (Alvarado-Esquivel *et al.*, 2016), 5% in India (Elhence *et al.*, 2010), 0.28% in Taiwan (Chiang *et al.*, 2012), 3.6% in Tehran, Iran (Ormazdi *et al.*, 2010) and between 0 to 5.47% in Iran (Karimi *et al.*, 2016). Furthermore, the percentage of low avidity among seropositive blood donors for anti- *T. gondii* (IgG) has been reported 0 to 16.6% (Sundar *et al.*, 2007; Elsheikha *et al.*, 2009; Chiang *et al.*, 2012; Karakas *et al.*, 2012).

The seroprevalence of anti-*T. gondii* antibodies IgG and IgM in different populations in Babol, northern Iran, were from 60.6% to 82.5% (Bayani *et al.*, 2013; Kalantari *et al.*, 2014; 2015) and 6.3%, respectively (Kalantari *et al.*, 2015). However, there is no information about the scale of *T. gondii* infection in blood donors in north of Iran. This study was conducted to evaluate the seroprevalence and molecular detection of *T. gondii* infection and the associated risk factors among young healthy blood donors in the central part of Mazandaran province, northern Iran.

MATERIALS AND METHODS

Population study and sample size

Blood samples were collected from healthy blood donors attending to Babol Blood Transfusion Organization, Babol, northern Iran, from 1 Sep 2016 to 1 July 2017. Babol is one of the most important cities in the north of Iran. It is located on the northern slopes of the Alborz Mountains and southern coast of the Caspian Sea. Babol is about 20 kilometers south of the Caspian Sea and receives abundant annual rainfall. It is very humid, and the mean annual temperature varies between 12.5 to 20°C. Its population was 219,467 in 66,944 families at the 2012 census (https://sco.wikipedia.org/wiki/Mazandaran_Province).

As prevalence of toxoplasmosis increased by age and the studied area was considered as an endemic region (Sharif *et al.*, 2016), samples were taken from individuals under 30-years old. All samples were taken from donors voluntarily accepting to participate in the study. Furthermore, seropositive participants for hepatitis B virus surface antigen (HBsAg), HIV, HCV and *T. pallidum* infection were excluded from the current study.

Socio-demographic, clinical, and behavioural data were obtained by a questionnaire through an interview. Age, gender, residence and educational level were considered as socio-demographic data, and clinical data included health status, presence or history of lympha-

denopathy, surgery, blood transfusion, or organ transplantation. Behavioural data included contact with animals and cats, soil contact, eating raw or undercooked meat, and eating uncooked milk and eggs.

The number of sample size was calculated by the sample size determination formula as described below:

$$N = \frac{Z^2 \times P(1-P)}{d^2}$$

The minimum sample size for the determination of prevalence rates of anti-*T. gondii* antibodies (IgM) was 384 if Z=1.96, P=50%, 1-P= 50% and d=0.05 were used. Power estimation was not calculated for the prevalence rate of immunoglobulin IgM in the current study.

Diagnostic techniques

Serology

Enzyme-Linked Immunosorbent Assay (ELISA)

Two mL blood samples were taken from each participant, and the serum was separated. The serum and blood cells were stored at -20°C until used. After sample collection, the frozen sera were thawed at room temperature and assessed for the presence of anti-*T. gondii* antibodies (IgG) using a commercial ELISA kit (Euroimmun, *Toxoplasma* IgG, Canada). Additionally, the sera that was positive for anti-*T. gondii* IgG antibodies were further analyzed for *anti-T. gondii* IgM antibodies by a commercially available ELISA kit (Euroimmun, *Toxoplasma* IgM, Germany). The results were interpreted according to the manufacturers' instruction.

IgG avidity test

Positive samples (n=142) for anti-*T. gondii* (IgG) antibodies which had antibody titre over 20 IU/mL were randomly selected to evaluate the avidity of the anti-*T. gondii* antibodies. Anti-*Toxoplasma* IgG avidity was measured by the *T. gondii* IgG avidity ELISA (Euroimmun, Germany), and results were interpreted according to the manufacturer's instruction.

DNA extraction and Polymerase Chain Reaction

DNA was extracted from whole blood of all IgM positive and 66 randomly selected IgG positive samples by a DNA extraction mini kit (Yektatajhiz, Iran). Also, DNA was extracted from the tachyzoites of *T. gondii* RH strain using a method described elsewhere (Kalantari *et al*., 2016) and used as positive control. The extracted DNA from patients' blood and the tachyzoites were kept at -20°C until used. PCR was carried out for all extracted DNA using a set of primers to amplify a 182 fragment of the *T. gondii* REP-529 genes. Human Bcl-2 was used as the internal control. The oligonucleotide primer pairs used in the present study were: REP-529, 5-TGTGCTT GGAGCCACAGAAG3' (F) and 5-GCAGCC AAGCCGGAAACAT3' (R) (Rahumatullah *et al*., 2012), and Bcl-2, 5'TTGCTTCAGGG TTTCATCCA3' (F) and 5'TGGCCTCTCTT GCGGAGTA3' (R) (Liu *et al*., 2014). Water was used as negative control.

Statistical analysis and ethical consideration

Chi-square, logistic regression and T-student tests were used to analyse the data by SPSS version 19.0., and a 95% confidence level and a P value of less than 0.05 were considered statistically significant. The aim of the study was explained for each blood donor, and all participants voluntarily confirm his or her willingness to participate in the study. This study was approved by the Ethics Committee of Babol University of Medical Sciences, Babol, Iran; through grant number 9441638.

RESULTS

The mean age and standard deviation of the participants were 25.16±3.1 years. A total of 488 out of 500 individuals (97.6%) were male, and 12 (2.4%) were women. Anti-*T. gondii* antibodies (IgG) were found in 316

(63.2%) members of the studied population. Three cases (0.95%) were positive for of anti-*T. gondii* antibodies (IgM). Mean and standard deviation titre of anti-*T. gondii* antibodies (IgG) were 99.33±90 IU/mL ranging from 0.1 to 267, and for IgM were 0.2±.27 IU/mL ranging from 0.01 to 1.22. The seroprevalence rate of toxoplasmosis was statistically different among various age groups as the 26-30-year-old age group had the highest level of seropositivity (P=0.002). The seropositivity rate of *Toxoplasma* was higher among blood donors living in the rural areas in comparison to those living in the urban areas (P=0.000). Blood donors with a history of soil and animal contact had a significantly higher frequency of infection than those without contact (P<0.05) (Table 1). Seroprevalence of *Toxoplasma* infection in association with other risk factors is also shown in Table 1.

Findings obtained from IgG-avidity test showed that 23.2% (33 out of 142) of the IgG positive blood donors had low avidity. The mean of IgG titer was significantly different among low and high IgG-avidity groups. There was no meaningful difference between the mean of IgG titre in link with age (Table 2). Furthermore, low avidity antibodies were more prevalent in cases living in urban areas (20 out of 33) in comparison with participants living in rural regions (13 out of 33). This difference was not statistically significant (P= 0.09).

Results obtained from PCR of REP-529 gene showed that *T. gondii* DNA was found in 21 out of 45 samples (46.7%). Figure 2 shows of PCR analysis of *T. gondii* REP-529 gene from whole blood of IgG positive samples.

DISCUSSION

Toxoplasmosis is considered a blood-borne disease and can be of critical importance in blood recipients, mainly in immuno-suppressed cases, multiple transfusion recipients and transfusion-dependent patients (Khurana & Batra, 2016). The absence of entirely effective therapies and the lack of safe and effective vaccines are essential reasons in making efforts to reduce toxoplasmosis transmission. However, leukoreduction and leukodepletion techniques have been developed to minimize febrile nonhemolytic transfusion reactions. These methods can also reduce blood-borne parasite infections such as *T. gondii* but it is not practically possible to implement this programme, especially in developing countries and other under-resourced nations (Sharma & Marwaha, 2010).

The current study found that 63.2% and 0.95% of the participants were positive for anti-*T. gondii* antibodies (IgG) and for both IgM and IgG, respectively. Compared to other regions of Iran, the overall seroprevalence was higher than reports from blood donors from Gonbad (Ferdowsi *et al*., 2013), Rafsanjan (Zainodini *et al*., 2014), Kerman (Mahmoudvand *et al*., 2015), Shiraz (Shaddel *et al*., 2014), Fars province (Sarkari *et al*., 2014) and Tehran (Ormazdi *et al*., 2010), where the seroprevalences vary from 18.3% to 56.4%. It was also higher than the published data of blood donors from other countries including Taiwan (Chiang *et al*., 2012), New Zealand (Zarkovic *et al*., 2007), Turkey (Eser & Yay, 2006), Egypt (Elsheikha *et al*., 2009) and India (Elhence *et al*., 2010), where the seroprevalences vary from 9.3% to 51.8%. The high seroprevalence in blood donors may possibly be a result of differences in climate conditions, sociocultural habits, and transmission routes or other factors in the studied populations (Mansouri *et al*., 2017). However, the studied area has the suitable conditions which are essential for sporulation of oocytes such as sufficient aeration, humidity, and warm temperature. Our results revealed that significant risk factors for *T. gondii* seropositivity were consumption of raw vegetables, contact with animals and soil, which exhibits that the ingestion of oocysts was the main route of infection among the blood donors in the present study. These findings were supported by other studies indicating that oocyst ingestion is one of main routes of human infection. It has been well

Table 1. *T. gondii* seroprevalence and related risk factors among healthy blood donors in Northern Iran

Variable	N (%)	Seropositivity (%)	Odds ratio	95% CI	P-value
Gender					
Male	488 (97.6)	308 (63.1)	1.17	0.347–3.936	0.53
Female	12 (2.4)	8 (66.7)			
Age Group					
18-22	119 (23.8)	56 (54.6)	2.16	1.341–3.478	0.002
22-26	183 (36.6)	108 (59)	1.8	1.176–2.771	0.007
26-30	198 (39.6)	143 (72.2)			
Cat contact					
Yes	20 (0.4)	15 (75)	0.56	0.200–1.568	0.52
No	480 (99.6)	301 (62.7)			
Animal contact					
Yes	77 (15.4)	56 (73.4)	0.598	0.349–1.025	0.038
No	423 (84.6)	260 (61.5)			
Soil contact					
Yes	220 (44)	159 (72.3)	0.49	0.336–0.715	0.000
No	280 (56)	157 (56.1)			
Education					
Under Diploma	109 (21.8)	75 (68.8)	0.73	0.463–1.146	0.12
Upper Diploma	391 (78.2)	241 (61.6)			
Location					
Urban	306 (61.2)	173 (56.5)	2.2	1.457–3.189	0.000
Rural	194 (38.8)	143 (73.7)			
Blood groups					
A	148 (29.6)	91 (61.4)	0.97	0.506–1.864	0.93
B	141 (28.2)	81 (57.4)	1.15	0.597–2.208	0.68
O	160 (32)	113 (70.6)	0.65	0.334–1.244	0.19
AB	51 (10.2)	31 (60.8)			
History of blood transfusion					
Yes	32 (6.4)	20 (62.5)	1.03	0.493–2.164	0.536
No	468 (93.6)	296 (63.2)			
Raw vegetable					
Yes	404 (80.8)	263 (65.1)	0.66	0.421–1.038	0.047
No	96 (19.2)	53 (55.2)			
Raw meat					
Yes	48 (9.6)	34 (53.1)	0.68	0.356–1.310	0.16
No	452 (90.4)	282 (64.7)			
Raw milk/eggs					
Yes	83 (16.6)	52 (62.7)	1.029	0.632–1.675	0.501
No	417 (83.4)	264 (63.3)			

established that in environmental conditions with sufficient aeration, humidity, and warm temperature oocysts sporulate and become infective within 1-5 days. These oocysts of *T. gondii* are very resistant to environmental conditions and can survive in moist soil or sand for up to 18 months (Tenter *et al*., 2000).

Results obtained from the present study showed that the seroprevalence of *T. gondii* in female donors was not significantly higher than that in males. Similar findings

Table 2. Anti-*Toxoplasma* IgG avidity in the sera of IgG-positive blood donors in association with age and Anti-*Toxoplasma* IgG titer, in Northern Iran

Avidity	Low	Border line	High	95% CI	P-value
Number (%)	33/142 (23.2)	9/142 (6.3)	100/142 (70.4)	–	–
IgG titre	184±43	189.4±45.2	159.1±68.5	-50.04259-0.20368	P=0.00*
Age	25.5±3.1	25.7±3.5	25.2±3.1	-1.55766-0.92856	P=0.63*

* P value reffer to comparison of IgG titre and age among low and high avidity groups.

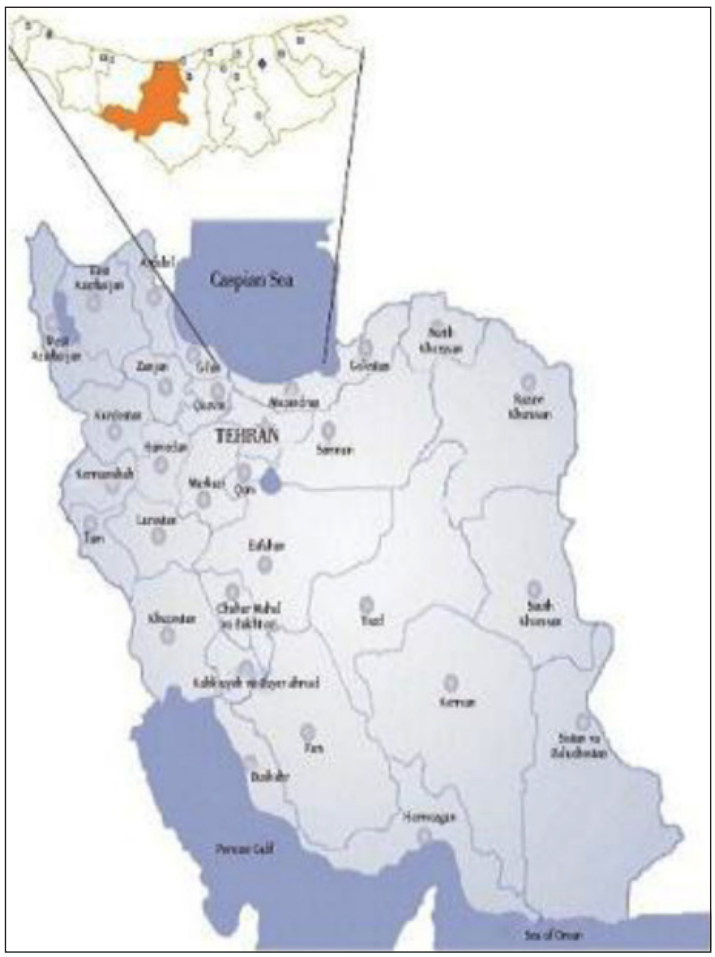

Figure 1. Geographical locations of Babol in Mazandaran province where this study was carried out. This map was adapted from https://sco.wikipedia.org/wiki/Mazandaran_Province and https://en.wikipedia.org/wiki/Babol.

were obtained by other studies conducted by some researchers (Sarkari *et al*., 2014; Zainodini *et al*., 2014; Ormazdi *et al*., 2010). But, it was in contrast to the results of others (Elhence *et al*., 2010; Mahmoudvand *et al*., 2015). In the present work, similar to other studies conducted in blood donors, there were a small number of female donors, and therefore the results should be confirmed on a larger sample size. Furthermore, this

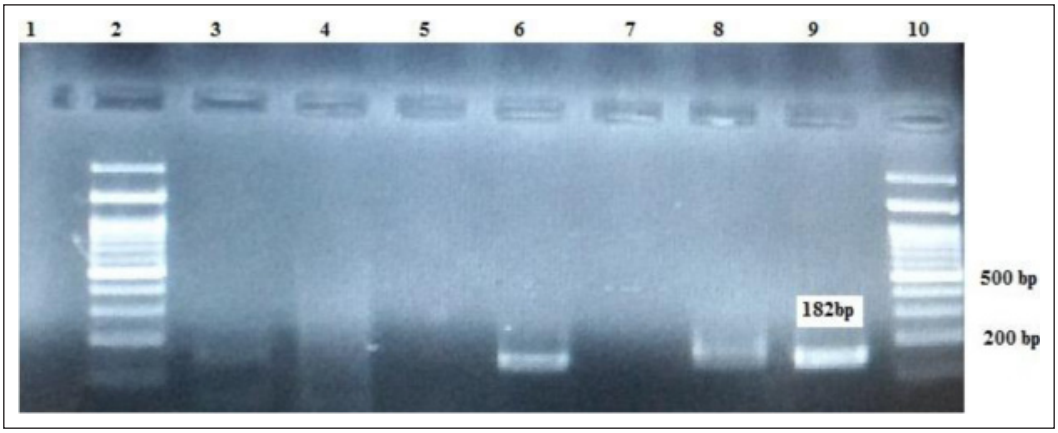

Figure 2. Gel electrophoresis of PCR product of *T. gondii* REP-529 gene from whole blood of cases having *anti-T. gondii* IgG antibodies. Lanes: 1&10, negative control; 2, 100 bp ladder DNA size marker; 3-8 samples number 3, 1, 2, 6, 11; 16; 9, positive control (*T. gondii* RH strain).

research showed that the seropositivity rate was significantly increased by age. This finding was in agreement with the results of other studies (Sarkari *et al*., 2014; Alvarado-Esquivel *et al*., 2016). Additionally, it was found that the seroprevalence rate of *T. gondii* was significantly higher in the donors living in rural areas than those living in urban regions. This noteworthy difference could be attributed to occupational activities including farming and gardening which relate to contact with animals and lower hygienic lifestyle levels as described elsewhere (Elhence *et al*., 2010; Mahmoudvand *et al*., 2015; Sharif *et al*., 2010). Furthermore, the present study identified that soil and animal contact, and the consumption of raw vegetables, the potential risk factors for acquiring toxoplasmosis, are linked with the seropositivity of *T. gondii*. Moreover, no relationship was found between raw/half cooked meat consumption, raw milk/egg consumption, education level, blood groups and blood transfusion and the presence of anti-*T. gondii* antibodies. There is controversial data on the association of these factors and seropositivity of *T. gondii* in blood donors in literature.

Moreover, the IgG avidity test and then PCR of REP-529 gene were performed to identify possible recent infections of toxoplasmosis in blood donors and the risk of transmission by blood transfusion. The avidity of IgG antibodies against *T. gondii* separate the low-avidity (LA) antibodies produced in the initial phase of the infection from the high-avidity (HA) antibodies produced in chronic infection first described by Hedman *et al.* (1989). The avidity test value in pregnancy and new-borns is being established by several studies (Villard *et al*., 2016; Fonseca *et al*., 2017; Laboudi & Sadak, 2017). The IgG avidity detection in blood donors was evaluated by few studies (Chiang *et al*., 2012). The results obtained in this assay revealed that 23.2% (33/142) of the selected samples for the IgG avidity test had LA antibodies which represent new infections.

Additionally, molecular analysis has developed to detect *Toxoplasma* infection in several body fluids, including blood that could detect the presence of circulating parasites which may lead to the diagnosis of primary, reactivated or chronic toxoplasmosis (Liu *et al*., 2015). The results obtained from PCR analysis of REP-529 gene showed that 31.8 of the samples were positive for *T. gondii* DNA. The presence of intermittent parasitemia with low parasite burden in patients with latent toxoplasmosis, and the over expecting of the period of parasitemia in the chronic phase of the disease are possible explanations (Kompalic-Cristo *et al*., 2007). Although

the PCR analysis was repeated for some of samples and the same outcomes were obtained but the possibility of the false positive results cannot be ruled out. Therefore, a more sensitive molecular method such as real time PCR is suggested (Liu *et al*., 2015).

However, limitations of the present study were that the avidity test and PCR analysis were not performed for all seroposite individuals. Furthermore, the PCR positive samples were not evaluated by mouse bioassay or cell culture and qPCR.

In conclusion, this study found that *T. gondii* infection had high prevalence among young healthy blood donors under 30 years old in the north of Iran with an overall seroprevalence rate of 62.8%. Moreover, it showed that the seroprevalence of anti-*Toxoplasma* IgM was low and the ingestion of oocysts was the main route of *Toxoplasma* infection in young blood donors in this region. To reduce the risk of parasite transmission, leukofilteration method is recommended for donated blood used for immunosuppressed patients.

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Conflict of interest

The authors announce that there is no conflict of interests.

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