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Original paper

High occurrence of *Toxoplasma gondii* infection among blood donors in Ardabil Province as main focus of zoonotic visceral leishmaniosis, northwestern Iran

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ABSTRACT, Toxoplasmosis, as cosmopolitan parasitic disease, is considered as one of the transfusion-transmitted parasites. The true burden of *Toxoplasma gondii* (*T. gondii*) amongst blood donors remains undisclosed around the world. Since there was no evidence on the prevalence of *T. gondii* in blood donors in Ardabil Province, as main focus of zoonotic visceral leishmaniosis (ZVL), northwestern Iran, current research was therefore conducted to estimate the seroprevalence of *T. gondii* and PCR assay among them for the first time. In the present study, 462 plasma samples from asymptomatic blood donors of Ardabil Province, northwestern Iran, were tested for IgM and IgG anti-*T. gondii* antibodies levels using ELISA test. Moreover, the buffy coat of all seropositive subjects was screened for *T. gondii* DNA by conventional PCR. Also, the data sheet consisting of characteristic information was registered for all the applicants. Overall, anti-*T. gondii* antibodies were found in 36% (166/462) of asymptomatic blood donors. Anti-*T. gondii* IgM and IgG seroprevalence was 1.5% and 32.5%, respectively. Only nine subjects (2%) were found to be positive for both IgM and IgG. Moreover, *T. gondii* DNA was identified in 18% (30/166) of seropositive donors. The logistic regression analysis showed a significant correlation between *T. gondii* seropositivity and contact with cats, agricultural activities, history of consumption of undercooked meat and being non-educated (P=0.001). The high prevalence (about one-third) of anti-*T. gondii* antibodies and possibly active infection using conventional PCR test represents that asymptomatic carriers of *T. gondii* are quite common in the study areas and pose a potential threat to the blood safety and hemovigilance program.

Keywords: *Toxoplasma gondii*, seroprevalence, PCR, blood donors, Iran

Introduction

Toxoplasmosis, a cosmopolitan disease, is caused by *Toxoplasma gondii* (*T. gondii*) protozoan in humans and a wide range of animals [1–3]. One-third of the world's population is believed to be infected with this parasite. But it rarely induces serious symptoms in immunocompetent humans [1,4]. Humans are infected with *T. gondii* mostly through oral transmission, congenital transmission, organ transplantation, and blood transfusion [5]. Therefore, *T. gondii* is a transfusion-transmissible pathogen and often blood donors with immunoglobulin M (IgM) positive serum can be a risk factor for the susceptible humans and various animal recipients [4,6]. In this regard, according to a systematic review and meta-analysis of the prevalence of toxoplasmosis in Iran, organ transplant recipients were more at risk than other human hosts studied [7]. *T. gondii* could be viable

for several weeks in stored blood at 5°C [8]. Accordingly, the preservation of blood bags in the cooling chain can not prevent the transmission or delay of infection. Toxoplasmosis has a poor prognosis and can during the acute stage cause serious complications such as pneumonitis, encephalitis, myocarditis and even death in immunocompromised individuals, particularly in AIDS patients [9–12]. Chronic asymptomatic toxoplasmosis is intensely related to autoimmune diseases [13]. Also, infection with this parasite during pregnancy will create complications such as hydrocephalus, microcephaly, mental retardation, brain calcification, jaundice, blindness, abortion and fetal death [14]. Serology methods such as indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) are routinely used to diagnose toxoplasmosis in order to determine the specific antibodies *T. gondii* [1]. The prevalence of this infection varies depending on age, eating habits and geographical regions in Iran, and it was approximately 18% to 70% [15].

According to the results of a meta-analysis study, the prevalence of *T. gondii* in blood donors was estimated at 33% worldwide [4]. On the average, *T. gondii* prevalence in Iranian blood donors ranged from 12.3% to 52.8% [16]. There is limited information available on the global true burden of *T. gondii* infection among blood donors. Unfortunately, screening for this parasite in blood banks is not obligatory worldwide; therefore, there was no evidence of the prevalence of *T. gondii* in blood donors in Ardabil Province, as old focus of zoonotic visceral leishmaniosis (ZVL) [17], in the Northwest of Iran. ZVL is highly endemic in the province [17] and Asfaram et al. [18] showed 3.8% of blood donors were seropositive in the province. This has led to neglect of *Toxoplasma* infection in the blood donors of this region. Thus, the current research attempts to determine the prevalence and risk factors of *T. gondii* using ELISA and approve the *T. gondii* DNA in blood donors using polymerase chain reaction (PCR).

Materials and Methods

Sample collection

Between July 2017 and September 2017, the present study was conducted in five blood transfusion centers including Ardabil as a core unit and Meshginshahr, Pars Abad, Khalkhal, and Namin districts as non-core (by mobile unit) in Ardabil Province, northwestern Iran. Peripheral blood samples were gathered from 462 adult donors (449 males and 13 females; 18–65 years old) without the history of common viral and bacterial infections such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and syphilis, as well as human T-lymphotropic virus (HTLV) type 1 and type 2. Each donor completed a questionnaire such as data on gender, age, residential place, blood group, educational level, contact with cats, agricultural activity and history of consumption of undercooked meat, after being agreed by the Research Ethics Committee at Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1398.536). From each donor, a 3–5 ml blood sample was obtained, into vacutainer tubes containing ethylene diamine tetraacetic acid (EDTA), and immediately centrifuged at 5000 rpm for 5–10 min. Plasma was used for detecting *Toxoplasma*-specific IgM and IgG antibodies and buffy coat was transferred into 1.5 ml microtubes at −20°C for PCR assay.

Serological assay

All plasma was screened for anti-*T. gondii* IgM and IgG antibodies using a commercial ELISA kit (EUROIMMUN, Germany) according to the manufacturer's instructions. The value of the index was gained for both IgG and IgM. The value ≤ 0.8 IU/ml was considered to be a negative result, the value greater than 1.1 IU/ml was considered to be a positive result for both IgG and IgM.

PCR assay

As previously stated by Fakhar et al. [19], the phenol-chloroform extraction technique was used to extract DNA from the buffy coat of each seropositive sample. The conventional PCR was set up for detection of *T. gondii* DNA in an absolute volume of 25 µl, containing 12.5 µl of 2x Master Mix (Fermentas, Hundred pM),1 µl of each 20-picomole of forward primer TOXOF (5-CAGGGA GGAAGACGAAAGTTG-3) and TOXOR (5-CAG ACACAGTGCATCTGGATT-3), 6 µl of DNA, and 4.5 µl sterile water. The PCR program is followed by a 30 cycle thermal cycler (Bio-Rad CFX96, USA), set at 94°C for 5 min, 94°C at 30 s, annealing at 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. In all experiments, positive controls including *T. gondii* DNA (RH strain) and negative control were free of DNA. 10 µl of the PCR amplicons were run on 1.5% agarose gel

Table 1. Demographic data and risk factors associated with *Toxoplasma gondii* seroprevalence among blood donors in Ardabil Province, northwest Iran

Variables	No. of examined (%)	No. of IgG positive (%)	No. of IgM positive (%)	No. of IgM and IgG pos. (%)	Odds ratio 95% CI	*P*-value
Male	449 (97.2)	144 (32)	3 (0.6)	9 (2)	0.26 (0.3–2.26)	0.19
Female	13 (2.8)	6 (46)	4 (30.8)	0 (0)	1	
Age group (years)						
18–30	112 (24.3)	34 (30.3)	4 (3.6)	3 (2.7)	0.99 (0.23–4.23)	0.61
31–40	185 (40)	55 (29.7)	1 (0.5)	5 (2.7)	1.78 (18.01–17.55)	0.99
41–50	100 (21.7)	43 (43)	2 (2)	1 (1)	0.66 (0.04–10.68)	0.76
> 50	65 (14)	18 (27.7)	0 (0)	0 (0)	1	
Residential place						
Urban	141 (30.5)	42 (29. 8)	2 (1.4)	3 (2.1)	1.14 (0.28–4.63)	0.85
Rural	321 (69.5)	108 (33.6)	5 (1.6)	6 (1.9)	1	
Districts						
Meshginshahr	154 (33.3)	48 (31.2)	2 (1.3)	3 (2)	0.53 (0.05–5.17)	0.58
Namin	50 (10.9)	19 (38)	0 (0)	1 (2)	0.52 (0.03–8.42)	0.64
Pars Abad	117 (25.3)	32 (27.3)	3 (2.6)	4 (3.4)	0.30 (0.33–2.70)	0.25
Khalkhal	45 (9.7)	13 (28.9)	0 (0)	0 (0)	0.47 (0.03–7.74)	0.59
Ardabil	96 (20.8)	38 (39.6)	2 (2)	1 (1)	1	
Blood group						
A	180 (39)	54 (30)	4 (2.2)	5 (2.8)	2.17 (0.42–11.52)	0.90
B	86 (18.6)	33 (38.4)	1 (1.2)	2 (2.3)	1.82 (0.25–13.23)	0.54
AB	41 (8.9)	16 (39)	0 (0)	0 (0)	1.87 (0.16–21.93)	0.61
O	155 (33.5)	47 (30.3)	2 (1.3)	2 (1.3)	1	
Agricultural activities						
Yes	341 (73.8)	124 (36.4)	6 (1.8)	7 (2.1)	3.14 (1.18–10.60)	0.001
No	121 (26.2)	26 (21.6)	1 (0.8)	2 (1.6)	1	
Contact with cats						
Yes	301 (65.2)	126 (41.9)	7 (2.3)	9 (3.0)	7.42 (4.18–13.20)	0.001
No	161 (34.8)	24 (14.9)	0 (0)	0 (0)	1	
Educational levels						
Educated	171 (37.1)	22 (12.9)	1 (0.58)	1 (0.6)	10.25 (8.18–23.20)	0.001
None-educated	291 (62.9)	128 (44.0)	6 (2.1)	8 (2.7)	1	
History of consumption of undercooked meat						
Yes	281 (60.8)	138 (49.1)	6 (3.6)	9 (6.5)	14.15 (9.18–23.10)	0.001
No	181 (39.2)	12 (6.6)	1 (8.3)	0 (0)	1	
Total	462	150 (32.5)	7 (1.5)	9 (1.9)		

containing SYBR Green and the results were recognized by the PCR Gel Documentation System. Positive samples were expected to have a product size of 529-base pair.

Data analysis

The statistical program IBM SPSS version 20 was used to process the data. The frequency of the dependent variables was thoroughly explained. The relationship between variables and seropositivity of *T. gondii* in blood donors was compared using Chi-squared and Fisher exact tests ($P<0.05$). The logistic regression model was used to calculate odds ratios and confidence intervals (CI 95%) among the variables.

Results

Demographic characteristic

In total, 462 eligible blood donors were enrolled in this study. The mean age of the participants was 39.3±11.7 years, with the youngest and the oldest being 18 and 65 years, respectively. The majority of blood donors were male (97.2%) and 31–40 years of age. The blood group A was found in the majority of the donors (39%). The full demographic characteristics and associated risk factors are briefly listed in table 1.

ELISA test

The overall prevalence of anti-*T. gondii* antibodies were identified in the plasma of 166 (36%) out of 462 blood donors that 150 (32.5%) were seropositive only for IgG, 7 (1.5%) were seropositive only for IgM, and among these 9 (2%) cases were positive for both IgM and IgG. Totally, considering the living place of the donors, serum levels of IgG and IgM anti-*T. gondii* was in Meshginshahr 34.4% (53/154), Namin 40% (20/50), Pars Abad 33.3% (39/117), Khalkhal 28.9% (13/45) and Ardabil 42.7% (41/96) districts. However, the highest and lowest infection rates were in Ardabil and Khalkhal, respectively.

Contact with cats (P=0.001), agricultural activities (P=0.001), history of consumption of undercooked meat and being non-educated (P=0.001) were the risk factors significantly associated with *T. gondii* seropositivity. There were no statistically significant differences between *T. gondii* infection and other variables (P>0.05) (Tab. 1).

PCR test

Out of 166 seropositive donors, *T. gondii* DNA was detected in 18% (30/166) of them, which was identified as 529 bp band by PCR method (Fig. 1). Of these, 28.6% (2/7) donors, with IgM positive antibody and 16.7% (25/150) donors with IgG positive antibody and 33.3% (3/9) with both IgM and IgG positive were positive by this method.

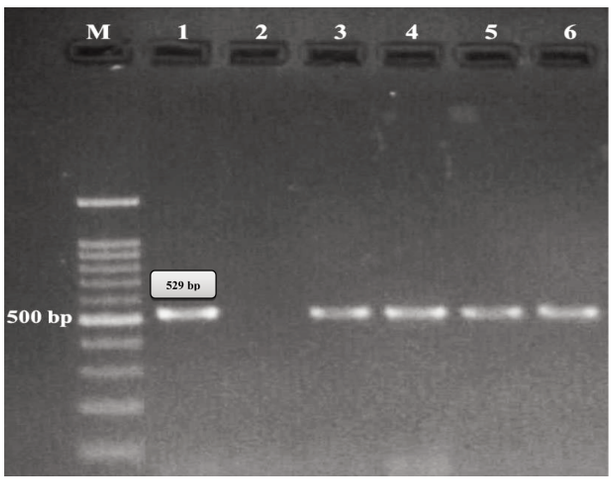

Figure 1. 2% agarose gel electrophoresis of PCR products from buffy coat DNA of blood donors. M: standard marker (100 bp), lane 1: positive control (*Toxoplasma gondii*; RH strain; 529 bp), lane 2: negative control (H_2O), lanes 3–6: seropositive blood donors

Discussion

Both anti-*T. gondii* antibodies found in the blood donors suggest *Toxoplasma* infection can be a potential risk for subjects with suppressed immune systems, including thalassemia and hemodialysis patients or children with aplastic anemia that are recipients of whole blood or blood components [8,20]. Therefore, assessment of both IgG and IgM anti-*T. gondii* antibodies in blood donors are very valuable. The current study is the first investigation to assess the serological and molecular prevalence of *T. gondii* infection amongst blood donors in different geographical areas of Ardabil province, particularly rural areas, northwestern Iran. According to our results, of the total 36% seroprevalence positive donors, 1.5%, 2% and 32.5% of blood donors were found seropositive for only IgM, both IgM and IgG, and only IgG antibodies respectively, which indicated a significant prevalence of *T. gondii* antibodies among blood donors. The IgG positive results obtained in this study were comparable to those obtained in other studies from most areas in Iran, including Saki et al. [21] in Ahvaz county (34.4%), Sadooghian et al. [22] in Razavi, Khorasam province (37.5%), Zainodini et al. [23] in Rafsanjan city (34 %), Bahhaj et al. [24] in Tabriz city (38.6%), and Hazrati Tappeh et al. [25] in Urmia city, northwest of Iran (37.8%), whereas reported a low seroprevalence of the infection by Moshfe et al. [26] in Boyer-Ahmad county (16.3%), Sarkari et al. [27] in Fars province (12.3%), Modrek et al. [28] in Zahedan city (25%) and Mahmoudvand et al. [29] in Kerman (28.8%). A meta-analysis study on blood donors worldwide found that Asia (29%; 95% CI 23%–35%) and Africa (46%; 95% CI 14%–78%) had the lowest and highest seroprevalence of

Toxoplasma infection, respectively; also, among different countries, Brazil (75%) and Ethiopia (73%) had the highest seropositive antibody, and Namibia had the lowest (1%) [4].

Dissimilarities in *T. gondii* seropositivity rates around the world can be attributed to various risk factors such as geographical conditions, food habits, and climate because *T. gondii* oocysts cannot live in cold climates for long periods of time, resulting in a lower prevalence of this parasite in cold highland environments [26] .

Various studies exhibited a significant association between *T. gondii* infection and several variables such as gender, living place and blood group, and contact with cats, etc. [27–33].

In the present study, data analysis showed that seroprevalence of *T. gondii* was not statistically different between male and female (P=0.19). A similar finding was reported previously [27,34]. The small number of women enrolled in this study may be due to the fact that Iranian women are infrequently involved in blood donation due to cultural issues and their beliefs. Also, the present study identified a significant relationship between contact with cats (P<0.0001) and the risk of *T. gondii*. This finding is agreed with previous research [4,29,34].

The risk of *T. gondii* was also significantly (P<0.0001) higher in non-educated subjects and those who had agriculture activities and also a history of consumption of undercooked meat. We believe that these significant differences could be attributed to lower socioeconomic level and frequent exposures to soil, lack of knowledge about the transmission route of toxoplasmosis which supports the evidence of high occurrence of *T. gondii* by significant association. Our findings are consistent with similar studies which were performed on blood donors [29,34].

Most studies in Iran have used the ELISA method by different commercial diagnostic kits to identify either IgG or IgM antibodies, which has high specificity and sensitivity and can be used to differentiate between the acute and chronic phases of *T. gondii* infections. However, in some cases, ELISA as a screening method cannot detect acute infection [35,36], so DNA-based approaches for infection detection have higher validity for screening blood donors in blood banks in countries with a high prevalence of infection among the general population [36]. In this study, 18% of blood donors tested positive for *T. gondii* using PCR, and *Toxoplasma* DNA was found in blood samples from two and three subjects who tested positive for both IgM and IgG, respectively. These findings are more significant because they warrant the spread of *Toxoplasma* infection via blood transfusion. However, in PCR positive samples, the parasite's viability was not verified, thus confirming the potential for blood to cause infection in the person who is going to receive it.

Our findings indicate quite a high prevalence (about one-third) of *Toxoplasma* infection among volunteer donors in Ardabil Province, known as the main VL focus in Iran. This data shows that asymptomatic carriers of *T. gondii* are quite common among blood donors in Ardabil province and pose a potential threat to the blood safety and hemovigilance program. Regarding the possibility that IgM-positive and or PCR positive donors have *T. gondii* tachyzoites in their blood, this will serve as an alert to blood transfusion organizations to encourage *Toxoplasma* infection screening, at least in the blood used by pregnant and immuno-suppressed patients.

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