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Original Article

Molecular and Serological Detection of Acute and Latent Toxoplasmosis Using Real-Time PCR and ELISA Techniques in Blood Donors of Rafsanjan City, Iran, 2013

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Abstract

Background: The differentiation between acute and latent forms of the *Toxoplasma gondii* (*T. gondii*) infection is still considered as a complicated issue. This study was aimed to elucidate the status of infection in the blood donors and the probable importance of blood transfusion in the transmission of the infection through detecting both immunological and genetic markers of acute and latent infection.

***Methods*:** Totally 235 blood samples from blood donors were collected. The levels of anti-*T. gondii* IgG and IgM antibodies were examined by specific ELISA kits. cDNA were synthesized from total extracted mRNA molecules from the serum samples and SAG1 gene, specific for tachyzoite form, were amplified using Real-Time PCR technique. Demographic information of study subjects including their gender, age, job, and habitat were recorded.

***Results*:** Out of 235 serum samples, 80 (34.04%) and 4 (1.71%) were positive regarding anti-*T. gondii* IgG and IgM antibodies, respectively. Real-Time PCR results showed that 14 out of 200 (6.97%) of blood donor had mRNA molecules of SAG1 gene. The positive results of Real-Time PCR of SAG1 in female gender and housekeepers were significantly higher than those of male gender and other job categories.

***Conclusion*:** The prevalence of chronic and acute infection is high in Iranian blood donors. Additionally, evaluation of antibodies could not be reliable, because several donors negative for anti-*T. gondii* IgM antibodies had detectable SAG1 mRNA molecules. Hence, it seems that molecular diagnostic tests are essential to detect acute infections.

Introduction

Toxoplasma gondii , the causative agent of toxoplasmosis is a parasitic protozoan which infects a wide range of warm-blooded vertebrates. The parasite infection in human, as intermediate host, involves early motile and quickly multiplying tachyzoites in nucleated cells resulting in an acute and short time systemic phase, and later slow growing bradyzoites in tissue cysts leading to the condition known as latent toxoplasmosis (1-3).

The human infections are prevalent in many countries around the world, with an average prevalence varying between 30-60% (3). Investigations on various Iranian populations using serological methods have showed a high prevalence rate of infection in nationwide scale (4-12), including our study area i.e. Rafsanjan (13).

Human infection is mainly developed by either oral ingestion of water and foods contaminated with parasite oocysts excreted by cat feces as final host, or eating raw and undercooked meat of intermediate hosts containing tissue cysts. Moreover, the infection can be transmitted through placenta, milk, organ transplantation, and blood transfusion (14, 15).

Toxoplasma could survive up to 50 days in blood and its components in 4°C (the temperature of blood bank refrigerators) (16). There is evident that leukemic patients have been infected by *T. gondii* after receiving packed leukocytes (17). There are reports on the presence of anti-*T. gondii* IgM immunoglobulins in healthy blood donors around the world, so that 3.6% of blood samples in India (18), 2.4% in Czech Republic (19), 1.9% in Mexico (20) and 3.6% in Tehran, Iran (21) have been positive for IgM antibodies against *T. gondii*. Since anti-*T.gondii* IgM is known as an indicator of acute toxoplasmosis, the presence of tachyzoite form in blood circulation, and according to the fact that some blood recipients are immunosuppressed, hence, it appears that *T. gondii* can be transmitted to these patients and these reports may be warning and noticeable (21).

In the present time, serological tests detecting different classes of anti-*T. gondii* immunoglobulins, especially IgG and IgM are the main diagnostic method for toxoplasmosis. However, the differentiation between acute and latent forms of the infection still is a complicated issue. The problem is partially due to this fact that in some infected individuals, the persistent IgM antibodies against *T. gondii* survive after acute phase ended, and also some of acute *T. gondii* infected patients are unable to produce IgM against the parasite immediately (22). This may impede the precise diagnosis of acute toxoplasmosis, especially in pregnant women, immunosuppressed patients and blood donors (23, 24).

In recent years a great progress has been occurred in the field of differential diagnosis of acute and latent toxoplasmosis and their respective parasitic stages i.e. tachyzoites and bradyzoites, by evaluation of SAG1 and BAG1 mRNAs, respectively (2, 23, 25-28).

This study was aimed to investigate healthy blood donors in Rafsanjan, regarding both immunological and genetic markers of acute and latent *T. gondii* infection using ELISA and Real-Time PCR methods, as well to elucidate the probable importance of routine blood transfusion in the transmission of *T. gondii* infection.

Material and Methods

Study area

This study was performed in Rafsanjan City, province of Kerman, southeastern Iran. The city has a population of approximately 250,000 and is located around 56° east longitude and 31° north latitude with an average height of 1470 meters above sea levels and a mean annual precipitation of about 100 mm.

Samples

According to anti-*Toxoplasma* IgM seroprevalence of 3.8% in blood donors in Tehran, Iran (21) and anti-*Toxoplasma* IgG seroprevalence of 48% in pregnant women in Rafsanjan city, Iran (13) and using the following formula, totally 235 blood samples were collected from healthy and voluntary blood donors referring to Rafsanjan center of Blood Transfusion Organization (BTO):

$$n = \frac{z_{(1-\alpha/2)}^{2} \times pq}{d^{2}}$$

The center's physician verified general health status of study subjects. Demographic information of study subjects including their gender, age, job, and habitat were also recorded. Serum samples were transferred to our laboratory in Immunology of Infectious Diseases Research Center, and stored in -20 °C until used.

ELISA

Anti-*T.gondii* IgG and IgM antibody levels of 235 serum samples were examined by commercial ELISA kits (Pishtaz Teb Zaman Diagnostics, Tehran, Iran) as detailed by manufacturer's protocol. According to the manufacture's announcement, the sensitivity and specificity of the kits were 100 and 99 percents, respectively. To assess the reliability of the kits, inter- and intra-assay were evaluated and produced scores of CV<14% and CV<3%, respectively.

Real-Time PCR

All mRNA molecules in 200 serum samples were extracted by RNX solution (CinnaGen Co, Tehran, Iran) and total cDNA of were synthesized using oligo-(dt) primer and cDNA synthesis kit (Parstous, Mashhad, Iran). Then, the synthesized cDNA molecules were amplified in a thermal cycler (Bio-Rad CFX96, USA) using a SYBR Green PCR master mix (Parstous, Mashhad, Iran) and a protocol which is described elsewhere (28), except using the specific primer for SAG1 gene amplifying a product of 355 bp, as follow (28):

SAG1F: 5'-GCTGTAACATTGAGCTCCTTGATTCCT-G-3'

SAG1R: 5'-CCGGAACAGTACTGATTGTTGTCTT-GAG-3'

According to the fact that SAG1 gene is expressed in the acute form, hence, SAG1 mRNA positive samples were considered as acute toxoplasmosis infections.

Result

Four out of 235 (1.71%) blood donors were as positive for anti-*T.gondii* IgM antibodies. The results also demonstrated that 80 (34.04%) of evaluated blood donors had detectable anti-*T. gondii* IgG antibodies.

The dissociation stages, quantitative and melting curves analyses of Real-Time PCR results, using CFX manager software version 1.1.308.111 (Bio-Rad, Foster City, USA), revealed that the data were valid and 14 out of 200 (6.97%) blood donors had detectable mRNA molecules of SAG1.

IgG and IgM results had no significant association with the gender, mean age, job and habitat of the blood donors. However, the positive results of Real-Time PCR of SAG1 in female gender (Table 1) and housekeepers (Table 2) were significantly higher than those of male gender and other job categories ($P<0.01$).

Table 1: The frequency of SAG1 positivity in blood donors in Rafsanjan City in 2013 according to gender

SAG1/Gender	Male n(%)	Female n(%)	Total n(%)
Negative	185 (93.9)	1 (33.3)	186 (93.0)
Positive	12 (6.1)	2 (66.7)	14 (7.0)

Table 2: The frequency of SAG1 positivity in blood donors in Rafsanjan City in 2013 according to job

SAG1/Job	Free n(%)	Student n(%)	Farmer n(%)	Officer n(%)	Housewife n(%)	Total n(%)
Negative	92 (95.8)	27 (93.1)	34 (94.4)	32 (88.9)	1 (33.3)	186 (93.0)
Positive	4 (4.2)	2 (6.9)	2 (5.6)	4 (11.1)	2 (66.7)	14 (7.0)

Discussion

We evaluated 235 blood donors for acute and chronic toxoplasmosis by using anti-*Toxoplasma* IgG and IgM immunoglobulins and tachyzoite-specific mRNA molecules (SAG1). The study showed a relatively high prevalence of acute *T. gondii* infection in blood donors as a potential source for infection transmission, as well as, the necessity of molecular methods for precise diagnosis of acute toxoplasmosis.

Diagnosis of human toxoplasmosis is mainly based on detecting of anti-*Toxoplasma* antibodies in serum samples using various serological methods. ELISA has been accepted as a sensitive and specific method for the routine laboratory diagnosis of both acute and chronic *Toxoplasma* infection in humans. An IgM-EILSA positive test alone or an increase of IgG concentration in two consecutive IgG-ELISA tests in a fortnight interval is regarded as an indicative of acute toxoplasmosis and the relevant parasitic stage i.e. tachyzoites.

The seroprevalence of *Toxoplasma* infection in Rafsanjan area based on our IgG-ELISA and IgM-ELISA results was 34.4% and 1.71%, respectively. While in the only other study in the area, which performed in 1993, the positive serums for IgG and IgM against *Toxoplasma* has been reported 48.3% and 0.0%. These differences may be resulted from using different diagnostic method (ELISA versus Indirect Fluorescent-Antibody, IFA) and from different study populations (blood donors versus pregnant women).

Recent reports of positive IgM-ELISA tests in healthy blood donor may be recognized as the worrying probability that blood transfusion could be a potential source for *Toxoplasma* transmission in human populations, especially immunosuppressed individuals.

However, there is evidence that due to the presence of the persistent anti-*Toxoplasma* IgM antibodies, any positive IgM-ELISA could not definitely be interpreted as the acute infection (23, 24). PCR-based techniques for detecting the specific genetic markers of tachyzoites such as SAG1 in tissues (30-32) or its expression in RNA level in blood samples could overcome the above-mentioned problem.

The remarkable higher positive results in Real-Time PCR compared to IgM-EILSA (6.97% versus 1.71%) may be suggesting that IgM-ELISA is not capable to diagnose all acute infections, and hence, there is a high potential risk of *Toxoplasma* transmission through blood transfusion among human population, especially immunosuppressed individuals.

Moreover, the positive results of Real-Time PCR of SAG1 in female gender and housekeepers were significantly higher than those of male gender and other job categories ($P < 0.01$). The finding may be considered as an important health concern of congenital toxoplasmosis, but regarding to the typical paucity of females among blood donor populations, e.g. three women versus 197 men in our study, the conclusion need to further investigation.

Conclusion

The prevalence of *T. gondii* infection is high in Iranian blood donors, which is confirmed by positivity of anti-*T. gondii* IgG and IgM antibodies as well as SAG1 mRNA. Additionally, evaluation of anti-*T. gondii* antibodies could not be reliable, because several donors negative for anti-*T. gondii* IgM antibodies had de-

tectable SAG1 mRNA. Hence, it seems that molecular diagnostic tests are essential to detect acute *T. gondii* infection.

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References

1. Pusch L, Romeike B, Deckert M, Mawrin C. Persistent *Toxoplasma* bradyzoite cysts in the brain: incidental finding in an immunocompetent patient without evidence of a toxoplasmosis. Clin Neuropathol. 2009; 28(3):210-2.
2. Di Cristina M, Del Porto P, Buffolano W, Beghetto E, Spadoni A, Guglietta S, et al. The *Toxoplasma gondii* bradyzoite antigens BAG1 and MAG1 induce early humoral and cell-mediated immune responses upon human infection. Microbes Infect. 2004; 6(2):164-71.
3. Flegr J, Havlicek J, Kodym P, Maly M, Smahel Z. Increased risk of traffic accidents in subjects with latent toxoplasmosis: a retrospective case-control study. BMC Infect Dis. 2002; 2:11.
4. Ahmad D, Mehdi S, Sayed HH, Sayed AK, Shirzad G. Serological survey of *Toxoplasma gondii* in schizophrenia patients referred to Psychiatric Hospital, Sari City, Iran. Trop Biomed. 2010; 27(3):476-82.
5. Fallah M, Rabiee S, Matini M, Taherkhani H. Seroepidemiology of toxoplasmosis in primigravida women in Hamadan, Islamic Republic of Iran, 2004. East Mediterr Health J. 2008; 14(1):163-71.
6. Hashemi HJ, Saraei M. Seroprevalence of *Toxoplasma gondii* in unmarried women in Qazvin, Islamic Republic of Iran. East Mediterr Health J. 2010; 16(1):24-8.
7. Keshavarz H, Nateghpour M, Eskandari S. A Seroepidemiologic Survey Of Toxoplasmosis In Islamshahr District Of Tehran, Iran. Modares Journal of Medical Sciences (Pathobiology). 2004; 6(2):111-9.
8. Saeedi M, Veghari G, Marjani A. Seroepidemiologic evaluation of anti-*Toxoplasma* antibodies among women in north of Iran. Pak J Biol Sci. 2007; 10(14):2359-62.
9. Sharif M, Daryani A, Barzegar G, Nasrolahei M. A seroepidemiological survey for toxoplasmosis among schoolchildren of Sari, Northern Iran. Trop Biomed. 2010; 27(2):220-5.
10. Sharif M, Ziaei H, Daryani A, Ajami A. Seroepidemiological study of toxoplasmosis in intellectual disability children in rehabilitation centers of northern Iran. Res Dev Disabil. 2007; 28(3):219-24.
11. Ali Mohammadi H, Fouladi N, Amani F, Safarzadeh M, Pourfarzi F, Mazaheri E. Sero Epidemiological Toxoplasmosis In Pre Marriage Women On The Basis Of Remarriage Tests 2007. Journal of Ardabil University of Medical Sciences (Jaums). 2009; 8(4):408-13.
12. Keshavarz Valian H, Horri H. Seoprevalence of toxoplasmosis in pergnant women in Kerman City. J Med Council Iran. 1995; 4:322-328.
13. Keshavarz Valian H, Zare Ranjbar M. Toxoplasmosis in pregnant women and its transmission to infant in Rafsanjan County. Journal of Gilan University of Medical Sciences. 1993; 2(6 &7):28-34.
14. Montoya J, Boothroyd J, Kovacs J. Section H - Protozoal Diseases, Chapter 279: *Toxoplasma gondii*. Mandell, Douglas & Bennett's Principles and Practice of Infectious Diseases. 7th ed: Churchill Livingstone, An Imprint of Elsevier; 2009.
15. Asgari Q, Sarnevesht J, Kalantari M, Sadat SJ, Motazedian MH, Sarkari B. Molecular survey of *Toxoplasma* infection in sheep and goat from Fars province, Southern Iran. Trop Anim Health Prod. 2011; 43(2):389-92.
16. Vasina SG, Dunaeva ZV. [On the length of survival of *Toxoplasma* outside the host organism]. Med Prom SSSR. 1960; 29:451-4.
17. Siegel SE, Lunde MN, Gelderman AH, Halterman RH, Brown JA, Levine AS, et al. Transmission of toxoplasmosis by leukocyte transfusion. Blood. 1971; 37(4):388-94.

18. Sundar P, Mahadevan A, Jayshree RS, Subbakrishna DK, Shankar SK. *Toxoplasma* seroprevalence in healthy voluntary blood donors from urban Karnataka. Indian J Med Res. 2007; 126(1):50-5.
19. Svobodova V, Literak I. Prevalence of IgM and IgG antibodies to *Toxoplasma gondii* in blood donors in the Czech Republic. Eur J Epidemiol. 1998; 14(8):803-5.
20. Alvarado-Esquivel C, Mercado-Suarez MF, Rodriguez-Briones A, Fallad-Torres L, Ayala-Ayala JO, Nevarez-Piedra LJ, et al. Seroepidemiology of infection with *Toxoplasma gondii* in healthy blood donors of Durango, Mexico. BMC Infect Dis. 2007; 7:75.
21. Ormazdi H, Sanikhani N, Hadighi R, Akhlaghi L, Memar A, E R. Investigation of antibodies (IgG And IgM) against *Toxoplasma Gondii* In blood donors referred to Tehran Blood Transfusion Organization by Elisa. Urmia Medical Journal. 2010; 2(21):212-6.
22. Contini C. Clinical and diagnostic management of toxoplasmosis in the immunocompromised patient. Parassitologia. 2008; 50(1-2):45-50.
23. Gross U, Holpert M, Goebel S. Impact of stage differentiation on diagnosis of toxoplasmosis. Ann Ist Super Sanita. 2004; 40(1): 65-70.
24. Press C, Montoya JG, Remington JS. Use of a single serum sample for diagnosis of acute toxoplasmosis in pregnant women and other adults. J Clin Microbiol. 2005; 43(7):3481-3.
25. Lyons RE, McLeod R, Roberts CW. *Toxoplasma gondii* tachyzoite-bradyzoite interconversion. Trends Parasitol. 2002; 18(5): 198-201.
26. Su C, Shwab EK, Zhou P, Zhu XQ, Dubey JP. Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology. 2010; 137(1):1-11.
27. Contini C, Giuliodori M, Cultrera R, Seraceni S. Detection of clinical-stage specific molecular *Toxoplasma gondii* gene patterns in patients with toxoplasmic lymphadenitis. J Med Microbiol. 2006; 55(Pt 6):771-4.
28. Mahittikorn A, Wickert H, Sukthana Y. *Toxoplasma gondii*: Simple duplex RT-PCR assay for detecting SAG1 and BAG1 genes during stage conversion in immunosuppressed mice. Exp Parasitol. 2010; 124(2):225-31.
29. Sajadi SMA, Mirzaei V, Hassanshahi G, Khorramdelazad H, Daredor HY, Hosseini SMH, et al. Decreased Expressions of Toll-Like Receptor 9 and Its Signaling Molecules in Chronic Hepatitis B Virus-Infected Patients. Arch Pathol Lab Med. 2013; 137(11):1674-9.
30. Hohlfeld P, Daffos F, Costa JM, Thulliez P, Forestier F, Vidaud M. Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. N Engl J Med. 1994; 331(11):695-9.
31. Montoya JG, Parmley S, Liesenfeld O, Jaffe GJ, Remington JS. Use of the polymerase chain reaction for diagnosis of ocular toxoplasmosis. Ophthalmology. 1999; 106(8): 1554-63.
32. Gianotti N, Cinque P, Castagna A, Novati R, Moro M, Lazzarin A. Diagnosis of toxoplasmic encephalitis in HIV-infected patients. AIDS. 1997; 11(12):1529-30.