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 sero-prevalence of 3.8% in blood donors in Tehran, Iran (21) and anti-Toxoplasma IgG sero-prevalence 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 manufacturer'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 (CiniaGen 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’-GCTGTAACCAGCTCCTGATTCCT-3’
SAG1R: 5’-CCTGAACACGTACGTGTCTTT-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).

| 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 n(%) | 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-ELISA 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-ELISA (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-

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

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