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Original paper

Comparison of nested PCR and ELISA techniques in diagnosis of toxoplasmosis infection in thalassemia patients by targeting the RE gene

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Introduction

Thalassemia is an inherited (i.e., passed from parents to children through genes) blood disorder leading to severe anemia. Due to the recent therapeutic advances, the life expectancy of patients with major thalassemia has significantly increased. This incidence rate of this disease varies in different countries [1].

Toxoplasmosis is a global zoonotic disease caused by an intracellular coccidian parasite, *Toxoplasma gondii*. Approximately, one-third of the world's human population is infected with this parasite [2–4]. Transmission of *T. gondii* occurs via eating tissue cysts in raw or undercooked meat, ingestion of sporulated oocytes shed in cats' faeces, and blood transfusion [5].

The fundamental medical conditions related to thalassemia are severe tiredness, weakness, shortness of breath, pounding, fluttering or irregular heartbeats, and pale skin brought about by the absence of hemoglobin [6].

The immune system changes after blood transfusion in all people consist on immunization and reduction immune system. These problems are caused by the regular blood transfusions used to treat anemia and can cause problems with the heart, liver, and hormone levels if untreated [7].

Toxoplasma can also be transmitted through blood or leukocytes from people with normal immune systems to people with defective immune systems; it can provoke one of the most potent innate, pro-inflammatory responses of all infectious disease agents [8,9]. Due to the dysfunction and decrease of lymphocytes, thalassemia can lead to a weakened immune system, impaired immune response such as phagocytosis and chemotaxis, and various infections [7,10]. Thus, an immuno-suppressive factor can lead to an increase in toxoplasmosis infection and death in the late stages of thalassemia.

In Iran, the gene frequency of thalassemia is between 4% and 8%, and it depends on loci [11]. Detection of toxoplasmosis in this group of patients is critical and can help them with the infection. In these patients, several serological methods such as enzyme linked immunosorbent assay (ELISA) have been used to detect antibodies against *T. gondii* [12–14]. Some molecular methods, such as nested PCR, have been used to increase DNA detection sensitivity in *Toxoplasma* infection based on multicopy genes [15,16].

In the current study, the sensitivity and specificity of ELISA and nested PCR techniques were tested for the diagnosis of toxoplasmosis in thalassemia patients.

Materials and Methods

Study design and population

This cross-sectional study included 110 thalassemia patients from Imam Khomeini Hospital of Zabol and Razmjomoghaddam Hospital of Zahedan in Sistan Baluchestan province, Iran during October 2019 to December 2019. Meanwhile, 110 healthy individuals were used simultaneously as controls. To collect samples, 2 ml blood samples (without anti-clotting agent) and 2 ml of blood in a special CBC tube containing 2% EDTA anti-coagulant solution were taken from 110 thalassemia patients approved by an oncologist, and transferred to a parasitological lab. All patients had a special thalassemia insurance. Sera separated stored at –20°C.

Anti-Toxoplasma IgM and IgG assays

In order to evaluate the titer of serum antibodies against *T. gondii*, the immunoassay enzyme method was used. For this purpose, the serum titer of IgG and IgM antibodies were determined by preparing the commercial kits of Pishtazat® following the instructions provided by the manufacturer. Optical absorption of controls and samples was read with the help of ELISA reader (DANA®/ IRAN) at a 450 nm.

DNA extraction and amplification

Following the manufacturer's instructions, DNAs were extracted using the DNA blood mini extraction kit (DNADynaBio™ Blood/Tissu DNA Extraction Mini Kit), and stored at −20°C. A frozen vial containing *T. gondii* RH strain DNA was prepared as positive control. Nested PCR consists of two repeated PCR amplification steps with two pairs of inner and outer primers. RE gene was used in this study. For the first step, the conventional PCR was done by a pair of outer primers. The total volume of reaction was 15 µl, containing 7.5 µl of PCR Master Mix (Ampliqon) with an $MgCl_2$ concentration adjusted to 1.5 mM, 1 µl of each primer at a concentration of 10 pmol/µl, 2 µl of DNA, and 4.5 µl of distilled water was prepared. PCR reactions were initiated at 95°C for an initial denaturation for 5 min and then cycled for 30 cycles with denaturation at 94°C for 20 s, followed by annealing at 53°C for 20 s and 55°C for 20 s, and finally an extension step at 72°C for 20 s. The yield of PCR products were approximately 529-bp fragments. Extracted DNA from RH strain of *T. gondii* s used as positive control.

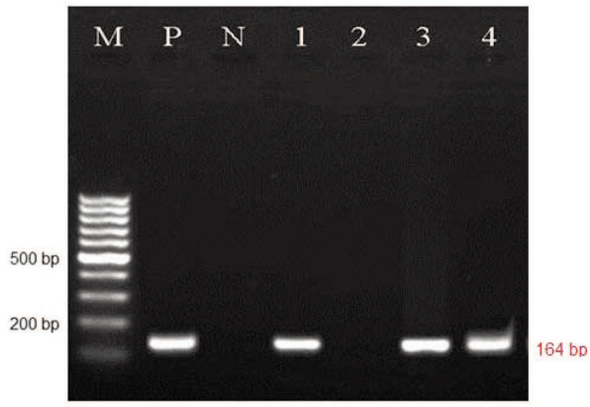

Figure 1. Agarose gel electrophoresis showing the results of nested-PCR tests based on RE gene in detection of *T. gondii* of thalasemic patient. The PCR products are as follows: lane 1, 3, 4 as positive; lane 2 as negative; N as negative control; lane P represents positive control. Marker size is 100-bp

In the second PCR, the 1:25 diluted of first run production was used for nested PCR by two inner primers. Similar to the first step, the reaction volume was prepared at 95°C for 5 min in initial denaturation, 35 cycles at 94°C for 20 s, 53°C for 20 s, and 72°C for 20 s for denaturation, annealing, and extension, respectively and followed by a final extension for 10 min at 72°C. DNA fragments were analyzed by 1.5% agarose gel electrophoresis. Finally, the yield of PCR products were 164-bp fragments (Fig. 1). The DNA fragments of each target region used in PCR amplification are as follow: first PCR: 5'CTGCAGGGAGGAAGAC GAAAGTG-3' and 5'CAGTGCATCTGGATTCC TCTCC-3'; second PCR: 5'GTGCTTGGAGCCA CAGAAGGGAC-3' and 5'GTGCTTGGAGCCAC AGAAGGGAC-3'.

Statistical analysis

Data were analyzed by SPSS software version 17, using paired-test and Spearman test. *P*-value less than 0.05 was considered as statistically significant.

Ethical considerations

The study protocol was approved by the Ethics Committee of Zabol University of Medical Sciences, Iran. Moreover, an informed written consent was obtained from all the participants.

Results

In this study, out of 110 patients with thalassemia,

Table 1. Comparison of serology results and nested-PCR techniques results in thalassemia patients

	Serology results					
	IgG+		IgG–		Total	
Nested PCR results	Patient	Control	Patient	Control	Patient	Control
Positive	2 (16.6%)	0	10 (83.4%)	4 (3.6%)	12 (10.9%)	4 (3.6%)
Negative	13 (13.2%)	29 (26.3%)	85 (86.8%)	77 (72.6%)	98 (89.1%)	106 (96.3%)
Total	15 (13.6%)	29 (26.3%)	95 (86.4%)	81 (73.6%)	110 (100%)	110 (100%)

15 (13.6%) cases were positive for IgG anti-*Toxoplasma* antibody and there was no positive case for IgM anti-*Toxoplasma* antibody. In concurrent control group, 29 (26.3%) cases were positive for IgG anti-*Toxoplasma* antibody and one case was positive for IgM anti-*Toxoplasma* antibody.

Results of nested PCR using RE gene to detect toxoplasmosis on blood samples showed that among 110 samples, 12 (10.9%) thalassemia patients and 4 (3.6%) control cases were positive for toxoplasmosis. The results of comparing ELISA and nested PCR techniques in both groups have been shown in table 1. The predictive values of nested PCR for sensitivity, specificity, positive, and negative predictive values are 17%, 87%, 13%, and 89%, respectively.

Discussion

Blood transfusion has various adverse effects, including allergic reactions, chills, fever, hives, and transmission of infectious diseases. Thalassemia is a global disease, which is most prevalent in Southeast Asia, Africa, and Mediterranean countries [17].

Severe opportunistic infections like *Toxoplasma* may be associated with immune deficiency and chronic immune stimulation following repeated blood transfusions, iron overload, and splenectomy [12,18,19].

Although the serological method is one of the most common laboratory techniques for diagnosing *Toxoplasma* infection in patients and is very suitable and cost-effective, it might cause serious problems in some high-risk patients [20,21].

The most common laboratory method for diagnosing toxoplasmosis has been serology for decades. However, the onset of diseases associated with decreased immune levels revealed various clinical signs of toxoplasmosis infection and manifested itself as a deadly disease, with irreversible complications. There are several drawbacks, such as the possibility of the absence of antibodies early in the disease, or the possibility of IgM antibodies in the patient's blood for a long time. Due to the prevalence of toxoplasmosis in patients with low levels of immunity, it is necessary to provide an alternative to the high-sensitivity serological method. Nested-PCR has been shown to have a high sensitivity and specificity of up to 94% and compared to PCR method has more ability and capacity in detecting laboratory tests and is significantly more cost-effective [22].

In this study, using serological method, we found that the prevalence of *Toxoplasma* infection in thalassemia patients was lower than healthy individuals. El-Tantawy et al. [23] showed that seroprevalence of *T. gondii* infection among β-thalassemia major in children was 23.2% and 53.6% for IgM and IgG anti-*Toxoplasma* antibodies, respectively; in addition, the prevalence of *T. gondii* infection in the control group was 5% and 18% for IgM and IgG anti-*Toxoplasma* antibodies, respectively. In another research carried out in Turkey, the IgM antibody in β-thalassemia major patients was higher than normal subjects [24]. The difference between such results could be due to the lack of antibody production or primary infections, especially in immunocompromised people, including thalassemia patients. Even with the nutritional limitations of patients, it can affect the way *Toxoplasma* is transmitted through diet. Accordingly, the antibody ranges could be affected.

In the current study, we attempted to find the sensitivity of RE-nested PCR in blood samples of thalassemia patients. Using this method, we observed that *Toxoplasma* infection in thalassemia patients was higher than healthy individuals. *Toxoplasma* DNA was found using nested PCR

from 10 (83.4%) patients with thalassemia and 4 (3.6%) subjects in the control group whose IgG anti-*Toxoplasma* test result was negative. It is likely that in these people the *Toxoplasma* infection occurred recently and sampling was done before increasing the serum antibody titer. As witnessed, the antibody titer was low in thalassemia patients but the positive cases in the molecular test were higher than the control group; this could be due to the weakness of the humoral immune system in these people, which results in a reduced antibody titer. In these people, due to the weakness of cellular immune system, the infection is reactive, and the parasite is present in the blood as tachyzoites. Hence, the molecular test in these people shows more positive cases than the control group with a normal cellular immune system.

Hanifehpour [18] reported the serological and molecular diagnosis of *T. gondii* infections in thalassemia patients; in this study, 9.78% of the thalassemia patients were positive for *T. gondii*, indicating a non-significant difference with the control group. However, 51.9% of thalassemia patients were positive for anti-*Toxoplasma* IgG. Moreover, using SAG1-LAMP, 5 (2.1%) patients with thalassemia and 3 (1.2%) patients in the healthy group had negative serological results.

The nested PCR method shows the sensitivity and specificity for detecting toxoplasmosis in normal individuals [15,25]. Different factors, including DNA marker features, the volume of the sample, extraction, and molecular methods affect the technique sensitivity [26]. Since the sensitivity and specificity of diagnostic nested PCR are much higher than the conventional molecular diagnostic methods such as serology tests, nested PCR method can be used to identify acute toxoplasmosis in humans and animals. Detection of *T. gondii* using nested PCR can be valuable in alignment with serological methods [5,15].

Several genes are used in the diagnosis of *Toxoplasma*, but B1 and RE *Toxoplasma* is more widely used [27]. In addition, the RE gene has a large number of copies and is more sensitive than the B1 fragment, so this genomic target has a better effect on the PCR method for detecting *Toxoplasma* DNA in suspected individuals, especially those at risk, including patients with systemic weakness. However, studying and comparing these two markers in the diagnosis of toxoplasmosis in thalassemia patients should be recommended.

Positive results using nested PCR in thalassemia patients with a negative ELISA test along with other pathogenic disorders could be harmful to their lives. Moreover, positive results in healthy individuals shows a negative serological effect with ELISA method. Due to the absence of *T. gondii* and no infection with toxoplasmosis, more accurate complementary tests should be performed.

As Mumcuoglu et al. [28] expressed, a diagnostic algorithm for non-thalassemia patients should be implemented to consider its cost-effectiveness. We believe that choosing the correct molecular detection method can increase the scope of health development.

In conclusion, although serological method is currently the most common laboratory technique for diagnosing toxoplasmosis in thalassemia patients, molecular techniques such as nested PCR have better efficiency and performance in the diagnosis of active and reactive cases of toxoplasmosis.

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