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Molecular Evolution of *Toxoplasma gondii* in Thalassemic Patients Using Nested PCR Targeting B1 and RE Genes

[1]Raghad N. Shihab, **[2]Israa Kasim Al-Aubaidi**

[1]Departmentof Cancer Research, Iraqi Center for Cancer and Medical Genetic Research, MustansiriyahUniversity, Baghdad, Iraq
[2]Department of Biology, College of Education for pure science (Ibn- Al- Haitham), University of Baghdad, Baghdad, Iraq.

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Abstract : Toxoplasmosis, caused by *T gondii*, is a parasitic disease affecting various mammalian hosts. Thalassemia, an inherited microcytic hemolytic anemia, is characterized by abnormal hemoglobin production. This study aimed to establish a molecular detection method using nested polymerase chain reaction (PCR) targeting the B1 and RE genes of *T. gondii* in thalassemic Iraqi patients. The study samples comprised 165 thalassemic patients and 80 healthy individuals, aged from 2 to 45 years (mean age 15.387 ± 0.627 years). Samples were collected from Al-Karma Teaching Hospital in Baghdad, Iraq, between March and June 2022. Two diagnostic tests were used for toxoplasmosis IgM/IgG antibodies immunochromotography rapid test which found 44(26.67%) for IgG and chemiluminescent microparticle immunoassay (CMIA). Key findings include: Anti-Toxoplasma IgG antibodies were found in 36.4% (60/165) of thalassemic patients, with an average titer of 41.475 ± 9.193 IU/mL.*T. gondii* DNA was detected in 60% of thalassemic patients using the B1 gene, compared to a 30% positivity rate in the control group. For the RE gene, only 5% of thalassemic patients tested positive, while the control group showed no positive results. These results highlight the significant presence of *T. gondii* DNA in thalassemic patients and demonstrate the effectiveness of nested PCR in detecting toxoplasmosis. The higher prevalence of toxoplasmosis in thalassemic patients compared to healthy individuals underscores the need for vigilant screening and management in this vulnerable population.

Keywords: *Toxoplasmagondii,Thalassemia,B1 gene, RE gene, Nested PCR.*

Corresponding author: (Email: raghd.naji1102a@ihcoedu.uobaghdad.edu.iq).

Introduction

The single-celled parasite *Toxoplasma gondii* is the source of toxoplasmosis infections. It affects almost every warm-blooded species, including people and animals, and is one of the most widespread parasitic diseases(1-4). This parasite could be vertically transferred to the fetus during pregnancy, which can result in a range of clinical issues for the baby. Recurrence of the latent infection due to an opportunistic pathogen could be fatal, in patients with impaired immune systems, newborns, and fetuses infected congenitally(5). There are three morphological stages of *T. gondii*: tachyzoites, bradyzoites andsporozoites, while the extra intestinal phase affects all intermediate hosts, the intestinal phase regarding the parasite life cycle

takes place in the small intestine of cats, humans contract the infection by eating raw or undercooked meat that contains tissue cysts (final host), or by eating unwashed vegetables that contain oocysts, or by coming into contact with the excrement of infected cats. the parasite affects every important organ in the body, particularly during the acute phase of the illness (6-8). This parasite could be vertically transferred to the fetus during pregnancy, which can result in a range of clinical issues for the baby. Recurrence of the latent infection due to an opportunistic pathogen could be fatal, in patients with impaired immune systems, newborns, and fetuses infected congenitally (9). Toxoplasmosis infection can change the behavior of chronic infections (10). As a highly prevalent immunological type of this parasitic infestation, it elicits humoral immunity (HI) and cell-mediated immunity (CMI) responses, which are considered characteristic of this infestation (11).

Thalassemia is a type of inherited autosomal recessive blood condition characterized by defect hemoglobin production (12-14). Thalassemia is classified in two varieties: alpha and beta. Alpha thalassemia can lead to consequences including hemolytic anemia or lethal hydrops fetalis. Beta thalassemia major is an inherited disorder that may affect general health, gene mutations that result in low level and/or malfunctioning globin protein respectively, the root causes of these disorders. One of these proteins might occasionally not exist at all carriers of alpha or beta thalassemia trait exhibit minor symptoms depending on how severe the disease is, beta thalassemia major is an inherited disorder that may affect general, the human beta globin (HBB) gene, sickle cell-beta thalassemia (HbS/-thal) is a good example of a combination of two frequent genetic anemia (15-17).

Nested polymerase chain reaction is used to enhance the specify of PCR technique by amplify the region required in DNA template and to deleting the non-specific amplified region with other two primers. This method of PCR employed two sets of primers. The sensitivity regarding PCR is increased when two pairs of oligonucleotides are used since more cycles can be performed. Response specificity is boosted when two distinct primer sets bind to the same target template. (18,19). The B1 gene of *T. gondii*, has 35 copies in the parasite's genomic DNA. It is now considered the most used gene in the field of *T. gondii* nucleic acid detection (20). Similarly, the 529 RE gene fragment, with a repetition rate ranging from 200 to 300 copies in the *T. gondii* genome, is thought to contribute to the improved sensitivity and specificity of the detection (21). This study was aimed to establish molecular detection tools by using nested PCR targeting B1 and RE genes in *T.gondii* parasite among thalassemic Iraqi patients.

Materials and Methods

1.Subjects

Blood sample were collected from 165 thalassemia patients who attended to the Al-Karma Teaching Hospital in Baghdad, Iraq. Eighty healthy individuals which considered as negative control group was also involve in this study, their ages range from (2-45years) with mean (15.387±0.627) years. During the period from March to June 2022, 5 ML of venous blood were withdrawn via using a sterile syringe from each patient. Three ml was transferred into a fully labeled gel tube to separate the serum by centrifuge at 3000 rpm for 5 minutes and stored at -20 C° until used in chemiluminescent

microparticle immunoassay CMIA detection. Two ml of whole blood was collected in a labeled EDTA tube in order to the molecular investigation. After doctor's examination with application a necessary blood tests to detect thalassemia.

2. *T. gondii* Diagnosis

2. 1. Serological Diagnosis

The diagnostic kit (Architect Toxo IgM/IgG kit - Abbott GmbH, Germany) using (CMIA) was utilized to detect both anti-Toxoplasma IgG and IgM antibodies in the serum samples.

2.2 Genetic Diagnosis

A. DNA Extraction

DNA was isolated from whole blood using a DNA extraction kit (Bioneers, Korea) according to the manufacturer's instructions.

B. Detection of *T. gondii* DNA by Nested PCR Technique

A set of primers targeting the B1 and RE genes of *T. gondii* were used in the nested PCR experiment. The primers produced 287 bp and 194 bp products for the B1 gene, and 520 bp and 243 bp products for the RE gene, as shown in Table (1).

The nested PCR procedure consisted of two rounds:

First Round:

1. One µL of forward primer and 1 µL of reverse primer were added.
2. Five µL of DNA sample was added.
3. Thirteen µL of deionized nuclease-free water were added to a PCR tube containing master mix (Bioneers).
4. The mixture mixed using an exispin.
5. The tube placed in the PCR device according to the parameters listed in table (2).

Second Round:

1. One µL of forward primer and 1 µL of reverse primer were added.
2. Five µL of DNA sample was added.
3. Thirteen µL of deionized nuclease-free water were added to a PCR tube containing master mix (Bioneers).
4. The mixture mixed using an exispin.
5. The tube placed in the PCR device according to the parameters listed in table (2).

All PCR products were analyzed by electrophoresis on a 1.5% agarose gel and examined under UV light.

Table(1): Nucleotidessequencesof B1 and REgenesprimers

Primer	Sequence		Amplicon
B1	F1	5-TCAAGCAGCGTATTGTCGAG	287bp
	R1	5-CCGCAGCGACTTCTATCTCT	
	F2	5-GGAACTGCATCCGTTCATGAG	194bp
	R2	5-TCTTTAAAGCGTTCGTGGTC	
RE	F1	5-CTGCAGGGAGGAAGACGAAAGTTG	520bp
	R1	5-CAGTGCATCTGGATTCCTCTCC	
	F2	5-GTGCTTGGAGCCACAGAAGGGAC	243bp
	R2	5-GAGGAAAGCGTCGTCTCGTCTGG	

Table(2): Cycling conditions of first and second amplification

TEMP.	TIME	NO. of Cycles
95 C°	5 MIN	1 cycle
95 C°	30 SEC.	35 cycles
Annealing TM.	30 SEC.	
72 C°	1 MIN	
72 C°	5 MIN	1 cycle

Statistical Analysis

The Statistical Analysis System (SAS), 2018) program was used to assess the effects of various factors on the study parameters (22). Analysis of Variance (ANOVA) was performed, and the Least Significant Difference (LSD) test was used for multiple comparisons between means. Additionally, the Chi-square test was employed to determine significant differences in the findings, with significance levels set at $P \leq 0.01$ and $P \leq 0.05$.

Results and Discussion

Toxoplasmosis can cause various clinical disorders. In immunocompetent individuals, it typically presents without symptoms, but in immunocompromised individuals, it can lead to severe complications and potentially life-threatening illness (23, 24).

Table 3 shows the results of the immunochromatography rapid test for Toxoplasma IgM and IgG antibodies. Among the thalassemic patients, 44 out of 165 (26.67%) tested seropositive for anti-Toxoplasma IgG antibodies. In the non-thalassemic control group, 33 out of 80 (41.25%) tested seropositive for anti-Toxoplasma IgG antibodies, with significant differences observed between these results ($P \leq 0.01$). No thalassemic patients tested positive for Toxoplasma IgM antibodies, while 1 out of 80 (1.25%) of the non-thalassemic control group tested positive for anti-Toxoplasma IgM antibodies.

Table(3): Immunochromatography Rapid Test Results for Toxoplasma IgM/IgG Antibodies in Thalassemic and Non-Thalassemic Groups

Group name	Total No. of samples for each group	IgG +ve No %	IgM +ve No %	IgG and IgM +ve No %	-Ve No %	P-value
Thalassemia Patients	**165**	**44 26.67%**	**0 0.0%**	**1 0.60%**	**120 72.73%**	**0.0001 ****
Non-Thalassemia Control	**80**	**33 41.25%**	**1 1.25%**	**2 2.5%**	**44 55%**	**0.0001 ****
P-value	**---**	**0.078 NS**	**0.802 NS**	**0.711 NS**	**0.0063 ****	**---**

**($P \leq 0.01$) indicates highly significant differences.
NS non-significant

According to Figure 1, the thalassemic group with toxoplasmosis had an IgG titer of 41.475 ± 9.193 IU/mL. The non-thalassemic positive control group recorded an IgG titer of 35.59 ± 8.336 IU/mL. However, no response was recorded for IgM antibodies in any study group.

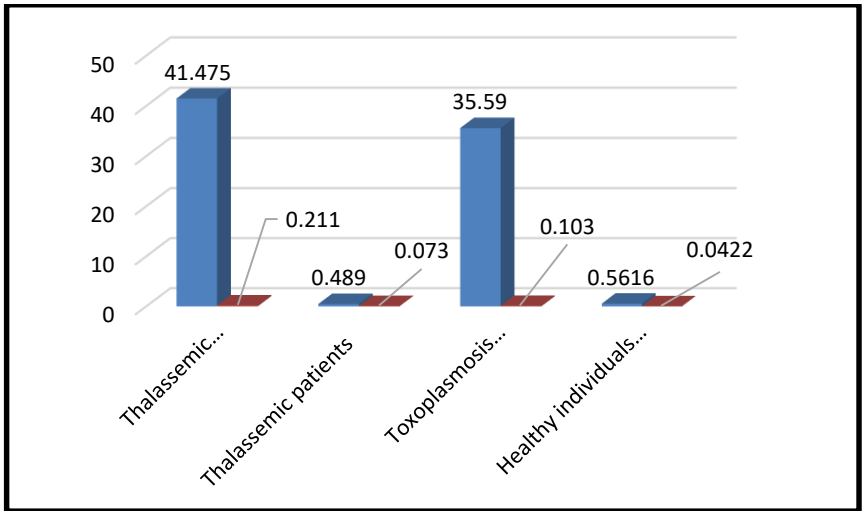

Figure (1) :Mean values ofanti- Toxoplasma IgG/IgM IU/mLinstudiedgroups.

The molecular weight of IgM is 970 Kd, with an average normal serum concentration of 1.5 mg/mL. It is predominantly produced during the first immunological response to pathogens, acting as a pentamer that activates the classical complement pathway. IgG, with a molecular weight of approximately 146 Kd and a normal serum concentration of 9.0 mg/mL, is primarily produced during the secondary immune response. IgG can activate the classical complement pathway and provides significant protection (25-27).

The immunochromatography rapid test for IgM/IgG antibodies is quick and simple, serving as a viable screening tool for toxoplasmosis detection. Results are obtained within 15 minutes without the need for specialized equipment or expertise. However, due to its qualitative or semi-quantitative nature, false positives and negatives can occur (28, 29).

The results are similar to those reported by Yousef *et al.*(30) who found that 30.76% (36/117) of thalassemic patients and 20% (41/205) of healthy controls were seropositive for anti-Toxoplasma IgG antibodies. The same study noted a low rate of anti-Toxoplasma IgM detection: 1.70% (2/117) in thalassemic patients and 0.48% (1/205) in healthy controls. Hanifehpour*et al.*(31) reported that 55.31% of thalassemic patients and 37.02% of healthy individuals had anti-*Toxoplasma* IgG antibodies.

Many patients with sickle cell anemia and severe types of thalassemia require blood transfusions to improve their clinical condition. Without comprehensive serological screening for all microorganisms, there is a risk of transmission of infections, including viruses, bacteria, and parasites, through transfusions (32). Toxoplasmosis in immunocompromised individuals is often caused by the reactivation of a latent infection (33).

Nested PCR, widely used in the literature to identify *T. gondii* parasitemia, targets the conserved B1 gene, which has multiple copies in the *T. gondii* genome, making it ideal for PCR amplification (34). Results from Table 2 indicate that *T. gondii* DNA was found in 60% of thalassemic patients with toxoplasmosis, while 30% of the positive control group tested positive, see Figures 2 and 3.

Table(4):Result of Polymerasechainreactiontechnique bygeneB1inthestudiedgroups.

Groups	No. of samples	Positive No +ve (%)	Negative No -ve (%)	P-value
Thalassemic patients withtoxoplasmosis	25	15 (60.00%)	10 (40.00%)	0.317 NS
Thalassemicpatients	20	0 (0.00%)	20 (100%)	0.0001 **
Toxoplasmosis patients (controlpositive)	20	6 (30.00%)	14 (70.00%)	0.0073 **
Healthy individuals (controlnegative)	20	0 (0.00%)	20 (100%)	0.0001 **
P-value		0.0001 **	0.0074 **	0.0001 **

**(P≤0.01) highly significant.
NS non-significant

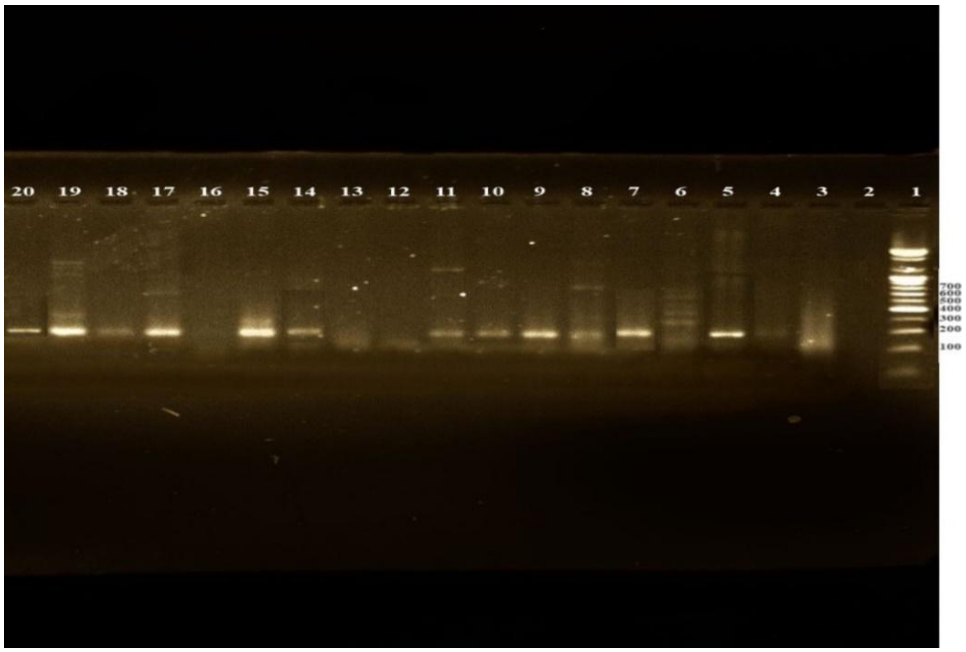

Figure (2):PCR product for detection *T. gondii* B1 gene(194bp) following electrophoresis in thalassemic patients with toxoplasmosis.

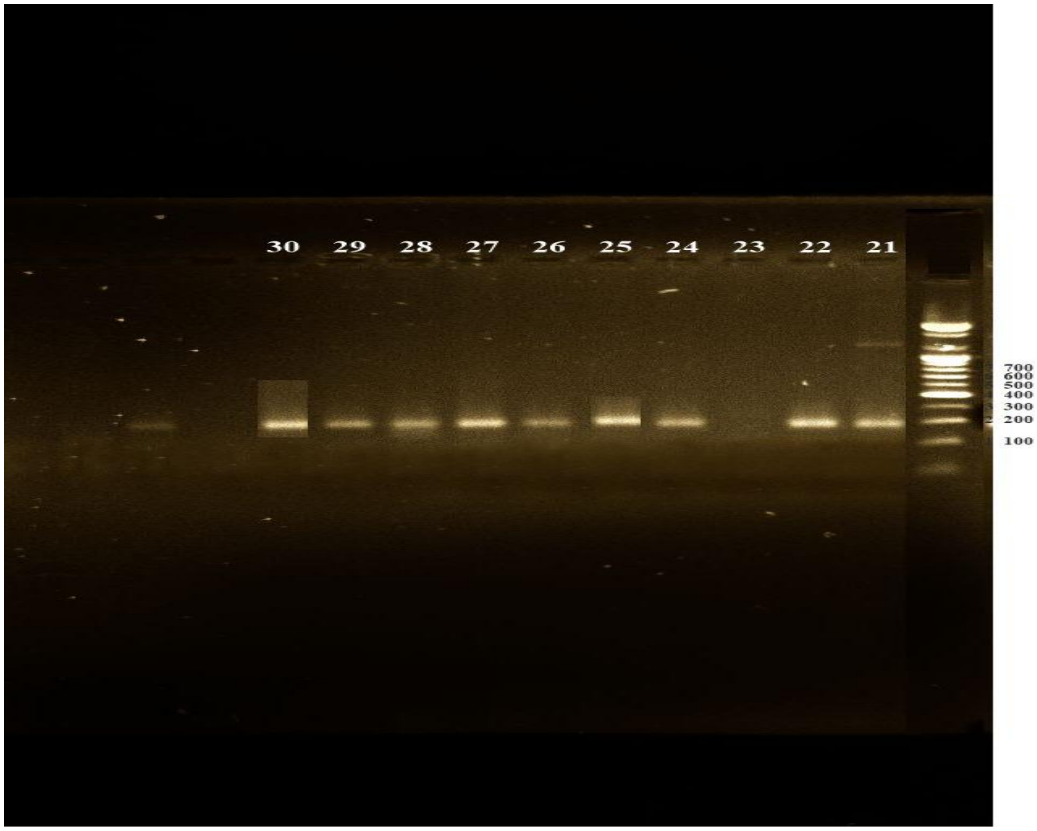

Figure (3):PCR product for detection *T. gondii* B1 gene(194bp) following electrophoresis in toxoplasmosis patients (control positive).

Similar studies have shown varying rates of *T. gondii* detection using nested PCR. Mohammed *et al.*(35) reported a 77.77% positivity rate in married samples, with a 96 bp PCR product for the B1 gene. Bakre(36) found that 10% of premarital female blood samples tested positive for *T. gondii* using PCR. Nakashima *et al.*(37)detected*T. gondii* in 10.8% of blood donor samples using nested PCR for the B1 gene.

One possible explanation for the higher detection rate in thalassemic patients compared to non-thalassemic individuals is the increased susceptibility to infections due to frequent blood transfusions. These transfusions can introduce pathogens, including *T. gondii*, into the bloodstream, especially if the blood products are not adequately screened (32). Additionally, the compromised immune system of thalassemic patients may contribute to the reactivation of latent toxoplasmosis, leading to higher detection rates (33).

Results from Table 5 indicate that the RE gene was detected in only 5% of thalassemic patients with toxoplasmosis, with no positive results in other groups, see Figure 4.

Table(5):Results of the nested PCR assay targeting RE gene in studied groups.

Groups	No. of samples	Positive No +ve (%)	Negative No -ve (%)	P-value
Thalassemic patients with toxoplasmosis	25	4 (5.00%)	21 (95.00%)	0.0007 **
Thalassemic patients	20	0 (0.00%)	20 (100%)	0.0001 **
Toxoplasmosis patients (controlpositive)	20	0 (0.00%)	20 (100%)	0.0001 **
Healthy individuals (controlnegative)	20	0 (0.00%)	20 (100%)	0.0001 **
P-value		0.137 NS	0.902 NS	---
****(P≤0.01) highly significant.** NS non-significant				

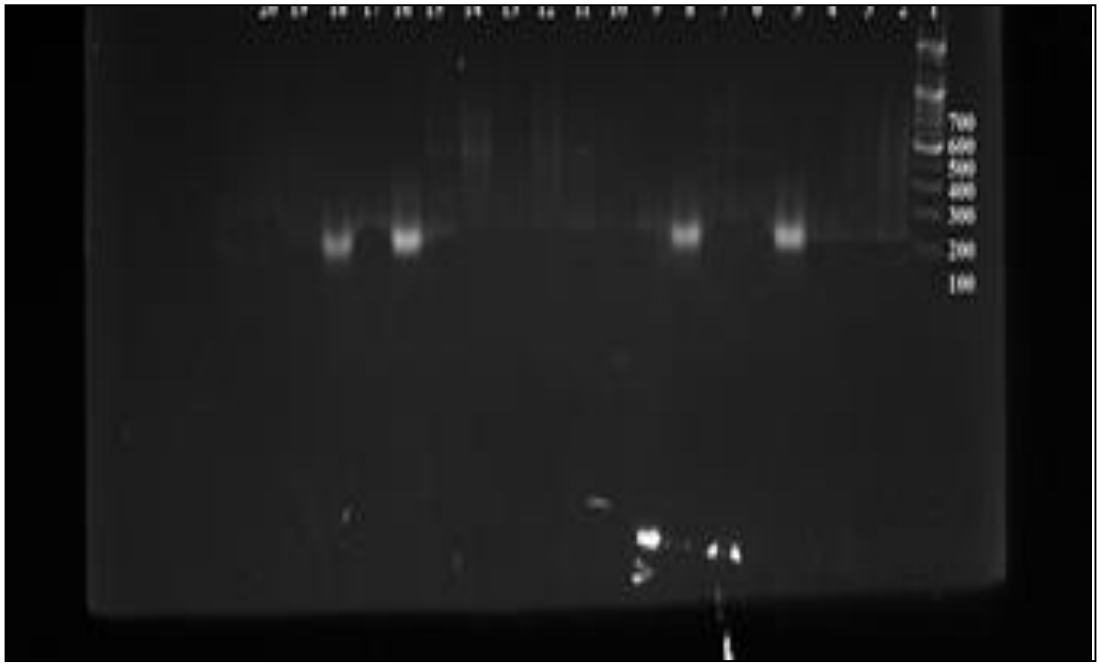

Figure (4) :PCR product for detecting *T. gondii* RE gene after electrophoresis

Mala and Bakre(38) found a 1.56% positivity rate for the RE gene in blood samples from diabetic patients using nested PCR. The prevalence of toxoplasmosis in diabetic patients was 67.8% for the B1 gene and 57.1% for the RE gene (39).

In this study, primers specific to the *T. gondii* genome (two genomic repeated targets) did not interfere with the human genome, indicating high specificity for *T.gondii*detection. The resultssuggest that the B1 gene is more effective than the RE gene in detecting toxoplasmosis. The effectiveness of PCR techniques for identifying *T. gondii* DNA in at-risk individuals, such as thalassemic patients, was demonstrated. Our findings align with those of Cardona *et al.*(40) and Wahab

et al.(41) who reported that targeting the B1 gene was more efficient than the RE gene. Conversely, Reischl *et al.*(42),Cassaing*et al.*(43) and Fallahi *et al.*(44) found that the RE gene may be a preferred diagnostic target over the B1 gene due to its higher sensitivity. Fallahi *et al.* assessed the analytical sensitivity of nested PCR assays and suggested that the sensitivity of PCR targeting the B1 gene is 10 to 100 times lower than targeting the RE gene.

One possible explanation for the differing effectiveness between the B1 and RE genes could be the presence of gene deletions or variations in different *T. gondii*strains, which may affect the detection sensitivity. The higher copy number of the B1 gene in the *T. gondii*genome might contribute to its greater detection rate in nested PCR assays (44).

However, a subset of 72.5% of thalassemia patients were positive for circulating *T. gondii* DNA by PCR, indicating an inability to effectively control parasites proliferation. In contrast, immunocompetent controls displayed an intact capability to prevent disease reactivation, as evidenced by asymptomatic parasitemia (45).The results suggest that thalassemic patients may have an impaired immune response, making them more susceptible to toxoplasmosis reactivation and persistent infection.

Conclusion

This study highlights the increased prevalence and detection rates of *T. gondii* in thalassemic patients compared to non-thalassemic controls. Serological tests revealed a significantly higher seropositive rate for anti-Toxoplasma IgG antibodies in both groups, with absence of IgM antibodies. Nested PCR targeting the B1 gene demonstrated superior sensitivity, detecting *T. gondii* DNA in 60% of thalassemic patients, compared to 30% in the positive control groups. The RE gene showed lower detection rates, with only 5% positivity in thalassemic patients. These findings suggest that the B1 gene is a more effective marker for *T. gondii* detection in thalassemic patients, who appear to be at higher risk for toxoplasmosis reactivation and persistent infection. Further research is warranted to understand the mechanisms underlying this increased susceptibility and to develop targeted interventions for this vulnerable population.

Declarations

Availability of data and materials

Data and materials are available on request.

Competing interests

The authors declare no competing interests relevant to this article.

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Not applicable

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