Serological Detection of *Toxoplasma gondii* in Packed Red Blood Cells Using Soluble Recombinant Dense Granule Antigen (rGRA7), with Confirmatory Nested-PCR Performed on ELISA-Positive Samples

Hadi Mirahmadi [1,2], Marziye Shahraki Zare [2], Ali Reza Salimi-Khorashad [2], Ebrahim Alijani [1], Abolhassan Safdari [3], Ahmad Mehravaran [2,4], Reza Shafiei [5], Seyed Ghader Azizi [1*]

1. Clinical Immunology Research Center, Zahedan University of Medical Sciences, Zahedan, Iran
2. Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran
3. Department of Infectious Disease, School of Medicine Infectious Diseases and Tropical Medicine Research Center, Zahedan, Iran
4. Infectious Diseases and Tropical Medicine Research Center, Research Institute of Cellular and Molecular Science in Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran
5. Vector-borne Diseases Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran

Corresponding Authors*: Seyed Ghader Azizi
Address: Khalij -e- Fars Blvd, Zahedan, Sistan & Balouchestan Province, Islamic Republic of Iran
Tel: +98-54-33372013
Email: s.ghaderazizi@yahoo.com

ORCID:

Hadi Mirahmadi: 0000-0002-6423-2578

Marziye Shahraki Zare: 0000-0003-1986-5867

Ali Reza Salimi-Khorashad: 0000-0002-4542-3109

Ebrahim Alijani: 0000-0001-9921-8947

Abolhassan Safdari: 0000-0001-6793-1596

Ahmad Mehravaran: 0000-0002-2079-7972

Reza Shafiei: 0000-0001-9604-2982

Seyed Ghader Azizi: 0000-0003-3751-8906

Abstract:

Introduction: Toxoplasmosis is a prevalent and severe but significantly overlooked parasitic disease, which can be transmitted via blood products. The current study aimed to assess the possible contamination of packed red blood cells (PRBCs) with *Toxoplasma gondii* (*T. gondii*) and the applicability of combined serological (ELISA) and molecular (Nested-PCR) methods for its detection.

Material and methods: After plasmid transformation and GRA7 protein production, the purified protein was used to develop an ELISA kit. IgG and IgM antibodies against *T. gondii* were detected via a GRA7 antigen-based ELISA. In serologically positive samples, Nested-PCR was used to verify the parasite by amplifying a 164 bp fragment within a larger 529 bp repetitive element (RE) sequence of *T. gondii*.

Results: Out of all tested samples, 35 (1.75 %) were positive for IgM, 402 (20.1 %) were positive for IgG, and 14 (0.7 %) samples showed simultaneous positivity for both IgG and IgM**.** Notably, within the 423 antibody-positive PRBCs, 82 units (4.1%) underwent leukocyte reduction. Four PRBC units that were non leukocyte reduced were positive for *T. gondii* DNA.

Conclusion: Due to the limited sensitivity of serological assays in detecting *T. gondii*, the use of complementary molecular diagnostic techniques is essential for accurate identification. Also, practical procedures such as using leukocyte reducing filters for high-risk blood recipients and implementing a strict donor screening process are necessary to mitigate the transmission of Toxoplasma.

Key words: ELISA, Packed RBC, PCR, rGRA7, *Toxoplasma gondii*

1. Introduction:

Toxoplasmosis is a prevalent yet severe infection often underestimated. Notably, it is well-established that *T. gondii*-can persist more than 30 days at 4°C [1-3]. Given the possibility that asymptomatic blood donors may be carriers, it is believed that there is an association between

toxoplasma infection and blood transfusions, highlighting the need for routine screening protocols [4].

Studies have shown that anti-*Toxoplasma* antibodies are abundant in rural areas where individuals do not receive proper education [5, 6]. In clinical settings, toxoplasmosis is diagnosed by detecting serum antibodies against specific antigens (somatic or excreted-secreted) where the ELISA is used. To be specific, in high-risk conditions (HIV, fetal infection, cancer, brain abscess in immunosuppressed patients, and pregnant women) where both speed and accuracy of diagnosis are critical to start treatment, there are some challenges in data interpretation in serological methods. As such, these methods are not very helpful for a definitive diagnosis [7-9].

It is thought that ELISA-based commercial kits that mainly use antigens purified from parasites cultured in cell media or mouse peritoneal fluid often suffer from poor-quality or contamination with antigens, which in turn may lead to low sensitivity and inconsistency in the diagnostic results. Of note, this inability may be related to the lack of sufficient antibodies in the early episodes of the disease or a severe decrease in antibodies in immunosuppressed patients. Under such conditions, designing natural antigens is laborious and expensive [7, 8]. It seems that recombinant antigens such as GRA7 and ROP2, derived from dense and rhoptry granules, have demonstrated strong diagnostic and immunogenic potential in both ELISA and vaccine development so can be a suitable replacement for native tachyzoite antigens in serological methods [10, 11].

In recent years, molecular diagnostic methods have significantly advanced for detecting various diseases. Among these methods, Nested-PCR, which targets multi-copy sequences on DNA, has emerged as a technique with high sensitivity and cost-effectiveness for diagnosing *T. gondii*, especially for high-risk patients. One of the most frequently used and recently established genomic targets in diagnosing *T. gondii* is the RE. This 529 bp fragment is widely used because it is highly repeated (200 and 300 times) in the genome, which can be readily identified and amplified [12].

This study investigated possible contamination with *T. gondii* in 2000 units of PRBCs in Zahedan, Iran. At first, ELISA using the recombinant GRA7 (rGRA7) antigen was used to evaluate specific IgG and IgM antibodies against *T. gondii* in plasma isolated from these products. Then, positive serological samples were confirmed by the Nested PCR method using a 164 bp fragment within the larger 529 bp RE sequence specific to *T. gondii*.

2. Materials and Methods

Figure 1 shows a summary of the workflow performed in the study.

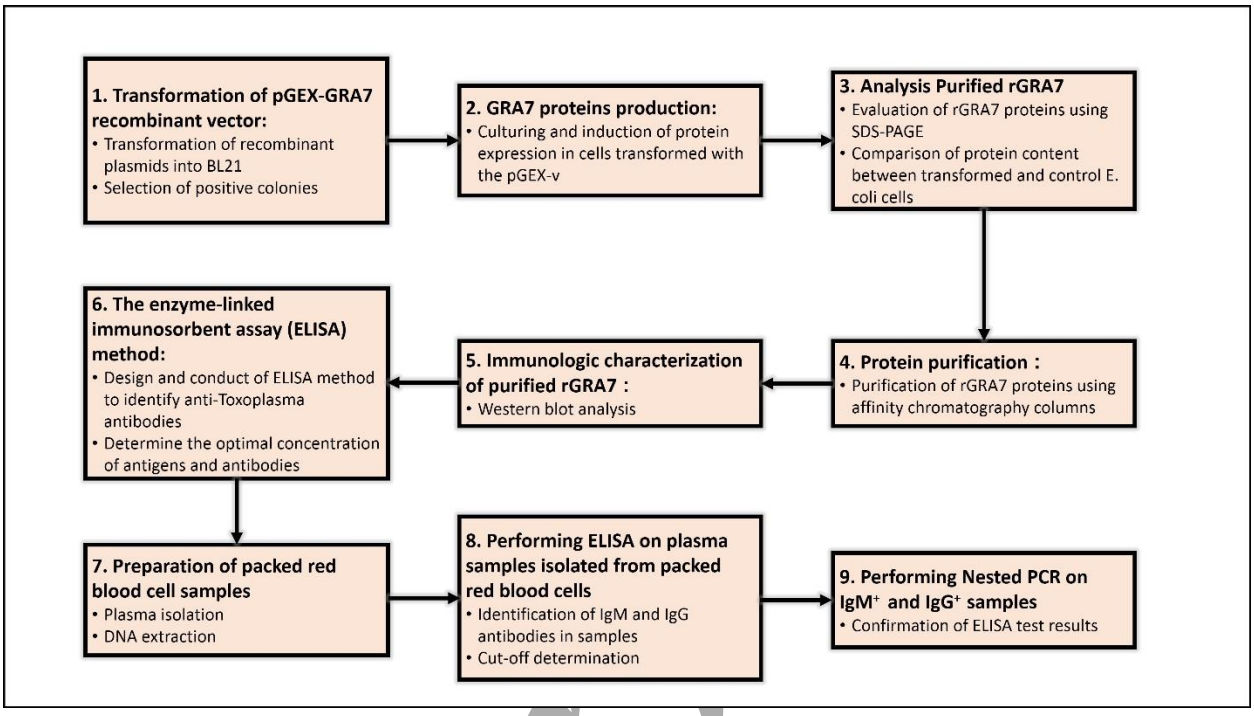

Figure 1. Work flow (study flow) diagram

2.1. Ethics Statement and Study Population

The current study involving human subjects was conducted in accordance with the principles outlined in the Declaration of Helsinki as revised in 2000. Approval for this study was obtained from the Ethical Committee of Zahedan University of Medical Sciences, Zahedan, Iran (IR.ZAUMS.REC.1402.381).

The study pertained to an examination of 2000 units of PRBCs (410 units leukocyte reduced and 1590 units non leukocyte reduced) sent by Zahedan Blood Transfusion Organization to Imam Ali Hospital, Zahedan. Zahedan County, with its administrative center located in Zahedan city, serves as the center of Sistan and Baluchestan province (Figure 2).

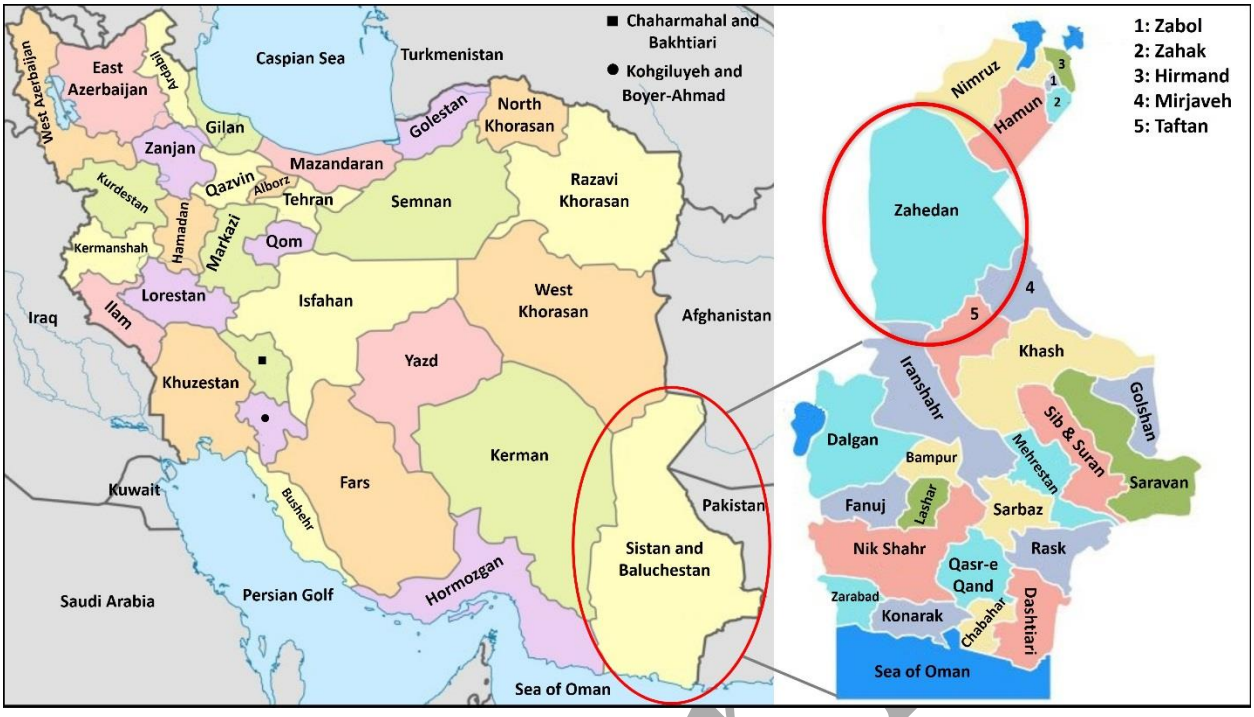

Figure 2. Sistan & Bauchestan province and Zahedan location in Iran

It should be noted that the PRBCs were meticulously maintained in the blood bank section of the clinical laboratory of Imam Ali Hospital at a controlled temperature of 2-6°C, which is consistent with the standards stipulated by the Iranian Blood Transfusion Organization (IBTO). The contents of the cords were initially separated from the main bag. Afterwards, centrifugation (at 3000 g for 5 minutes) was performed to isolate the plasma. The separated plasma was stored at -20°C for further processing. Furthermore, the centrifuged contents of the cords yielded the buffy coat, which was stored at -20°C for DNA extraction.

2.2. Plasmid Transformation and GRA7 Proteins Production

Plasmid transformation was performed using *Escherichia coli* (*E. coli)* Bl21-competent cells and the BamHI restriction enzyme, as described in a previous study [13]. Also, GRA7 protein production was done according to Arab-Mazar's study [14].

2.3. Analysis of Purified rGRA7 by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The rGRA7 protein was assessed by SDS PAGE (Laemmli) with 15% acrylamide stacking and resolving gels. Coomassie Brilliant Blue dye was used for gel staining and subsequent analysis.

2.4. Protein Purification

We used a Glutathione Sepharose 4B kit in strict accordance with the manufacturer's guidelines (Pharmacia Biotech Code No: 17-070755-1) for the purification of rGRA7 protein. After carefully packing into the column, the gel was thoroughly washedthoroughly washed with 77 bed volumes of phosphate-buffered saline (PBS). We used PBS to eliminate alcohol as a preservative. Then we equilibrated the gel with three bed volumes of PBS. After protein expression, we applied the sonicated samples to the column and discarded the eluent. The column was washed comprehensively with 7 bed volumes of PBS. Ultimately, an elution buffer (10 mM Glutathione in 50 mM Tris-HCl (pH 8.0)) was applied to elute the bound material and collect the resulting fractions.

2.5. Immunologic Characterization of Purified rGRA7

Serological investigations were conducted using human sera from both healthy individuals and *T. gondii*-infected patients to determine the immunological efficacy of purified recombinant protein following the method of Arab-Mazar [14].

2.6. Western Blot Analysis

After SDS-PAGE, we transferred the proteins from the gel onto nitrocellulose membranes. Immobilization was done via a UV crosslinker (UV Tec, EEC). After cutting the nitrocellulose membranes into vertical strips, blocking with bovine serum albumin (BSA) was done at 22°C with continuous agitation (2 hours). After washing, the strips were incubated with positive human serum at a 1:100 dilution for 1 hour. Afterward, the strips were washed and then treated with rabbit anti-human IgG and IgM antibodies (1:2000) (Sigma, USA) conjugated to horseradish peroxidase (HRP) for 1 hour at 22°C. Then we washed strips and treated sequentially with deionized distilled water (ddH2O), hydrogen peroxide (H2O2), Tris, and 3,3’-diaminobenzidine (DAB) (Merck, Germany). Subsequent strips incubation was done at 22°C (15 min). The reaction was then

terminated through rinsing with ddH2O, and the strips were subsequently analyzed for brown bands [13, 15].

2.7. ELISA

An initial ELISA was conducted to demonstrate the most effective concentrations of recombinant antigens and antibodies for coating and to evaluate the presence of Toxoplasma antibodies. The titration of antigens and antibodies was carried out using a checkerboard method. We coated 96-well plates (fat bottom) with 100 µl of rGRA7. The wells were diluted serially from 2 µg/ml (200 ng/well) to 0.2 µg/ml in a coating buffer (sodium carbonate with pH=9.6) per well. A moist chamber was used for incubating coated plates at 4°C for overnight. Then, washing the plates was applied via PBS-Tween 20 (PBS-T) three times. Already sera with positive (1:10, 1:100, 1:500, and 1:1000) and negative results were added to the covered wells with rGRA7. A humid chamber was used for a one-hour incubation at 22°C, and then the wells were washed with PBS-T three times. Any unbound materials were rinsed away using PBS with 0.05% Tween after a one-hour incubation at room temperature. Following this, we added rabbit anti-human IgG and rabbit anti-human IgM antibodies (IgG at 1:15,000; IgM at 1:10,000; both from Sigma, USA) conjugated to HRP, respectively, to the wells. After 1 hour of incubation at 22°C, we removed any remaining anti-human antibodies by rinsing with PBS containing 0.05% Tween. The reaction was then initiated using OPD (O-Phenylenediamine dihydrochloride) with H2O2 (Sigma, USA). The resulting plates were analyzed at a wavelength of 450 nm (ELISA Tecan, Austria; Cross linker Uvitec, EEC/ELISA reader, Helsinki, Finland) [16].

Upon comparative analysis with the manufacturer's instructions, the most optimal concentrations of antibody and antigen were established for the commercially available ELISA kit (Toxoplasma-IgG/IgM-lot No: 95001–Pishtazteb Iran). The determined cut-off for IgG and IgM was 0.262 and 0.214, respectively. The sensitivity and specificity were set to 93% and 96%, respectively, for IgG and 90% and 92% for IgM, compared to commercial kits. The calculated cut-off points for IgG and IgM were 0.324 and 0.293, respectively [17].

The serum from a patient displaying the highest IgG and IgM levels was utilized as a positive control in triplicate wells across all tested plates. Following the pilot test, the optimum concentration for the combined antigens was 2 µg/ml of rGRA7 in a sodium carbonate buffer with a pH of 9.6 per well. On the other hand, the most effective dilution for detecting Toxoplasma

antibodies was also identified as a 1:100 dilution. During this investigation, the sera under examination were diluted to an optimal 1:100 ratio and subsequently applied to ELISA plates coated with rGRA7 to bind all specific antibodies to the antigen. An indirect ELISA methodology was employed to identify antibodies targeted against rGRA7 [17, 18].

We examined each sample in triplicate, and we used the mean OD value for analysis. Demographic and clinical characteristics of 50 patients with toxoplasmosis and 50 without toxoplasmosis who were hospitalized at the Children's Cancer Medical Center in Tehran, Iran, and participated in this study have been previously reported [19].

The ELISA method was performed on the isolated plasma from PRBCs in the next stage. The IgM- and IgG-positive samples were selected for the molecular method.

2.8. DNA Extraction

DNA was extracted via the DNGTM-Plus commercial protocol (SinnaGen Inc, Tehran, Iran) from the samples that were positive for IgM and IgG antibodies.

2.9 Nested-PCR Amplification

PCR amplification targeting a 529 bp segment of *T. gondii* was carried out using specific forward (5'-CAGGGAGGAAGACGAAAGTTG-3') and reverse (5'-CAGACACAGTGCATCTGGATT-3') primers. We mixed a 25 µL mixture comprising Taq DNA polymerase (1.25 units), extracted DNA (1 µL), MgCl2 (1.5 mM), forward primer (100 pmol), reverse primer (100 pmol), dNTP (0.2 mM), and PCR reaction buffer ×10. The thermal profile included a first denaturation phase (5 minutes at 94°C), 35 thermal cycles, a separation step (35 seconds at 94°C), an annealing step (1 minute at 56°C), an extension step (1 minute at 72°C), and a terminal extension (10 minutes at 72°C). We separated the products by electrophoresis in an agarose gel (1.5%), followed by staining with a RedSafe [20].

We diluted the initial PCR product 1:25 in ddH2O to 2 µL and used it as a template for the next PCR reaction. The total volume of the second PCR was 15 µL, and it amplified a 164 bp segment of *T. gondii* DNA. This amplification was achieved by utilizing two specific primers: 5´-AGGGACAGAAGTCGAAGGGG-3´ and 5´-GCAGCCAAGCCGGAAACATC-3´ [20].

The second thermal profile included the first denaturation phase (5 minutes at 94 °C), 35 thermal cycles included, separation (20 seconds at 94°C), annealing (20 seconds at 53°C), extension (20

seconds at 72°C), and a terminal extension (5 minutes at 72°C). Then, we separated the 164 bp PCR products on an agarose gel (1.5%). Control *T. gondii* DNA (GenBank: AF146527.1) was employed to confirm the PCR results. Negative controls contained ddH2O instead of template DNA [20].

3. Results

3.1. Western Blot Analysis

Western blot results for purified rGRA7 and GST (Gluthathion-S-Transferases) antigen are shown in Figure 3.

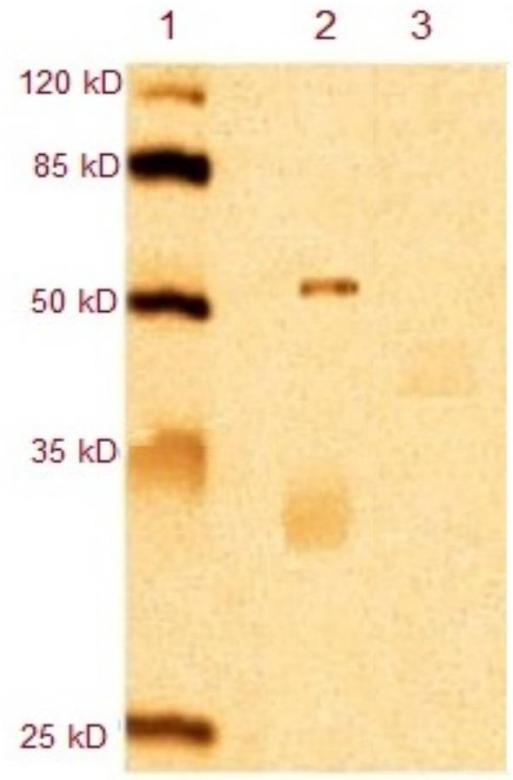

Figure 3. Western blot results with a concentration of 20 µg/mL of each of the purified rGRA7 and GST antigens and a 1/100 dilution of the patient's serum; 1: 25 to 120 kDa protein standard marker; 2: rGRA7 57 kDa (29 kDa + GST 28 kDa) (20 µg/ml) which reacted positively with Toxoplasma positive human serum; 3: GST 28 kDa (Gluthasion-S-Transferases) (20 µg/ml), which reacted negatively with Toxoplasma positive human serum.

3.2. ELISA Method

Table 1 details the results of our extensive assessment of 2000 samples. Among these, 423 samples were positive for antibodies, while 1577 samples were negative. Of all positive cases, 35 samples exhibited IgM antibodies, of which 21 were exclusively IgM-positive, while 14 showed both IgG and IgM antibodies (mixed type). Among the 423 positive cases, 402 were IgG-positive, including 388 cases that were exclusively IgG-positive

Table 1. Frequency of Positive IgM, IgG and Mix antibodies samples in PRBC

Antibody Type	**Case**	**Antibody concentrations***		
		(3+) >200 IU/mL	(2+) 100-200 IU/ mL	(1+) 10-100 IU/mL
IgM	21	5	10	6
IgG	388	49	181	158
Mix (IgM+IgG)	14	-	5	9

(IgG mean OD450 = 1.040, cut-off 0.324), (IgM mean OD 450nm = 0.585, cut-off 0.293)

* Samples were classified based on the intensity of the reaction as follows: +1 for antibody concentrations ranging from 10 to 100 IU/µL, +2 for concentrations between 100 and 200 IU/µL, and +3 for concentrations exceeding 200 IU/µL.

3.3. Nested-PCR Amplification

Subsequent molecular investigations identified the parasite genome in only four samples (Figure 4). Specifically, 3 IgM-positive cases exhibited parasite DNA upon molecular analysis. Among the 14 cases with positive mixed test results (IgM+ and IgG+), only one demonstrated parasite DNA. On the other hand, out of a total of 2000 PRBCs, 410 (20.5%) samples were leukocyte reduced, and 1590 (79.5%) samples were not. Of the 423 cases with positive antibodies, 341 samples (17.05%) were associated with units lacking leukocyte reduction, while 82 cases (4.1%) were associated with leukocyte reduced products. Furthermore, all PRBCs containing parasite DNA were non leukocyte reduced. All positive molecular test results underwent duplicate testing to prevent potential errors in the molecular findings. Table 2 shows a summary of the comparison

between the results of serological and molecular methods in PRBC units with and without leukocyte reduction.

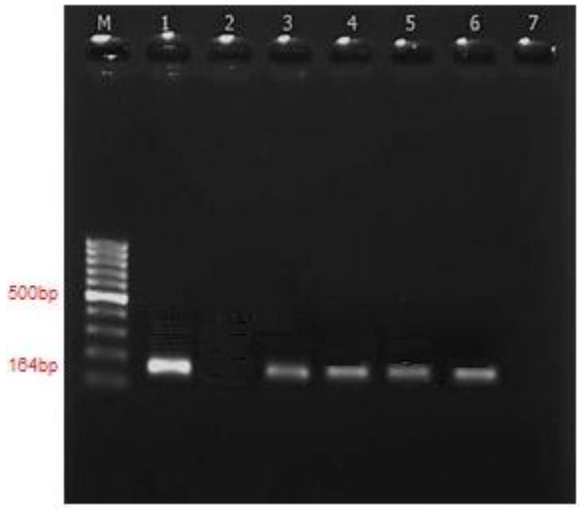

Figure 4. Electrophoresis results for the products obtained from the amplification of the RE gene sequence, M: marker 100bp; 1; positive control; 2: negative control; 3, 4, 5 and 6: positive sample.

Table 2. Comparison between detection anti-*Toxoplasma gondii* antibody and molecular positive cases in PRBC

Antibody	Positive n (percent)	Leukocyte Reduced	Non Leukocyte Reduced	PCR Positive n (percent)
IgM+	21 (1.05 %)	5 (0.25%)	16 (0.8%)	3 (0.15 %) (Non Leukocyte Reduced)
IgG+	388 (19.4%)	74 (3.7%)	314 (15.7%)	-
Mix	14 (0.7%)	3 (0.15%)	11 (0.55%)	1 (0.05%) (Non Leukocyte Reduced)
Total (Positive)	423 (21.15%)	82 (4.1%)	341 (17.05%)	4 (0.2%) (Non Leukocyte Reduced)

All positive PRBC units for Toxoplasma DNA were leukocyte reduced.

4. Discussion:

This study provides evidence for the potential contamination of PRBCs with *T. gondii*. Meanwhile, considering the sensitivity and specificity calculated in a previous study, recombinant rGRA7 antigen can be utilized as an efficient tool in the serological diagnosis of toxoplasmosis. Moreover, the performance of this antigen was confirmed by western blot and immunochemical methods, indicating that rGRA7 has great potential for the development of more accurate and economical diagnostic kits [17].

Another highlight of this study was the use of Nested-PCR to directly confirm the presence of parasite DNA in serologically positive samples. This method, targeting the 529-bp multicopy gene, had high sensitivity and could directly confirm parasite presence in some serologically positive units. Since serological methods may be misleading or inadequate in high-risk cases (such as immunocompromised patients, pregnant women, or blood recipients), PCR-based methods can be crucial for diagnosis in these groups.

These findings highlight Toxoplasma contamination in blood products, as assessed by recombinant antigen-based ELISA and Nested-PCR. According to the results obtained from the present study and other studies, developing diagnostic kits based on recombinant antigens and their integration into routine screening protocols can effectively reduce the risks of blood transfusion and improve public health [21].

Discrepancies between serological (ELISA) and molecular (Nested-PCR) results arise from the different targets detected by each method. Since ELISA indicates the presence of antibodies and PCR indicates the presence of parasite DNA, complete agreement between the two methods is not expected, particularly in chronic infections or immunocompromised individuals. On the other hand, ELISA may yield false positive results due to cross-reaction with similar antigens or previous contamination. Therefore, a combination of the diagnostic methods and interpretation of results is strongly recommended, taking into account the donors' clinical context [22, 23].

Another noteworthy point is the importance of antibody levels in relation to molecular results. In the present study, many of the IgG-positive cases had low to moderate levels of IgG (Table 1). Several studies have evaluated IgG, particularly through avidity testing, and examined its simultaneous use with PCR as a diagnostic tool. In our study, three cases with positive parasite DNA had no detectable IgG, and one case was mixed positive, with low IgG levels, suggesting an early stage of IgG seroconversion. On the other hand, a 2020 study by Nakashima et al. showed

that serum IgG concentration in healthy donors did not necessarily correlate with nested-PCR results. Even in the presence of high IgG titers, PCR may yield negative results [24]. Therefore, interpreting serological results based solely on antibody titer may be misleading, and the simultaneous use of molecular methods and consideration of the donor's clinical condition are of particular importance.

The majority of blood recipients are highly vulnerable to toxoplasmosis through blood transfusion. Studies have shown a high prevalence of positive serological results for *T. gondii* infection in patients with hematological conditions such as cancer, and thalassemia, as well as the possibility of parasite transmission via blood products [25]. Numerous studies have been conducted on Iranian blood donors related to *T. gondii*. These studies have yielded conflicting results. For example, in a survey conducted in Shiraz, Shaddel et al. analyzed fresh frozen plasma (FFP) and PRBCs and concluded that donor screening for *T. gondii* should be considered for blood products [26]. Karimi et al. authored a critical letter to the editor regarding Shaddel's study on *T. gondii* in the same year. The correspondence highlighted several significant points, including the challenge of diagnosing acute infection due to the persistence of IgM in the serum for up to 12 years following the initial infection. Moreover, the letter underscored the potential for false-positive results due to rheumatoid factors or nonspecific antibody binding, thereby questioning the reliability of antibody-based assays with absent confirmatory methods. While theoretical transmission of Toxoplasma through blood products is plausible, documented instances of infection via this route are limited. Given these perspectives and the non-recommendation of Toxoplasma screening by esteemed organizations such as the World Health Organization (WHO), the Association for the Advancement of Blood & Biotherapies (AABB), and the European Council, screening for Toxoplasma in Iran may be considered optional [27].

Also, Karimi (2016) and Mardani (2020) provided various reasons for not conducting screening tests among donors. These reasons included the moderate prevalence of infections, a significant challenge in distinguishing between recent and past infections, lack of cost-effectiveness, reduction in donor numbers due to the exclusion of individuals with positive serological results, absence of licensed serological tests for screening donors, and the absence of global consensus on whether to include transfusion-transmitted toxoplasmosis (TTT) screening tests as a routine practice in blood donation centers [28, 29].

In light of the aforementioned data, two contrasting perspectives exist concerning *T. gondii* infection in blood donors. The prevailing stance, advocated by the IBTO, WHO, and AABB, asserts that donor screening is not imperative. Instead, the use of leukocyte reducing filters is recommended for high-risk individuals. Conversely, an alternative viewpoint underscores the necessity of screening tests. The identification of *T. gondii* DNA and antibodies in PRBCs in the present study underscores the need for further investigation in this domain. Furthermore, all four positive samples of parasite DNA in the present study were observed in PRBCs without leukocyte reduction, which was largely consistent with the findings of Karimi and Mardani's studies. It is clear that a more sensitive and thorough donor screening process, including the evaluation of donors for clinical indicators in Toxoplasma-endemic regions, along with the use of leukocyte reducing filters for high-risk blood recipients, is currently the most effective approach to mitigate the transmission of blood product-associated Toxoplasma to recipients.

One limitation of this study was the absence of clinical follow-up for donors. No data were available on the clinical status or history of infection in the donors, making it difficult to analyze the correlation between serological or molecular findings and the actual disease status of donors. Additionally, the inability to confirm transmission through blood transfusion was another limitation of this study. Although the presence of the parasite was confirmed in 4 blood samples, due to the lack of follow-up of blood recipients, it cannot be definitively claimed that the disease was transmitted through blood products. This is a common limitation in studies based on blood product samples, so these limitations can be considered in future studies.

5. Conclusion

It seems that conventional serological methods for toxoplasmosis screening in blood donors and blood products are unreliable and inefficient, particularly in certain populations, as antibodies against Toxoplasma appear only after some time. In this context, PCR, especially when targeting high-copy gene sequences such as RE, is a valuable adjunct to serological methods in diagnosing *T. gondii* infections. Given the high mortality rate and heavy complications associated with this pathogen, Nested-PCR is particularly significant for diagnosing active toxoplasmosis, especially in comparison with more costly molecular techniques such as loop-mediated isothermal amplification (LAMP) and real-time PCR. Due to its sensitivity, reliability, and reduced reliance

on specialized personnel and expensive equipment, Nested-PCR can promptly and accurately identify active infectious agents such as Toxoplasma, particularly within high-risk populations. Such features make Nested-PCR particularly useful in low-income countries.

Acknowledgments:

The office of the Vice-Chancellor financially supported this study for the research of Zahedan University of Medical Sciences (Grant No. 11177). Consent for this study was received from the Ethical Committee of Zahedan University of Medical Sciences, Zahedan, Iran (IR.ZAUMS.REC.1402.381).

Authorship contribution statement

HM: Writing – original draft, Validation, Methodology. **MSZ:** Methodology, Formal analysis, Investigation. **ARSK:** Methodology. **EA**: Software, Resources. **AS:** Resources. **AM:** Date curation. **RS:** Formal analysis. **SGA:** Writing – review, Editing, Supervision.

Conflict of interests:

The authors declare no conflict of interests.

Ethics approval:

Approval for this study was obtained from the Ethical Committee of Zahedan University of Medical Sciences, Zahedan, Iran (IR.ZAUMS.REC.1402.381).

Funding Sources:

The office of the Vice-Chancellor financially supported this study for the research of Zahedan University of Medical Sciences (Grant No. 11177). The study has been the subject of an MSc thesis in medical parasitology (Marziye Shahraki Zare).

Data availability

Data will be made available on request.

References:

1. El-Tantawy N, Darwish A, Eissa E. Seroprevalence of *Toxoplasma gondii* Infection Among B-Thalassemia Major Pediatric Population: Implications for Transfusion Transmissible Toxoplasmosis. Pediatr Infect Dis J. 2019;38(3):236–40. https://doi.org/10.1097/inf.0000000000002111.
2. Hussain Al- Zubaidei HH, Abed ND, Hassan HB. Epidemiological Study of Toxoplasma infection in Humans and Sheep Using Molecular, Serological, and Traditional Techniques in Central Iraq. Iran J Vet Med. 2025:–. https://doi.org/10.22059/ijvm.2025.399894.1005865.
3. Alseady HH. Molecular Prevalence of *Toxoplasma Gondii* in Butchers and Slaughtered Cattle in Middle Euphrates, Iraq. Iran J Vet Med. 2024;18(3):407–14. https://doi.org/10.32598/ijvm.18.3.1005460.
4. Wesołowski R, Pawłowska M, Mila-Kierzenkowska C. The Medical Relevance of Toxoplasma Infections in Terms of the Safety of Blood Recipients under Immunosuppression—A Meta-Analysis. Microorganisms. 2023;11(8):1980. https://doi.org/10.3390/microorganisms11081980.
5. Yılmaz A, Yazıcı E, Turk C. Assessment of seroprevalence of *Toxoplasma gondii* in blood donors applied to the blood center of Gazi university hospital. Iran J Microbiol. 2021;13(2):243–7. https://doi.org/10.18502/ijm.v13i2.5986.
6. Mehrabi F, Rassouli M, Emadi Chashmi SH. Molecular Detection of *Toxoplasma gondii* in Chicken Meats and Eggs in Semnan City, Iran. Iran J Vet Med. 2023;17(2):167–72. https://doi.org/10.32598/ijvm.17.2.1005238.
7. Mirahmadi H, Mehravaran A, sani Haidari M, Rahmati-Balaghaleh M, Raissi V, shafiei R. Serological and Molecular Survey of *Toxoplasma gondii* Infection in Hemodialysis Patients with Chronic Renal Disease in Zahedan, Iran J Kerman Univ. Med. Sci. 2021;28(4):391–8. https://doi.org/10.22062/jkmu.2021.91720.
8. Dehghan Haghighi J, Hosseini A, Shafiei R, Mehravaran A, Alijani E, Mirahmadi H. Evaluation of *Toxoplasma gondii* Antibodies in Addicted and Non-Addicted Women in Zahedan, Southeast of Iran. Int J High Risk Behav Addict. 2020;9(3):e106458. https://doi.org/10.5812/ijhrba.106458.
9. Firooz Jahantigh F, Rasekh M, Ganjali M, Sarani A. Seroprevalence of *Toxoplasma Gondii* Infection Among Pregnant Women and Small Ruminant Populations in Sistan Region, Iran. Iran J Vet Med. 2020;14(3):239–49. https://doi.org/10.22059/ijvm.2020.294216.1005048.
10. Sabukunze S, Gu H, Zhao L, Jia H, Guo H. Comparison of the performance of SAG2, GRA6, and GRA7 for serological diagnosis of *Toxoplasma gondii* infection in cats. Front Vet Sci. 2024;Volume 11 - 2024. https://doi.org/10.3389/fvets.2024.1423581.
11. Ferra BT, Holec-Gąsior L, Gatkowska J, Dziadek B, Dzitko K, Grąźlewska W, et al. The first study on the usefulness of recombinant tetravalent chimeric proteins containing fragments of SAG2, GRA1, ROP1 and AMA1 antigens in the detection of specific anti-*Toxoplasma gondii* antibodies in mouse and human sera. PLoS One. 2019;14(6):e0217866. https://doi.org/10.1371/journal.pone.0217866.
12. Mirahmadi H, Nozari B, Metanat M, Solgi R, Shahraki E, Alijani E, et al. Evaluating the performance of LAMP diagnostic test in the detection of toxoplasmosis in hemodialysis patients. Gene Rep. 2022;26:101430. https://doi.org/https://doi.org/10.1016/j.genrep.2021.101430.

13. Arab-Mazar Z, Seyyed-Tabaei S, Mirahmadi H. Cloning of Dense Granular (GRA) 7 Gene of *Toxoplasma gondii* into pTZ57RT Vectors. Novel Biomed. 2014;2(4):114–9.
14. Arab-Mazar Z, Fallahi S, Koochaki A, Haghighi A, Seyyed Tabaei SJ. Immunodiagnosis and molecular validation of *Toxoplasma gondii*-recombinant dense granular (GRA) 7 protein for the detection of toxoplasmosis in patients with cancer. Microbiol Res. 2016;183:53–9. https://doi.org/https://doi.org/10.1016/j.micres.2015.11.006.
15. Arab-Mazar Z, Fallahi S, Koochaki A, Mirahmadi H, Tabaei SJ. Cloning, expression and immunoreactivity of recombinant *Toxoplasma gondii* GRA5 protein. Iran J Microbiol. 2016;8(5):331–7.
16. Mirahmadi H, Fallahi S, Seyyed Tabaei SJ. Soluble recombinant merozoite surface antigen-142kDa of Plasmodium vivax: An improved diagnostic antigen for vivax malaria. J Microbiol Methods. 2016;123:44–50. https://doi.org/10.1016/j.mimet.2016.02.003.
17. Jafari Modrek M, Hasanzadeh R, Foroutan M, Mirahmadi H, Rahmati-Balaghaleh M, Zarean M. Seroprevalence and molecular evaluation of *Toxoplasma gondii* in Schizophrenic patients hospitalized in Sistan and Baluchestan province, Southeast of Iran. Trop Biomed. 2019;36(2):422–9.
18. Rahmati-Balaghaleh M, Hosseini Farash BR, Zarean M. Diagnosis of acute toxoplasmosis by IgG avidity method in pregnant women referred to health centers in south-eastern Iran. J Parasit Dis. 2019;43(3):517–21. https://doi.org/10.1007/s12639-019-01120-8.
19. Fallahi S, Seyyed Tabaei SJ, Pournia Y, Zebardast N, Kazemi B. Comparison of loop-mediated isothermal amplification (LAMP) and nested-PCR assay targeting the RE and B1 gene for detection of *Toxoplasma gondii* in blood samples of children with leukaemia. Diagn Microbiol Infect Dis. 2014;79(3):347–54. https://doi.org/https://doi.org/10.1016/j.diagmicrobio.2014.02.014.
20. Fallahi S, Kazemi B, Seyyed tabaei SJ, Bandehpour M, Lasjerdi Z, Taghipour N, et al. Comparison of the RE and B1 gene for detection of *Toxoplasma gondii* infection in children with cancer. Parasitol Int. 2014;63(1):37–41. https://doi.org/10.1016/j.parint.2013.08.005.
21. Hosseini SA, Golchin E, Sharif M, Sarvi S, Ahmadpour E, Rostamian A, et al. A serological investigation and genotyping of *Toxoplasma gondii* among Iranian blood donors indicates threat to health of blood recipients. Transfus Apher Sci. 2020;59(3):102723. https://doi.org/10.1016/j.transci.2020.102723.
22. Souza I, Siqueira VDS. Molecular and serological diagnosis of toxoplasmosis: a systematic review and meta-analysis. Rev Inst Med Trop Sao Paulo. 2023;65:e19. https://doi.org/10.1590/s1678-9946202365019.
23. Danehchin L, Razmi G, Naghibi A. Molecular detection of *Toxoplasma gondii* infection in aborted fetuses in sheep in Khorasan Razavi province, Iran. Iran J Vet Med. 2017;11(2):147–54. https://doi.org/10.22059/ijvm.2017.61709.
24. Nakashima F, Pardo VS, Miola MP, Murata FHA, Paduan N, Longo SM, et al. Serum IgG Anti-*Toxoplasma gondii* Antibody Concentrations Do Not Correlate Nested PCR Results in Blood Donors. *Front Cell Infect Microbiol*. 2020;Volume 9 - 2019. https://doi.org/10.3389/fcimb.2019.00461.
25. Zhou N, Fu H, Wang Z, Shi H, Yu Y, Qu T, et al. Seroprevalence and risk factors of *Toxoplasma gondii* infection in children with leukemia in Shandong Province, Eastern China: a case-control prospective study. PeerJ. 2019;7:e6604. https://doi.org/10.7717/peerj.6604.
26. Shaddel M, Mirzaii-Dizgah I, Hoshangi M. Anti-*Toxoplasma gondii* Antibody Levels in Blood Supply of Shiraz Blood Transfusion Institute, Iran. Iran J Parasitol. 2014;9(1):120–4.

27. Karimi G, Mardan A, Zadsar M. Toxoplasma and blood transfusion. Iran J Parasitol. 2014;9(4):597–8.
28. Karimi G, Mardani A, Zadsar M. Prevalence of Toxoplasma gondii among Iranian Blood Donors: A Narrative Review Article. Iran J Parasitol. 2016;11(1):10–8.
29. Mardani A. Prevention strategies of transfusion-transmitted parasitic infections (TTPIs): Strengths and challenges of current approaches, and evaluation of the strategies implemented in Iran. Parasite Epidemiol Control. 2020;9:e00141. https://doi.org/10.1016/j.parepi.2020.e00141.