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Comparison of the RE-529 sequence and B1 gene for *Toxoplasma gondii* detection in blood samples of the at-risk seropositive cases using uracil DNA glycosylase supplemented loop-mediated isothermal amplification (UDG-LAMP) assay

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ABSTRACT

Diagnosis of toxoplasmosis is an important issue, especially in at-risk patients. The molecular methods showed a promising future for such diagnosis; however, the method itself and the target sequence to be detected is an important part of accurate detection of the infection. The aim of the present study was to evaluate the RE-529 sequence and B1 gene for *Toxoplasma gondii* detection in blood samples of the at-risk seropositive cases using uracil DNA glycosylase supplemented loop-mediated isothermal amplification (UDG-LAMP) assay. In this study, 110 *T. gondii* seropositive at-risk individuals (pregnant women and immunocompromised patients) and 110 seronegative controls were enrolled. The two most studied sequences (RE-529 and B1) were used and compared for accurate and reliable detection of *T. gondii* in blood samples using UDG-LAMP assay and compared with real-time PCR method. The detection limit, accuracy, and reliability of UDG-LAMP for the parasite's DNA were also studied. Among 110 studied cases, 39 (35.45%) and 36 (32.7%) were positive for *T. gondii* DNA with the RE-LAMP and B1-LAMP, respectively. The seronegative cases remained negative for *T. gondii* DNA with the studied genes, however, there were few false negatives compared with real-time PCR method. The detection limit of the UDG-LAMP for both DNA targets was 0.16 tachyzoite's DNA per reaction tube. Based on the results of this study, the RE-529 sequence has a better detection rate compared to the B1 gene for toxoplasmosis among at-risk people. UDG-LAMP is a highly sensitive, accurate, and reliable method with no false-positive results for the diagnosis of *T. gondii* infection in blood specimens, however few cases may be missed.

1. Introduction

Toxoplasma gondii is a parasitic organism causing infection in humans and animals worldwide. It has been estimated that one-third of the world's population has been exposed to this protozoan parasite [1,2]. Although the primary infection is usually benign in immunocompetent patients, the infection can cause serious complications in a child from an infected mother [3] and immunocompromised patients [4]. Primary infection in pregnant women can lead to congenital toxoplasmosis, and in immunocompromised patients, it can cause severe neurological symptoms such as diffuse encephalopathy and extensive brain mass. Reactivation of latent toxoplasmosis is another critical aspect in patients with the diseased immune system [5–10]. Given the importance of infection with *T. gondii*, a rapid, sensitive and specific diagnostic tool for the detection and discrimination of acute and chronic toxoplasmosis is necessary, especially in at-risk people. The diagnosis of toxoplasmosis can be attained by different methods. Although serological methods are often used to detect anti-*T. gondii*

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antibodies among suspected individuals [11,39], yet it is not able to differentiate between past and present infection and is not appropriate in immunocompromised patients with the deficient immune system [12]. In recent years, rapid, sensitive, and specific genetic amplification methods have become an essential tool for medical research and diagnostics [13]. Molecular diagnosis of *T. gondii* has been developed in many types of research on different biological samples [14–16]. Among various molecular techniques, loop-mediated isothermal amplification (LAMP) has developed as a popular technique because of its high sensitivity and specificity, and isothermal reaction conditions, which avoid the need for specialized thermal cycling equipment [17]. The highly potent amplification mechanism in the LAMP assay makes it highly sensitive to contamination from previous LAMP reactions that become templates for re-amplification, causing inaccurate false-positive results [18]. Uracil DNA glycosylase supplemented-LAMP (UDG-LAMP), has recently been developed, which is a convenient and effective approach for avoiding carryover contamination in the LAMP assay [19]. This method was designed for eliminating LAMP carryover contamination by utilizing deoxyuridine triphosphate (dUTP) instead of deoxythymidine triphosphate (dTTP) in all LAMP reactions. In this method, the amplicons are labeled with uracil base making them chemically discriminated from target DNA. Before the subsequent LAMP reactions, any uracil-containing LAMP amplicons from the previous reactions (carryover contaminants) consumed with uracil-DNA-glycosylase (UDG) that specifically removes uracil bases in uracil-containing DNA, while has no effect on the natural, thymine-containing ones. This will allow eliminating false-positives resulted from carryover contaminants [20,21].

It is critical to diagnose opportunistic infections such as toxoplasmosis in at-risk hosts like pregnant women and immunocompromised patients for proper and prompt treatment to prevent a serious complication. In the present study, we performed UDG-LAMP assay targeting a repetitive 529 bp DNA fragment and B1 gene in *T. gondii* for the detection of *T. gondii* in blood samples of seropositive at-risk cases.

2. Materials and methods

2.1. Parasite preparation and DNA extraction

Tachyzoites forms of the virulent *T. gondii* strain, RH, obtained from the Toxoplasmosis Research Laboratory, Tehran University of Medical Sciences, Tehran, Iran. Parasites were suspended in sterile phosphate-buffered saline (PBS) containing 100 IU/mL penicillin and 100 μg/mL streptomycin and were then injected to female BALB/c mice intraperitoneally (i.p.). Three to 5 days post-injection, when clinical signs were observed, tachyzoites were harvested from peritoneal exudates by aspiration and centrifuged for 3 min at 3500 g, washed three times with cold PBS (2 mL each, pH 7.2) [22]. The pellets were then re-suspended in cold PBS, and the parasites were counted by a counting-chamber (hemocytometer) under a light microscope. DNA was extracted from the purified parasite using a commercial DNA extraction kit (Roche, Rotkreuz, Switzerland) according to the manufacturer's instructions. The genomic DNA from the counted amounts of *T. gondii* tachyzoites (RH strain) was used as a standard calibrator in the LAMP assay. Negative controls were also included to avoid contamination.

2.2. Clinical samples

During 11 months (August 2016 to July 2017), 110 blood samples were collected from two groups of cases, including 55 anti-*T. gondii* IgM positive individuals (25 pregnant women and 30 immunocompromised patients) and 55 anti-*T. gondii* IgG positive people (28 pregnant women and 27 immunocompromised patients). All immunocompromised cases were HIV positive patients and were selected based on $CD4^+$ T-lymphocyte counts (below 200 cells per μL). In addition to them, 110 seronegative blood samples for anti-*T. gondii* antibodies from 55 pregnant women and 55 immunocompromised patients were also collected as negative controls. All cases admitted to the different medical centers of Tehran University of Medical Sciences, Tehran, Iran (Table 2). Blood sera were used for serological testing and whole blood for DNA extraction and subsequent molecular examinations. All samples had tested for *Toxoplasma* DNA previously by real-time PCR method on the same sequences and those results were used as a gold standard for calculating the sensitivity and specificity of UDG-LAMP assay [38].

2.3. Enzyme immunoassays

Anti-*Toxoplasma* IgM and IgG antibodies were analyzed by VIDAS anti-*T. gondii* IgG and IgM Kits (bioMe'rieux, France). The assay combined an enzyme immunoassay method by immunocapture with a final fluorescent detection (Enzyme-Linked Fluorescent Assay; ELFA). Analysis of the serum antibodies was assessed according to the Kit manufacturers' instruction. The test results were interpreted as follows: for IgM, < 0.55 IU/mL: negative, $0.55 \leq$ to < 0.65 IU/mL: equivocal, ≥ 0.65 IU/mL: positive and for IgG, < 4 IU/mL: negative, between $4 \leq$ to < 8: equivocal and ≥ 8: positive.

2.4. DNA extraction from clinical samples

DNA was extracted from 110 seropositive cases of both groups (55 IgM +, IgG + and 55 IgM-, IgG+) and 110 seronegative cases using commercial extraction kit according to the company's protocol (Roche, Rotkreuz, Switzerland). DNA was extracted from the whole blood and kept frozen at −20 °C for further use in UDG-LAMP reactions. As a positive control, genomic DNA was extracted from 100 μl of RH strain tachyzoites suspension containing 3×10^6 parasites/mL.

2.5. The UDG- LAMP reaction

The UDG- LAMP assay was carried out based on highly conserved and repeated region, RE and B1, of *T. gondii* genome. Six

Table 1
Primers used for the UDG-LAMP assay.

Target	Oligonucleotide sequence	Size	Reference
B1 (AF179871)	BIP-5′-TCGCAACGGAGTTCTTCCCAGTTTTGGCCTGATATTACGACGGAC-3′ FIP-5′-TGACGCCTTTAGCACATCTGGT TTTTGATGCTCAAAGTCGACCGC-3′ F3-5′-GGGAGCAAGAGTTGGGACTA-3′ B3-5′CAGACAGCGAACAGAACAGA-3′	202 bp	[24]
529 bp RE (AF146527)	BIP-5′-TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAGCCAAG-3′ FIP-5′-TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC-3′ LF-5′-TCCAAGACGGCTGGAGGAG-3′ LB-5′-CGGAGAGGGAGAAGATGTTTCC-3′ F3-5′-CCACAGAAGGGACAGAAGTC-3′ B3-5′-TCCGGTGTCTCTTTTTCCAC-3′	212bp	[23]

Table 2
Regents and concentrations used for *Toxoplasma* B1 and RE UDG-LAMP.

Regents	Primary Concentration	Final concentration	Volume
Thermopol buffer	10 x	1 x	2.5 μl
$MgSO_4$	100 mM/μl	8 mM/μl	2.0 μl
dUTP mix	10 mM/μl	1.4 mM/μl	3.5 μl
F3 Primer	500 pmol/μl	5 pmol/μl	0.25 μl
B3 primer	500 pmol/μl	5 pmol/μl	0.25 μl
FIP Primer	500 pmol/μl	40 pmol/μl	2.0 μl
BIP primer	500 pmol/μl	40 pmol/μl	2.0 μl
F-Loop primer	500 pmol/μl	20 pmol/μl	1.0 μl
B-Loop primer	500 pmol/μl	20 pmol/μl	1.0 μl
Betaine	5 M	0.8 M	4.0 μl
Bst2 DNA Polymerase	8000 U	8 U	1.0 μl
Template	100 ng	4 ng	1.0 μl
UDG	200U	1U	1.0 μl
UDG Buffer	10 x	1 x	2.5 μl
DW	–	–	1.0 μl
Final volume			25 μl

oligonucleotide primers targeting eight conserved regions within the 200- to 300-fold repetitive 529 bp [23] and four primers targeting six conserved regions of the B1 gene were used [24]. All used primers sequences are listed in Table 1.

The LAMP reaction mixture (25 μl), contained 1 μL of template DNA, 40 picomol of each of FIP and BIP primers, 20 pmol of each LF and LB primers (only for RE-LAMP), 5 pmol of each of F3 and B3 primers, 8 U of *Bst2* DNA polymerase (New England Biolabs, Ipswich, MA, USA), 1.4 mmol/L of deoxyuridine triphosphates (dUTP) instead of dTTP and 2X reaction buffer, containing 1.6 mol/L betaine (Sigma-Aldrich, St. Louis, Missouri, United States), 40 mmol/L Tris-HCl (pH 8.8), 20 mmol/L of KCL, 20 mmol/L of $(NH_4)_2SO_4$, 16 mmol/L of $MgSO_4$ and 0.2% tween 20). The regents and their concentration and the used volumes are summarized in Table 2. The reactions were carried out at 63 ˚C for 60 min. For visual assessment of the LAMP amplicons in the reaction, 3 μL of fluorescent detection reagent, SYBR Green I (Invitrogen, Thermo Fisher Scientific Invitrogen lot, California, United States), was diluted in dimethyl sulfoxide (DMSO) and then was added to each reaction tube and the fluorescent signals of each solution were observed under daylight and UV light. Furthermore, LAMP positive reactions could be detected by visual endpoint result of turbidity of the magnesium pyrophosphate that was produced in the reaction mixture. Gel electrophoresis was performed on the LAMP products in a 1.5% agarose gel stained with DNA Safe Stain (1 μg/mL) and visualized under UV light. The assay reproducibility was evaluated by the duplicated reactions of the samples simultaneously. A positive control (*T. gondii* RH strain DNA) and negative control (doubled distilled water) were also included in each LAMP reaction.

2.5.1. The analytical sensitivity and specificity of UDG-LAMP

In order to determine the analytical sensitivity of the UDG-LAMP assay for the detection of *T. gondii* DNA, 10-fold serial dilutions of *T. gondii* tachyzoites ranging from 10000 to 1 tachyzoite were prepared and DNA was extracted from each dilution using a DNA extraction kit (Roche, Rotkreuz, Switzerland) and the same LAMP reaction targeting the both described target sequences were carried out for all dilutions. The DNA samples of other parasites, including *Leishmania major*, *Cryptosporidium* spp., and *Fasciola hepatica* were used to determine the specificity of the LAMP technique. *Cryptosporidium* was chosen because *Toxoplasma* and *Cryptosporidium* are both coccidia, and the two other control parasites were chosen because they were conveniently available.

2.5.2. UDG-LAMP

In this modified LAMP reaction, replacing dTTP with dUTP results in the production of uracil integrated DNA. If this uracil-integrated DNA leads to the carryover contamination of the subsequent LAMP reactions, it can be eliminated using UDG enzyme that cleaves uracil bases from any contaminating LAMP amplicons while uracil-free target DNA (template DNA) remains completely unaffected [20]. In order to approve the ability of *Bst2* DNA polymerase in incorporating dUTP instead of dTTP in the LAMP reaction, the reaction was done in an optimized reaction mixture containing 1.6 μL of 10 mM dUTP (New England Biolabs, Ipswich, MA, USA) instead of 10 mM dTTP [21].

2.5.3. UDG treatment

In this stage, which applies to all subsequent reactions, UDG digestion was done before the amplification; we treat the reaction mixture with a heat-labile UDG enzyme. For evaluation of UDG ability to eliminate carryover contamination in a LAMP reaction, 5U of UDG (New England Biolabs, Ipswich, MA, USA) was added to the optimized reaction mixture containing 1 μL of LAMP product from the previous reaction executed using dUTP instead of dTTP prepared at the original concentration and at 1/10 and 1/50 dilutions. Because of similarities between the two reaction buffer components for *Bst2* DNA polymerase and UDG enzyme, the UDG reaction buffer was not added to the reaction mixture. Before the primary LAMP reaction, 2-min incubation at 37 ˚C was done for the UDG treatment [20,21].

2.6. Data analysis

The obtained data were analyzed using the SPSS software version 21 for Windows (IBM Corp., Armonk, NY, United States).

Ethical approval

This study was appraised and approved by the Ethics Committees of Tehran University of Medical Sciences, Tehran, Iran (No: 32018).

3. Results

3.1. Analytical sensitivity of UDG-LAMP reaction

Serial dilutions, 1, 5, 50, 500, 5000 tachyzoites per mL evaluated for the sensitivity of UDG-LAMP assay based on two target sequences. The detection limit of the assay was as low as five tachyzoites per mL of blood or 0.16 tachyzoite's DNA per reaction tube (Figs. 1 and 2). The

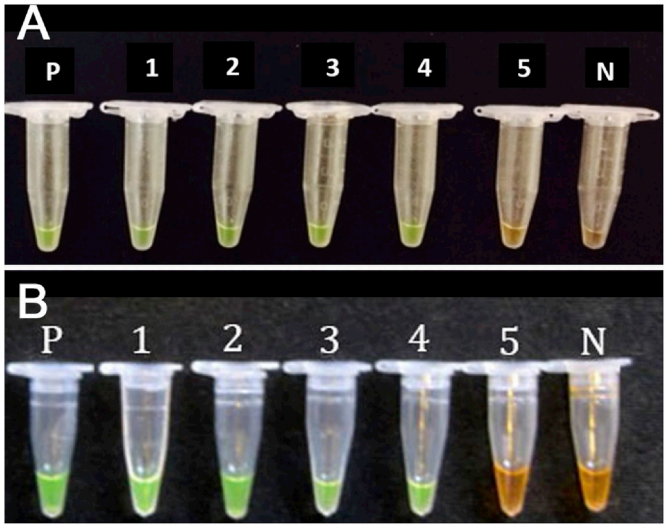

Fig. 1. RE-LAMP (A) and B1-LAMP (B) products of the different tachyzoite numbers stained with SYBR Green under UV light. P, positive control1, 5000 tachyzoites; 2, 500 tachyzoites; 3, 50 tachyzoites; 4, five tachyzoites; 5, one tachyzoite; N, negative control.

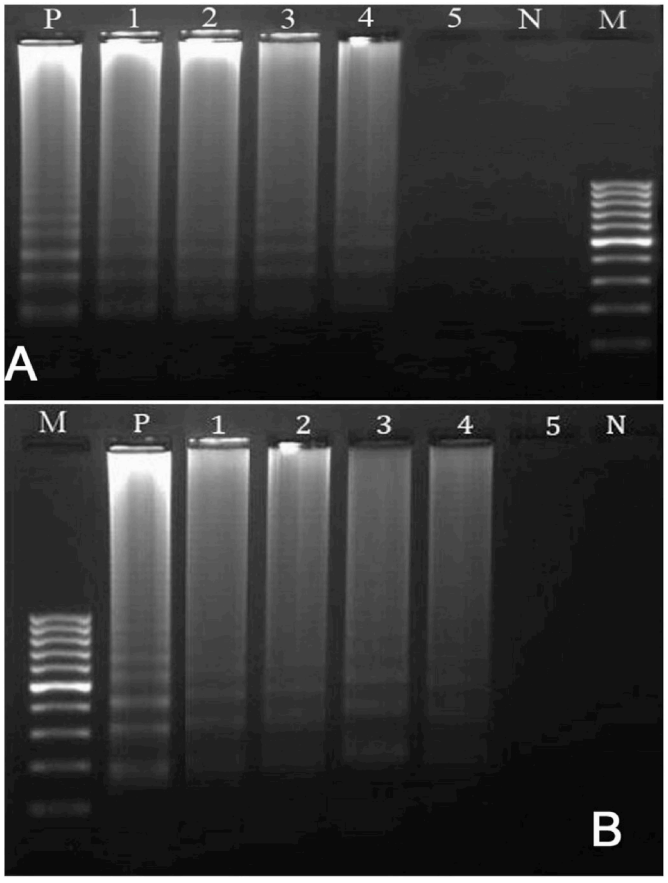

Fig. 2. RE-LAMP (A) and B1-LAMP (B) products of the different tachyzoite numbers. P, positive control; N, negative control; M, 100 bp DNA Marker; lanes 1–5: 5000 tachyzoites, 500 tachyzoites, 50 tachyzoites, five tachyzoites, one tachyzoite.

negative controls and other used organisms (*Leishmania major*, *Cryptosporidium* spp., and *Fasciola hepatica*) did not yield positive amplification in the LAMP reaction. The turbidity of the positive and negative samples was not distinguishable by the visual examination.

3.2. UDG-LAMP on clinical samples

Among 110 studied cases, 39 (35.45%) and 36 (32.7%) were positive for *T. gondii* DNA with the RE-LAMP and B1-LAMP, respectively (Table 1). The results of the same sequences were known with the real-time PCR method, when compared with UDG-LAMP, no false-positive was observed. However, there were false negatives, especially in the RE-LAMP (Table 3). The positive tubes showed a fluorescent signal after adding SYBR Green I and, yet the negative ones did not show any color change (Fig. 3). The seronegative cases remained negative for *T. gondii* DNA with the studied genes.

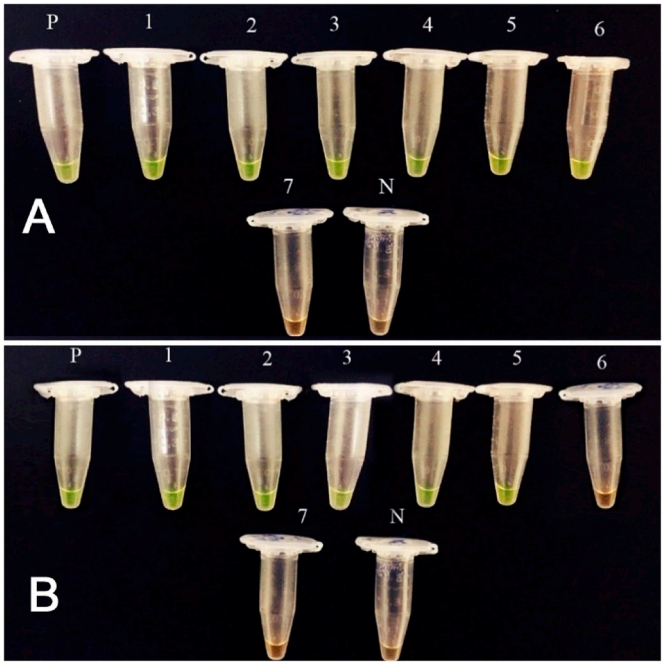

Fig. 3. Tubes with LAMP reaction (A, RE-LAMP; B, B1-LAMP) stained with SYBR Green. P, positive control; N, negative control; 1–7, different patients with positive titers of anti-*Toxoplasma* antibodies.

4. Discussion

In the present study, the B1 gene and RE sequence of *T. gondii* are evaluated for the detection of the parasite's DNA in the blood samples of seropositive and seronegative individuals. The sensitivity of UDG-LAMP for the detection of *T. gondii* was also studied. This is the first report of using UDG-LAMP on the detection of *T. gondii* infection in immunocompromised patients and pregnant women in Iran. The detection limit of UDG-LAMP for *T. gondii* tachyzoites was five parasites/mL of sample (0.16 tachyzoite's DNA/reaction tube) for both studied genes, which seems quite reasonable. On the other hand, none of the seronegative cases were yielded positive results considering *T. gondii* DNA, which shows the method's high specificity; however there were few false negatives, especially in RE-LAMP among IgG^+/IgM^+ patients. Thus the method needs more modification to roll out any false negative, which is critical, especially in pregnant women, and losing even one patient is not ideal. Furthermore, the RE repetitive sequence of *T. gondii* could result in high positive rates, which may show its high sensitivity.

There are different molecular techniques for the detection of *T. gondii* in different clinical samples, which provided great diagnostic and research tool for *T. gondii* [25,40]. LAMP is an alternative method to the conventional PCR [26]. LAMP is one of the recently introduce methods

Table 3
The positivity of *T. gondii* DNA among studied patients using the UDG-LAMP method compared to the results of real-time PCR as a gold standard.

Studied population	Serology	Target sequence	LAMP no (%)	Coincidence RE with B1	False Positive*	False Negative*	Sensitivity	Specificity
Pregnant women	IgM+, IgG+	RE	14 (60)	14 (100%)	0	3	85%	100%
N = 53	N = 25	B1	14 (60)		0	0	100%	100%
	IgM-, IgG+	RE	6 (21.4)	5 (83.33%)	0	1	86%	100%
	N = 28	B1	5 (17.8)		0	1	83%	100%
Immunocompromised patients	IgM+, IgG+	RE	17 (56.6	15 (88.23%)	0	3	87%	100%
N = 57	N = 30	B1	15 (50)		0	2	89%	100%
	IgM-, IgG+	RE	2 (7.4)	2 (100%)	0	0	100%	100%
	N = 27	B1	2 (7.4)		0	0	100%	100%

Real-time PCR of the same sequence used as a gold standard.

for DNA amplification in an isothermal condition that amplifies DNA sequence with elevated productivity, specificity, and promptness. LAMP works with a *Bst* DNA polymerase and a set of four primers that recognize six distinct sequences on the DNA sample. This method initially developed by Notomi et al., in 2000 [17]. LAMP advances by dislocating and freeing a single-strand DNA fragment. The amplification initiated by an inner primer continues by strand displacement by an outer primer. The amplification products can be revealed by direct fluorescence using fluorescent dyes, e.g., SYBR Green [26]. LAMP assays targeting the SAG1 and SAG2 genes, RE-529 sequence and B1 gene of *T. gondii* were developed and used in different studies [23,27–31]. LAMP is reported to have higher sensitivity compared with nested PCR for the detection of *T. gondii* DNA in the blood samples [29,30].

In the present study, the parasite's DNA was detected in 60% of anti-*T. gondii* IgG and IgM positive pregnant women, which is a considerable number considering the risk of congenital toxoplasmosis [3]. The presence of parasite DNA in the blood of pregnant women may represent acute toxoplasmosis [26]. On the other hand, 21.4% of the IgM-, IgG + pregnant women, and 7.4% of immunocompromised patients had *T. gondii* in their blood samples, or in the other word they may have parasitemia. The same results are reported from several studies [30,32,33]. The reason for this can be the reactivated chronic toxoplasmosis [34]. Thus, it could be inferred that even with the negative anti-*T. gondii* IgM serological result, there can be a chance of parasitemia and may be problematic, especially in immunocompromised patients. However, the free parasitic DNA in the blood serum during the chronic phase should not be ignored.

In the present research, the RE-529 sequence showed slightly higher positive frequency compared with the B1 gene using the UDG-LAMP method. It may show the superiority of the RE-529 sequence for the detection of *T. gondii* DNA in different samples. The similar results are reported from different studies using other molecular methods [30,35,36]. Despite the preference of molecular methods over the serological test for toxoplasmosis, choosing the suitable target sequence for diagnostic proposes seems to be critical. In this study, few patients were missed by the RE-529 and B1 gene; thus, if it happens in diagnostic labs on at-risk patients, it would have serious consequences.

In 2008, Sotiriadou and Karanisac evaluated the LAMP for the detection of *T. gondii* in water samples and compared it with conventional PCR. LAMP was assessed with B1 and TgOWP *T. gondii* genes. They reported a sensitivity detection limit of 0.1 tachyzoite's DNA for both studied genes, which is very close to our findings (0.16). The detection sensitivity of LAMP and nested PCR was reported 100% and 53.8%, respectively. Their data suggest that LAMP could be a rapid, sensitive, and specific tool for the detection of contamination of water with *T. gondii* [24].

Zhang et al. (2009) determined the detection limit of *T. gondii* RE-529 LAMP as one pg/μL of *T. gondii* DNA. Furthermore, they compared LAMP and PCR for the detection of *T. gondii* in pigs' lymph nodes and reported 76.9% and 85.7% of the samples having the parasite with PCR and LAMP analyzes, respectively. They suggested the LAMP assay as an alternative molecular tool for the detection of toxoplasmosis from veterinary samples [23].

Lau et al. (2010) performed three LAMP assays on the SAG1, SAG2, and B1 genes of *T. gondii* and compared with nested PCR. Their finding suggests that the LAMP assay is highly sensitive and had a detection limit of 0.1 tachyzoite's DNA per reaction, and also it does not have any cross-reactivity with the DNA of other parasites. Their results and ours are alike considering our detection limit of 0.16 tachyzoite's DNA per reaction and no cross-reactivity with the other parasitic organisms' DNA. They also tested the LAMP assay on 105 individuals (40 with active toxoplasmosis, 40 negative controls, and 25 with other parasitic infections). Their finding showed that the SAG2-LAMP had 87.5% sensitivity compared with the SAG1-LAMP with 80%, B1-LAMP 80%, and nested PCR 62.5% of sensitivity. The specificity of LAMP and nested PCR were reported 100% [29].

Fallahi et al. (2014) compared the LAMP and nested-PCR assays targeting the RE-529 sequence and B1 gene for the detection of *T. gondii* DNA in the blood samples of children with leukemia. They reported the detection of parasite's DNA among 50 anti-*T. gondii* IgM^+, IgG^+ patients in 92% and 86% on RE- and B1-LAMP, and 82% and 68% on RE- and B1-nested PCR analyses, respectively. Contrary to our findings, they found three, two, and one positives by RE-LAMP, B1-LAMP, and RE-nested PCR methods among 50 seronegative individuals, respectively; however no positive was found by the B1-nested PCR. According to the results of our study, none of the seronegative controls were positive by the B1 or RE-529 UGD-LAMP assay. Furthermore, none of their 10 IgM^-, IgG^+ samples was positive with LAMP and nested-PCR assays, yet we had such positive individuals who were IgM^- and IgG^+ with the positive UGD-LAMP result [30].

Considering the high sensitivity of the LAMP assay, the false-positive results can be produced by even minuscule amounts of contaminants [37]. Consequently, preventing carryover contamination is crucial to achieving accurate and reliable detection. In the present study, UDG-LAMP, a new version of the LAMP technique supplemented with an initial UDG treatment to prevent carryover contamination, has been applied to detect the *T. gondii* DNA in blood samples of the highly at-risk seropositive cases.

5. Conclusion

According to the results of the present study, the RE-529 sequence showed slightly higher positive rates and may have superiority upon the B1 gene for the diagnosis of *T. gondii* infection using the UGD-LAMP method, however, few cases would be missed with both sequences. UGD-LAMP possessed a very low detection limit, which a fraction of one *T. gondii* tachyzoite's DNA can be detected by the LAMP. This improved UGD-LAMP assay makes an accurate, rapid, and reliable diagnosis of toxoplasmosis possible, yet it needs further modification on false-negative results. The anti-*T. gondii* IgM^- and IgG^+ serological pattern can not exclude parasitemia in pregnant women and immunocompromised patients, which shows the importance of diagnostic methods for such at-risk people.

CRediT authorship contribution statement

Hossein Keshavarz Valian: Methodology, Supervision, Funding acquisition, Writing - review & editing. **Hossein Mirhendi:** Methodology, Supervision, Writing - review & editing, Investigation. **Mehdi Mohebali:** Methodology, Supervision, Writing - review & editing. **Saeedeh Shojaee:** Methodology, Supervision, Writing - review & editing. **Shirzad Fallahi:** Data curation, Methodology, Writing - review & editing. **Rasool Jafari:** Investigation, Data curation, Writing - original draft. **Farnaz Kheirandish:** Investigation, Data curation, Writing - original draft. **Parisa Mousavi:** Methodology, Data curation, Validation, Investigation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that have no conflict of interests.

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