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Challenging loop-mediated isothermal amplification (LAMP) technique for molecular detection of *Toxoplasma gondii*

Shirzad Fallahi[1*], Zahra Arab Mazar[2], Mehrdad Ghasemian[3], Ali Haghighi[2]

[1]*Department of Medical Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran*
[2]*Department of Medical Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[3]*Department of Medical Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Objective: To compare analytical sensitivity and specificity of a newly described DNA amplification technique, LAMP and nested PCR assay targeting the RE and B1 genes for the detection of *Toxoplasma gondii (T. gondii)* DNA. **Methods:** The analytical sensitivity of LAMP and nested-PCR was obtained against10-fold serial dilutions of *T. gondii* DNA ranging from 1 ng to 0.01 fg. DNA samples of other parasites and human chromosomal DNA were used to determine the specificity of molecular assays. **Results:** After testing LAMP and nested-PCR in duplicate, the detection limit of RE-LAMP, B1-LAMP, RE-nested PCR and B1-nested PCR assays was one fg, 100 fg, 1 pg and 10 pg of *T. gondii* DNA respectively. All the LAMP assays and nested PCRs were 100% specific. The RE-LAMP assay revealed the most sensitivity for the detection of *T. gondii* DNA. **Conclusions:** The obtained results demonstrate that the LAMP technique has a greater sensitivity for detection of *T. gondii*. Furthermore, these findings indicate that primers based on the RE are more suitable than those based on the B1 gene. However, the B1-LAMP assay has potential as a diagnostic tool for detection of *T. gondii*.

1. Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite capable of infecting humans and all warm-blooded animals worldwide[1]. Despite the high rate of infection in human, infections often remain asymptomatic in immunocompetent individuals. Congenital toxoplasmosis can lead to mild or severe sequela, and life-threatening infections can occur in immunocompromised patients including patients with AIDS or organ transplant recipients[2,3]. An effective, rapid and accurate diagnosis is crucial and desirable to initiate adequate treatment in the immunocompromised patients. Traditionally, toxoplasmosis diagnosed by identifying the anti-*Toxoplasma* specific antibodies in the serum with serological techniques such as Enzyme-linked immunosorbent assay (ELISA) and Immunofluorescence antibody Assay (IFA)[4,5]. Diagnosis of toxoplasmosis by IFA and ELISA is laborious, time-consuming, expensive with variable sensitivity and specificity[6]. Since the *T. gondii* specific antigens and antibodies may not be present early, especially in the patients with immunodeficiency and specific antibodies, particularly IgM, may not arise during reactivation of the encysted form of the parasite, serological methods have some limitations[7]. Mouse inoculation or tissue cultures of the clinical samples may confirm the infection by parasites but they are still impractical, labor-intensive and require several days to obtain results[8,9]. The acute cases of toxoplasmosis in humans could be alternatively identified on the basis of the detection of *T. gondii* DNA in amniotic fluids, tissues, or blood by using the molecular assays such as polymerase chain reaction (PCR)[10,11]. During the last decade, PCR methods have considerable progress for the detection of toxoplasmosis. Among these methods, nested-PCR followed by hybridization has been reported as the most sensitive diagnostic technique for the diagnosis

*Corresponding author: Shirzad Fallahi, Department of Medical Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran.
Tel&Fax: +98 661 33120133
E-mail: Shfupdate@gmail.com, Fallahi.Sh@lums.ac.ir
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of toxoplasmosis[12,13]. However, due to the expensive cost of the apparatus and long reaction time, the PCR assays are not widely used[14,15]. Therefore, development of accurate, simple, rapid and cost effective detection methods for direct detection of *T. gondii* is still needed.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification assay with extremely high sensitivity and specificity which was originally developed by Notomi *et al*[16]. This easy-to-performed technique using a DNA polymerase with strand displacement activity and four to six specially designed primers that recognize six to eight regions of the target DNA (which eliminates nonspecific binding) can amplify up to 10^9 copies in less than an hour under isothermal conditions (63 ℃-65 ℃)[17,18]. Due to the isothermal conditions in the LAMP assay, a simple incubator, such as a water bath or block heater, is sufficient for the DNA amplification, making use of incubators under field conditions feasible[19,20]. Amplification and detection of a gene can be completed in a single step. DNA amplification can be easily detected by visual inspection of the turbidity or fluorescence of the reaction mixture or by a Loopamp real-time turbidimeter[16,17]. As a consequence, visual inspection alleviates the need for gel electrophoresis, reduces the assay time, and thus, makes the method suitable for field tests. These characteristics enable the LAMP to be applied as an accurate and rapid molecular diagnostic tool for the detection of *T. gondii*. LAMP has been successfully developed and applied in detection of various pathogens including West Nile virus[21], Corona virus[22], *Trypanosoma* species[23,24], *Babesia gibsoni*[25], *Mycobacterium* species[26], *Cryptosporidium* species[27], *Entamoeba histolytica*[28], *Plasmodium falciparum*[29,30], *Taenia* species[31], *Schistosoma japonicum*, *Schistosoma haematobium* and *Schistosoma mansoni*[32,33], *Fasciola hepatica* and *Fasciola gigantica*[34], and *T. gondii*[35-37].

Challenging the analytical (sensitivity and specificity) and technical performances of the LAMP and nested-PCR assays targeting the two repetitive conserved regions (RE and B1) in the *Toxoplasma* genome for the molecular diagnosis of *T. gondii* infection, was the main purpose of the present study.

2. Materials and methods

2.1. Preparation of the T. gondii tachyzoites

T. gondii tachyzoites were prepared as previously described[38]. In brief, a vial of frozen in nitrogen *T. gondii* tachyzoites (RH strain) was injected to the mice intra-peritoneally. When the symptoms of disease (lethargy, ascites, and bristle hair) were appeared in the mice, 3-5 mL of cold sterile saline was injected intra-peritoneally. Thereafter peritoneal exudate was collected following a few gentle impulses, and centrifuged for 3 min 3 500 *g*, afterwards the supernatant was discard and the precipitate washed three times with 3 mL cold phosphate-buffered saline (PBS, pH 7.2). In order to rupture the infected macrophages and purification of the tachyzoites, the final pellet was filtered three times through a 30-gauge needle syringe. The treated samples were then centrifuged at 4 ℃ (3 min 3 500×*g*), after discarding the supernatant, 1 mL of cold PBS was added to the precipitate until the DNA was extracted from the parasites. 100 μL of the purified parasites solution, containing 3×10^6 tachyzoites was used to extracting the DNA.

2.2. DNA extraction

Extraction of the DNA was performed using the classical phenol-chloroform extraction, and two commercial DNA extraction kits (DNP™ DN8115C, and DNG-Plus™, DN8118C, Cinnaclon Co. Iran) according to the manufacturer's instructions. DNA extraction using DNP and DNG commercial kits previously was described[38]. As the positive control, genomic DNA was extracted from the *T. gondii* tachyzoites RH-strain using the three above mentioned DNA extraction methods. A Nano-spectrophotometer (WPA-Biowave Ⅱ, England) was used to determine the concentration and quality of the DNA. Each *T. gondii* one 80-Mbp genome was assumed equal about 80 fg DNA[39].

2.3. Analytical sensitivity and specificity of LAMP and nested-PCR

In order to determine the analytical sensitivity of LAMP and nested-PCR assays targeting the two different genes, ten-fold serial dilutions of *T. gondii* DNA ranging from 1 ng to 0.01 fg, were prepared. To determine the least amount of template DNA in a reaction which can be detected by each protocol, the dilution series were tested in duplicate with LAMP and nested-PCR assays. DNA samples of other parasites including, *Cryptosporidium parvum*, *Giardia duodenalis*, *Echinococcus granulosus* (protoscolex and germinal layer), *Acanthamoeba*, *Leishmania tropica*, *Leishmania major*, *Leishmania infantum*, *Plasmodium falciparum*, *Plasmodium vivax*, *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moskovski* and human chromosomal DNA were subjected in the PCR and LAMP to determine the specificity of the assays.

2.4. Nested-PCR

The selected genomic targets (B1 gene and 529 bp repeated element (RE)) for nested-PCR and LAMP methods are two commonly used repetitive sequences in the genome of *T. gondii*. Nucleotide sequences of the primers were used in nested PCR assay are shown in Table 1[13, 40]. PCR amplification of the B1 gene and RE targets in *T. gondii* was carried out as previously described[38]. A PCR negative-control sample omitted template DNA, which was replaced by sterile water and a positive-control sample that used extracted DNA from *T. gondii* tachyzoites RH-strain. The PCR products of 164 and 194 bp for the positive reactions for RE and B1 gene respectively appeared on the 1.5% agarose gel stained with ethidium bromide solution (1 μg/mL). The results of nested-PCR assays were

compared to each other and with the results of LAMP technique.

2.5. LAMP

All the used primer sequences in the LAMP technique are listed in Table 1[41-43]. The LAMP reaction mixture contained 1 μL of template DNA, 40 pmol each of primers FIP and BIP, 20 pmol each of primers LF and LB (were used only in RE-LAMP), 5 pmol each of primers F3 and B3, 8 U of *Bst* DNA polymerase (New England Biolabs, USA), 1.4 mM deoxynucleoside triphosphates (dNTP), and 2 reaction buffer (1.6 M betaine (Sigma-Aldrich), 40 mM Tris–HCl (pH 8.8), 20 mM KCl, 20 mM $(NH_4)_2SO_4$, 16 mM $MgSO_4$, and 0.2% Tween 20) in a final volume of 25 μL. To ensure that the used loop primers in the RE-LAMP assay did not induce self-amplification of primers, reaction was carried out in the absence of any template. As well as to find the optimum time and temperature conditions for LAMP assay, the reactions were carried out at 60 ℃ to 67 ℃ for 30, 45, 60 and 75 min. As positive and negative controls, *T. gondii* RH-strain genomic DNA and double-distilled water, respectively, were included in each run. The reaction products were analyzed by electrophoresis on a 1.5% ethidium bromide-stained agarose gel (1 μg/mL) and visualized under a UV transilluminator. LAMP-positive results are characterized by the appearance of ladder-like pattern on agarose gel electrophoresis. Positive responses were also visually established by the whitish coloration resulting magnesium pyrophosphate, a byproduct of the LAMP assay in the reaction mixture. As well as visual inspection of the LAMP amplicons in the reaction tube was performed by adding 1 μL of 1/10 dilution of SYBR Green Ⅰ (Invitrogen lot: 49743A, USA) after the reaction, and the fluorescent signals of the solutions were observed under UV light. To avoid carryover contamination with amplified products, upon opening the tube and before adding the SYBR Green I, the reaction tubes were centrifuged at 12 000 rpm for 3 min and then were frozen at -20 ℃ for 10 min. In order to determine the reproducibility of the nested-PCR and LAMP, each sample was amplified in duplicate.

2.6. Specificity of LAMP primers

Each outer LAMP primer pair (F3 and B3; Table 1) was initially tested for *Toxoplasma* specificity using the PCR. The PCR reaction mixtures (20 μL) contains 10 mM Tris-HCl, pH 8.3 (at 25 ℃), 50 mM KCl, 1.5 mM $MgCl_2$, 5 μM of each primer F3 and B3 (Table 1), 250 μM each dNTP, 0.1 U *Taq* DNA polymerase, and 1 μL of extracted DNA. Each PCR was consisted of 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 49 ℃ and 50 ℃ for 30 s for B1 gene and RE respectively and extension at 72 ℃ for 30 s, with an initial denaturation step consisting of incubation at 94 ℃ for 5 min and a final extension step consisting of incubation at 72 ℃ for 5 min also included. After PCR amplification, 10 μL aliquots of the PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide (1 μg/mL) for visualization under UV light. PCR products were purified by a DNA extraction kit from agarose gel (Fermentas Co.), according to the manufacturer's instructions. Purified PCR products were sequenced using an ABI 377 automated DNA sequencer (using Big Dye Terminator Chemistry) employing the same primers (individually) as used in the PCR. The sequences were aligned with the target one using the Basic Local Alignment Search Tool (BLAST).

Table 1
Nucleotide sequences of the used primers for nested-PCR and LAMP assays.

Molecular assay	Target	Nucleotide sequence	Amplicon*	Reference or source
Nested PCR	B1	B1F1-5′-TCAAGCAGCGTATTGTCGAG-3′	194 bp	Wiengcharoen *et al*, 2004
		B1R1- 5′-CCGCAGCGACTTCTATCTCT-3		
		B1F2- 5′-GGAACTGCATCCGTTCATGAG-3′		
		B1R2-5′- TCTTTAAAGCGTTCGTGGTC -3′		
Nested PCR	RE	REF1-5′-TGACTCGGGCCCAGCTGCGT-3′	164 bp	Kong *et al*, 2012
		RER1-5′-CTCCTCCCTTCGTCCAAGCCTCC-3′		
		REF2-5′-AGGGACAGAAGTCGAAGGGG-3′		
		RER2-5′-GCAGCCAAGCCGGAAACATC-3′		
LAMP	B1	BIP- 5′-TCGCAACGGAGTTCTTCCCAGTTTTGGCCTGATATTACGACGGAC-3′	212 bp	Sotiriadou *et al*, 2008
		FIP-5′-TGACGCCTTTAGCACATCTGGT TTTTGATGCTCAAAGTCGACCGC -3′		
		F3- 5′-GGGAGCAAGAGTTGGGACTA-3′		
		B3- 5′CAGACAGCGAACAGAACAGA -3′		
LAMP	RE	BIP- 5′-TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAGCCAAG-3′	202 bp	Zhang *et al*, 2009
		FIP-5′-TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC-3′		Lin *et al*, 2012
		LF- 5′-TCCAAGACGGCTGGAGGAG-3′		
		LB- 5′-CGGAGAGGGAGAAGATGTTTCC-3′		
		F3- 5′-CCACAGAAGGGACAGAAGTC-3′		
		B3- 5′-TCCGGTGTCTCTTTTTCCAC-3′		

*In the LAMP technique, the length between F3 and B3.

2.7. LAMP and nested-PCR on blood samples from infected mice

BALB/c mice (Male, six-week old) were purchased from the Experimental Animal Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Five mice were infected by intraperitoneal injection of 10 virulent tachyzoites of the *T. gondii* RH-strain. Blood samples were collected from the vena orbitalis posterior plexus blood at 1-9 day post infection (dpi). The blood samples DNA was extracted by using the three above-mentioned DNA extraction methods. The purified DNA from 100 μL blood samples was dissolved in 30 μL of the solvent buffer or double-distilled water and 1 μL of the resulting supernatant was used as the template. As negative control, the DNA was extracted from the blood samples of five uninfected mice. In addition, the diagnostic sensitivity and specificity of LAMP and nested-PCR for the detection of *T. gondii* in blood samples was analyzed by examining 20 blood samples obtained from 10 infected (with 10 virulent *T. gondii* RH-strain tachyzoites) and 10 normal mice.

3. Results

3.1. Detection and verification of LAMP product

Successful LAMP reaction was carried out under optimal conditions. The best results were obtained when the reaction was done at 63 C and 65 C for 60 min for RE and B1 genomic targets respectively (data not shown). Positive LAMP reaction yields a characteristic ladder of multiple bands of different sizes up to the loading well on a 1.5% ethidium bromide-stained agarose gel. The positive results were also confirmed by visual establishment of the resulting magnesium pyrophosphate whitish coloration of the reaction mixture. Furthermore, the positive reaction turned green upon addition of SYBR Green I to the reaction products whilst, the negative remained orange.

PCR amplification and subsequent sequencing of the products sequences using the two outer primers, B3 and F3 (Table 1) was done to confirm the specificity of LAMP primers for the detection of Toxoplasma. Ultimately the obtained partial sequences were identical to the corresponding B1 and 529 bp RE sequences reported in GenBank (accession numbers AF179871 for B1 and AF146527 for 529 bp RE). The used loop primers in RE-LAMP reaction did not induce self-amplification in the absence of any template.

3.2. Analytical sensitivity and specificity of LAMP and nested-PCR

The detection limit of the RE-LAMP, B1-LAMP, RE-nested PCR and B1-nested PCR assays was one fg (Figure 1A), 100 fg (Figure 1B), one pg (Figure 1C) and 10 pg (Figure 1D) of the *T. gondii* DNA respectively. For the specificity test, only the *T. gondii* DNA was amplified, whereas no amplification was observed in the DNA samples of other parasites and human chromosomal DNA (data not shown). DNA extraction using the different methods did not affect the results of LAMP and nested-PCR.

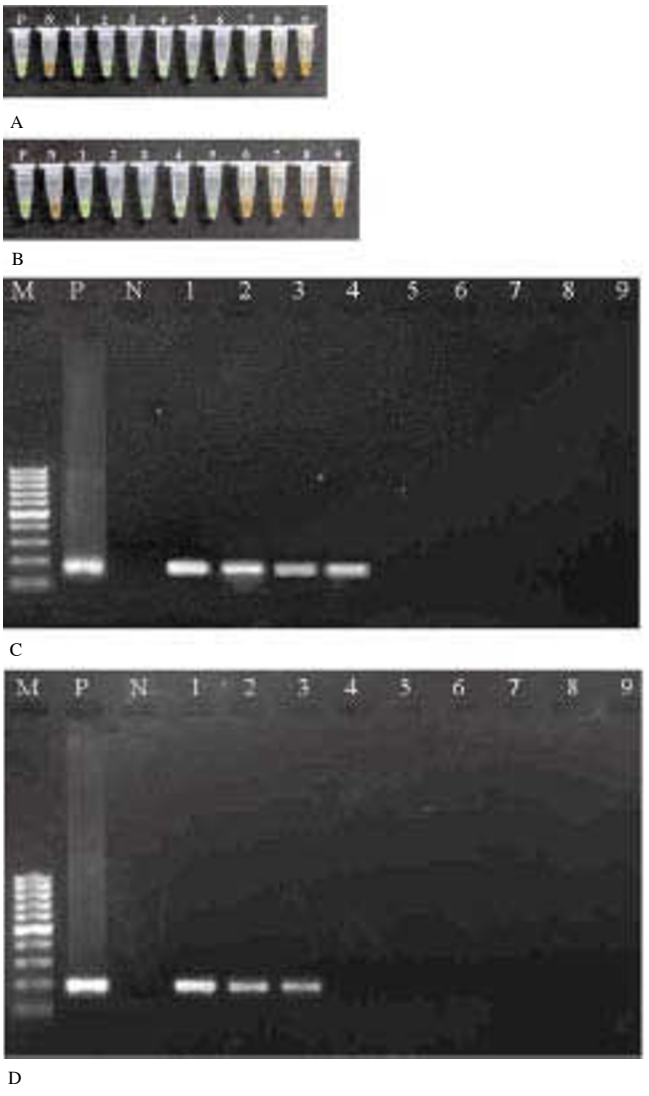

Figure 1. Comparative sensitivities of LAMP and nested-PCR for the specific detection of *T. gondii* DNA targeting the RE and B1 gene.
(A) RE-LAMP analysis of *T. gondii* DNA (B) B1-LAMP analysis of *T. gondii* DNA (C) RE-nested PCR reaction with *T. gondii* DNA (D) B1-nested PCR reaction with *T. gondii* DNA. M, 100 bp molecular-weight marker; P, positive control; N, negative control; tubes/lanes 1-9 represent 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg of DNA respectively.

3.3. LAMP and nested-PCR on blood samples from infected mice

The earliest detection of parasite DNA by RE and B1-LAMP assays in infected mice with ten *T. gondii* tachyzoites was at 2 dpi (Figure 2C and 2D) while, by RE and B1-nested PCR assays the parasites were first detected at 4 (Figure 2A) and 5 dpi (Figure 2B) respectively demonstrated the efficiency of LAMP technique

for earlier diagnosis of Toxoplasma infection. There was no amplification of DNA fragments by LAMP and nested PCR in the negative control uninfected mice. Out of 20 blood samples five and three were positive by RE-nested PCR and B1-nested PCR respectively while eight and six were RE-LAMP and B1-LAMP-positive respectively.

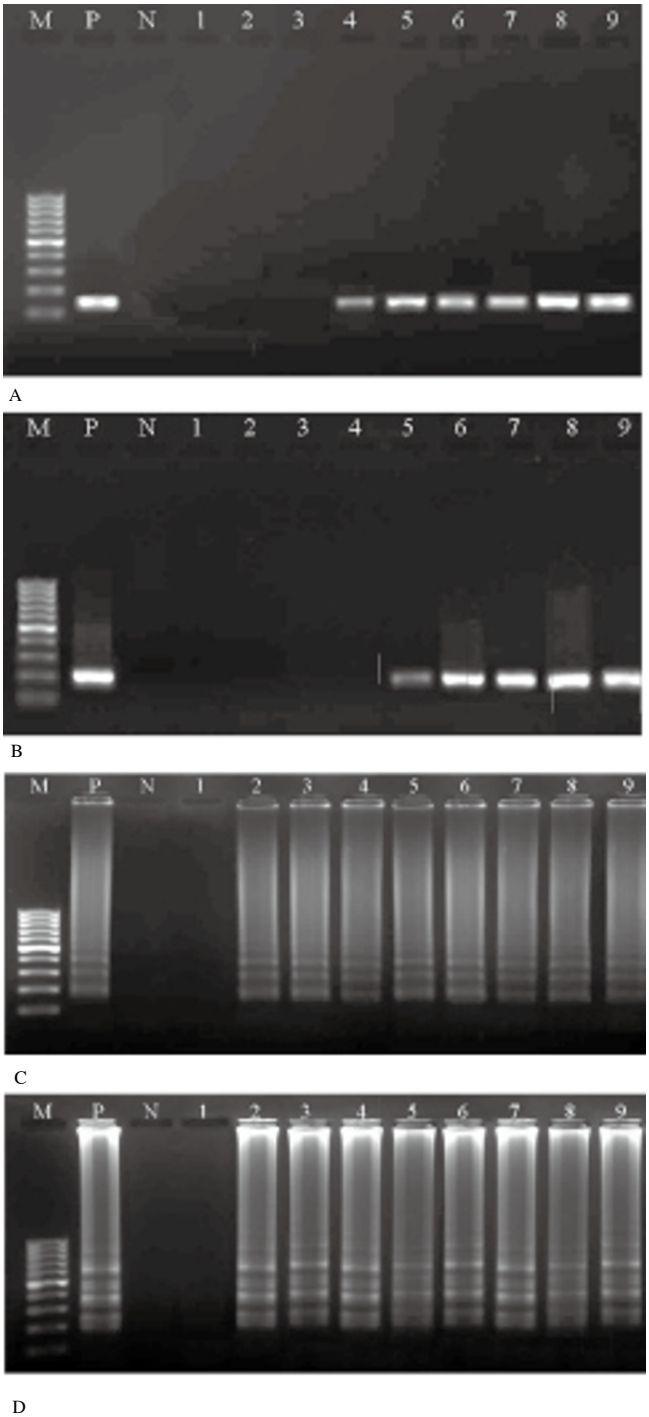

Figure 2. Analysis results of blood samples from *T. gondii* infected mice. (A) RE-nested PCR. (B) B1-nested PCR. (C) RE-LAMP. (D) B1-LAMP. M, 100 bp molecular-weight marker; P, positive control; N, negative control; lanes 1-9 represent mice blood samples collected 1-9 days post injection.

4. Discussion

Several diagnostic molecular assays based on different targets and procedures have been evaluated and described for the detection of *T. gondii*. In the present study, the sensitivity and specificity of LAMP and nested-PCR assays targeting the 529 bp RE and B1 gene were evaluated for the detection of *T. gondii* DNA. Due to the different studies reported a good sensitivity and specificity for the molecular markers targeting the repeated region in the *T. gondii* genome for detecting the parasite[6,38,39,44,45], these genomic targets were selected.

The optimal time and temperature conditions for the LAMP reaction were at 63 ℃ and 65 ℃ for 60 min for RE and B1 genomic targets respectively (data not shown). The analytical sensitivity of the LAMP reaction targeting the B1 gene and RE against 10-fold serial dilutions of purified Toxoplasma DNA was comparable to that obtained with the nested-PCR. Based on to the reported results in the study by Reischl and colleagues, one 80-Mbp genome equivalent of *T. gondii* was assumed equals about 80 fg[39]. According to this hypothesis, the detection limit of the RE-LAMP assay was about 0.01 *T. gondii* tachyzoite in less than an hour While, the detection limit of B1-LAMP was about one *T. gondii* tachyzoite took nearly an hour. In contrast, the limit of the RE-nested PCR was about 12 *T. gondii* tachyzoites and the detection limit of B1-nested PCR was 125 tachyzoites of *T. gondii*. Thus, these findings show that the sensitivity of the RE-LAMP assay was 10^2, 10^3 and 10^4-fold higher than the B1-LAMP, RE-nested PCR and B1-nested PCR assays respectively. Relying on these results it is concluded that the primers based on the RE are more suitable than those based on the B1 gene. It should be noted that, these incidents are not always happening at any case of genes and the results are case by case. In our study, the obtained detection limit for RE-LAMP, B1-LAMP and RE-nested PCR assays was slightly higher than that was reported by Kong *et al*[13]. The reported detection limit for 529 bp-LAMP, B1-LAMP and 529 bp-nested PCR assays in their study was 0.6 fg, 60 fg and 600 fg of the DNA template respectively. Conversely, our detection limit for LAMP and nested PCR was lower than those reported by Zhang *et al*[41]. They reported that the detection limit of LAMP and nested-PCR assay targeting the 529 bp repetitive element was one pg and 10 pg of the *T. gondii* DNA respectively.

Early diagnostic potency of LAMP technique for the detection of *T. gondii* DNA in the blood samples of mouse should be considered as a powerful aspect. In the infected and control mice, the LAMP technique had a higher sensitivity and specificity than nested PCR in the diagnosis of DNA of parasite in mouse blood sample.

The major potential pitfall of the LAMP is extremely easy to contaminate a laboratory by simply opening a reaction tube. Measurement of the turbidity[17], changing color using the Calcein or Hydroxy naphthol blue[46,47], entrapping the fluorescent compound into the wax beads that melt at 80 ℃ and released it in the last step of the reaction[48] are several methods which have been developed to obtain results without even opening the tube. To avoid the post-amplification contamination, the strict spatial separation of reagent preparations and test procedures is also required. Regularly change

the gloves and application of the sterile pipetting techniques during the entire LAMP experiment is also helpful. In our study for the first time, upon completion of the LAMP assay, the reaction tubes were centrifuged at 12 000 rpm for 3 min and then were frozen at -20 ℃ for 10 min thereafter, SYBR Green Ⅰ was added to the reaction tubes at 4 ℃. This process will greatly prevent carryover contamination with post-amplification products.

For the specificity test, only *Toxoplasma* DNA was amplified, whereas DNA samples of other parasites and human chromosomal DNA was negative by PCR or LAMP. Could be concluded that the primer sets of the LAMP and nested-PCR targeting the B1 gene and RE was highly specific for the detection of *T. gondii*. Sequencing and aligning of the PCR product resulted from amplification with two B3 and F3 outer primers revealed that the PCR products were identical to the corresponding B1 and 529 bp repeated element sequences reported in GenBank, proved that the two genomic targets LAMP primers are highly specific for the detection of *T. gondii* (data not shown).

In conclusion, our results demonstrating that, the LAMP assay based on the RE and B1 gene had a greater sensitivity than the conventional PCR based on the same genomic targets for the detection of *T. gondii* DNA. Because of its simplicity, sensitivity, and specificity, LAMP is suggested as an appropriate method for the routine molecular diagnosis of toxoplasmosis.

Conflict of interest statement

We declare that we have is no conflict of interest.

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