Research Article · Open Access

Development of Sensitive Nested Real-time PCR for Diagnosis of Acute and Chronic Phases of Toxoplasmosis in Mice Model

Parisa Mousavi[1], **Hossein Mirhendi**[2], **Hossein Keshavarz Valian**[1*], **Saeedeh Shojaee**[1], **Shirzad Fallahi**[3,4,] **Armin Farhang**[2], **Mohammad-Ali Mohaghegh**[5] and **Rasool Jafari**[2]

[1]*Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

[2]*Department of Medical Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*

[3]*Department of Medical Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran*

[4]*Razi Herbal Medicines Research Center, Lorestan University of Medical Science, Khorramabad, Iran*

[5]*Department of Laboratory Science, Torbat Heydariyeh University of Medical Sciences, Torbat Heydariyeh, Iran*

[*]**Corresponding author:** Hossein Keshavarz Valian, Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, E-mail: hkeshavarz@tums.ac.ir

Abstract

Toxoplasma gondii is an intracellular parasite that causes a variety of clinical manifestations. Acute and chronic phases of toxoplasmosis are considered as the presence of actively proliferating tachyzoites in the nucleated cells of mammalian hosts such as humans, and spread through blood to other parts of the body, which subsequently forms tissue cysts. The present study was aimed to evaluate the diagnostic value of nested real-time polymerase chain reaction (PCR) for the acute and chronic phases of toxoplasmosis in the laboratory mice using compared to conventional real-time PCR. To induce acute toxoplasmosis, 103 tachyzoites of *Toxoplasma gondii* RH strain were intraperitoneally inoculated to 25 BALB/c mice. In order to induce chronic toxoplasmosis, the mice were subcutaneously infected by the parasite and then treated with sulfadiazine from day one to day 14 post-injection. Genomic DNA was extracted from blood and brain tissues. Real-time and nested real-time PCR targeting 529 bp repeated element (RE) was performed. All mice with acute infection were positive for *Toxoplasma gondii* using nested real-time PCR and 21 were positive by real-time PCR. In the chronic phase, all blood samples were negative with real-time PCR and three were positive using nested real-time PCR. However, of the 25 brain samples, 28%, 52% and 72% were positive with the microscopic, real-time PCR and nested real-time PCR methods, respectively. The results of the present study showed that the molecular methods have high sensitivity for the diagnosis of acute toxoplasmosis. In the chronic phase, a blood sample is not suitable for the detection of the infection, and other tissue samples may be used instead. Also, nested real-time PCR has even higher sensitivity compared to the conventional real-time PCR.

Keywords: Acute toxoplasmosis; Chronic toxoplasmosis; Nested real-time PCR; Diagnosis; BALB/c mice

Introduction

Toxoplasma gondii is an intracellular coccidian parasite that recognized as a cause of most prevalent food-borne parasitic diseases [1]. Toxoplasmosis is generally transmitted orally when *T. gondii* oocysts or tissue cysts are eaten. Oocysts become matured in a humid environment and, as a result, it is much prevalent in humid areas [2]. The prevalence of human toxoplasmosis is variable in different countries and is estimated from 0.3% to 68% [3]. *Toxoplasma gondii* causes a variety of clinical manifestations in humans as the intermediate host [2], and is categorized into five clusters including; immune-competent patients during pregnancy, congenitally acquired, reactivated in immuno-deficient patients and ocular toxoplasmosis [4,5]. Clinical manifestations are not specific in any group and differential laboratory techniques are required for the diagnosis of the infection [6]. Acute and chronic phases of toxoplasmosis are considered as the presence of tachyzoites in human body fluids (mostly nucleated blood cells) and cysts formation in different tissues, respectively [4].

Lymphadenopathy and reticular cell hyperplasia are the main clinical manifestations of acute toxoplasmosis [7] and it is diagnosed commonly based on the clinical signs and some laboratory tests [8]. Chronic phase of the infection is usually asymptomatic [9], yet the cyst may tear to pieces and cause an active infection like the acute phase of disease [10]. Moreover, recent studies revealed that not only chronic toxoplasmosis is unsafe, but also could cause different mental disorders such as schizophrenia, depressive [11,12] epilepsy, Alzheimer's disease, Parkinson's disease, mental retardation [13], suicide attempts [14,15] and risk of traffic accidents [16]. Because of these problems, diagnosis of chronic toxoplasmosis is vitally crucial.

Serological techniques such as antibody detection and IgG avidity test have been established to diagnose the acute and chronic toxoplasmosis, but these methods are not specific enough in immunodeficient patients [17-19]. Molecular techniques, mostly PCR has been broadly used as a fast, sensitive and specific diagnostic technique in comparison with conventional parasitological and serological methods [20] and different molecular methods have been developed to increase the diagnostic accuracy of the several forms of toxoplasmosis [21,22]. Nested PCR is a modification of PCR intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites and heighten the specificity of the test.

Although, nested PCR has increased the sensitivity and specificity of conventional PCR technique, the method is laborious and have a risk of laboratory contamination *via* short amplified DNA fragments [23]. Nested real-time PCR with a high sensitivity as a combination of conventional PCR and sensitive real-time PCR has recently used for diagnosis of tuberculosis [24] malaria [25] and various other infections.

It was shown that nested q-PCR improved the sensitivity of the detection of *Plasmodium falciparum* in its' blood stage from clinical Dried Blood Spot (DBS) samples to use as an effective surveillance of the disease in areas of submicroscopic asymptomatic infections. During the chronic phase of Toxoplasma infection, the parasite shifts into the tissues to form the tissue cysts and having fewer activities in blood, therefore a more sensitive diagnostic tool is necessary to diagnose the infection.

The aim of the present study was to evaluate the nested real-time PCR as a novel technique that is the combination of nested real-time PCR and real-time PCR, to detect the presence of Toxoplasma DNA in blood and brain tissues in experimentally infected mice. Therefore, diagnosis of infection in the primary step, especially in chronic phase is important for successful treatment and reducing the damaging effects.

Materials and Methods

Experimental infections

Tachyzoites of *Toxoplasma gondii* RH strain were obtained from Toxoplasmosis Research Center, Tehran University of Medical Sciences, Tehran, Iran. Parasites were inoculated into peritoneum cavity of female BALB/c mice, collected from days three to five post-infection and counted by light microscope [26] to use in subsequent experiments.

For induction of acute toxoplasmosis, 25 female BALB/c mice were inoculated with 103 tachyzoites *via* intraperitoneal injection. After five days, the mice were anesthetized and 2 ml of their heart blood was collected and used for DNA extraction. For the chronic phase of the infection, 25 female BALB/c mice were subcutaneously infected with 103 tachyzoites of *Toxoplasma gondii*.

One Sulfadiazine tablet (SDZ) (300 mg/l) and NaHCO$_3$ (3 g) were dissolved in 2 litters of mice drinking water from day one to day 14 after infection [27]. Sulfadiazine as an anti-toxoplasma drug was used to prevent causing acute phase of toxoplasmosis. After 40 days, all infected mice were anesthetized, and Blood samples were collected from the heart.

Brain tissues were removed, and samples were homogenized with Phosphate-buffered saline (PBS) for microscopic examination in order to determine the presence of tissue cysts and finally DNA was extracted from tissues and whole blood for molecular study in order to evaluate the presence of parasite DNA. Also, 25 mice were subjected to the subcutaneous inoculation with PBS and considered as negative controls.

Amplification of repeated element by nested real-time PCR

Blood samples for acute phase (taken four days after injection) and both blood samples and brain tissues (taken 40 days after injection) were collected. The genomic DNA was extracted and purified using genomic DNA purification kit (GeneAll Korea) according to the manufacturer's instructions.

Nested real-time PCR consists of two repeated PCR amplification steps with two pairs of inner and outer primers that the second step is replaced by real-time PCR assay. For the first step, the conventional PCR was done by a pair of outer primers sequences. Primer sequences are as follows: forward primer 5′- TGACTCGGGCCCAGCTGCGT3′-and reverse primer 5′- CTCCTCCCTTCGTCCAAGCCTCC3′-and the second run was done by a pair of inner primer sequences as follows: forward primer 5′-AGGGACAGAAGTCGAAGGGG3′-) and reverse pimer5′-GCAGCCAAGCCGGAAACATC5′- [28].

PCR reaction was carried out in a final volume of 20 μL containing 100 ng of genomic DNA, 2 μL of 10X buffer, 0.2 mM dNTP, 2 mM MgCl$_2$, 0.5 pM each outer primer and 1 unit of Taq DNA polymerase. Amplification program was started with an initial denaturation at 95ºC for 5 min, followed by 30 cycles with denaturation at 94ºC for 20 sec, annealing at 55ºC for 1 min and an extension step at 72ºC for 30 sec followed by 5 min of final extension at 72ºC.

For second PCR, 2 μL of the 1:50 diluted product of the first PCR was added to real-time PCR mixture containing 10 μL of SYBR green Ampliqon premix, 0.4 μL of each inner primer (10 pmol/μL) and 7.2 μL of sterile distilled water.

Reaction was started using a Step One™ Real-Time PCR System (Applied Bio systems, Massachusetts, USA) and carried out as follows: 10 min hot start at 95°C and 40 cycles of 95°C for 10 sec, 60°C for 30 sec, followed by 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec.

The primers amplified 164 bp fragment of 529 bp region that is repeated 200-300 times in the parasite genome. Melting curve analysis was done to confirm that the correct products had been amplified in positive samples and also to evaluate the primers specificity. This study is proved by ethical committee of Isfahan University of Medical Sciences.

Results

Assessment of parasite infection in the acute phase

After 3-5 days of *Toxoplasma gondii* inoculation (RH strain), acute infection was identified in mice using real-time and nested real-time PCR on blood specimen. Twenty-one (84%) out of 25 mice were positive with the real-time PCR whereas 100% were positive using the nested real-time PCR based on *RE* genes in the acute phase of toxoplasmosis.

Identification was proven by amplification plot curve based on cycle threshold value (Ct) so that all samples were positive according to amplification plot which surpassed the threshold line by nested real-time PCR (Figures 1-6).

Effects of SDZ in the treated mice

All of the 25 subcutaneously infected and SDZ-treated mice were survived, and brain tissue cysts were microscopically observed in (7 cases) 28% of them (Figure 3). In the control group, which were not infected by Toxoplasma gondii, all the mice were negative for both microscopically and molecular tests.
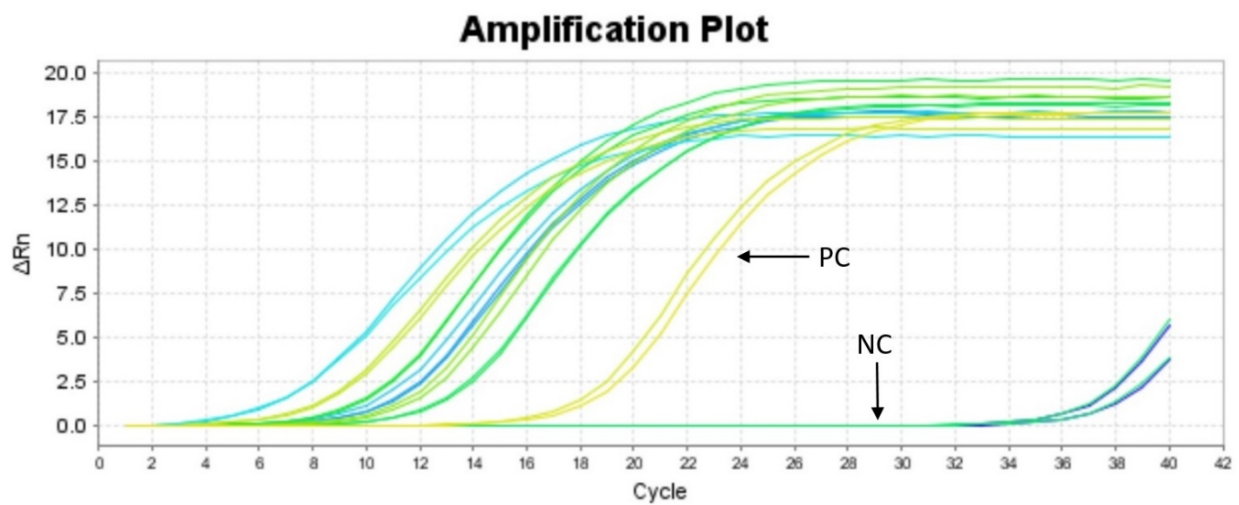
Figure 1: Real-time amplification plot for *RE* gene of *Toxoplasma gondii* in acute infection group of mice (blood samples). PC and NC stand for positive and negative control, respectively. Based on this curve, the cycle threshold value (Ct) was detected. All tests were done for each sample in duplicate.
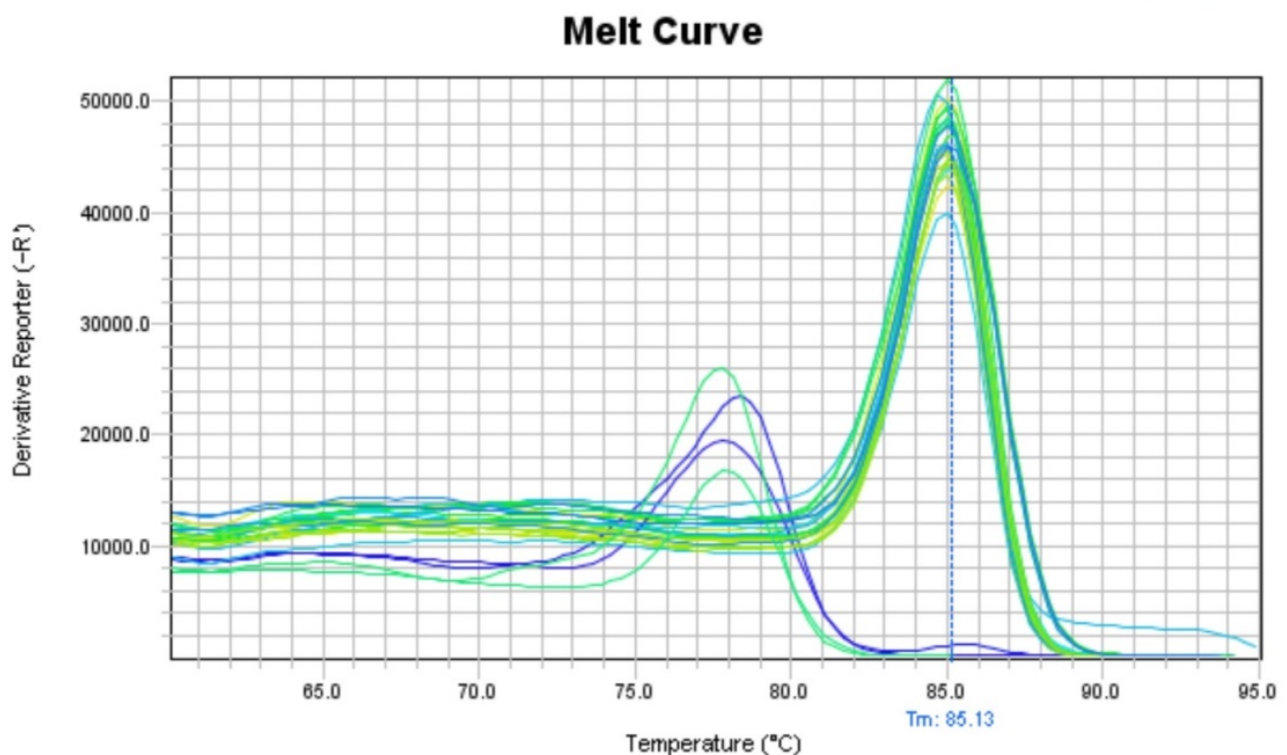

Figure 2: The melting curve of Real-time PCR for *RE* gene of *Toxoplasma gondii* in the infection group of mice with acute-phase (blood samples), the positive and negative control were done in duplicate.
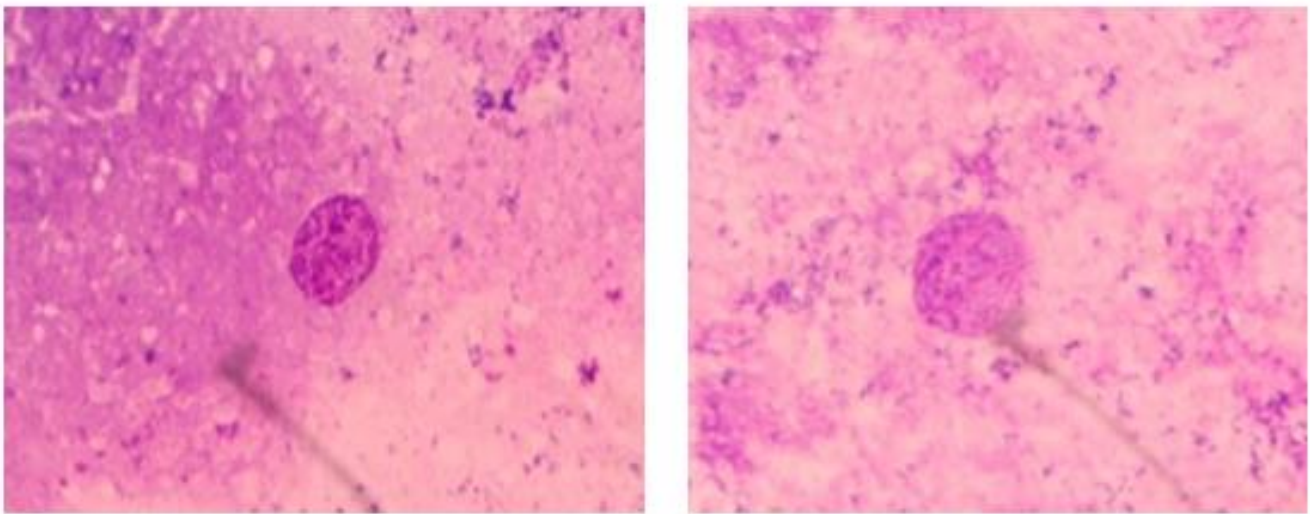
Figure 3: Tissue cyst in the brain of mice injected subcutaneously with tachyzoites of *Toxoplasma gondii*, RH strain and treated with SDZ (Giemsa staining).
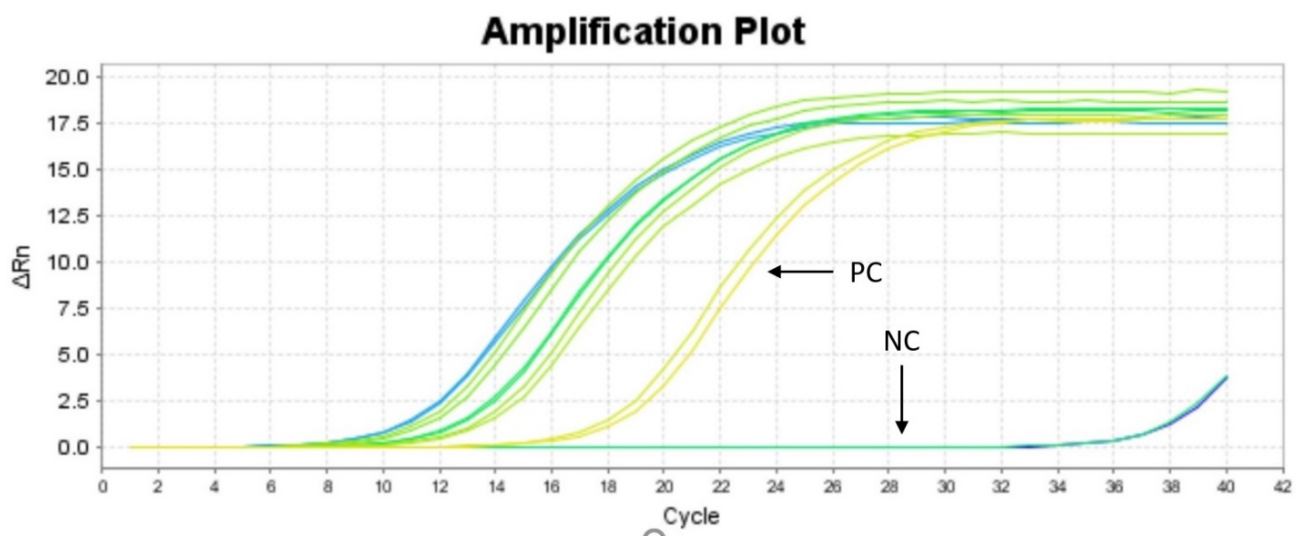

Figure 4: Real-time amplification plot for *RE* gene of *Toxoplasma gondii* in chronic infection group of mice (brain tissue). PC and NC stand for positive and negative control, respectively. Based on this curve, the cycle threshold value (Ct) was detected. All tests were done for each sample in duplicate.

Assessment of parasite infection in the chronic phase

In the chronic phase, all 25 blood samples were negative for Toxoplasma gondii with the real-time PCR method, however; 3 cases were positive by nested-real time PCR. Brain samples were assessed by microscopic examination, real-time and nested real-time PCR methods and the results showed that 7 (28%), 13 (52%) and 18 (72%) samples were positive for Toxoplasma, respectively (Table 1).

To evaluate detection limit of the methods, six serial dilutions of Toxoplasma gondii DNA ranging from one to 10000 tachyzoites per reaction were used to show the capability of real-time PCR to detect the presence the RE gene as low as one tachyzoites in a 20 μl reaction volume (Ct=21.49).
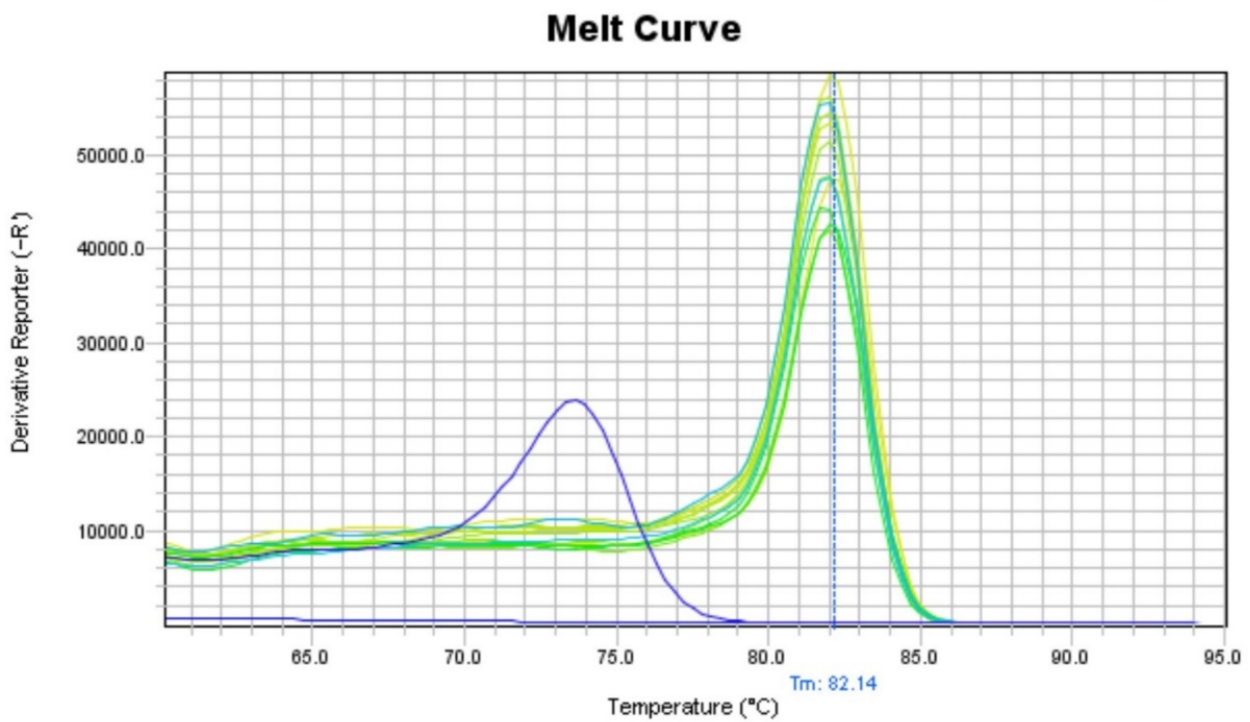
Figure 5: The melting curve of Real-time PCR for *RE* gene of *Toxoplasma gondii* in the infection group of mice with chronic-phase (brain tissue), the positive and negative control were done in duplicate.
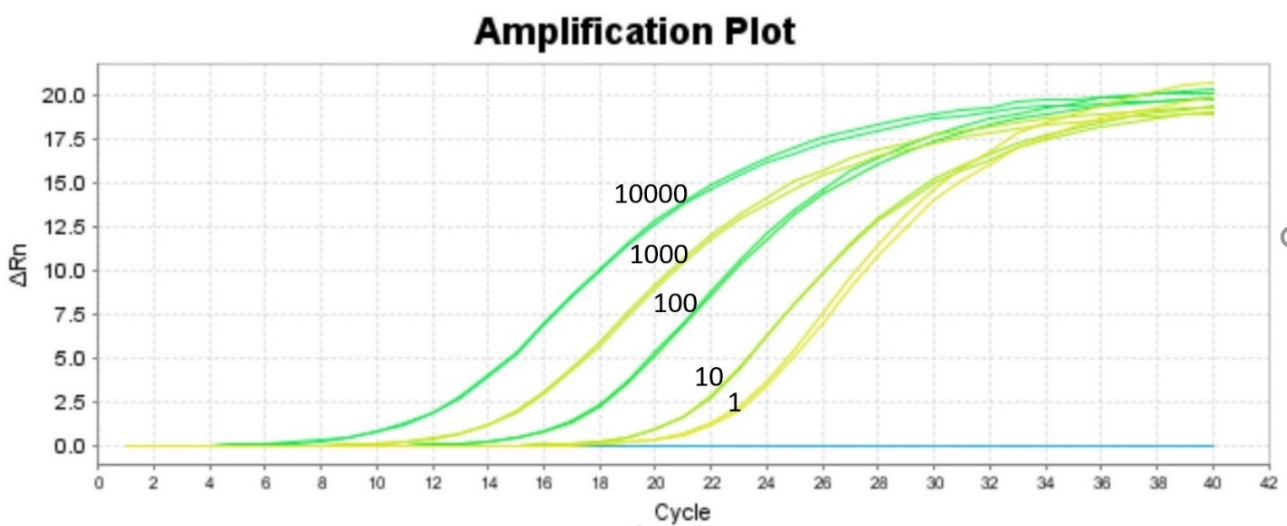

Figure 6: Real-time amplification plot to detect the presence of *Toxoplasma gondii*. DNA dilutions ranging from 1 to 10000 tachyzoites per reaction, all tests were done in duplicate.

Diagnosis of acute and chronic phase of toxoplasmosis	Blood sample		Brain tissue		
			Microscopic examination	Real Time PCR	Nested- real time PCR
	Real Time PCR	Nested- real time PCR			
Acute phase (n=25)	21 (84%)	25 (100%)	0 (0%)	0 (0%)	0 (0%)
Chronic phase (n=25)	0 (0%)	3 (12%)	7 (28%)	13 (52%)	18 (72%)
Negative group (n=25)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 1: Results of microscopic real time and nested-real time PCR techniques for diagnosis of acute and chronic phase of toxoplasmosis in blood and brain tissue samples.

Discussion

Nowadays, the sensitivity of laboratory tests is not quite 100%. Serological findings only show the infection, but molecular tests by finding the DNA of pathogens, confirm the existence of parasites [29]. Molecular methods such as conventional PCR and real-time PCR have been developed for accurate detection of *Toxoplasma gondii* in different forms of toxoplasmosis, especially in immunodeficient patients and pregnant women [30,31].

Acute and chronic toxoplasmosis was assessed by using real-time and nested real-time PCR. Enzyme-linked immuno-sorbent assay (ELISA) test on blood donors indicated that analysis of anti-Toxoplasma antibodies could not be trustworthy because numerous cases were reported to be negative for anti-*Toxoplasma gondii* IgM antibodies, those negative cases were positive for Toxoplasma mRNA by real-time PCR [3]. In a similar study by Villard et al., PCR and ELISA were used for diagnosis of toxoplasmic chorioretinitis on aqueous humor samples and the result revealed that PCR technique diagnosed 28% of patients with active lesions [32].

Zainodini et al. used real-time PCR and ELISA tests for the detection of acute and latent toxoplasmosis in blood donors and indicated that molecular diagnostic tests are more sensitive to detect acute infections. Their results suggesting that anti-Toxoplasma IgM ELISA results are not capable of diagnosing all acute infections, and there is a high potential risk of Toxoplasma transmission through blood transfusion among the human population, especially in immunosuppressed individuals [33].

In the present study, active toxoplasmosis was diagnosed by a nested real-time PCR method on the blood samples after five days of experimental inoculation. This study indicated a high rate of parasitemia in the acute stage of the infection, whereas only 3 cases in chronically infected mice show positive results with this method.

The latter happens when the parasite shifts from blood to the tissues in the chronic phase of the infection. It results in a low parasite load in peripheral blood and that is why blood samples are not suitable for detection of *Toxoplasma gondii* in the chronic phase of toxoplasmosis. In a similar research carried out by Kompalic-Cristo et al., they detected Toxoplasma DNA in four out of 110 patients with chronic infection. Thus, it suggests that patients with chronic toxoplasmosis have low parasite burden in their blood [34].

In this study, brain samples were collected from the chronic group and assessed using microscopic and molecular methods. Results indicated that out of 25 mice, 7 (28%), 13 (52%) and 18 (72%) samples were positive for microscopic, real-time and nested real-time PCR, respectively. In a study performed by Hade, they detected acute toxoplasmosis in 76.5% of sheep serum samples with the nested PCR method. Additionally, Jassem reported the diagnostic rate of 90.3% in rats eight weeks post-infection using real-time PCR [35]. In a similar study conducted by Aigner et al., real-time PCR technique was used for detection of *Toxoplasma gondii* DNA in brain and heart samples of serologically positive outdoor chickens. Parasite DNA was detected in 92.3% of samples with real-time PCR and also no significant differences between tissues were indicated [36].

Finally, the results of current and similar works indicated that serological and molecular tests are not sensitive for detection of chronic toxoplasmosis on a blood sample and also it is suggested tissue samples are the better choice for detection of the infection in the chronic phase. Another important factor to accurately detection is to use suitable techniques such as nested PCR and real-time PCR, that in the current work, it's appeared that nested real-time PCR was sensitive than the conventional real-time PCR. Appropriate selection of target genes is an important parameter to enhance the sensitivity of molecular diagnosis. Since the *B1* gene has 35 and *RE* gene has 200 to 300 copies in parasite genome, it can be expressed that the use of RE fragment as a PCR target would increase the analytical sensitivity.

Conclusion

According to the results of the present study, nested real-time PCR is a highly sensitive method for detection of acute toxoplasmosis. The suitable molecular methods for diagnosis of chronic toxoplasmosis are yet to be introduced. Further population-based studies among seropositive and seronegative humans with the molecular methods can also be suggested to determine its capability for clinical use.

Acknowledgment

Conflicts of Interest

The authors have no conflicts of interest.

References

1. McAllister MM (2005) A decade of discoveries in veterinary protozoology changes our concept of "subclinical" toxoplasmosis. Veterinary parasitology 132: 241-247.

2. Mehlhorn H (2015) Host Manipulation by Toxoplasma gondii. In: Mehlhorn H. (eds) Encyclopedia of Parasitology. Springer, Berlin, Heidelberg, p. 191.

3. Edrissian G, Rokni MB, Mohebali M, Nateghpour M, Mowlavi G, et al. (2016) History of Medical Parasitology and Parasitic Infections in Iran. Arch Iran Med 19: 601-607.

4. Dubey JP (2016) Toxoplasmosis of animals and humans (2ndedn) CRC press p. 185.

5. Rahimi-Esboei B, Zarei M, Mohebali M, Valian HK, Shojaee S, et al. (2018) Serologic Tests of IgG and IgM Antibodies and IgG Avidity for Diagnosis of Ocular Toxoplasmosis. Korean J Parasitol 56: 147.

6. Dard C, Chemla C, Fricker-Hidalgo H, Brenier-Pinchart M-P, Baret M, et al. (2017) Late diagnosis of congenital toxoplasmosis based on serological follow-up: A case report. Parasitol Int 66: 186-189.

7. Weiss LM, Dubey JP (2009) Toxoplasmosis: A history of clinical observations. Int J Parasitol 39: 895-901.

8. Remington JS, Miller MJ, Brownlee I (1968) IgM antibodies in acute toxoplasmosis: I. Diagnostic significance in congenital cases and a method for their rapid demonstration. Pediatrics 1968, 41: 1082-1091.

9. Montoya JG (2002) Laboratory diagnosis of Toxoplasma gondii infection and toxoplasmosis. J Infect Dis 185 Suppl 1: S73-S82.

10. Dalimi A, Abdoli A (2012) Latent toxoplasmosis and human. Iran J Parasitol 7: 1.

11. Brown AS (2011) The environment and susceptibility to schizophrenia. Prog Neurobiol 93: 23-58.

12. Yolken RH, Bachmann S, Rouslanova I, Lillehoj E, Ford G, et al. (2001) Antibodies to Toxoplasma gondii in individuals with first-episode schizophrenia. Clin Infect Dis 32: 842-844.

13. Stern H, Booth J, Elek S, Fleck D (1969) Microbial causes of mental retardation the role of prenatal infections with cytomegalovirus, rubella virus, and Toxoplasma. The Lancet 294: 443-448.

14. Arling TA, Yolken RH, Lapidus M, Langenberg P, Dickerson FB, et al. (2009) Toxoplasma gondii antibody titers and history of suicide attempts in patients with recurrent mood disorders. J Nerv Ment Dis 3: 905-908.

15. Okusaga O, Langenberg P, Sleemi A, Vaswani D, Giegling I, et al. (2011) Toxoplasma gondii antibody titers and history of suicide attempts in patients with schizophrenia. Schizophr Res 133: 150-155.

16. Flegr J, Havlícek J, Kodym P, Malý M, Smahel Z (2002) Increased risk of traffic accidents in subjects with latent toxoplasmosis: a retrospective case-control study. BMC Infect Dis 2: 11.

17. Dard C, Fricker-Hidalgo H, Brenier-Pinchart MP, Pelloux H (2016) Relevance of and New Developments in Serology for Toxoplasmosis. Trends Parasitol 32: 492-506.

18. Pomares C, Zhang B, Arulkumar S, Gonfrier G, Marty P, et al. (2017) Validation of IgG, IgM multiplex plasmonic gold platform in French clinical cohorts for the serodiagnosis and follow-up of Toxoplasma gondii infection. Diagn Microbiol Infect Dis 87: 213-218.

19. Dhakal R, Gajurel K, Pomares C, Talucod J, Press CJ, et al. (2015) Significance of a positive Toxoplasma immunoglobulin M Test Result in the United States. J Clin Microbiol 53: 3601–3605.

20. Novais EA, Commodaro AG, Santos F, Muccioli C, Maia A, et al. (2014) Patients with diffuse uveitis and inactive toxoplasmic retinitis lesions test PCR positive for Toxoplasma gondii in their vitreous and blood. Br J Ophthalmol 98: 937-940.

21. Abdul-Ghani R (2011) Polymerase chain reaction in the diagnosis of congenital toxoplasmosis: more than two decades of development and evaluation. Parasitol Res 108: 505-512.

22. Robert-Gangneux F, Gavinet M-F, Ancelle T, Raymond J, Tourte-Schaefer C, et al. (1999) Value of prenatal diagnosis and early postnatal diagnosis of congenital toxoplasmosis: retrospective study of 110 cases. J Clin Microbiol 37: 2893-2898.

23. Abravaya K, Esping C, Hoenle R, Gorzowski J, Perry R, et al. (2000) Performance of a multiplex qualitative PCR LCx assay for detection of human immunodeficiency virus type 1 (HIV-1) group M subtypes, group O, and HIV-2. J Clin Microbiol 38: 716-723.

24. Takahashi T, Nakayama T (2006) Novel Technique of Quantitative Nested Real-Time PCR Assay for Mycobacterium tuberculosis DNA. J Clin Microbiol 36: 1029-1039.

25. Taun MT, Amirali A, Shanping Li, Ongoiba A, Kayentao K, et al. (2014) A nested real time PCR assay for the quantification of Plasmodium falciparum DNA extracted from dried blood spot. Malar J 13: 393.

26. Derouin F, Mazeron MC, Garin YJ (1987) Comparative study of tissue culture and mouse inoculation methods for demonstration of Toxoplasma gondii. J Clin Microbiol 25: 1597-1600.

27. Selseleh MM, Keshavarz H, Mohebali M, Shojaee S, Modarressi MH, et al. (2012) Production and evaluation of Toxoplasma gondii recombinant surface antigen 1 (SAG1) for Serodiagnosis of Acute and Chronic Toxoplasma infection in Human Sera. Iran J Parasitol 7: 1-9.

28. Kong QM, Lu SH, Tong QB, Lou D, Chen R, et al. (2012) Loop-mediated isothermal amplification (LAMP): early detection of Toxoplasma gondii infection in mice. Parasit Vectors 5: 2.

29. Bastien P (2002) Molecular diagnosis of toxoplasmosis. Trans R Soc Trop Med Hyg 96: S205-S215.

30. Lin MH, Chen TC, Kuo TT, Tseng CC, Tseng CP (2000) Real-Time PCR for Quantitative Detection of Toxoplasma gondii. J Clin Microbiol 38: 4121-4125.

31. Costa JM, Ernault P, Gautier E, Bretagne S (2001) Prenatal diagnosis of congenital toxoplasmosis by duplex real time PCR using fluorescence resonance energy transfer hybridization probes. Prenat Diagn 21: 85-88.

32. Villard O, Filisetti D, Roch-Deries F, Garweg J, Flament J, et al. (2003) Comparison of enzyme-linked immunosorbent assay, immunoblotting, and PCR for diagnosis of toxoplasmic chorioretinitis. J Clin Microbiol 41: 3537-3541.

33. Zainodini N, Zare-Bidaki M, Abdollahi SH, Afrooz M, Ziaali N, et al. (2013) Molecular and serological detection of acute and latent toxoplasmosis using real-time PCR and ELISA techniques in blood donors of Rafsanjan City, Iran, 2013. Iran J Parasitol 9: 336–341.

34. Kompalic-Cristo A, Frotta C, Suárez-Mutis M, Fernandes O, Britto C (2007) Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of Toxoplasma gondii in human peripheral blood. Parasitol Res 101: 619-625.

35. Jassem GA (2015) Use of real time polymerase chain reaction (PCR) and histological changes for detection of the Toxoplasma gondii parasite in male rats. J Bacteriol Res 7: 8-13.

36. Aigner CP, Silva AV, Sandrini F, Osório Pde S, Poiares L, et al. (2010) Real-time PCR-based quantification of Toxoplasma gondii in tissue samples of serologically positive outdoor chickens. Mem Inst Oswaldo Cruz 105: 935-937.