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Molecular Approach for Detection of *Toxoplasma gondii* Virulent RH Strain Using Conventional and Real Time PCR Based Assays

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Abstract: *Toxoplasma gondii* is one of the most protozoa's on earth known to date with potential zoonotic impact, all species of warm-blooded animals and birds. The main objective of the current study was to apply different assays for sensitive detection of *T. gondii* in both acute and chronic toxoplasmosis. For this purpose, a total of 40 albino mice were divided into two groups and used in the experimental infection (acute and chronic experimental infection). Tachyzoites were isolated from peritoneal cavity of the mice 3 days post infection (PI), brain cysts were detected at the 4th week PI (immature cysts) and mature brain cysts were also detected at the 8th week PI.In conventional PCR assay, by using primer sets (B1, B2) and (IP1a, IP2a) for B1and SAG1 genes, the expected PCR products 115 bp and 521 bp, respectively, were amplified with DNA extracted from peritoneal fluid. On the other hand, the conventional PCR could not detect the parasite in blood. The relative sensitivity of the PCR assay was analyzed; the PCR detection limit using the first primer set (B1, B2) was 0.1 tachyzoite per assay, while the detection limit using the second primer set (IP1a,IP2a) was 10 tachyzoites per assay. Real-time quantitative PCR test with SYBRGreen I for gene B1 on blood samples of acutely infected mice was applied to detect the parasite numbers in each sample. A standard curve with linear range across at least 6 logs of DNA concentrations was obtained. As little as 0.0007 *T. gondii* tachyzoite were quantitively detected in an assay. In addition, the detection limit of *T. gondii* DNA using the primer set (IP1a, IP2a) was 1 tachyzoite per assay. We are concluded that the application of quantitative PCR has a sensitive specific and rapid test for detection the parasite in blood.

Key words: *Toxoplasma gondii* · Real time- PCR · Albino mice · Zoonosis

INTRODUCTION

Toxoplasmosis is a widely prevalent zoonosis caused by the facultative two-host protozoan *Toxoplasma gondii* [1]. *T. gondii* occupy superior medical position among opportunistic pathogens that cause human latency. Presently, *T. gondii* becomes one of the most successful obligate intracellular tissue cyst-forming protozoan, with potential zoonotic impact among humans, all animals species and birds [2].

T. gondii is an obligate intracellular protozoan that belongs to the phylum Apicomplexa, subclass coccidia. It has a worldwide distribution with a high incidence of infection depending on the geographic location, 15 to 85% of the human populations are asymptomatically infected [3,4].

Members of cat family are definitive hosts and all mammals including man, birds and reptiles are intermediate hosts. It is unique, even among the cyst forming coccidia, in its range of intermediate hosts [5,6]. Hosts, including felids can acquire *T. gondii* by ingesting either tissues of infected animals or food or drink contaminated with sporulated oocysts, or by transplacental transmission [3,7].

T. gondii has evolved a complex life cycle results in four infectious stages; the tachyzoite, bradyzoite, merozoite and sporozoite. Each of the infectious stages has a distinctive biological role to play. The tachyzoites and merozoites proliferate within a host to increase parasite density. The difference between them is that tachyzoites undergo indiscriminate proliferation in all nucleated host cells and disseminate to all tissues of

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the body compared to the merozoites whereas proliferation is limited to a few (2-4) generations within the enterocytes of the cat gut. In addition, a sub-population of tachyzoites can undergo stage conversion to initiate bradyzoite development, while the merozoites give rise to the sexual (microgametocytes and macrogametocytes) stages. The role of bradyzoites and sporozoites is to transmit infection between hosts and as such they have to survive the rigors of the external environment and/or the digestive tract of the new host. On entering a new host cell, they appear unable to produce new generations of bradyzoites or sporozoites, but default immediately to tachyzoite development [8, 9].

T. gondii is a well-adapted parasite which generally causes very little disease unless the host's immune system is compromised [10]. The course of infection by *Toxoplasma* is generally benign, as the vast majority of the infected human population remains asymptomatic or present only mild symptoms. However, the infection can cause significant morbidity and mortality in the developing fetus on the one hand and in immuno-compromised individuals on the other hand [11]. Sever disease is caused by *T. gondii* in many species of animals. In sheep and goats, embryonic death and resorption, fetal death and mummification, abortion, still birth and neonatal death occur resulting in economical losses of veterinary importance [2,3].

For the diagnosis of toxoplasmosis in animals and men; a number of serological tests are used: indirect haemagglutination test (IHAT), indirect immunofluorescence assay test (IFAT), enzyme-linked Immunosorbent assay (ELISA). Because of the great importance of *T. gondii* as a causative agent of a zoonosis, public health organizations, such as the World Health Organization (WHO), have repeatedly advised the collection of accurate epidemiological data on this parasite [1].

Routine diagnosis is based on serology. However, serological diagnosis of active infection is unreliable because reactivation is not always accompanied by changing in antibody levels and the presence of IgM does not necessarily indicate recent infection [4]. Developments of molecular diagnostic methods, particularly those utilizing the polymerase chain reaction (PCR) for the detection of parasitic protozoan were potentially very useful. PCR based methods have advantages over traditional methods for the diagnosis of Toxoplasmosis, especially when serology fails and clinical symptoms are not evident, but there was a need for laboratory procedure to be refined before PCR-based assay were accepted as the tool of choice for the routine detection of *T. gondii* [12,13]. The locus most often routinely used for PCR detection was the tandemly arrayed 35- fold repetitive B1 gene. Targets such as the B1 and P30 (SAG-1) and ribosomal DNA, which are the most conserved genes sequences among different strains of *T. gondii*, have shown to be potential candidates to assure quality for clinical diagnosis of Toxoplasmosis [14 - 16]. Recently, real time quantitative PCR techniques have been implemented for the detection of a variety of pathogens including *Toxoplasma*. Application of quantitative PCR has evolved as a sensitive, specific and rapid method for the detection of *T. gondii* DNA in amniotic fluid, blood, tissue samples and cerebrospinal fluid [4,17].

The current study aimed to apply different assays for sensitive detection of *T. gondii* in both acute and chronic toxoplasmosis.

MATERIALS AND METHODS

Strain and Experimental Design: *T. gondii* RH strain was obtained from the Department of Zoonotic Diseases, National Research Center, Dokki, Giza. A total of 40 albino mice obtained from Laboratory Animals House, National Research Center weighing from 25-30 g were used in both acute and chronic infection experiments. For acute infection experiment, 10 mice were intraperitoneally injected by 10^3 tachyzoites each; after 3 days, mice were scarified, peritoneal fluid and blood were collected. For chronic infection experiment, 30 mice were subcutaneously injected by 100 tachyzoites each; three mice were scarified weekly and blood and brains were collected until reach the 8^{th} week [18].

DNA Extraction: DNA extracted from peritoneal fluid as previously described [19]. Briefly, to each prepared sample, 1% and 0.3 mg/ml final concentration of SDS and proteinase K, respectively were added and the mixture incubated at 37°C over night. DNA was extracted by phenol–chloroform-isoamyl alcohol followed by ethanol precipitation and resuspended in TE buffer.

Extraction of DNA from blood was performed by DNA Mini kit (GENOMIX) following the instruction of the manufacturer. The purity of the DNA was spectrophotometery determined by reading OD at 260 and 280 nm [19].

Table 1: Sequence primer pairs used in either PCR or Real-Time PCR

Primer	Sequence 5"–3"	Position gene	(bp)	Reference
B1–B22	AACGGGCGAGTAGCACCTGAGGAGA	1793-818	115	Bretagne *et al.*, 1993
B1–B23	TGGGTCTACGTCGATGGCATGACAAC	1907-1882		
SAG1-IP1[a]	CGACAGCCGCGGTCATTCTC	503-522	521	Odaert *et al.*, 1996
SAG1-IP2[a]	GCAACCAGTCAGCGTCGTCC	1024-1005		

DNA Amplification by Conventional PCR Assay: PCR test was targeted at the *T. gondii* B1and SAG-1 genes. Table 1 shows the upstream and downstream primer sequences targeting a portion of two genes.

Different PCR assays were performed according to Contini *et al.* [20] using 25 µl PCR reaction volume. The amplification was performed in a programmable heating block, (Primus thermal Cycler, MWG Biotech, Germany). All PCR reactions were repeated at least twice with positive (DNA extracted from RH strain of *T. gondii*) and negative (reaction mixtures without DNA) controls for each run.

All solutions were thawed and kept cool, gently mixed and brief centrifuged, added in a thin walled PCR tube on ice. The following PCR components were added in each PCR tube: 5 X PCR master mixes (Jena Bioscience), 25 pmol from each primer, 5 µl of DNA template and volume completed by nuclease free water. The Amplification program include, Initial denaturation at 95°C for 5 minutes. Followed by Thirty five cycles each cycle included a denaturation step at 93°C for 1 minute, a primer annealing step at 60 °C for1 minute and a chain elongation step at 72°C for 3 minutes. The final elongation step was prolonged by 10 min to ensure a complete extension of the amplified DNA. Aliquots (10 µl) of each PCR product were layered on 1.5% agarose gels

PCR Detection Limit of *T. gondii* DNA: To determine the sensitivity of the PCR assay, 10 fold serial dilution from *T. gondii* DNA was performed and processed by PCR as described above using B1 and SAG1 primers.

DNA Amplification by Real-Time PCR Assay: The Real-time quantitative -PCR test was targeted at the *T. gondii* B1 and SAG-1genes. All runs included one negative DNA control consisting of PCR-grade water and a DNA sample extracted from *T. gondii* RH strain. Reactions were performed in a final volume of 20 µl, using the 2X DNA Master SYBR Green I mix (Roche Molecular Biochemicals), each primer at the concentration: 0.5 µM for B1 gene, 0.5 µM for SAG1 and 5 µl of extracted DNA [13].

Amplification was carried out in a RotorGene 6000 real time detection system (Corbett Research, Australia) and a single fluorescence reading for each sample was taken at the extension step. Results were expressed by determination of the detection threshold (CT), or the crossing point (Cp), which marked the cycle when the fluorescence of a given sample significantly exceeded the baseline signal. The standard temperature profile included a holding step at 95°C for 15 minutes, followed by 45 cycles of denaturation at 95°C for 10 s, annealing 62 for 20 s and extension at 72°C for 30 s with fluorescence monitoring.

Melting Step: Melt (72-95°C), hold 90 seconds on the 1st step, hold 20 seconds on next steps, melt A [1].

Standard Curve and Detection Limit: To determine the detection limit of the SAG1 and B1 PCR method and to establish a standard curve that could be used for quantification, a serial dilution of *T. gondii* DNA with a final concentration from 100.000 tachyzoites per assay was subjected to analysis. It was calculated that one tachyzoite corresponded to 0.1 pg of DNA [21].

RESULTS

Results of Experimental Mice Infection: Tachyzoites were successfully isolated from peritoneal cavity of the mice 3 days post infection and experimentally infected mice were survived until 8 weeks, but it was notified that 4 mice were dead at 4th week PI. Brain cysts were detected at the 4th week PI (immature cysts) using brain print method and Giemsa stain (Photo 2). Mature brain cysts were also detected at the 8th week PI (Photo 3).

PCR: Results of amplification of *T. gondii* DNA using primer set (B1, B2) for B1 gene are shown in Fig. 1. Expected PCR product (115 bp) was amplified with DNA extracted from peritoneal fluid from infected mice. Amplification using primer set (IP1a, IP2a) for SAG1 gene is shown in Fig. 2. expected PCR product was 521 bp.

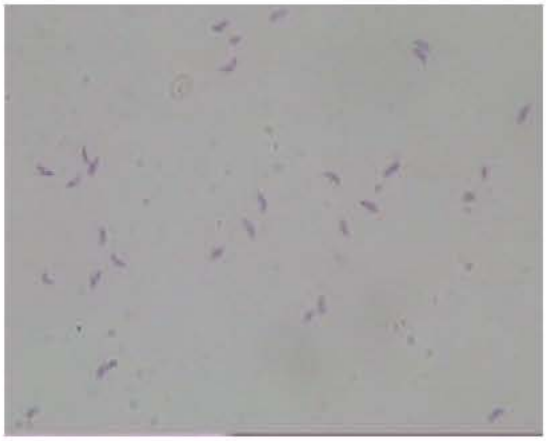

Photo 1: Demonstration of Giemsa stained tachyzoites obtained from peritoneal fluid of infected mice which appear crescent-shaped and 2 ìm - 6 ìm in size with nucleus located more centrally(X200).

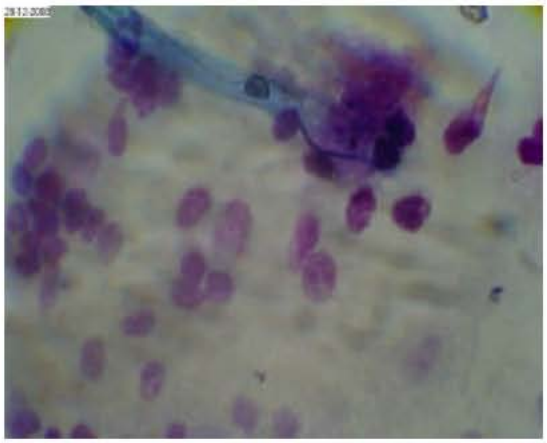

Photo 2: Demonstration of immature brain cysts at 4th week PI using brain print and Giemsa stain. The cysts appear small size with few number of bradyzoites The tissue cyst wall is elastic, thin (<0.5 ìm) and enclose crescent-shaped slender bradyzoites each measuring 7 ìm - 1.5 ìm. Bradyzoites having a nucleus situated toward the posterior end.(X400)

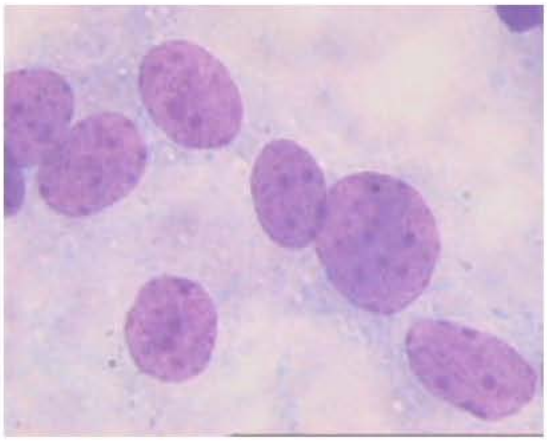

Photo 3: Demonstration of mature brain cysts at 8th week PI using brain print and Giemsa stain. The brain cyst wall is elastic, thin (<0.5 ìm) and enclose hundreds crescent-shaped slender bradyzoites each measuring 7 ìm - 1.5 ìm. Bradyzoites having a nucleus situated toward the posterior end,(X=1000).

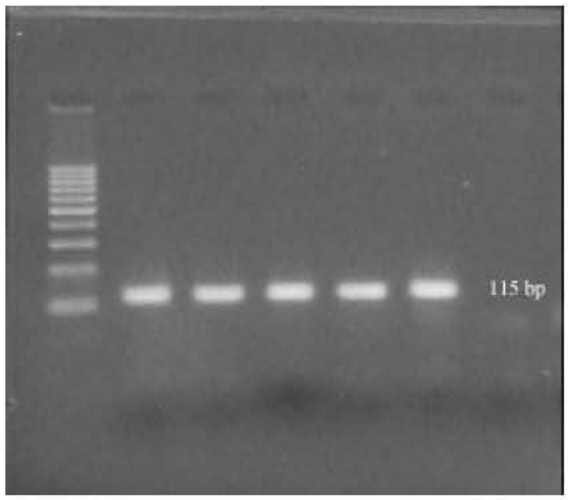

Fig. 1: PCR amplification of *T.gondii* DNA from peritoneal fluid using primer set (B1, B2) for B1 gene. Lane 1:100 bp molecular weight marker; lane 2: positive control; lanes 3-6: samples and lane 7: negative control

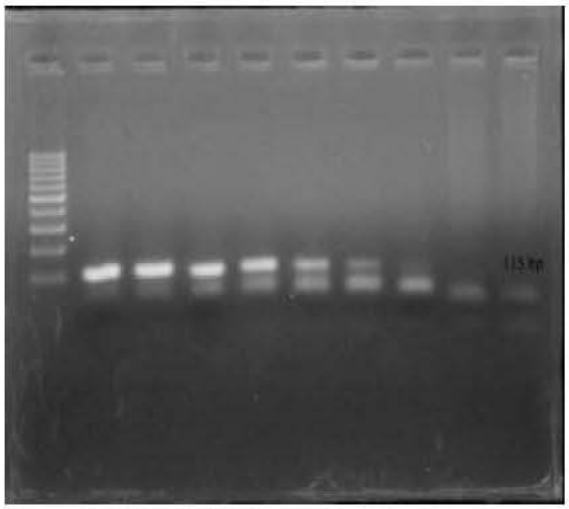

Fig. 3: Detection limit of *T.gondii* DNA by conventional PCR using primer set (B1, B2) for B1 gene. Lane M: 100 bp molecular weight marker; lanes2-9: different concentrations from 10^5 to 10^{-2} tachyzoites and lane N: negative control

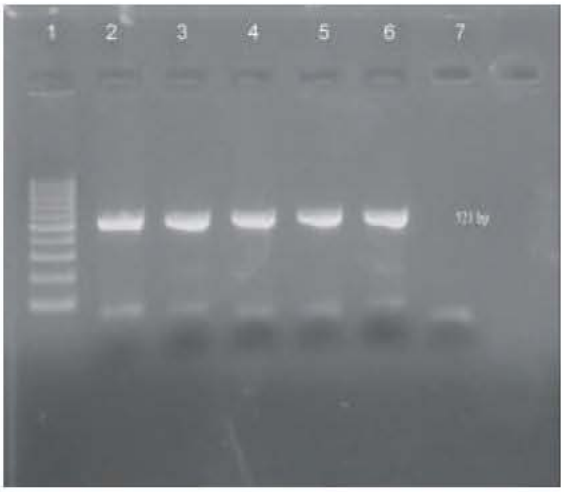

Fig. 2: PCR amplification of *T.gondii* DNA from peritoneal fluid using primer set (IP1a, IP2a) for SAG1 gene. Lane 1:100 bp molecular weight marker; lane 2: positive control; lanes 3-6: samples and lane 7: negative control

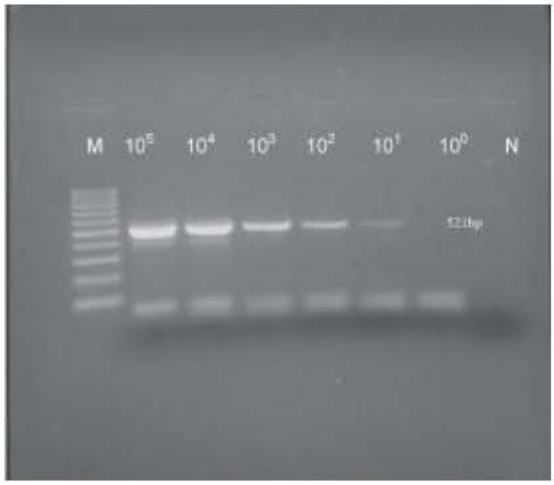

Fig. 4: Detection limit of *T.gondii* DNA by conventional PCR using primer set (IP1a, IP2a) for SAG1 gene. Lane M:100 bp molecular weight marker; lanes2-7: different concentrations from 10^5 to 1 tachyzoite and laneN: negative control.

Results of amplification of *T. gondii* DNA in blood using primers sets (B1, B2) and (IP1a, IP2a) were negative and the PCR cannot detect the parasite.

PCR Detection Limit of *T. gondii* DNA: The relative sensitivity of the PCR assay was determined by amplification of *T. gondii* DNA products of 10-fold dilutions using 2 different sets of primers (B1, B2) and (IP1a, IP2a). The PCR detection limit using the first primer set (B1, B2) was 0.1tachyzoite per assay as shown in Fig. 3. While, the detection limit using the second primer set (IP1a, IP2a) was10 tachyzoites per assay as shown in Fig. 4.

Rreal-Time PCR

Sensitivity and Specificity of B1-B2: By determining the detectability and the linearity of each real-time PCR assay using primer set (B1, B2) for B1 gene in blood samples of

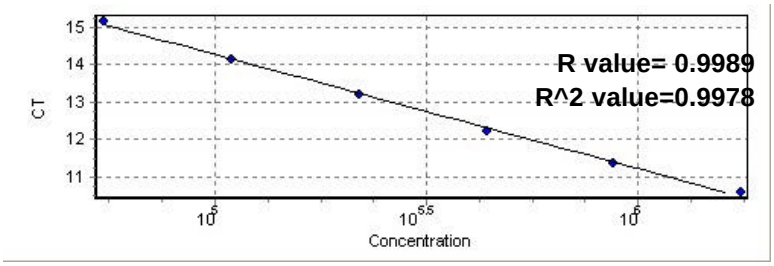

Fig. 5: a).Standard curve of the real-time PCR assay for *T.gondii* from the same experiment as in (a). The threshold cycle is plotted against the log of the starting copy number

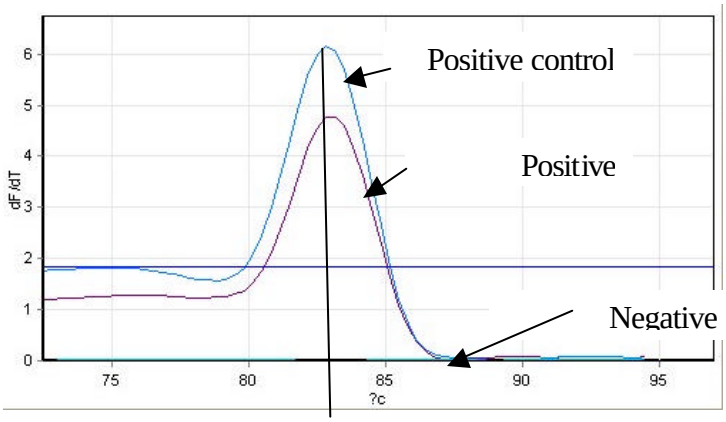

Fig. 5: b). Melting curves of the real-time PCR assay for *T.gondii* from the same experiment as in (a). The *x*-axis indicates the melting temperatures while the *y*-axis indicates the fluorescence intensity.

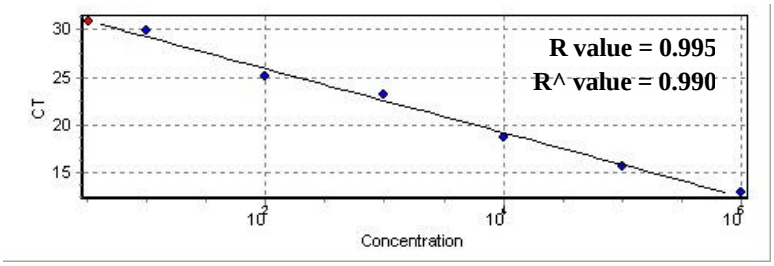

Fig. 6: a). Standard curve of the real-time PCR assay for *T. gondii* from the same experiment as in (a). The threshold cycle is plotted against the log of the starting copy number

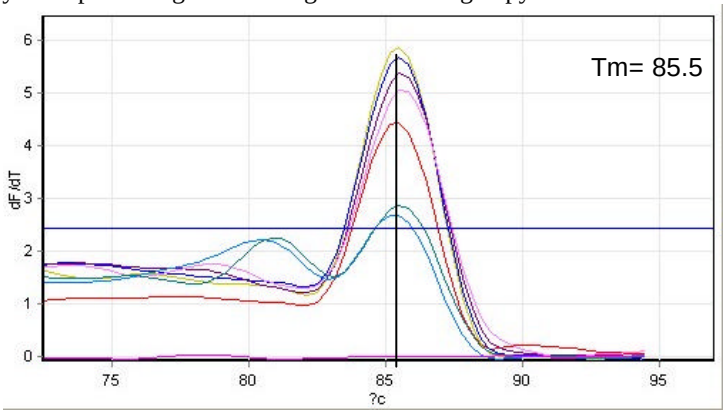

Fig. 6: b). Melting curve of the real-time PCR assay for *T. gondii* from the same experiment as in (a). The *x*-axis indicates the melting temperatures while the *y*-axis indicates the fluorescence intensity

acute experiment, two-fold serial dilutions of positive control DNA (pure tachyzoites DNA) were tested. Ct values, which correlate with the copy numbers of the nucleic acid templates, were measured in the experiments and were plotted against the known copy number of the positive control DNA. The generated standard curve covered a linear range of six orders of magnitude in each real-time PCR, providing an accurate measurement over a very large variety of starting target amounts and correlation coefficient was 0.998. The mean intra assay coefficient of variation (cv) was 4.8%. Melting curve analysis showed the melting point of each PCR product, including *T.gondii* PCR [83°C]. The results of the standard curves and dissociation curves of real-time PCR of *T. gondii* are shown in Fig. (5 a and b). It was possible to detect 0.061 copies of parasitic DNA per reaction of each real-time PCR. The lowest detection limit for genomic DNA was also 0.0007 copies per reaction.

Sensitivity and Specificity of IP1a, IP2a: By determining the detectability and the linearity of each real-time PCR assay using primer set (IP1a, IP2a) for SAG1 gene, ten-fold serial dilutions ranged from 10^{5} to 10^{-2} parasite / reaction of positive control DNA (pure tachyzoites DNA) were tested. Results of the amplification curves, standard curves and dissociation curves of real-time PCR of *T. gondii* are shown in Fig. 6 a and b. It was possible to detect one copy of parasitic DNA per reaction of each real-time PCR. Also the lowest detection limit for genomic DNA was also one copy per reaction.

DISCUSSION

This coccidian protozoan infects up to a third of the world's population with an obligate intracellular tissue cyst-forming characters, with subsequence ranks among the 10 most commonly occurring opportunistic pathogen that cause latency in human and animal's hosts, however represent the third leading cause of death among food-borne diseases in the immunocompromised individuals For this is reason the major of trials how to detect the parasites before became more dangerous and more spreading, so the fast and accurate diagnosis the main way to solve this problems.

Current diagnosis of toxoplasmosis relies on serological detection of specific anti-*Toxoplasma* immunoglobulin [1,11,22]. Although serological testing has been one of the major diagnostic techniques for toxoplasmosis, it has many limitations. For example, it may fail to detect specific anti-*Toxoplasma* immunoglobulin G (IgG) or IgM during the active phase of *T. gondii* infection, because these antibodies may not be produced until after several weeks of parasitemia. Therefore, the high risk of congenital toxoplasmosis of a fetus may be undetected because the pregnant mother might test negative during the active phase of *T. gondii* infection. Furthermore, the test may fail to detect *T. gondii* infection in certain immunocompromised patients due to the fact that the titers of specific anti-*Toxoplasma* IgG or IgM may fail to rise in this type of patient [23]. An alternative method of identifying *T. gondii* by mouse inoculation or tissue culture of the clinical specimen may confirm the infection by parasites. However, this method usually requires several days to obtain results and is labor-intensive [24]. Thus, a more efficient method is needed to provide rapid and quantitative results for the diagnosis of *T. gondii* infection.

A milestone in the field of molecular biology was set in the 1980s with the development of polymerase chain reaction (PCR) [25]. Several PCR-based techniques have been developed for the diagnosis of toxoplasmosis using various clinical specimens, including amniotic fluid [26] blood [27], cerebrospinal fluid [28] and tissue biopsy [29]. Among these techniques, nested PCR followed by hybridization of PCR products has been the most sensitive method. However, the major disadvantage of these methods is that they are quite time-consuming and do not provide quantitative data. For this purpose, [30] had developed competitive nested PCR to quantify the amount of *T. gondii* tachyzoites. However, this method not only is labor-intensive but also provides only semi-quantitative data, with a narrow linear range of 2 to 3 logs of DNA concentrations.

Thisstudy shows that Real-time PCR based fluorescence assays have advantages over nested or single-step gel based assays. Although both molecular assays were highly specific, real-time PCR requires less manipulation, reducing the potential for amplification product carryover associated with conventional PCR. It is much less labor-intensive, since there is no need for post-PCR handling, such as agarose gel electrophoresis of the PCR product [13].

SYBR green is one of the most frequently used Real-time PCR chemistries. The dye binds to the minor grove of ds-DNA. Its affinity to ds-DNA is more than 100-fold than that of ethidium bromide and when bound, the fluorescence signal increase about 1000-fold [31]. In the current study, SYBR green was used as it is cheap compared to hybridization probes and this chemistry can be used together with any primer pair, which is not the case of hybridization probes where specific primers

and probes work in synergy. SYBR green has no particular sequence specificity. Hence, the specificity of fluorescence signal relies on the design of the primers. So primer dimmers may give rise to ds-DNA fragment, which are detected by SYBR green however primer dimmer or co-amplification of two different DNA targets can be identified using melting curve analysis [32].

In this study *T. gondii* DNA was detected in blood specimens from five mice with acute toxoplasmosis. it was demonstrated that the Real-time PCR of the B1 gene is extremely sensitive at very low concentrations (detection limit from $6x10^{-1}$ to $7x\ 10^{-3}$ parasites genome/ml) and these results similar to Contini *et al.* [13] who reached to detection limit ranged from 10^{2} to 10^{-3} parasite/ml.In the other hand, by comparing these results with previous findings, *T. gondii* has been detected by various real time PCR sequence detection systems. The results varied in sensitivity and reproducibility (detection limit from 100 to 10 parasites/ml) [17, 33 -37]. Also Real-time PCR with SYBR Green I fluorescence (Applied Biosystems) with B1 gene was used during *Toxoplasma* posterior uveitis and the detection limit was less than 10 organisms for sample [38]. Moreover, Kompalic- cristo *et al.* [39] was able to accurately quantify parasite range from $9.92x10^{-3}$ to $8.73x10^{-1}$ tachyzoite genome/ml blood and this was probably because of the different amplification fragment produced. Results show that the sensitivity limit of SAG-1 gene was lower compared with the B1 gene and equivalent to 1 parasite/assay. Because the B1 gene is present in 35-fold repeats the fluorescent signal of each dilution appeared earlier in the B1 PCR method than in the P30 PCR method and comparing with previous finding, Buchbinder *et al.* [36] reached to 10 parasite genome per assay as a detection limit using SAG-1 gene.

Conventional PCR, by contrast, was found negative for the B1 and SAG-1 genes in all specimens collected for the period of active disease and these results in agreement with Savva *et al.*[40] using rodent experimentally infected with virulent or avirulent *Toxoplasma* strains as a model and. Ho-Yen *et al.* [41] using humans representing with acute toxoplasmic lymphadenopathy as another model. This may explained by the low parasite numbers in blood as primary infection in immunocompetent subjects seems to be associated with parasitemia in low concentrations [17].

In the current study, the detection limits of both genes by conventional PCR were 0.1 and 10 tachyzoites genome per assay, respectively and this considered low sensitivity limit as compared with that obtained with Real-time PCR and this may explain the negative result obtained for blood samples.

Results of detection of *T. gondii* DNA in blood of chronically infected mice were found negative for both conventional and Real-time PCR assays and these results may illustrated by findings of. Bou *et al.* [42] and Figueroa *et al.* [43] which have shown that PCR performed with B1 gene yields a greater sensitivity when specimens are collected before undertaking specific antiparasitic therapy. In this study chronic infected group had received low doses of sulfonamides during the first week, which may lead to the negative PCR results and B1 or SAG-1 genes were not detected. In this setting, primers targeting *T. gondii* bradyzoite-specific genes may have a potential for diagnostic and prognostic value in monitoring *T. gondii* infection [20, 44].

CONCLUSION

It could be concluded that application of quantitative PCR has evolved as a sensitive, specific and rapid method for the detection of *T. gondii* DNA in blood and by the use of a standard curve for the target of interest, relative copy number values can be determined for any unknown sample, while ELISA and immumo blotting can be used for detection of chronic infection due to absence of specific antibodies during acute infection.

The significance of this experiment particularly for determining *T. gondii* load in potential *Toxoplasma* carriers, especially for epidemiologic studies or for monitoring the effect of therapy in treatment trials.

REFERENCES

1. Barakat, A.M.A., M.M. Abd Elaziz and H.A. Fadaly, 2009. Comparative Serodiagnostic Tests for Toxoplasmosis Among Sheep and Goats In Giza Governorate, Egypt. Global Veterinarial, 3(2): 07-14.
2. Tenter, A.M., A.R. Heckeroth and L.M. Weiss, 2000: *Toxoplasma gondii:* from animals to humans. International. J. Parasitolol., 30: 1217-1258.
3. Dubey, J.P. and C.P. Beattie, 1988. Toxoplasmosis of animals and man. Boca. Raton, flumequine. CRC Press, pp: 1-220.
4. Reischl, M., S. Bretagne, D. Kruger, P. Ernanlt and J.M. Costa, 2003. Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC-infectious. Diseases, 3(7): 2.

5. Tadros, W. and J.J. Laarman, 1982. Current concepts on the biology, evolution and taxonomy of tissue cyst-forming eimeriid coccidia. Advanced Parasitolol., 20: 293-468.
6. Dubey, J.P., 1993. *Toxoplasma, Neospora, Sacrocystis* and other tissue cyst-forming coccidian of humans and animals. In: J.P. Kreier, (Ed.), Parasitic Protozoa, Academic Press, New York, 6: 1-158.
7. Wendy, J. and S. Joseph, 1992. Toxoplasmosis infection in sheep. Compend. Contin Education, 14: 1543-1549.
8. Dubey, J.P., 1997. Bradyzoite induced murine toxoplasmosis: stage conversion, pathogenisis and tissue cyst formation in mice fed bradyzoites of different strains of *Toxoplasma gondii*. J. Eukaryotic Microbiol., 44: 592-602.
9. Dubey, J.P., C.A. Speer, S.K. Shen, O.C.H. Kwon and J.A. Blixt, 1997. Oocyst-induced murine toxoplasmosis: life cycle, pathogenicity and stage conversion in mice fed *Toxoplasma gondii* oocysts. J. Parasitol., 83: 870-872.
10. Navia, B.A., C.K. Petito, J.W. Gold, E.S. Cho, B.D. Jordan and R.W. Price, 1986. Cerebral toxoplasmosis complicating the acquired immune deficiency syndrome: clinical and neuropathological findings in 27 patients. Annual Neurol., 19: 224-238.
11. Remington, J.S., R. McLeod and G. Desmonts, 1995. Toxoplasmosis. In: J.S. Remington, J.O. Klein, editors Infectious diseases of the fetus and newborn infant. 4th ed. Philadelphia, Pa: The W.B. Saunders Co., pp: 140-267.
12. Morgan, V.M. and R.C.A. Thomapson, 1998. Molecular detection of parasitic protozoa. Parasitol., 117: 573-585.
13. Contini, C., S. Seraceni, R. Cultrera, C. Incorvaia, A. Sebastiani and S. Picot, 2005. Evaluation of a Real-Time PCR-based assay using the light cycler system for detection of *Toxoplasma gondii* bradyzoite genes in blood specimens from patients with toxoplasmic retinochoroditis.International J. Parasitol., 35: 75-283.
14. Boothroyd, J.C., 1987. Antigen and tubulin genes of *Toxoplasma gondii*. UCLA Symposium Molecular Cell Biol., 42: 237-250.
15. Bretagne, S., J.M. Costa, F. Foulet, L. Iabot Lestang, F. Baud Camus and C. Cordonnier, 2000. Prospective study of *Toxoplasma* reactivation by polymerase chain reaction in allogenic stem-cell transplant recipiente. Transplant Infectious Diseases, 2: 127-132.
16. Kupferschmidt, O., D. Kruger, T.K. Held, H. Ellerbrok, W. Siegert and K. Janitschke, 2001. Quantitative detection of *Toxoplasma gondii* DNA in human body fluids by TaqMan polymerase chain reaction. Clinical Microbiolgical and Infection, 7: 120-124.
17. Costa, J.M., C. Pautas, P. Emanlt, F. Foulet, C. Cortonnier and S. Bietagne, 2000. Real-time PCR for diagnosis and follow up of *Toxoplasma* reactivation after allogenic stem cell transplantation using fluorescence resonance energy trandfer hybridization probes J. Clinical Microbiol., 38(8): 2929-2932.
18. Johnson, M.A., P.J. Mc Donald and H.S. Neoh, 1979. Kinetics of the growth of *Toxoplasma gondii* (RH strain) in mice. International J. Parasitol., 9: 55-56.
19. Sambrook, J., D. Russell and J. Gola, 2001. Molecular Cloning, Third ed, a Laboratory Manual, 1: 32-34.
20. Contini, C., R. Cultrera, S. Seraceni, D. Segala, R. Romani, E. Fainardi, P. Cinque and A.S. Lazzarinand Delia, 2002. The role of stage-specific ologonucleotide primers in providing effective laboratory support for the molecular diagnosis of reactivated *Toxoplasma gondii* encephalitis in patients with AIDS. J. Med. Microbiol., 51: 879-890.
21. Cornelissen, A.W.C.A., 1984. Determination of nuclear DNA of five cucoccidian parasites, *Isospora* (*Toxoplasma) gondii*, Sarcocystis cruzi, eimeria tenella, E. acervulina and Plasmodium berghei, with special reference to gamotogenesis and meiosis I. (T) gondii. Parasitol., 88: 531-553.
22. James, G.S., V.G. Sintchenko, D.J. Dickeson and G.L. Gilbert, 1996. Comparison of cell culture, mouse inoculation and PCR for detection of *Toxoplasma gondii*: effects of storage conditions on sensitivity. J. Clinical Microbiol., 34: 1572-1575.
23. Porter, S.B. and M.A. Sande, 1992. Toxoplasmosis of the central nervous system in the acquired immunodeficiency syndrome. the New England J. Med., 327: 1643-1648.
24. Luft, B.J. and J.S. Remington, 1992. Toxoplasmic encephalitis in AIDS. Clinial of Infectous Diseases, 15: 211-222.
25. Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich and N. Arnheim, 1985. Enzymatic amplification of beta-globulin genomic sequences and restriction sites analysis for diagnosis of sickle cell anemia. Sci., 230(4732): 1350-4.

26. Costa, J.M., M.L. Dardé, B. Assouline, M. Vidaud and S. Bretagne, 1997. Microsatellite in the beta-tubulin gene of *Toxoplasma gondii* as a new genetic marker for use in direct screening of amniotic fluids. J. Clinical Microbiol., 35: 2542-2545.
27. Bergstrom, T., A. Ricksten, N. Nenonen, M. Lichtenstein and S. Olofsson, 1998. Congenital *Toxoplasma gondii* infection diagnosed by PCR amplification of peripheral mononuclear blood cells from a child and mother. Scandinavian J. Infectious Diseases, 30: 202-204.
28. Roberts, T.C. and G.A. Storch, 1997. Multiplex PCR for diagnosis of AIDS-related central nervous system lymphoma and toxoplasmosis. J. Clinical Microbiol., 35: 268-269.
29. Johnson, J.D., P.D.Butcher, D. Savva and RE. Holliman, 1993. Application of the polymerase chain reaction to the diagnosis of human toxoplasmosis. J. Infectious, 26: 147-158.
30. Lee, P.Y.C., J. Mangan, R.E. Holliman and P.D. Butcher, 1999. Quantitation of *Toxoplasma gondii* DNA in a competitive nested polymerase chain reaction. J. Clinical Pathol., 52: 61-64.
31. Wilhelm, J. and A. Pingoud, 2003. Real-time polymerase chain reaction. Chembiotechnology, 4(11): 1120-8.
32. Ririe, K.M., R.P. Rasmussen and C.T. Wittwer, 1997. Product differentiation by analysis of DNA melting curves during polymerase chain reaction. Annual Biochemistry, 245(2): 154-60.
33. Jones, C.D., N. Okhravi, P. Adamson, S. Tasker and S. Liightman, 2000. Comparison of PCR detection methods for B1, P30 and 185 rDNA genes of *T.gondii* in aqueous humor. Invest Ophthalmology Vis Science(IOVS), 413(3): 634-644.
34. Lin, M.H., T.C. Chen, T.T. Kuo, C.C. Tseng and C.P. Tseng, 2000. Real-Time PCR for quantitative detection of *Toxoplasma gondii*. J. Clinical Microbiol., 11: 4121-4125.
35. Botterel, F., P. Ichai, C. Feray, P. Bouree, F. Saliba, R. Tur Raspa, D. Samuel and S. Romand, 2002. Disseminated Toxoplasmosis, resulting from infection of allograft, after orthopic liver transplantation: usefulness of quantitative PCR J. Clinical Microbiol., 5: 1648-1650.
36. Buchbinder, S., R. Blatz and A.C. Rodloff, 2003: Comparison of Real-time PCR detection methods for B1 and P30 genes of *Toxoplasma gondii*. Diagnostic. Microbiolog. Of Infectious. Diseases., 45: 269-271.
37. Menotti, J., G. Vilela, S. Romand, Y.J.F. Garin, L. Ades, E. Gluckman, F. Derouin and P. Ribaud, 2003. Comparison of PCR-Enzyme-Linked immunosorbent assay and Real-Time PCR assay for diagnosis of an unusual case of cerebral toxoplasmosis in a stem cell transplant recipient. J. Clinical Microbiol., 11: 5313-5316.
38. Dworkin, L.L., T.M. Gibler and R.N. Van Gelder, 2002. Real-Time quantitative polymerase chain reaction diagnosis of infectious posterior uveitis. Archives Ophthalmol., 120: 1534-1539.
39. Kompalic- Cristo, A., C. frotta, M. Suarez - Mutis, O. Fernands and C. Brittp, 2007. Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of *Toxoplasma gondii* in human peripheral blood. Parasitological. Res., 101(3): 619-625.
40. Savva, D., J.C. Morris, J.D. Johonson and R.E. Holliman, 1990. Polymerase chain reaction for detection of *T. gondii*. J. Medical. Microbiol., 32: 25-31.
41. Ho-Yen, D.O., A.W.L. Joss, A.H. Balfour, E.T.M. Smyth, D. Baird and J.M.W. Chatterton, 1992. Use of the polymerase chain reaction to detect *Toxoplasma gondii* in human blood samples. J. Clinical Pathol., 45: 910-913.
42. Bou, G., M.S. Figueroa, E. Marti`-Belda, Navas and A. Guerrero, 1999. Detection of *Toxoplasma gondii* in aqueous humor and blood samples from immunocompetent patients with ocular toxoplasmosis. J. Clinical. Microbiol., 37: 3465-3468.
43. Figueroa, M.S., G. Bou, P. Marti-Belda, R. Lopez-Velez and A. Guerrero, 2000. Diagnostic value of polymerase chain reaction in blood and aqueous humor in immunocompetent patients with ocular toxoplasmosis. Retina, 20: 614-619.
44. Cultrera, R., S. Seraceni and C. Contini, 2002. Efficacy of a novel reverse transcriptase-polymerase chain reaction (RT-PCR) for detecting *Toxoplasma gondii* bradyzoite gene expression in human clinical specimens. Molecular Cell Probes, 16: 31-39.