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Research Article

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Diagnosis and quantitative detection of *Toxoplasma gondii* in male and female laboratory rats using qReal -Time PCR

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Abstract The protozoan *Toxoplasma gondii* is one of the most common infectious pathogenic parasites and can cause severe medical complications in infants and immune-compromised individuals. In the current study, *Toxoplasma gondii* was isolated from the placenta of aborted women and the suspension of placenta was inoculated into the peritoneal cavity of male and female laboratory rats. The acute toxoplasmosis in male and female laboratory rats was diagnosed using qReal-Time PCR and the ratio of diagnosis 96.55% in males and 98.85% in females. In chronic infection after two months of infection, the diagnostic rate was 100% in the males and females. The rate of diagnosis was 100% in male brain and in testis was 71.43%, while it reached 90.48% in female brain and 100% in ovary. The present study showed that the positive diagnosis of infection of *Toxoplasma gondii* in male and female rats infected experimentally by using the impression smear method for organs and stained with Giemsa, were 95.24% (42 / 40) in males and 100% (42 / 42) in females. The study also detected the load of DNA of the *Toxoplasma gondii* in blood samples (acute infection) and tissue samples (chronic infection) of experimentally infected male and female rats. Load of DNA of parasite in males with acute infection reached $0.65x10^4$ and $8.76x10^4$ in chronic infection ($p < 0.001$), while in females, the load of DNA of parasite in acute infection reached $54.70x10^4$, while it appeared higher in chronic infection $437.00x10^4$ ($p < 0.001$). We carried out a rapid, sensitive, and quantitative real-time PCR for detection of *T. gondii*. The advantages of this technique for the diagnosis of toxoplasmosis in a clinical laboratory are discussed.

Keywords *Toxoplasma gondii*, qReal -Time PCR

Introduction

Toxoplasma gondii is a zoonotic parasite capable of causing disease in all warm-blooded animals. There are plenty of animal hosts of this parasite resulting in high prevalence of it. Felidae, as the only final host of the parasite, excrete the oocyst form of the parasites in their feces. Asexual forms of the parasite such as tachyzoite and bradyzoite are seen in intermediate hosts [1].

Toxoplasma infection in humans, especially in people with defective immune system, pregnant women, children caused serious damage. Thus, detection of this parasite in humans and animals closely related with humans, seems to be necessary [2-4].

Most cases of acquired toxoplasmosis are asymptomatic or may cause flu-like disease. However, in immune-compromised patients, such as HIV infected and organ transplant recipients, *Toxoplasma gondii* may cause severe life threatening disease, resulting in brain lesions or diffuse encephalitis. Reactivation of latent infection has been accounted for the cause of severe infection in such individuals although severe toxoplasmosis can also result from acute infection [5-6].

According to previous studies, maternal infection in the first and second trimester may be resulted in 5% prenatal death and 2 % stillbirth [7]. The majority of diagnostic assays for toxoplasmosis were serological. Molecular methods have been developed to improve the diagnosis accuracy of congenital toxoplasmosis [8]. Polymerase Chain Reaction (PCR)-based assay, to amplify a 200-300 fold repetitive 529-base pair (bp) DNA region, of *Toxoplasma* has a higher sensitivity than conventional serological tests [9]. Development of an accurate, sensitive and rapid method for the detection and identification of *T. gondii* is important in both diagnosis and treatment [10].

Several PCR-based techniques have been developed for the diagnosis of toxoplasmosis using various clinical specimens, including amniotic fluid, blood, cerebrospinal fluid, and tissue biopsy [11]. Among these techniques, nested PCR followed by hybridization of PCR products was the most sensitive method. However, the main disadvantage of these methods is that they are quite time-consuming and do not provide quantitative data. The real-time quantitative PCR technique has proven recently as a useful test for various applications, including pathogen detection, gene expression and regulation, and allelic discrimination [12]. Real-time PCR utilizes the 59 nuclease activity of *Taq* DNA polymerase to cleave a non-extendible, fluorescence-labeled hybridization probe during the extension phase of PCR. The fluorescence of the intact probe is quenched by a second fluorescent dye, usually 6-carboxy-tetramethyl- rhodamine (TAMRA). The nuclease cleavage of the hybridization probe during the PCR releases the effect of quenching resulting in an increase of fluorescence proportional to the amount of PCR product, and can be monitored by a sequence detector, such as the GenAmp 5700 Sequence Detection System [1]. Therefore this study was designed for diagnosis and quantitative detection of *T. gondii* infection in experimental male and female rats.

Materials and Methods

Laboratory animals

Mature male and female rats (*Rattus norvegicus*) were reared in plastic cages supplied with drinking water and food, and kept in animal house of the College of Education for pure science of Thi-Qar University. The animal house was supplied with ventilator fan and air conditioner in order to control the room temperature 21±2 ºC.

Isolation of *Toxoplasma gondii*

Parasite was isolated from placenta of aborted women with toxoplasmosis history. Placenta was cut into small pieces and mixed with an equal amount of normal saline and grinded by using mortar and pestle. This preparation (solution) was passed through a piece of gauze to avoid large particles and then centrifugation was done, 3000 rpm for 10 min. The supernatant was discarded and the sediment was suspended by normal saline and the process was repeated three times by washing the samples, 0.1 ml of 1,000 units of penicillin and streptomycin 100 mg was added to prevent contamination [13]. 0.3 ml suspension contains 100 tissues cyst of parasites. Animal was used for induction of experimental infection in 174 rats through intraperitoneal route, 30 rats were injected with saline to serve as control [14].

Diagnosis of *Toxoplasma gondii*

6-10 days after the inoculation, blood samples were taken from tails of animals. 8 weeks after induction, rats were anesthetized and tissue samples were taken from infected and control group to detect the parasite using impression smear and to detect B1 gene by qRT-PCR.

Extraction DNA parasite from blood samples and tissue samples

DNA of the parasite was extracted from the blood by the method of Gunel *et al*., (2012) [15] and from the tissue according to the method of Sutthikornchai *et al*., (2013) [16] by using reagent DNA extraction kit (Geneaid USA). The extracted DNA was checked by Nanodrop spectrophotometer.

qReal-Time PCR

Real-Time PCR based TaqMan probe was performed for rapid detection of *T. gondii* according to method described by Lin *et al*. (2000) [17]. Real-Time PCR TaqMan probe and primers were used for amplification of conserved region B1 gene in *T. gondii*. These primers were provided by Bioneer Company, Korea as shown in Table 1.

The Real-Time PCR amplification reaction was done by using (AccuPower® DualStarTM qPCR PreMix Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as shown in Table 2.

These qPCR master mix reaction components mentioned in Table 2 were added into AccuPower® DualStarTM qPCR PreMix tubes which contain Taq DNA polymerases, dNTPs, 10X buffer for TaqMan probe amplification. Then tubes were placed in Exispin vortex centrifuge at 3000 rpm for 3 min, after that transferred into MiniOpticon Real-Time PCR system and the thermocycler conditions shown in Table 3.

Table 1: Real-time PCR TaqMan probe and primers

Prime	Sequence
Forward primer	5'-TCCCCTCTGCTGGCGAAAAGT-3'
Reverse primer	5'-AGCGTTCGTGGTCAACTATCGATTG-3'
B1gene probe	FAM-TCTGTGCAACTTTGGTGTATTCGCAG-TAMRA

Table 2: The qPCR master mix

2X GreenStar Master Mix	25µl
PCR F-Primer(10 pmole)	1 µl
PCR R-Primer(10 pmole)	1 µl
50X Rox dye	1 µl
Template DNA	5 µl
DEPC-distilled water	17 µl
Total	50 µl

Table 3: Thermocycler conditions

qPCR	Temperature and Time	Repeat cycle
Pre-Denaturation	95 ºC 10-15 min.	1
Denaturation	95 ºC 5-20 sec.	40-45
Extention and Annealing	55-60 ºC 30-45 sec.	40-45
Detection	Scan	40-45
Melting	-	1

Results

Isolation of the parasite *Toxoplasma gondii*

Parasite was isolated from the placenta of aborted women to can be utilize for experimental animal infection, parasite was detected by impression smear that stained with Giemsa stain, which revealed the presence of tissue cyst containing bradyzoites of parasite as shown in Figure 1.

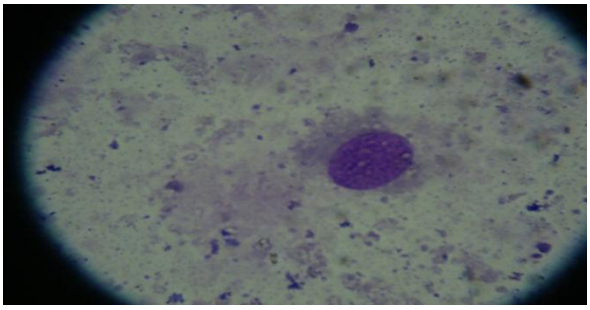

Figure 1: Tissue cyst of Toxoplasma gondii in placental tissue stained with Giemsa stain (100X)

Diagnosis of *Toxoplasma gondii* using qReal-Time PCR

1. Diagnosis of acute infection

The acute infection was diagnosed in male and female rats, 6-10 days after inoculation with parasite containing suspension using qReal-Time PCR. The total percentage of infection was 97.70% (174 /170 rat), the rate of diagnosis in males was 96.55% (87 / 84 rat) and in females 98.85% (87 / 86 rat). The diagnosis was evidenced by the curved of Amplification plot based on threshold cycler number (CT) value so that the threshold was positive when it exceed the threshold line (Table 4, Figure 2).

Table 4: Diagnosis rate of acute infection of *Toxoplasma gondii* in male and female laboratory rats using by qReal-Time PCR

%	Number of positively diagnosed rats	Number of inoculated rats	Sex
96.55	84	87	Males
98.85	86	87	Females
97.70	170	174	Total

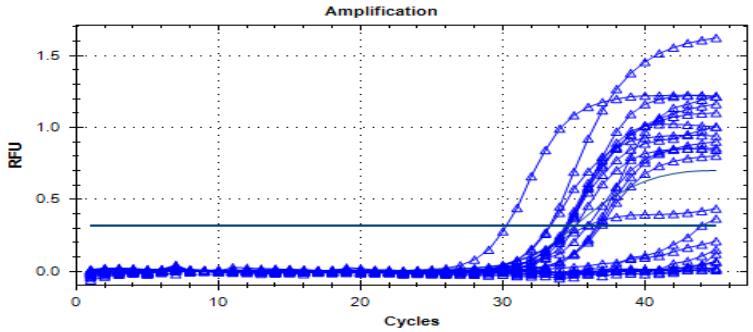

Figure 2: Real-Time amplification plot for B1 gene in Toxoplasma gondii of acute infection group

2. Diagnosis of chronic infection

a. using qReal-Time PCR:

The rate of diagnosis of chronic infection (2 months after inoculation of rats) was 100% in males 42/42 and females (Table 5, Figure 3).

Table 5: Diagnosis of chronic infection of *Toxoplasma gondii* in tissues of males and females laboratory rats using qReal-Time PCR

%	Number of positively diagnosed rats	Number of inoculated rats	Sex
100	42	42	Males
100	42	42	Females
100	84	84	Total

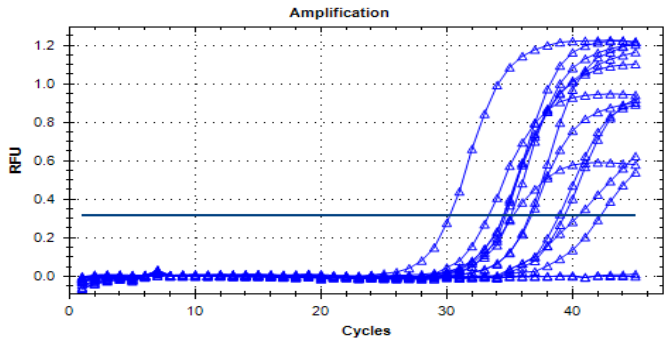

Figure 3: Real-Time amplification plot for B1 gene in Toxoplasma gondii in chronically infection group.

The study was also showed that the infection was diagnosed positively in 100 % (42/42) of male brains and 90.48 % (38/42) of female brains, while the rate of positive diagnosis from the male rate testis was 71.43 (30/40) and females ovary 100 % (42/42) (Table 6-7, Figure 3-6).

Table 6: The percentage of diagnosed *Toxoplasma gondii* infection according to organs in male laboratory rats using qReal-Time PCR

Total	Brain		Testis	
	Result positive	**%**	**Result positive**	**%**
Male(n=42)	42	100	30	71.43
control(n=15)	0	0	0	0

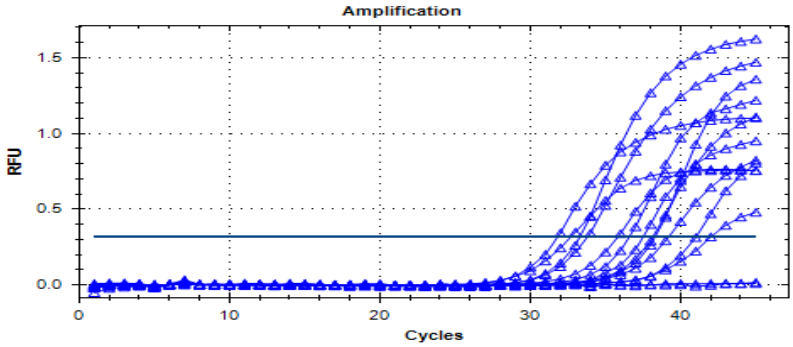

Figure 4: Real-Time amplification plot for B1 gene Toxoplasma gondii in male brain of laboratory rats

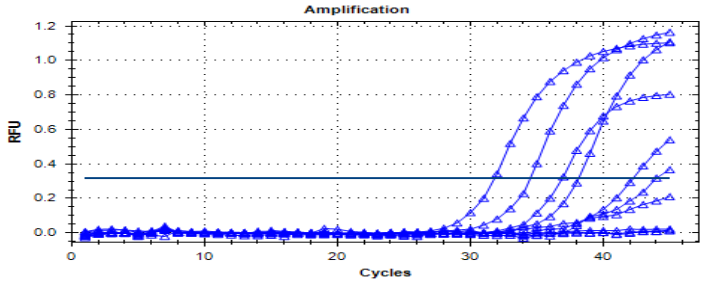

Figure 5: Real-Time amplification plot for B1 gene Toxoplasma gondii in male testis of laboratory rats

Table 7: The percentage of *Toxoplasma gondii* infection according organs in female laboratory rats using qReal-Time PCR

Total	Brain		Ovary	
	Result positive	**%**	**Result positive**	**%**
Female (n=42)	38	90.48	42	100
Control (n=15)	0	0	0	0

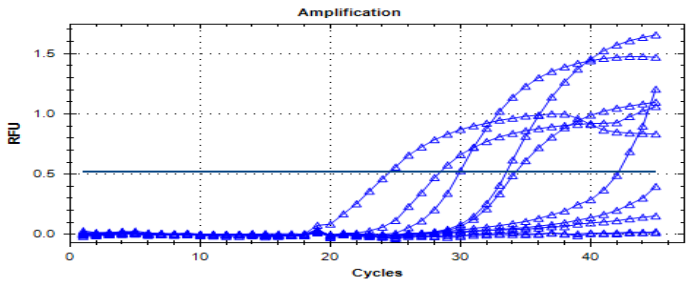

Figure 6: Real-Time amplification plot for B1 gene Toxoplasma gondii in female brain of laboratory rats

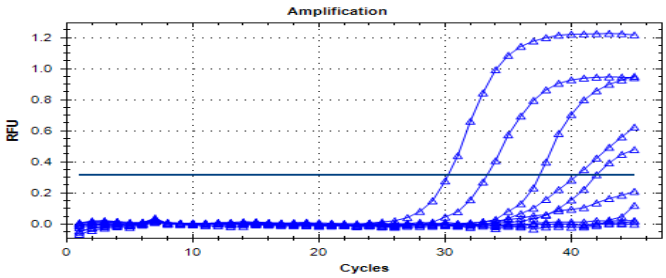

Figure 7: Real-Time amplification plot for B1 gene *Toxoplasma gondii* in female ovary of laboratory rats

b. Using impression smear

The present study showed that the positive diagnosis of infection of *Toxoplasma gondii* in male and female laboratory rats infected experimentally by inoculation of placenta suspension containing the tissue cysts of the parasite using the impression smear method for organs and stained with Giemsa, were 95.24% (42 / 40) in males and 100% (42 / 42) in females (Table 8).

Table 8: Diagnosis of chronic infection of *Toxoplasma gondii* in tissues of males and females rats using impression smear

%	**Number of positively diagnosed rats**	**Number of inoculated rats**	**Sex**
95.24	40	42	Male
100	42	42	Female
97.62	82	84	Total

Quantitative detection of *Toxoplasma gondii* using qReal-Time PCR in male and female laboratory rats

The study was also showed detection the load of DNA of the *Toxoplasma gondii* in blood samples (acute infection) and tissue samples (chronic infection) for male and female rats with experimentally infected via the peritoneal inoculation of placenta suspension. Load of DNA of parasite in males (acute infection) reached $0.65x10^4$ and $8.76x10^4$ in chronic infection and ($p < 0.001$), while in females, the load of DNA of parasite (acute infection) reached $54.70x10^4$ and chronic infection appeared higher $437.00x10^4$ ($p < 0.001$) (Table 9).

Table 9: Quantitative detection of *Toxoplasma gondii* using qReal-Time PCR in male and female laboratory rats

Significant (P value)	**Load of DNA of parasite in chronic infection $x10^4$**	**Load of DNA of parasite in acute infection $x10^4$**	**Sex**
($p<0.001$)	8.76±1.02	0.65 ±0.06	Male
($p<0.001$)	437.00±18.46	54.70±6.22	Female
0	0	0	Control

Discussion

The results of the current study revealed the possibility of isolation of *Toxoplasma gondii* from placenta of aborted women. Many previous studies also isolated the parasite from the placenta of aborted woman [18-20], while Al-Kennany and Hassan, (2010) isolated the parasite from the placenta of ewe [13].

As in our study, the parasite can isolated from the tissue of infected animals, Aghwan *et al*., (2010) [21] isolated the parasite from different organs of the rabbits (heart and brain and testis, kidney, spleen, liver, lung, muscle and ovary). Dzbenski *et al*., (1998) isolated the parasite from CSF after inoculation of rabbits with Tachyzoite of *Toxoplasma gondii* [22].

Gill *et al*., (2002) [23] also isolated the parasite from the mast cell in eye of rodent *Calomys callosus* (Rodentia: Cricetidae). The parasites were isolated from different organs included liver, brain, spleen, kidney, lung, heart and Sekletal muscles of the brown hares (*Lepus europaeus*), the placenta of ewes, goats and from aborted lamb embryos and milk and also from the placenta of the aborted women and semen of men with primary and secondary infertility

[24]. So toxoplasmosis, which infected human and many other animals, can subsequently invaded several tissues and can isolated from these numerous sources.

In the present study, the acute infection of *Toxoplasma gondii* in male and female rats can be diagnosed after 6-10 days after the inoculation with suspension of placenta containing the parasite, these results were in agreement with that recorded by Salibay and Calveria, (2006) [25], by which the diagnosis of acute toxoplasmosis in the blood of rabbits was achieved after a week of experimental infection using serological tests. However, Hade, (2013) diagnosed the acute infection in 76.5% of the sheep (51 / 39) in the serum using nPCR technique [26]. Acute infection of the parasite was diagnosed in the peritoneal fluid of laboratory mice after 10 days of inoculation. On the other hand, Cunha *et al*., (2004) diagnosed the infection in the blood samples of the laboratory mice infected experimentally, by the using of Indirect Fluorescent Antibody Test (IFAT) [27]. From the previous studies it was appeared that the diagnosis ratio of toxoplasmosis can be affected by the inoculation dose to the animal, immunity and resistance of the experimental animal for parasitic infection [28]. It was also appeared that females laboratory rats showed high susceptibility to parasitic infection than males [29].

The present study also showed the high rate of diagnosis of chronic toxoplasmosis in male and female rats experimentally infected. Jassem, (2015) recorded diagnostic rate of 90.3% in rats with experimental toxoplasmosis, two months after the infection using Real-Time PCR [19].

The appearance of false negative samples could be caused by mistakes in DNA extraction steps or due to the different conditions of keeping the sample [30-31]. As in our study, many other workers recorded high diagnostic rate from infected animals tissues such as brain, testis and ovaries [32-34].

According to our results, this diagnostic method was suitable for routine screening of *T. gondii* infection in the clinical laboratory investigation in conjunction with other diagnostic techniques, such as serological tests [17].

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