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Tissue tropism and parasite burden of *Toxoplasma gondii* RH strain in experimentally infected mice

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ABSTRACT

Objective: To evaluate parasite distribution and tissue tropism of *Toxoplasma gondii* tachyzoites in experimentally infected mice using real time QPCR. **Methods:** In this survey 16 Balb/c mice were inoculated with 1×10^4 alive tachyzoites of *Toxoplasma gondii* RH strain. After 1, 2, 3 days post infection and the last day (before death), different tissues of mice including blood, brain, eye, liver, spleen, kidney, heart and muscle were harvested. Following tissues DNA extraction, the parasite burden was quantified using real time QPCR targeting the B1 gene (451 bp). **Results:** It showed that *Toxoplasma* after intraperitoneal injection was able to movement to various tissues in 24 hours. Parasite burden was high in all tissues but the most number of parasites were observed in kidney, heart and liver, respectively. **Conclusions:** These data provide significant baseline information about *Toxoplasma* pathogenesis, vaccine monitoring and drug efficiency.

1. Introduction

Toxoplasma gondii (*T. gondii*), an intracellular protozoan parasite has a worldwide distribution and infects all warm-blooded animals including human. Toxoplasmosis is one of the most important public health problems and usually transmitted through ingestion of sporulated oocysts or tissue cysts[1,2]. *T. gondii* sporozoites are released from oocysts in the gut lumen, subsequently infect the intestinal epithelium and through the blood stream disseminated to several tissues including brain, heart, eye, kidney, liver, spleen and lung[2].

Although toxoplasmosis in immunocompetent individuals is mostly asymptomatic and self-limiting, in immunocompromised patients may cause opportunistic infection and life-threatening disease[3,4]. *T. gondii* is capable of invading several tissues of host and causes vary signs and symptoms depending on the site of infection. Therefore severity of disease manifestation may depend on the tendency and density of parasites. Also the pathological lesion in brain and eye tissues usually associated with multiplication of tachyzoites[1,5].

Until now, the parasitic load in tissues with *T. gondii* has been determined using mouse bioassay, histological examination and microscopic examination of samples. These methods have some disadvantages such as low sensitivity, time consumption and observation errors[6,7]. Quantitative PCR (QPCR) can be used to detect specific target DNA in samples quantitatively and qualitatively. This method is highly sensitive and rapid[8]. We previously investigate the parasite burdens in tissues of experimentally infected mice using Giemsa staining[9]. In continuation of our previous surveys, the present study was performed to precisely determine the parasite load and disseminate throughout the host different tissue samples after intraperitoneal infection in murine model using real time QPCR.

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2. Materials and Methods

Tachyzoites of the virulent RH strain of *T. gondii* harvested from the peritoneal cavity of Swiss-Webster mice 3-4 day after intraperitoneal (*ip*.) inoculation of parasite, which is maintained in Toxoplasmosis Research Center. Thereafter tachyzoites were washed twice in phosphate-buffered saline (PBS; pH=7.4), containing 100 IU/mL penicillin and 100 μg/mL streptomycin and counted using hemacytometer[10,11].

Sixteen inbred female C57BL/6 mice, 8-10 weeks of age and weighting 20 g, were obtained from Sari animal house (Mazandaran-Iran) and used for experiments. The project underwent ethical review and was given approval by the Ethics Committee of Mazandaran University of Medical Sciences. In order to evaluate parasite load in different tissues, twelve mice under sterile conditions were inoculated intraperitoneally with 1×10^4 live tachyzoites and randomly divided into 4 groups (n=3). Additionally, non-infected negative control group included mice inoculated with PBS (n=4).

Following infection, blood and tissues including the brain, eye, liver, spleen, kidney, heart and muscle were harvested from each mouse. The time points included 1, 2, 3 day post inoculation (dpi) and last day before death. Collected samples were stored at -80 ℃. For tissue DNA extraction, approximately 25-50 mg of disrupts organ pieces were homogenized and added 200 μL of tissue lysis buffer. Then 20 μL of proteinase K were added to the samples, mix by vortex and incubated at 60 ℃ until the tissue was completely lysed. Afterward blood and tissues DNA were extracted using tissue DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions and eluted in 200 μL Tris-EDTA buffer (TEB, pH=8.0) and stored at -20 ℃ until QPCR performance.

The 451 bp fragment of highly conserved B1 gene of *T. gondii* was used as the target gene[12]. Amplification was performed using real time QPCR and each amplification included positive control (total DNA extracted directly from tachyzoites of the RH strain) and negative control (distilled water or DNA extracts from a blood sample of a *Toxoplasma*-negative mouse). Real-time QPCR was performed in a final volume of 25 μL, using SYBR green master mix (12.5 μL) (Bioneer, Korea) that mixed with 1 μL Rox dye, 6 μL template DNA (10 ng), 6 μL DEPC distilled water and 1 μL of *T. gondii* 451 bp B1 gene specific primers at a concentration of 10 pmol/μL (forward primer 5′-CTCCTTCGTCCGTCGTAATATC-3′ and reverse primer 5′-TGGTGTACTGCGAAAATGAATC-3′). The following amplification protocol applied: 94 ℃ for 3 min, 40 cycles at 94 ℃ for 30 s, 62 ℃ for 40 s, 72 ℃ for 50 s, followed by a final extension 72 ℃ for 5 min. A SYBR green assay was used to optimize annealing temperature of the primer pair by a temperature gradient. Melting curve analysis was performed to verify the correct product size and did not result in formation of side products or primer dimmers.

QPCR was run in triplicate for each sample independently and values were averaged and means of parasitic load was calculated. The number of parasites in the infected mouse blood and tissues was quantified from the QPCR threshold cycle (C_T) value according to a standard known concentration. A standard curve generated from 8-fold serial dilution included 5×10^3 - 0.05 of RH strain tachyzoites per ml (Y=-3.954 7X+34.896; R^2=0.909). The results were expressed as *T. gondii* tachyzoite equivalents per mg of tissue.

In the different tissues, DNA quantification was compared by the ANOVA test and values were considered to be significant when P<0.05. Per tissue sample, the median number of parasites per gram in each day was calculated to check for statistically significant differences among various days by Repeated Measure ANOVA test using the SPSS software.

3. Result

Infection of female C57BL/6 mice with the *T. gondii* RH strain easily was measurable by QPCR after 24 h post inoculation (Figure 1). The acute infection follow the intraperitoneal injection of virulent strain, led to the fatality of infected mice 4-5 dpi. Analysis of blood and tissue samples collected on 1, 2, 3 dpi and last day before death using QPCR allowed to determine the parasite load and DNA copy number in all infected mice (Table 1). In this study we did not detect any *T. gondii* DNA in the control group (non infected mice).

Parasite load in tissues of *T. gondii* infected mice is shown in Figure 2. In the first day post infection, liver, brain and blood displayed high levels of parasite load, whereas amount of parasite DNA was particularly low in heart and spleen (1 411 and 5 870 parasites). On the second dpi parasite load in eye and heart tissues were drastically increased compared to the first day. In both 3 and 4 dpi high parasite load was seen in all tissues except the brain and liver. In the third day which reached a peak of parasite burden, difference was not significant, however other times (1, 2 dpi and last day) showed a significant difference among different tissues.

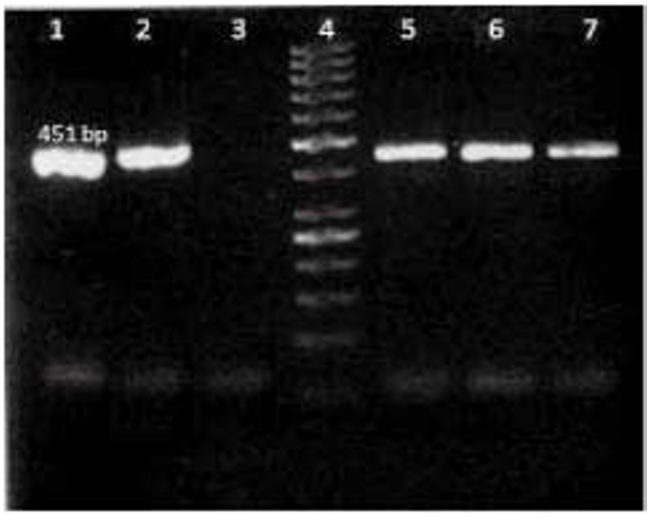

Figure 1. QPCR amplification products were analyzed by agarose gel electrophoresis (1.5%).
Lane 1: positive control (DNA extracted from tachyzoites), Lane 2, 5, 6, 7: unknown samples from tissues, Lane 3: negative control, Lane 4: DNA molecular weight marker (100 bp).

Table 1
Parasite burden of *Toxoplasma* tachyzoites in different tissues of mice.

Tissue	Days after infection (Parasite load)				*P*-value	*F*-value
	First	Second	Third	Last (before death)		
Eye	2.17×10^{7}	1.25×10^{9}	5.19×10^{7}	5.91×10^{6}	0.34	1.36
Muscle	1.73×10^{5}	6.67×10^{4}	1.73×10^{9}	5.55×10^{6}	0.001*	21.72
Kidney	1.71×10^{6}	4.89×10^{4}	1.03×10^{9}	2.44×10^{9}	0.002*	16.64
Heart	1.41×10^{3}	1.73×10^{9}	2.10×10^{9}	1.12×10^{9}	0.01*	9.35
Brain	1.51×10^{9}	1.33×10^{4}	2.03×10^{6}	1.98×10^{6}	0.02*	7.20
Spleen	5.87×10^{3}	4.81×10^{5}	1.76×10^{9}	1.63×10^{9}	0.04*	4.98
Blood	1.43×10^{9}	6.27×10^{7}	1.55×10^{9}	5.43×10^{7}	0.65	0.57
Liver	1.98×10^{9}	9.01×10^{7}	4.20×10^{4}	5.45×10^{7}	0.002*	18.13
P-value	0.004*	0.000*	0.138	0.03*		
F-value	4.867	7.538	1.888	3.055		

*Statistically significant difference.

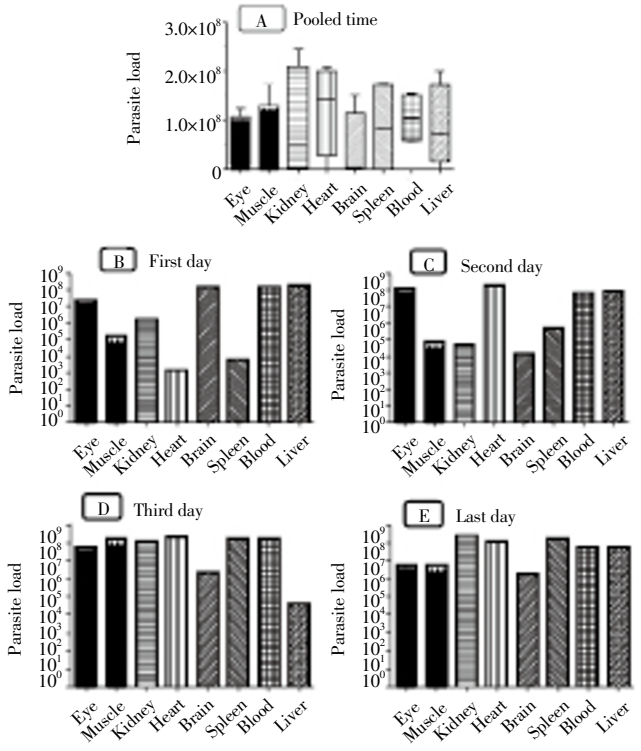

Figure 2. Comparative graph of the *Toxoplasma* parasite burden at different tissues in mice experimentally were *ip*. inoculated with 2×10^{4} RH strain tachyzoites.
A: pooled time, B-E: 1, 2, 3 dpi and last day (before death) respectively.

4. Discussion

In this study, we surveyed on distribution of *T. gondii* tachyzoites and parasitic load in intraperitoneally infected mice using real time QPCR. *T. gondii B1* gene as a target was amplified from blood and different tissues including eye, heart, brain, kidney, liver, spleen, muscle. All collected tissues from infected mice were positive during the experimental study. However, the highest parasite load was seen in the last day (before death) in the kidney tissue (2.44×10^{9}).

The research on movement trend of *Toxoplasma* and parasite load has been used mainly to assessment disease severity, anti-parasite drug and vaccine effect, and also suggested for diagnosis of ocular toxoplasmosis[13-15]. Preliminary, our research group applied Giemsa staining techniques for evaluation of presence and movement trend of *T. gondii* tachyzoites in different tissues of mice after intraperitoneal infection[9]. But staining method for determination of parasite load is generally unsatisfactory, inaccurate and time consuming, also has a low sensitivity. In contrast, molecular methods particularly real time QPCR has been described as a sensitive method for quantification and determination of *T. gondii* parasite load in blood and different tissues[8,15]. Hence, in the present study, we have utilized a formerly described real-time QPCR based *Toxoplasma B1* gene for quantitative detection of parasite DNA copy number[8]. This method is extremely sensitive and its main advantage is ability to quantify the parasite load in various tissue, as detect even 0.05 parasites per each reaction[8]. To date, there have been few published studies that investigated on the distribution of *Toxoplasma* and determination of parasite load in tissues using QPCR.

In a study on *Toxoplasma* burden in tissue samples of serologically positive chickens, using real time PCR, it has been reported that the parasite quantity did not show significantly difference between tissue types including brain and heart[16]. Juránková *et al* analysed the quantification of *Toxoplasma* in different tissues of goats such as brain, lung, liver, kidney, heart and spleen by magnetic capture and real time PCR[17]. The highest parasitic load was seen in lung; also brain tissue was the second most infected organ. In other tissues merely a low level of parasite burden was found, moreover in liver and kidney tissues the least parasite number was detected. Nevertheless, in the current study we found the high parasite load in all tissues and the peak of DNA copy number was observed in kidney, heart and liver. The probably basis for this tissue tropism would

be related to parasite strain and injection method as in the i.p inoculation adjacent tissues such as kidney and liver, are more likely to have infection. Also in kidney tissue, the large copy number of parasite may be associated with renal function and glomerular filtration. Jauregui *et al* used real time PCR for detection of *T. gondii* in experimentally infected pig and mouse tissues and confirmed that in large animal species because of sampling bias, parasite finding is difficult[18]. In addition the used primer pair influences on the results[19].

Djurkovic *et al* used real time PCR to examine the kinetics of the distribution of *Toxoplasma* in the murine model based on 529 bp gene fragment (gene bank AF487550.1)[20]. Recent study have described that 24 h post infection, parasite DNA was detected in all analysed tissues (blood, brain, lungs and liver) in cases of infected with 10^6 tachyzoites of *Toxoplasma* RH strain, and the highest parasite load was detected in the blood and the least in the brain tissue.

In conclusion, results show that *T. gondii* were able to movement to various tissues at 24 hours or may be even at earlier time. Therefore application of the real-time PCR for determination of parasite load in different tissues provide suitable and sensitive method for custom screening of *T. gondii* infection in high-risk patients such as immunocompromised and pregnant women. It is also noticeable to emphasize that the detection of *Toxoplasma* DNA in the eye and brain tissues, demonstrated parasite passage across the blood brain barrier from 1 dpi after experimental infection. Thus, present results provide significant baseline information about *Toxoplasma* pathogenesis in the acute phase of infection and monitoring of vaccine and drug efficiency.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet* 2004; **363**: 1965-1976.

[2] Weiss LM, Dubey JP. Toxoplasmosis: A history of clinical observations. *Int J Parasitol* 2009; **39**: 895-901.

[3] Chirch LM, Luft BJ, Michael J. Aminoff FoBDFSPP, Joseph RB. Cerebral toxoplasmosis in AIDS. *Handbook Clin Neurol* 2007;147-58.

[4] Hermanns B, Brunn A, Schwarz ER, Sachweh JS, Seipelt I, Schroder JM, et al. Fulminant Toxoplasmosis in a heart transplant recipient. *Pathol Res Pract* 2001; **197**: 211-215.

[5] Lachenmaier SM, Deli MA, Meissner M, Liesenfeld O. Intracellular transport of *Toxoplasma gondii* through the blood - brain barrier. *J Neuroimmunol* 2011; **232**: 119-130.

[6] Derouin F, Mazeron MC, Garin YJ. Comparative study of tissue culture and mouse inoculation methods for demonstration of *Toxoplasma gondii*. *J Clinical Microbiol* 1987; **25**: 1597-1600.

[7] Zenner L, Darcy Fo, Capron A, Cesbron-Delauw M-F. *Toxoplasma gondii*: Kinetics of the dissemination in the host tissues during the acute phase of infection of mice and rats. *Experimental Parasitol* 1998; **90**: 86-94.

[8] Lin MH, Chen TC, Kuo TT, Tseng CC, Tseng CP. Real-Time PCR for quantitative detection of *Toxoplasma gondii*. *J Clin Microbiol* 2000; **38**: 4121-4125.

[9] Dadimoghaddam Y, Daryani A, Kalani H, Ahmadpour E, Sharif M, Ziaei H, et al. Movement trend of *Toxoplasma gondii* in different tissues of Balb/c after immunization with excretory-secretory antigens. *J Mazandaran Univ Med Sci (JMUMS)* 2013; 23.

[10] Daryani A, Hosseini AZ, Dalimi A. Immune responses against excreted/secreted antigens of *Toxoplasma gondii* tachyzoites in the murine model. *Vet Parasitol* 2003; 113: 123-134.

[11] Saadatnia G, Haj Ghani H, Khoo BY, Rahmah N, Maimuna A. Research note optimization of *Toxoplasma gondii* cultivation in VERO cell line. Trop Biomed 2010; **27**: 125-130.

[12] Edvinsson B, Jalal S, Nord CE, Pedersen BS, Evengard B. DNA extraction and PCR assays for detection of *Toxoplasma gondii*. *Apmis* 2004; **112**: 342-348.

[13] Norose K, Tokushima T, Yano A. Quantitative polymerase chain reaction in diagnosing ocular toxoplasmosis. *Am J Ophthalmol* 1996; **121**: 441.

[14] Romand S, Chosson M, Franck J, Wallon M, Kieffer F, Kaiser K, et al. Usefulness of quantitative polymerase chain reaction in amniotic fluid as early prognostic marker of fetal infection with *Toxoplasma gondii*. *Am J Obstetrics Gynecol* 2004; **190**: 797-802.

[15] Bell AS, Ranford-Cartwright LC. Real-time quantitative PCR in parasitology. *Trends Parasitol* 2002; **18**: 338-342.

[16] Aigner CP, Silva AVd, Sandrini F, Osorio Pde S, Poiares L, Largura A. Real-time PCR-based quantification of *Toxoplasma gondii* in tissue samples of serologically positive outdoor chickens. Memórias do Instituto Oswaldo Cruz 2010; **105**: 935-937.

[17] Juránková J, Opsteegh M, Neumayerov H, , Kovarcik K, Frencova A, Balaz V, et al. Quantification of *Toxoplasma gondii* in tissue samples of experimentally infected goats by magnetic capture and real-time PCR. *Vet Parasitol* 2012.

[18] Jauregui LH, Higgins J, Zarlenga D, Dubey JP, Lunney JK. Development of a real-time PCR assay for detection of *Toxoplasma gondii* in pig and mouse tissues. *J Clin Microbiol* 2001; **39**: 2065-2071.

[19] Pelloux H, Weiss J, Simon J,Muet F, Fricker-Hidalgo H, Goullier-Fleuret A, et al. A new set of primers for the detection of *Toxoplasma gondii* in amniotic fluid using polymerase chain reaction. *FEMS Microbiol Lett* 1996; **138**: 11-15.

[20] Djurkovic-Djakovic O, Djokic V, Vujanic M, Zivkovic T, Bobic B, Nikolic A,et al. Kinetics of parasite burdens in blood and tissues during murine toxoplasmosis. *Experimental Parasitol* 2012; **131**: 372-376.