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EARLY DETECTION OF *TOXOPLASMA GONDII* INFECTION IN MONGOLIAN GERBIL BY QUANTITATIVE REAL-TIME PCR

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KEY WORDS

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ABSTRACT

Toxoplasmosis, caused by *Toxoplasma gondii*, is associated with several clinical syndromes, including encephalitis, chorioretinitis, and congenital infection. *Toxoplasma gondii* is a ubiquitous apicomplexan parasite found in both humans and animals. Mongolian gerbils, which are more susceptible to both high- and low-virulence *Toxoplasma* strains compared with mice, are considered useful models for assessing diagnosis and treatment methods for toxoplasmosis, as well as infection by and host defense to this organism. Here we established a quantitative real-time polymerase chain reaction (qPCR) method targeting the *B1* gene for early and specific detection of *T. gondii* infection in Mongolian gerbil. The detection limit of the developed qPCR was approximately 1 *T. gondii* tachyzoite. This method was also applied to detect *T. gondii* genomic DNA in experimentally infected Mongolian gerbils, with positive results in blood (66.7%), liver (73.3%), lung (80.0%), spleen (80.0%), and peritoneal fluid (66.7%) samples as early as 1 day postinfection. Specificity tests confirmed no cross-reactivity with DNA templates of *Neospora caninum, Cryptosporidium parvum, Eimeria tenella, Trypanosoma evansi, Schistosoma japonicum, Angiostrongylus cantonensis*, *and Strongyloides stercoralis*. This study first reports the use of Mongolian gerbils as an animal model for early diagnosis of toxoplasmosis by qPCR.

Toxoplasma gondii is an opportunistic pathogen responsible for severe human and veterinary diseases (Lindsay and Dubey, 2011). *Toxoplasma gondii* infection can be acquired by ingestion of raw and undercooked meat containing tissue cysts or food and water that are contaminated by oocysts (Dubey and Thulliez, 1993). In immunocompromised individuals, e.g., AIDS patients, toxoplasmosis may lead to life-threatening consequences such as encephalitis and ophthalmitis (Boothroyd and Grigg, 2002; Saadatnia and Golkar, 2012). *Toxoplasma gondii* infection may result in abortion or birth defects when infection is acquired by a pregnant woman during pregnancy.

Accurate and early diagnosis of toxoplasmosis is important for its treatment. Immunologic methods including enzyme-linked immunosorbent assays (Dai et al., 2013) and indirect fluorescent-antibody test (de Almeida et al., 2016) are widely applied for *T. gondii* detection, but may fail during the early stage of infection because of low antibody levels (Gross et al., 2000). Polymerase chain reaction (PCR)-based methods have been developed and applied for the detection of *T. gondii*, especially quantitative real-time PCR (qPCR) platforms that could provide more reliable detection data and allow quantification of PCR products (Ginzinger, 2002). Target sequence and primer selection are important factors affecting qPCR results. The multicopy *B1* gene and 529-base pair (bp) repetitive element (RE) of *T. gondii* are the most used sequences for parasite detection (Belaz et al., 2015). Indeed, 529-bp RE-PCR was reported to be more sensitive in *T. gondii* DNA detection than that when targeting the *B1* gene (Edvinsson et al., 2006; Belaz et al., 2015). On the other hand, *B1* gene-based PCR was reported to be more specific since there are strains of *T. gondii* in which either the whole or parts of the 529 RE fragment have been deleted or mutated (Wahab et al., 2010).

Thanks to biological mechanisms and characteristics similar to those of humans, Mongolian gerbils (*Meriones unguiculatus*) are increasingly used as infectious disease models, including parasitological diseases such as those caused by *Brugia pahangi* (Alworth et al., 2015), *Giardia duodenalis* (Amorim et al., 2010), *Babesia divergens* (Dkhil et al., 2014), and *Baylisascaris potosis* (Tokiwa et al., 2015). Mongolian gerbils are actually slightly more susceptible to *T. gondii* infection than mice, with various organs more suitable for extensive parasite proliferation than mouse counterparts (Suzuki and Tsunematsu, 1974). Of interesting, Mongolian gerbils were used to assess alterations of oxidative

stress, biochemical indices, and hematological parameters after *T. gondii* infection for a better understanding of toxoplasmosis pathogenesis (Atmaca et al., 2015). Lv et al. (2017) successfully identified differentially regulated proteins and specific signaling pathways in *T. gondii*-infected Mongolian gerbil brains via an iTRAQ-based strategy; their findings may provide a deeper understanding of injury mechanisms in the brain after *T. gondii* infection, and help develop efficient prevention and control methods for encephalitis. To date, reports focusing on the diagnosis of *T. gondii* infection in Mongolian gerbils are scarce. Therefore, in this study, the infection model of *T. gondii* in the Mongolian gerbil was established, and DNA detection was performed by conventional PCR and TaqMan qPCR targeting the *B1* gene.

MATERIALS AND METHODS

Ethics statement

Animals were maintained under pathogen-free conditions with food and water ad libitum according to Regulations for the Administration of Affairs concerning Experimental Animals of the People's Republic of China (1988.11.1). The animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Zhejiang Academy of Medical Sciences (Ethical Clearance Number: ZJAMS20140012).

Parasites and Mongolian gerbils

Tachyzoites of *T. gondii* RH strain were harvested from Vero cells 48–72 hr after infection, washed 3 times in cold phosphate-buffered saline, and then centrifuged at 1,500 *g* for 5 min. Genomic DNA from *T. gondii* was extracted with a universal genomic DNA extraction kit (Tiangen, Shanghai, China) according to the manufacturer's instructions.

A total of 60 Mongolian gerbils (6–8 wk old) was obtained from the Zhejiang Academy of Medical Sciences. Forty-five Mongolian gerbils were intraperitoneally infected with 1×10^3 tachyzoites of *T. gondii* RH strain. In addition, 15 Mongolian gerbils were intraperitoneally injected with physiological saline as negative controls. Blood, liver, lung, spleen, and peritoneal fluid samples were collected at 1, 3, and 5 days postinfection (dpi), respectively.

DNA extraction

Genomic DNA was extracted from the specimens (1 g or 1 ml) with a universal genomic DNA extraction kit (Tiangen) according to the manufacturer's instructions. Purified DNA was subsequently dissolved in 50 µl of double-distilled water for PCR and qPCR assays.

Conventional PCR and qPCR

PCR primers were designed on the basis of the multicopy *B1* gene of *T. gondii* (GenBank accession number AF179871) using the Primer 5.0 software to specifically amplify a 115-bp fragment. Forward and reverse primers were 5′-TGGCGAAAAGTGAAATTCATGAG-3′ and 5′-TCACGATCTTCTTCTCCTGTTCT-3′, respectively. The above primers and the probe FAM-CGATAGTTGACCACGAACGC were used for the qPCR.

For qPCR, each reaction mixture contained 12.5 µl of 2× TaqMan gene expression master mix (Applied Biosystems, Foster City, California), 0.5 µl of each primer (10 µM), 1 µl of probe (5 µM), 1 µl of DNA template, and 9.5 µl of sterile distilled water. All reactions were performed on a 7500 real-time PCR detection system (Applied Biosystems) as follows: initial incubation at 50 C for 2 min and 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min (data collection). Triplicate reactions containing no template were included in each run as negative controls. Threshold cycle values (Ct) were calculated on the basis of automatic adaptive baseline settings (threshold is 10 times the standard deviation of the baseline), and an unknown sample with a Ct value <35 was considered positive.

For conventional PCR, the 20-µl reaction mixture contained 0.5 µl of Taq DNA polymerase (5 U/µl) (TaKaRa, Shanghai, China), 2.0 µl of 10× Taq PCR buffer, 2.0 µl of deoxyribonucleotide triphosphate (2.5 mM), 0.5 µl of each PCR primer (10 mM), 2 µl of template DNA, and 12.5 µl of sterile distilled water. Amplification was performed at 95 C for 5 min, followed by 40 cycles of denaturation at 95 C for 15 sec, primer annealing at 60 C for 20 sec, and extension at 72 C for 30 sec, and a final extension at 72 C for 7 min.

Specificity and sensitivity assessment

The specificity of the qPCR assay was determined by assessing genomic DNA samples from *T. gondii*, *Neospora caninum*, *Cryptosporidium parvum*, *Eimeria tenella*, *Trypanosoma evansi*, *Schistosoma japonicum*, *Angiostrongylus cantonensis*, and *Strongyloides stercoralis*, with a negative control devoid of template included. All DNA samples used for the specificity test were diluted to a concentration of 50 ng/µl.

Sensitivities of the qPCR and conventional PCR methods were quantified by assessing 10-fold serial dilutions of purified *Toxoplasma gondii* DNA with the same primers. The resulting PCR products were analyzed by gel electrophoresis on a 2% agarose gel stained with GoldView™ solution (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) for visualization.

Application of the qPCR method

The 240 obtained DNA samples of blood, liver, lung, spleen, and peritoneal fluid from *T. gondii*-infected and uninfected Mongolian gerbils were assessed by the qPCR and conventional PCR methods. In addition, a total of 83 blood samples from naive Mongolian gerbils and 72 blood samples from naive mice randomly collected from the Laboratory Animal Centre of Zhejiang Academy of Medical Sciences or Laboratory Animal Centre of Hangzhou Normal University was used to determine the applicability of the established technique. *Toxoplasma gondii* DNA and sterile water were included in each test as positive and negative controls respectively.

RESULTS

As shown in Figure 1, a specific 115-bp fragment was amplified by PCR from genomic DNA of *T. gondii* targeting the *B1* gene.

DNA templates from *T. gondii*, *N. caninum*, *C. parvum*, *E. tenella*, *Trypanosoma evansi*, *Schistosoma japonicum*, *A. cantonensis*, *and Strongyloides stercoralis* were all diluted to a concentration of 50 ng/µl. All templates were applied with *B1*-

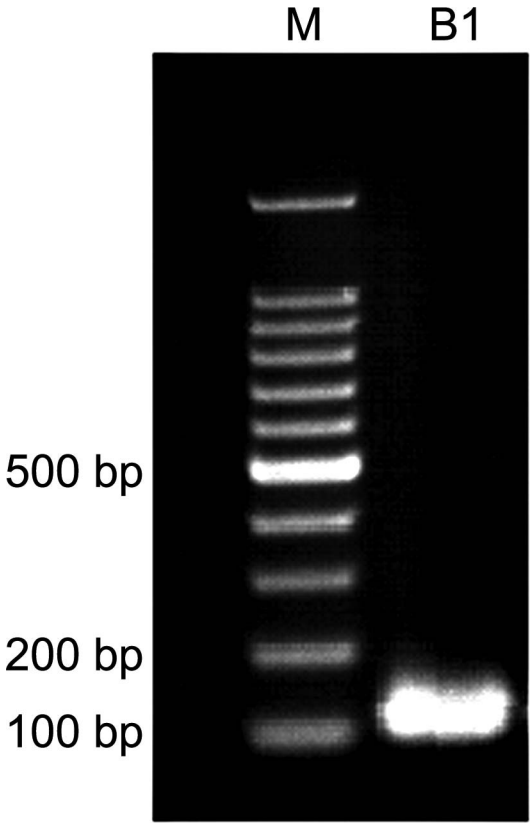

Figure 1. Polymerase chain reaction amplification of the *B1* gene of *Toxoplasma gondii*.

targeted PCR, but only *Toxoplasma gondii* DNA template was amplified (Fig. 2).

The same primers were used for qPCR methods. Genomic DNA of *T. gondii* was 10-fold serially diluted and used for sensitivity tests for both qPCR and conventional PCR. We found the detection limit of qPCR to be around 1 tachyzoite (Fig. 3A) and the detection limit of conventional PCR to be about 100 tachyzoites (Fig. 3B).

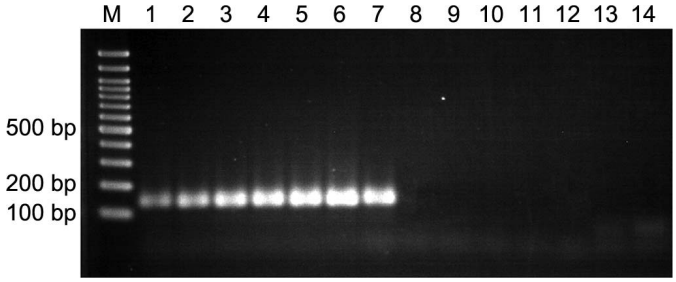

Figure 2. Specificity of the quantitative real-time polymerase chain reaction for *Toxoplasma gondii*. Lane M, DNA ladder marker; lanes 1 and 2, genomic DNA of *T. gondii* extracted from Vero cell culture; lanes 3 and 4, genomic DNA of *T. gondii* extracted from peritoneal fluid of mice; lanes 5–7, genomic DNA of *T. gondii* extracted from peritoneal fluid of Mongolian gerbil; lanes 8–14, genomic DNA of *Neospora caninum*, *Cryptosporidium parvum*, *Eimeria tenella*, *Trypanosoma evansi*, *Schistosoma japonicum*, *Angiostrongylus cantonensis and Strongyloide sstercoralis*, respectively. The concentrations of all the DNA templates used for specificity tests were diluted to 50 ng/μl.

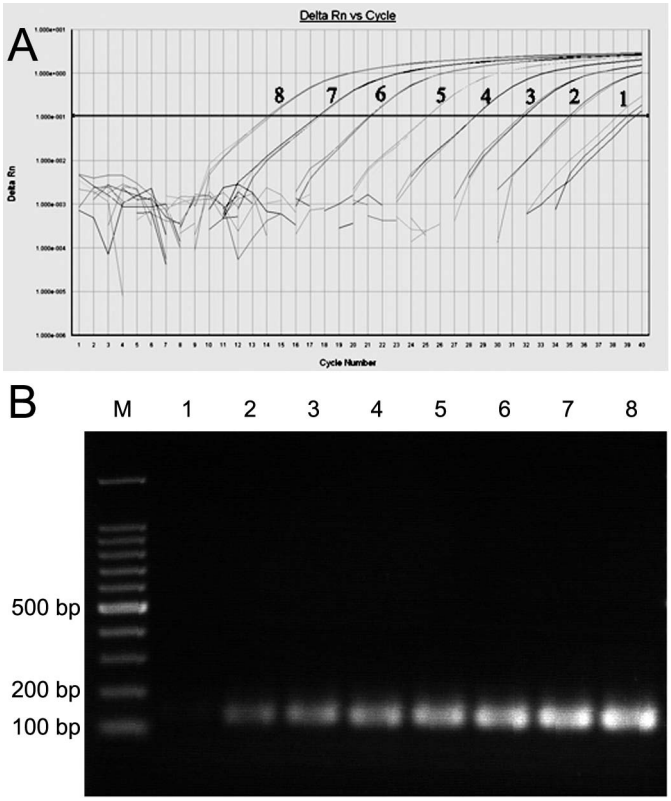

Figure 3. Sensitivity comparison of quantitative real-time polymerase chain reaction (qPCR) (**A**) and conventional PCR (**B**) for the specific detection of *Toxoplasma gondii* on the basis of *B1* gene amplification. **(A)** Detection limit of the qPCR assay. Lanes 1–8, *T. gondii* from 10^{-1} to 10^{6} parasites. (**B**) Detection limit of conventional PCR. Lane M, DNA ladder marker; lanes 1–7, *T. gondii* genomic DNA samples from 10 to 10^{7} parasites.

A total of 240 DNA samples from blood, liver, lung, spleen, and peritoneal fluid samples of *T. gondii*-infected or uninfected Mongolian gerbils was assessed by both qPCR and conventional PCR. As shown in Table I, *T. gondii* DNA was detected in blood (66.7%), liver (73.3%), lung (80.0%), spleen (80.0%), and peritoneal fluid (66.7%) samples as early as 1 dpi by the novel qPCR assay, and all samples were positive at 3 dpi. *Toxoplasma gondii* DNA was detected by conventional PCR only after infection for 3 days in blood (53.3%), liver (53.3%), lung (60.0%), spleen (60.0%), and peritoneal fluid (53.3%) samples; all samples were positive at 5 dpi.

A total of 83 blood samples from naive Mongolian gerbils and 72 blood samples from naive mice was analyzed by the established qPCR method. As shown in Table II, the positive rates of Mongolian gerbils and mice were both 0%.

DISCUSSION

The Mongolian gerbil is generally susceptible to both high- and low-virulence strains of *T. gondii* and is suitable for parasite isolation, disease diagnosis, immunoprophylaxis, and pathogenicity studies (Suzuki and Tsunematsu, 1974). The present report is the first to assess an infection model of *T. gondii* in the Mongolian gerbil by a novel qPCR method targeting the multicopy *B1* gene.

Table I. Comparison of the quantitative real-time polymerase chain reaction (PCR) and conventional PCR methods for the diagnosis of *Toxoplasma gondii* in experimentally infected Mongolian gerbils.

Group	Days postinfection	Sample (number)	Detection rate (no. positive reactions/no. performed reactions)	
			Real-time PCR	Conventional PCR
Infection	1	Blood (15)	66.7	0
		Peritoneal fluid (15)	66.7	0
		Liver (15)	73.3	0
		Lung (15)	80.0	0
		Spleen (15)	80.0	0
	3	Blood (15)	100	53.3
		Peritoneal fluid (15)	100	53.3
		Liver (15)	100	53.3
		Lung (15)	100	60.0
		Spleen (15)	100	60.0
	5	Blood (15)	100	100
		Peritoneal fluid (15)	100	100
		Liver (15)	100	100
		Lung (15)	100	100
		Spleen (15)	100	100
Control	5	Blood (15)	0	0
		Peritoneal fluid (15)	0	0
		Liver (15)	0	0
		Lung (15)	0	0
		Spleen (15)	0	0

The specific 115-bp PCR fragment was amplified, demonstrating that the designed *B1* gene primers were accurate. In addition, the specificity of these designed primers was conducted with genomic DNA templates from *T. gondii, N. caninum, C. parvum, E. tenella, Trypanosoma evansi, Schistosoma japonicum, A. cantonensis, and Strongyloides stercoralis* samples by conventional PCR assay. Only the products from templates of *Toxoplasma gondii* presented clear amplification bands, whereas no amplification products were found from other templates. These results indicated that there were no cross-reactions; hence, the designed primers targeting the *B1* gene are capable of specific amplification to produce an accurate detection of *T. gondii*. Sensitivity comparison between qPCR and conventional PCR was carried out using 10-fold serially diluted genomic DNA of *T. gondii*. We found that the detection limit of the qPCR method of around 1 tachyzoite was at least 100 times more sensitive than conventional PCR. Jones et al. (2000) evaluated a nested amplification of the *B1* gene that allows detection of as little as 50 fg of *T. gondii* DNA, which is in the same range level as the qPCR method established here, assuming that each tachyzoite yields about 80 fg of DNA (Reischl et al., 2003). The REP529-based qPCR method could possibly detect as little as 8 fg of *T. gondii* DNA, providing more sensitive diagnosis data than *B1*-based qPCR (Edvinsson et al., 2006). However, it was reported that either the whole or parts of the 529 RE fragment have been deleted or mutated in some strains of *T. gondii*, leading to a possibility of false-negative results when detected by PCR methods based on the 529 RE fragment.

Table II. Diagnosis of *Toxoplasma gondii* in blood samples.

Species	Positive	Negative	Total number	Positive rate (%)
Mongolian gerbil	0	83	83	0
Mice	0	72	72	0

The detection sensitivities of both qPCR and conventional PCR were further tested by detecting DNA samples extracted from blood, liver, lung, spleen, and peritoneal fluid specimens from *T. gondii*-infected and uninfected Mongolian gerbils. We found that the genomic DNA of *T. gondii* was detectable as early as 1 dpi by the novel qPCR assay, and all samples including blood, liver, lung, spleen, and peritoneal fluid were confirmed positive at 3 dpi. In contrast, *T. gondii* DNA was detected by conventional PCR only after infection for 3 days. Previously, Yu et al. (2013) established a qPCR method based on the *SAG1* gene. They found that *T. gondii* DNA remains undetectable in blood samples and is detected in lung (50%) and spleen (25%) specimens from mice at 3 dpi when assessed by conventional PCR; *T. gondii* DNA was detected in blood, lung, and spleen samples from mice at 1 dpi by qPCR, corroborating our findings. Relatively higher parasite loads were found in lung and spleen specimens from Mongolian gerbils, with a pattern similar to previous findings in mouse models (Derouin and Garin, 1991; Djurkovic-Djakovic et al., 2012). However, detection in this study was based on acute infection by *T. gondii* RH strain in the Mongolian gerbil model. Therefore, the sensitivity of clinical diagnosis could differ because of the various strains of *T. gondii*, and these findings should be interpreted with caution.

To prove the application of this established qPCR assay, 83 blood samples from naive Mongolian gerbils and 72 blood samples from naive mice randomly collected from the Laboratory Animal Centre of Zhejiang Academy of Medical Sciences or Laboratory Animal Centre of Hangzhou Normal University were analyzed (Table II). The positive rates of blood samples were both

0%, which indicated a low risk of *T. gondii* infection in experimental animals.

The 529 RE (AF146527) (Homan et al., 2000), *SAG1* (Yu et al., 2013), and *B1* (Kompalic-Cristo et al., 2007) genes are the most common target sequences used for the molecular detection of *T. gondii* DNA. Kasper et al. (2009) described a REP529-based qPCR assay that detects 1/30 to 1/50 of 1 parasite per reaction, nearly 30 times more sensitive than the qPCR method established in this study. However, Belaz et al. (2015) found 7 false negatives after the REP529 qPCR application compared with *B1* qPCR. A previous study revealed that the REP529 locus cannot be amplified in some samples from African patients, which may lead to false-negative results (Wahab et al., 2010). In addition, qPCR sensitivity is excellent, making the specificity of the selected target sequence a priority. Thus, in this study, the more suitable *B1* target gene was used to detect *T. gondii* DNA on the basis of the TaqMan probe.

In conclusion, we assessed early detection of *T. gondii* infection by *B1* gene-based qPCR in Mongolian gerbils. The results demonstrated that the Mongolian gerbil is a very useful animal model for assessing toxoplasmosis diagnosis. Because of its sensitivity and specificity, the novel assay presented here could be used as a reliable and alternative diagnostic tool for clinical and epidemiological investigations, as well as toxoplasmosis control in laboratory animal units.

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