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Development of triplex real-time PCR and detection of *Toxoplasma gondii* DNA in infected mice tissues and spiked human samples

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Abstract. *Toxoplasma gondii* is an important pathogen in veterinary and human medicine. In this study, a new multiplex *TaqMan* real-time PCR for detection of *T. gondii* DNA was developed. This assay consisted of new sets of primers and probes which targeted B1 gene and ITS-1 region of *T. gondii*, with *Vibrio cholera* gene as internal control. The B1 gene primers were designed to detect *T. gondii* RH strain, while the ITS-1 region primers detected most *T. gondii* strains. Specificity test using common protozoal and bacterial DNA revealed that the assay was very specific to *T. gondii*. Standard curves constructed using human body fluids spiked with *T. gondii* (RH and ME49 strains) showed that the sensitivity of the assay was one parasite, with R^2 value of 0.975 to 0.999 and efficiency of 97% to 99% for all types of samples. The assay performed on DNA extracted from tissues of mice infected with *T. gondii* showed that liver contained the highest parasite load for both strains of *T. gondii*. The multiplex real-time PCR developed in this study would be potentially useful for detection of *T. gondii* in human and animal samples.

INTRODUCTION

Toxoplasma gondii is estimated to infect as many as one-third of the human population (Dubey, 2004). Based on estimation of the disease burden of toxoplasmosis it ranks at the same level as salmonellosis or campylobacteriosis (Kijlstra & Jongert, 2008). Current diagnosis of toxoplasmosis mainly relies on serological methods that detect specific antibodies against *T. gondii*. However, there are limitations for use of serology in detecting acute infection since the peak concentrations of specific antibodies are found weeks after parasitemia has subsided (Gross *et al.*, 2000). Moreover, infections in immunocompromised patients are rarely accompanied by a significant rise in antibody. Mouse inoculation or tissue culture of clinical samples may also detect the infection; however, this approach is time consuming, impractical and showed only moderate sensitivity (Dubey & Jones, 2008). Thus, direct demonstration of the parasite in tissues or other biological fluids by PCR is a major breakthrough for the diagnosis of toxoplasmosis, especially in pregnant women and immunocompromised patients (Contini *et al.*, 2005).

Unlike conventional PCR, real-time PCR allows detection as well as quantification of the copy number of target DNA in a sample. The approach also eliminates the need to visualize the amplicons or PCR products by gel electrophoresis, thereby reducing the total time spent in an assay. Moreover, it also reduces the risk of false-positive due to contamination with previous amplified products. Quantitative real-time PCR is extremely useful to monitor the progression of infection under treatment and to diagnose low-level infection (Contini *et al.*, 2005). A

previous report also suggested that real-time PCR is useful in the diagnosis of ocular toxoplasmosis (Lin *et al.*, 2000). The adaptability of real-time PCR to a high-throughput 96-well format makes it useful for testing large number of samples.

Currently there are limited number of reports on the use of new real-time PCR primers and probes for detecting *T. gondii*. Thus, there is still room for improvement in molecular diagnosis of this parasite. The present study was aimed at developing a new specific and sensitive multiplex real-time PCR assay for *T. gondii*, which can also be used to determine the quantity of the parasite DNA in a sample. It is a continuation of a previous work on development of a triplex conventional PCR for detection of *T. gondii* (Anizah *et al.*, 2012).

MATERIALS AND METHODS

Statements of human and animal ethics

Approval for this study was obtained from the Human and Animal Research Ethics Committees of Universiti Sains Malaysia. All the animals used in this study were placed in a designated 24 h air conditioned and well-ventilated animal room with standard mice cages, according to the national guidelines for animal care. Mice food pellets and water were available *ad libitum* to the animals, and personnel were assigned to ensure that the animal bedding was changed regularly and the room was kept clean at all times. Efforts were made to minimize the number of animals used and their suffering throughout the experiments.

Culture of *T. gondii* strains

Two types of *T. gondii* strains were used in this study, RH and ME49. Both strains were maintained as previously reported (Anizah *et al.*, 2012).

DNA extraction

The DNA extraction method used for both *T. gondii* RH and ME49 strains has been described previously (Anizah *et al.*, 2012). The DNA was used as template for the optimization of real-time PCR amplification conditions or was stored at -20°C.

Animal infection

In total 20 mice were used for experimental animal infection using RH and ME49 strains. The method used for animal infection and the isolation and extraction of infected animal organs (brain, liver, spleen, heart and kidney) were similar as previously reported (Anizah *et al.*, 2012).

Assay development

Design of primer and probe

All primers and probes were designed using Primer Express 2.0 software (Applied Biosystem, USA). They were designed to amplify portions of B1 gene (AF179871), ITS-1 region of several strains (AY259045, AY2259044, AY143139, AB255454, AJ628253, AJ628252 and AJ628251) and internal control, *HemM* gene (AF227752).

Optimization of primer concentration

Concentrations of primers to detect B1 gene and ITS-1 region were optimized using increasing concentrations from 20 nM to 300 nM; and for the *HemM* gene, the primer concentrations were from 50 nM to 600 nM. The optimized PCR conditions were then used for the entire multiplex real-time PCR assay. All the reagents used for real-time PCR amplification were aliquoted in small volumes to avoid contamination and freeze-thawing that may affect the reaction efficiency. The PCR product of each primer pair was cloned, sequenced and analyzed using online BLAST (Basic Local Alignment Search Tool) program. Probe concentrations were standardized at 0.625 µM for the entire assay based on the manufacturer's recommendation.

Set-up of multiplex real-time PCR

Real-time PCR was performed using the Corbett Rotor Gene™6000 (Rotor GeneQ, Germany) which contain five different detection channels. In this study three different channels were used, and the reactions were performed using 0.2 ml PCR

tubes in a 36-well rotor. A total volume of 25 µl was set up for each reaction, which included *TaqMan*® Universal PCR Master Mix, primers and probes for the B1 gene, ITS-1 region and *HemM* gene, 1.4 mM $MgCl_2$ and 5 µl template. One µl plasmid containing *V. cholera HemM* gene was included in every reaction as heterologous control. Non-template control (NTC) using water served as the negative control in every reaction to monitor for possible cross contamination. The PCR reaction was performed at 95ºC for 15 min, followed by 50 cycles of denaturation at 95ºC for 9 sec and primer annealing at 59ºC for 60 sec. Each PCR reaction was conducted in triplicates.

Test of assay specificity

The assay specificity was tested using DNA of the following organisms: *Helicobacter pylori*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Giardia lamblia*, *Strongyloides stercoralis*, *Leptospira spp.*, *Plasmodium falciparum*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The DNA extracted from mice muscle was also included. *Toxoplasma gondii* RH and ME49 strains served as the positive controls.

Preparation of the human body fluids for standard curve construction

Whole blood

Blood samples were collected from 10 individuals who were negative for *T. gondii*-specific IgG and IgM antibodies by commercial serological assays (Platelia, BioRad, USA). Approximately 5 ml of whole blood in EDTA was collected individually, pooled, aliquoted and stored at 4ºC.

Cerebral spinal fluid (CSF)

CSF specimens from 5 individuals (with their consents) who were negative for *T. gondii* specific IgG and IgM antibodies were provided by the Department of Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia. The samples were pooled, aliquot and stored at -20ºC.

Amniotic fluid (AF)

Human AF specimens from 10 individuals (with their consents) who were negative for *T. gondii* specific IgG and IgM antibodies were obtained from the Department of Obstetrics and Gynecology, Hospital Universiti Sains Malaysia. They were then pooled, aliquot and stored at -20ºC.

Construction of standard curve

The limit of detection (LoD) of the assay was determined by constructing standard curves using the DNA extracted from human body fluids (whole blood, CSF and AF) spiked with *T. gondii* whole parasite. The concentration used for spiking ranged from 10^0 parasites to 10^5 parasites. The DNA extraction was performed as described in section 2.3. Linear regression of each series and the associated R^2 value were determined for each standard curve. The standard curves constitute the analytical sensitivity test for the developed assay. All Ct values were reported as mean ± SD.

RESULTS

Extraction of DNA

The DNA extracted from *T. gondii* RH and ME49 strains had high concentrations (1.3 µg/µl and 1.13 µg/µl respectively) and good purity (A260/A280 of 2.01 and 2.10 respectively). This was consistent with the strong intensity of the high molecular weight DNA band observed on the agarose gel electrophoresis (data not shown).

Design of primers and probes

The primers and probes designed to target B1 gene, ITS-1 region and HemM gene (internal control) are listed in Table 1. The B1 gene primers were designed to specifically detect *T. gondii* RH strain whereas the ITS-1 region primers were designed to detect most of *T. gondii* strains, such as RH, ME49, MAS, PGT, CTG, COUGAR, CN, SH, ZS and QH. The *V. cholera HemM* gene was used as an internal control. The

Table 1. Sequences of primers and probes used in the multiplex real-time PCR assay

Gene name		Sequence 5' to 3'	Size (bp)
HemM	F[a]	TAT TCT ACG TGC CAT TCC	61
	R[a]	ATT ACG AGT TGA ATG AAC AA	
	P[a]	Cy5 - ACA CTC TTG CCA CTC TCA CCA -BHQ2	
B1	F	GGT CCG GGT GAA ACA ATA GAG A	67
	R	GGC ATG ACA ACT GGG CAG TA	
	P[b]	6FAM - ACT GGA ACG TCGCCG -MGBNFQ	
ITS-1	F	ATT CGT GTC TCT GTT GGG ATA CTG	60
	R	GGT GTG GGA AAA AAG AAT GCA	
	P[b]	VIC - TTT CCA GGA GTT TCT TC -MGBNFQ	

green channel of the real-time PCR system detected FAM dye which targeted the B1 gene, the yellow channel detected VIC dye which targeted the ITS-1 gene; and the red channel detected Cy5 dye which targeted the internal control.

Optimization of primer concentrations

Amplification pattern of each target gene at different primer concentrations using the DNA of *T. gondii* RH strain as the template were recorded. The B1 gene and ITS-1 region primers showed constant Ct value from 60 nm to 300 nm. The first shift of Ct value was observed at 40 nm for both pairs of primer. Therefore, 40 nm was chosen as the optimal concentration for B1 gene and ITS-1 region primers. For internal control primers, the first change of Ct value was observed at 400 nm; therefore this was chosen as the optimal concentration for internal control primers. These optimized primer concentrations were used to perform the multiplex assay (data not shown).

Specificity analysis of the multiplex real-time PCR

The specificity analysis of multiplex real-time PCR showed no cross-reactivity with the tested organisms. There was no cross-reactivity with the DNA extracted from mouse muscle and *HemM* DNA. Successful amplifications were obtained with the positive control DNA samples from *T. gondii* RH and ME49 strains (data not shown).

Construction of standard curve

Tables 2 and 3 show the standard curves using the human body fluids spiked with whole parasites of both strains. For construction of each standard curve, real-time PCR was performed three times in triplicates under similar conditions to ensure reproducibility of results. All the data were reported as mean ± SD. The results showed that the multiplex real-time PCR was able to consistently detect as little as one parasite in all samples, thus the analytical sensitivity or detection limit of the assay was one parasite. The coefficient of correlation (R^2) ranged 0.975 to 0.999 and efficiency ranged from 97% to 99%, indicating a strong linear relation of the standard curve. This shows the accuracy and reproducibility of the dilution range used for the standard curve construction. B1 gene was observed to detect the target much faster compared to ITS-1 region. The internal control was amplified in the Ct value range of 24.98 to 25.67 for all samples (data not included in the table).

Detection of *T. gondii* DNA in tissues

Tables 4 and 5 show B1 gene and ITS-1 region amplifications in DNA extracted from tissues of experimental and control mice infected with RH or ME49 strains of *T. gondii*. The tissues used in this study comprised brain, heart, kidney, liver and spleen. Positive control (RH or ME49 DNA) and internal control (*V. cholerae* plasmid with HemM gene) were also included in all reactions. The

Table 2. Summary of mean C_t values obtained with DNA from RH strain-spiked human body fluids

No. parasites	Ct value (Mean ± SD)					
	Blood		CSF		AF	
	B1	ITS-1	B1	ITS-1	B1	ITS-1
100000	15.35±0.09	16.22±0.05	14.15±0.15	16.59±0.31	16.62±0.15	19.82±0.04
10000	18.59±0.04	20.19±0.06	18.40±0.35	21.12±0.09	20.60±0.07	20.68±0.07
1000	23.41±0.06	25.13±0.11	22.41±0.39	25.60±0.06	23.98±0.04	27.72±0.04
100	27.14±0.09	29.23±0.09	25.42±0.41	29.25±0.11	27.53±0.04	32.52±0.04
10	31.12±0.05	33.19±0.09	30.43±0.22	35.09±0.09	31.83±0.12	36.06±0.07
1	34.54±0.11	36.31±0.02	32.26±0.17	38.48±0.18	34.51±0.06	40.94±0.09

Ct value data are given as Mean ± SD

Table 3. Summary of mean C_t values obtained with DNA from ME49 strain-spiked human body fluids

No. parasites	Ct value (Mean ± SD)					
	Blood		CSF		AF	
	B1	ITS-1	B1	ITS-1	B1	ITS-1
100000	16.41±0.19	21.21±0.16	16.27±0.05	18.44±0.13	18.59±0.03	19.93±0.05
10000	19.74±0.16	25.18±0.04	21.33±0.07	25.30±0.09	24.50±0.06	25.56±0.05
1000	23.47±0.05	29.64±0.09	26.46±0.08	30.38±0.05	27.89±0.08	30.24±0.12
100	28.33±0.05	33.79±0.04	30.20±0.13	32.33±0.06	30.63±0.12	32.63±0.11
10	30.28±0.07	36.28±0.16	32.10±0.13	35.31±0.16	33.40±0.06	37.69±0.04
1	31.33±0.11	38.54±0.09	34.20±0.04	38.33±0.10	35.26±0.13	40.32±0.10

Ct value data are given as Mean ± SD

Table 4. Summary of mean C_t values obtained with DNA extracted from organs of RH strain infected mice using B1 and ITS-1 primers and probes

	Brain		Heart		Kidney		Liver		Spleen	
	B1	ITS-1	B1	ITS-1	B1	ITS-1	B1	ITS-1	B1	ITS-1
Mice 1	29.39±0.09	35.58±0.18	24.48±0.18	27.32±0.11	23.51±0.12	25.62±0.05	16.73±0.14	19.22±0.08	18.26±0.12	20.07±0.06
Mice 2	29.62±0.04	36.26±0.06	24.81±0.15	27.46±0.07	23.52±0.11	26.02±0.19	16.46±0.09	19.27±0.04	18.60±0.09	20.34±0.05
Mice 3	30.27±0.19	35.38±0.16	24.91±0.09	27.36±0.13	23.40±0.05	26.69±0.04	16.31±0.08	19.35±0.13	18.38±0.19	20.24±0.12
Mice 4	29.42±0.03	35.61±0.15	24.51±0.15	27.34±0.07	23.66±0.12	25.74±0.16	16.16±0.09	19.12±0.06	18.29±0.08	20.08±0.04
Mice 5	29.15±0.10	35.59±0.04	24.51±0.10	27.19±0.12	23.68±0.04	24.51±0.10	16.41±0.17	19.33±0.13	18.42±0.11	20.11±0.10
Positive control (RH)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Internal control	17.69	18.29	17.64	18.45	17.65	17.90	17.21	18.15	17.18	19.02

*NA: No amplification

Table 5. Summary of mean C_t values obtained with DNA extracted from organs of ME49 strain infected mice using B1 and ITS-1 primers and probes

	Brain		Heart		Kidney		Liver		Spleen	
	B1	ITS-1	B1	ITS-1	B1	ITS-1	B1	ITS-1	B1	ITS-1
Mice 1	28.34±0.09	33.07±0.11	28.96±0.10	32.32±0.14	30.89±0.02	34.17±0.05	26.30±0.05	32.05±0.09	28.62±0.10	38.81±0.05
Mice 2	28.26±0.08	32.47±0.11	28.84±0.05	32.40±0.09	31.88±0.08	35.17±0.05	26.58±0.08	32.41±0.17	28.18±0.09	38.84±0.09
Mice 3	28.80±0.08	32.74±0.18	29.29±0.03	32.56±0.08	31.52±0.07	34.76±0.19	26.71±0.12	32.03±0.17	28.29±0.06	39.30±0.06
Mice 4	28.46±0.08	32.65±0.16	29.09±0.12	32.50±0.17	31.14±0.09	35.13±0.07	26.46±0.08	31.97±0.05	28.82±0.04	39.17±0.06
Mice 5	28.30±0.14	32.45±0.06	29.30±0.05	32.45±0.10	31.25±0.09	34.27±0.14	26.30±0.18	32.11±0.06	28.60±0.07	39.20±0.16
Positive control (ME49)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Internal control	17.98	18.29	17.63	18.16	17.35	18.16	17.53	18.75	17.82	18.83

*NA: No amplification

DNA extracted from tissues of mice infected with *T. gondii* showed amplifications, while DNA of tissues from control mice showed no amplification. The average Ct values obtained for each organ from five mice for RH strain were as follows: brain: 29.57 (B1 gene), 35.08 (ITS-1 gene), heart: 24.66 (B1 gene), 27.33 (ITS-1 gene), kidney: 23.55 (B1 gene), 25.72 (ITS-1 gene), liver: 16.41 (B1 gene), 19.26 (ITS-1 gene) and spleen: 18.39 (B1 gene), 20.17 (ITS-1 gene). Meanwhile the average Ct values obtained for ME49 strain were as follows; brain: 28.43 (B1 gene), 32.68 (ITS-1 gene), heart: 29.10 (B1 gene), 32.45 (ITS-1 gene), kidney: 31.34 (B1 gene), 34.70 (ITS-1 gene), liver: 26.47 (B1 gene), 32.11 (ITS-1 gene) and spleen: 28.50 (B1 gene), 39.06 (ITS-1 gene). Liver of mice infected with RH strain showed the highest parasite load, followed by spleen, kidney, heart and brain. Similarly, the liver of mice infected with ME49 strain also showed the highest parasite load, followed by brain, spleen, heart and kidney.

Sequence analysis of the PCR amplicons

The BLAST analysis of the sequences of each PCR amplicons shows that amplified products for B1 gene were 100% identical to the sequences of RH (AF179871.1, AY259044.1), ME49 (AY259045), MAS (AY143140.1), CTG (AY143139.1), COUG (AY143138.1) and CAST (AY143137.1). For ITS-1 region, there were 100% identical to the sequences of B7 (AY143136.1), CN (AJ628254.1), SH (AY628253.1), ZS (AJ628252.1), QH (AJ628251.1), P (X75453.1), RH (X75429.1) and Sailie (X75430.1). For all sequences, the results showed score bits of 124, 0% gaps and E-values of 4e-26. Thus the target regions in DNA of *T. gondii* were accurately amplified by the newly designed real-time PCR primers and probes (data not shown).

DISCUSSION

After the first report on *T. gondii* DNA detection by PCR was published, several real-time PCR assays for quantifying *T. gondii* DNA were reported (Burg *et al.*, 1989; Lin *et al.*, 2000; Buchbinder *et al.*, 2003; Peyron *et al.*, 2004; Contini *et al.*, 2005). However, only a few of these studies involved development of new primers, and most of them focused on the comparison of target DNA rather than assay development for clinical use.

The molecular diagnosis of toxoplasmosis has been mainly performed using highly conserved regions such as the B1 gene (35 copies), 529 bp repetitive element (200-300 copies), and ITS-1 region which is part of 18S rDNA gene (110 copies). In the present study primers and probes were designed targeting the B1 gene region and ITS-1 region. Based on our previous study on triplex conventional PCR (Anizah *et al.*, 2012), we observed no difference between the B1

gene and 529 bp repetitive element in terms of diagnostic specificity and sensitivity. However, there was greater band intensity on agarose gel with B1 gene as compared to 529 bp repetitive element. Meanwhile ITS-1 region was included in the study due to its genus specificity, therefore the assay would be able to detect most strains of *T. gondii*.

In recent times, the 529 bp highly repetitive region has been increasingly used instead of B1 gene to detect *T. gondii* DNA (Homan *et al.*, 2000; Edvinsson *et al.*, 2005). Delhaes *et al.* (2013) evaluated three real-time PCR assays using 73 samples from congenital toxoplasmosis cases, using B1 gene and 529 bp repeat element as targets; and they reported that 529 bp repeat element was more sensitive than B1 gene. In another study, Reischl *et al.* (2003) designed two assays to quantitatively detect B1 gene and 529 repeat regions, and evaluated them using 51 *T. gondii* DNA-positive amniotic fluid samples. They reported that the detection limits for B1 gene and 529 repeat regions were 200 fg and 20 fg respectively. However based on the reports of several studies, we selected B1 gene over 529 repeat region as one of the target genes for detection of *T. gondii* DNA. One such study was a comparison done between 529 repeat regions and B1 gene using real-time PCR on 63 blood samples from *T. gondii* DNA-positive HIV patients, The results showed that the assay based on 529 repeat regions did not detect *T. gondii* DNA in three of the patients samples, whereas the assay targeting the B1 gene was able to detect the parasite DNA in all samples (Wahab *et al.*, 2010). Similarly in another study, performance of three different target genes, 18S rDNA, 529 repeat region and B1 gene was evaluated using amniotic samples from *T. gondii* IgM and IgG-positive pregnant women (Okay *et al.*, 2009). The results showed that the detection using B1 gene was far more sensitive than the 529 repeat region and 18S rDNA was the least effective. More importantly, the amplification of B1 gene has consistently shown to be 100% specific as compared to 529 repeat element which sometimes showed reduced specificity. In one study, a total of 135 amniotic fluid samples were analyzed by PCR, targeting both 529 bp repeat element and B1 gene (Kasper *et al.*, 2009). The sensitivity and specificity were 88% and 100% for B1 gene and 100% and 98.2% for 529 bp. In another study, 529 repeat region and B1 gene were compared using *TaqMan* based real-time PCR with blood and CSF samples from AIDS patients in Brazil (Mesquita *et al.*, 2010). The results showed that the 529 repeat region was more sensitive but less specific than B1 gene. Sensitivity and specificity are equally important for assay development; however, the latter is critical for diagnosis of congenital infection to avoid unnecessary treatment of pregnant women. Thus, although the high copy number of the 529 DNA target was desirable for higher sensitivity and that some reports showed B1 gene had lower sensitivity compared to 529 repeat element, the consistent high specificity of the former was pivotal in our choice of target DNA in the present study.

The relationship between the virulence of the parasite as measured by parasite load in amniotic fluid using real-time SYBR green PCR was reported by Peyron *et al.* (2004). In their study, the standard curve was constructed using plasmid containing the target sequence which was serially diluted in amniotic fluid from a seronegative woman. However, in the present study, the curve was constructed using whole parasites suspended in pooled amniotic fluid from seronegative women. Meanwhile Contini *et al.* (2006) developed a real-time fluorescence PCR, targeting multiple tachyzoite and bradyzoite stage-specific genes, and evaluated the assay with acute and follow-up blood specimens from immunocompetent patients with lymphadenitis. In the study, a standard curve using SAG-1 (P30) gene was constructed by real-time SYBR green using 10 fold dilutions from 1 to 10^7 parasites per ml. However P30 gene is not commonly used in standard curve construction because it is a single copy sequence and thus less sensitive to DNA amplification as compared to repetitive sequences like B1 and ribosomal DNA genes (Yai *et al.*, 2003; Jones *et al.*, 2000).

Although many real-time PCR studies have been reported, the sensitivity of the assays can still be improved. The present

assay could quantify as little as one parasite with reproducible results. Moreover, unlike most other reports, we determined the linear range of the assay in the presence of human DNA, which is more representative of a real biological sample. Besides, our multiplex *TaqMan* real-time PCR was tested with more than one strain of *T. gondii*.

A report by Kompalic-Cristo *et al.* (2007) also described an assay which used *TaqMan* probe to target B1 gene for the detection and quantification of *T. gondii* DNA in human peripheral blood. The real-time PCR was able to quantify the parasite load from 9.92×10^{-3} to 8.73×10^{-1} tachyzoites per ml of blood. Similar to this study, the assay was reported to detect as little as one parasite. However, in the present study, two target regions namely B1 gene and ITS-1 region were simultaneously amplified in a multiplex assay. Our assay was also tested using two other body fluids (amniotic fluid and CSF) besides blood sample. Most published reports targeted one region, i.e. either B1 gene or 529 repeat regions, for detection of several common *T. gondii* strains such as RH, ME49 and VEG. However, in the present study, ITS-1 region primers were included in order to target other common strains of *T. gondii*.

It was observed that the detection of RH strain was slightly earlier as compared to that of ME49 strain in all spiked samples even though the same primers and probes were used. This may imply that the primers used were more efficient at amplifying the RH strain, and may be related to the reported 1% difference in DNA sequences between the two strains (Dubey & Jones, 2008).

It is also important to emphasize that the use of heterologous internal control (*HemM* gene) in this study contributed to equal amplification of the targets. According to Pina-Vazquez *et al.* (2008), most of the previous studies on *T. gondii* quantification used homologous internal control generated by deletion of a fragment from the target sequence. The drawback of this method is the formation of heteroduplex molecules which accumulated in the plateau phase of the reaction and may interfere with the analysis and quantification of the products. Heterologous internal control avoids this problem; moreover since it was designed to have the same length and G/C content as the target, thus efficiencies of amplifications of both target and controls were similar. *V. cholera HemM* gene has also been successfully used in previous conventional PCR assays (Lalitha *et al.*, 2008; Anizah *et al.*, 2012).

The real-time PCR in this study showed the presence of tachyzoites in mice tissues infected with both strains of *T. gondii*. However there was uneven distribution of the parasite in different types of organs, as depicted by the variations in Ct values. Among the organs tested, liver of both strains of infected mice demonstrated the lowest Ct value, thus the highest infection intensity. *T. gondii* RH-infected mice generally showed higher parasite loads in all the organs as compared to ME49 strain-infected mice; the exception was with brain sample whereby *T. gondii* ME49-infected mice showed slightly higher infection intensity. This is consistent with the fact that *T. gondii* has a predilection for brain and the mouse-avirulent strain (ME 49) had ample time to develop many more brain cysts than mice infected with the mouse-virulent RH strain which died in a short period of time.

In conclusion, the newly developed real-time multiplex assay in this study showed high sensitivity and specificity for detection of *T. gondii* DNA in human and animal samples. The standard curves constructed can be used to compare results obtained at different times or by different laboratories.

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