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Optimization of Sybr Green Quantitative Real Time Polymerase Chain Reaction (qPCR) using Excreted-Secreted Antigens (ESAs) Genetik Marker for Detection Toxoplasma gondii

Optimalisasi Sybr Green Quantitative Real Time Polymerase Chain Reaction (qPCR) menggunakan Penanda Genetik Excreted-Secreted Antigens (*ESAs*) untuk Deteksi *Toxoplasma gondii*

Fitrine Ekawasti[1,2*], Agus Winarsongko[2], Farlin Nepho[2], Eko Setyo Purwanto[2], Didik Tulus Subekti[2], Harimurti Nuradji[2], NLP Indi Dharmayanti[2], Riza Zainuddin Ahmad[2], Siti Sa'diah[1], Umi Cahyaningsih[1*], Raden Wisnu Nurcahyo[3]

[1]The School of Veterinary Medicine and Biomedicine, IPB University, Bogor, Indonesia
[2]The Research Organization for Health, National Research and Innovation Agency, Bogor, Indonesia
[3]Department of Parasitology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia, 55281
*Email: fitrineekawasti@gmail.com

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Abstrak

Toxoplasma gondii merupakan parasit obligat intraseluler yang menyebabkan toksoplasmosis pada hampir semua hewan berdarah panas dan manusia di seluruh dunia. Toksoplasmosis adalah penyakit zoonosis yang menjadi perhatian kesehatan masyarakat yang serius. Invasi sel inang oleh takizoit *T. gondii* memiliki proses yang melibatkan sekresi berurutan dari *Excreted-Secreted Antigens* (*ESA*s). *T. gondi ESA* dapat menjadi kandidat yang berharga untuk diagnosis toksoplasmosis. Teknik deteksi *T. gondii* yang lebih akurat akhir-akhir ini dikembangkan metode bioteknologi yang saat ini digunakan, yaitu *Quantitative Real Time Polymerase Chain Reaction* (qPCR), dan konvensional. qPCR lebih banyak digunakan karena lebih sensitif dan spesifik. Tujuan dari penelitian ini adalah untuk mengoptimalkan Sybr Green qPCR pada gen wilayah berbeda berdasarkan *ESAs*, antigen permukaan tachyzoite dan antigen bradyzoite, kemudian mengadaptasi program PCR konvensional ke PCR real-time untuk mendeteksi *Toxoplasma gondii*. Optimasi diperlukan untuk mendapatkan kondisi PCR yang optimal sehingga mendapatkan hasil yang terbaik. Strain *T. gondii* RH berasal dari nitrogen cair dan DNA yang diekstraksi dengan DNAzol. Penanda genetik yang digunakan adalah *GRA1#1, GRA1#2, GRA7#1, GRA7#2, ROP1, MIC3, SAG1* dan *BAG1*. Hasil optimasi beberapa gen primer dapat beradaptasi dan digunakan secara optimal pada qPCR dengan menggunakan program siklus secara bersamaan dalam satu kali proses. Secara keseluruhan, qPCR untuk mendeteksi DNA *T. gondii* menunjukkan kesesuaian yang sangat baik dengan PCR konvensional. qPCR dengan analisis kurva leleh cepat dan sederhana sehingga memudahkan analisis *throughput* tinggi untuk mendeteksi *T. gondii.* Kondisi optimal yang diperoleh dari hasil optimasi dapat memudahkan penelitian lebih lanjut untuk mendeteksi *T. gondii*.

Kata kunci: antigen ekskretoris-sekretorik; Deteksi molekuler; toksoplasmosis

Abstract

Toxoplasma gondii is an obligate intracellular parasite, causing toxoplasmosis in almost all warm-blooded animals and humans worldwide. Toxoplasmosis is a zoonotic disease of serious public health concern. Host cell invasion by *T. gondii* tachyzoites has process involving the sequential secretion of *Excreted-Secreted Antigens* (*ESAs*). *T. gondi ESAs* could be a valuable candidate for the diagnosis of toxoplasmosis. Techniques to more accurately detection of *T. gondii* recently developed biotechnological methods that are currently being used, conventional and Quantitative Real Time Polymerase Chain Reaction (qPCR). qPCR is more widely used because it is more sensitive and specific. The aims of this study were to optimize the *Sybr Green qPCR*

in different region gene based on *ESAs*, tachyzoite surface antigen and bradyzoite antigen, then adapt the conventional PCR program to real-time PCR for detection *Toxoplasma gondii.* Optimization is necessary to get optimal condition of PCR to get the best results. *T. gondii* RH strains derived from liquid nitrogen and DNA extracted by DNAzol. The genetic marker used *GRA1#1, GRA1#2, GRA7#1, GRA7#2, ROP1, MIC3, SAG1* and *BAG1*. The results of the optimization of multiple primer genes can adapt and be used optimal in qPCR by using the same cycle program simultaneously in one run. Overall, qPCR for the detection of *T. gondii* DNA demonstrated excellent agreement with conventional PCR. qPCR with melting curve analysis is rapid and simple that facilitates high throughput analysis to detect *T. gondii*. The optimal conditions obtained from the optimization results can facilitate further research to detect *T. gondii*.

Keywords: Molecular detection; e*xcretory-secretory antigen*; toxoplasmosis

Introduction

Toxoplasma gondii is an obligate intracellular parasite, causing toxoplasmosis in almost all warm-blooded animals and humans worldwide. Toxoplasmosis is a zoonotic disease of serious public health concern (Opsteegh *et al.,* 2015; El Aal *et al.,* 2018). During *T. gondii* infection, the parasite releases a number of molecules called *T. gondii excretory secretory antigen* (*ESAs*) into the surrounding environment, which allows the organism to attack and survive inside the host cell through immunomodulation (Moncada and Montoya, 2012). *T. gondii* has set up secretory organelles involved in invasion and replication strategies, which include apical micronema, rhoptries, and dense granules (Zhu *et al.,* 2019; Franco *et al.,* 2018). Host cell invasion by *T. gondii* tachyzoites has been described as a process involving the sequential secretion of micronema, rhoptry and dense granule protein. Successful intracellular development is based on the process of active invasion and the formation of a new sub-cellular compartment named the parasitophorous vacuole (PV) (Beraki *et al.,* 2019; Braun *et al.,* 2013). PV is a specialized organelle in the host cell where the parasite replicates. The structure and stability of PV is maintained through the release of parasitic proteins from secretory vesicles called solid granules (Rommereim *et al.,* 2019; Gold *et al.*, 2015). *T. gondi ESAs* may be one of the first targets of the host immune system, and could be a valuable candidate for the diagnosis of toxoplasmosis (Wang *et al.*, 2017).

Several PCR-based methods have gradually improved the diagnosis of toxoplasmosis, especially the newly developed real-time PCR platforms (Brenier-Pinchart *et al.,* 2007). Many factors might affect the real-time PCR results: in particular, the choice of the target gene and designed primers (Yu *et al.,* 2013; Calderaro *et al.,* 2006). In fact, Toxoplasma parasite can circulate at low concentrations, or intermittently, thus might be detected in earlier phases of infection as circulating genomic materials which need a sensitive and specific technique to confirm infection in such critical time. PCR is the major breakthrough for the diagnosis of this infection due to *T. gondii.* Moreover, real-time PCR has recently emerged as an improvement in the reliability of PCR assays (Romand *et al.,* 2004). Molecular methods are reported to be effective enough to detect low concentrations of circulating genomic materials. Real-time visualitation in addition to the precise digital quantitation of the amplified DNA products without risk of contamination, greatly direct scientists to replace the traditional PCR multi-instruments by a single quantitative PCR machine. Real-time PCR is less time-consuming, less labour-intensive and also reduces the chance of contamination as there is no post-amplification procedure (Ramamurthy *et al.,* 2011; Brenier-Pinchart *et al.,* 2007).

Since quantitative real-time PCR (qPCR) does not require the tubes to be opened after amplification, the risk of contaminating environment and the risk of false positive results are decreased. Furthermore, real-time PCR gives quantitative data on the parasitic load (Ramamurthy *et al.,* 2011; Switaj *et al.,* 2005). Currently, the real time PCR technique with high resolution melting mechanism (qPCR-

HRM) is a simple, cost-effective, high-precision and sensitive assay, known for identifying and diagnosing of various pathogenic agents (Ghafari *et al.,* 2020). Few studies have been published about the detection of *T. gondii* by qPCR-HRM, and little research has been conducted on the comparing different gene regions to identify this infection via the predictive approach which determines DNA behavior and genotyping (Fehlberg *et al.,* 2013; Costa *et al.,* 2011). In qPCR, it is essential to select appropriate gene regions and design specific primers for detecting and diagnosing pathogens, and developing and improving this technique. Many factors could affect qPCR amplification efficiency, e.g., primer sequence, primer annealing temperature, primer concentration, and cDNA/DNA (template) concentration (Zhao *et al.,* 2021). However, optimization of these factors has often been overlooked in publications (Dai *et al.,* 2018; Nagy *et al.,* 2017). Here, we proposed an optimized method by combining the efficiency calibrated and curve methods to developed real-time PCR platforms to detect *T. gondii*. The aims of this study were to optimize the Sybr Green RT-PCR in different region gene based on *Excreted-Secreted Antigens* (*ESAs*), tachyzoite surface antigen (*SAG*) and bradyzoite antigen (*BAG*) then adapt the conventional PCR program to real-time PCR for detection *Toxoplasma gondii.*

Materials and Methods

Toxoplasma gondii isolate

The isolate of *T. gondii* RH strain was provided by Didik Tulus Subekti (Indonesia Research Center for Veterinary Sciences, Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, Indonesia) from Dominique Soldati-Favre (University of Geneva, Switzerland). Tachyzoite of *T. gondii* isolates stored in liquid nitrogen were utilized as samples.

Extraction of genomic DNA

The extraction in the Conventional and Real Time PCR methods is the same which is the process of separating the nucleic acid (DNA/RNA) of the pathogen from the host cell. In this study, DNA of *T. gondii* was extracted from an isolate stored in liquid nitrogen. The DNA template was extracted using DNAzol (Molecular Research Center, OH, USA) and extracts were stored at −20°C before PCR testing. Purification and concentration of the DNA extracts for DNA quantification were performed using Thermo Scientific™ NanoDrop™.

Genetic markers

The 8 genetic markers used in this study were *GRA1* (#1, #2), *GRA7* (#1, #2), *BAG1, SAG1, MIC3,* and *ROP1*. Primers listed in Table 1 were synthesized by Promega (Macrogen).

Table 1. The sets of *Toxoplasma gondii* gene primers.

No.	Primer	Sized (bp)	Nucleotides (5'-3' orientation)	References
1	GRA1#1	802	F CGGTTTGCTTGTGTTGTTTG R CATGGGGTACGATCACAACA	Ekawasti et al., 2021; Subekti et al., 2008
2	GRA1#2	226	F CGGCAATGTTAACGTGGAGG R CTGCACATCGTCGATCACCT	Ekawasti et al., 2023
3	GRA7#1	616	F GCGGATCCGCCACCGCGTCAGATGAC R CGGGATCCCTACTGGCGGGCATCCTC	Hou et al., 2019
4	GRA7#2	217	F TTGCCGCTGATAGACTTGTG R GATTCAGGCACCTCTTGCTC	Ekawasti et al., 2023
5	BAG1	460	F AGGAGAGAAGACCTCGAAAGAAG R TGAACGCTAGGTTTCTGGATACG	Apsari et al., 2012
6	SAG1	340	F CACACGGTTGTATGTCGGTTTCGCT R TCAAGGAGCTCAATG TTACAGCCT	Apsari et al., 2012
7.	MIC3	300	F GTGTGATATCCTTGTCCAACACTG GGTA R CACGAAGCTTTGCGAATGGGCG	Indrasanti et al., 2007
8	ROP1	1220	F CGTGACATATACTGCACTGAC R CATCGTCAAACTCGATCAC	Ekawasti et al., 2021; Kusumaningsih, 2018

Conventional Polymerase chain reaction (PCR) Assay

Conventional PCR assay was performed using MyTaq kit (Bioline, Meridian Bioscience) with 25 µL total volume each reaction, as follows: PCR reactions consisted of 12.5 µL MyTaq mix, 0.5 µL forward primer 20 µM, 0.5 µL reverse primer 20 µM, and 1 µL DNA as template. Temperature conditions consisted an initial denaturation time of 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, and a final extension period of 72 °C for 7 min. PCR was carried out in a Thermo Scientific™ Arktik™ Thermal Cycler (Thermo Fisher Scientific, Finland). PCR products were detected and visualised by gel electrophoresis using 1.5% agarose and SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Finland), run at 100 V for 40 minutes. The bands were visualized using a 312nm wavelength transilluminator.

DNA sequencing and analysis

PCR products from amplification of the gene target regions were sequenced and identified. PCR products were sequenced by Macrogen Sequencing. DNA sequences were analyzed using CLC Sequence viewer version 8.0 software (Qiagen, Denmark) (https://clcsequence-viewer.software.informer.com/8.0/).

Quantitative Real Time Polymerase Chain Reaction (qPCR) Assay

Real-time PCR SYBR green assay targeting the *T. gondii ESAs* (*GRA1, GRA7, MIC3* and *ROP1* gene) and tachyzoite surface antigen (*SAG1*), Bradyzoite antigen (*BAG1*). The programme concluded with melting curve analysis. No-template controls (NTC) and negative controls (NC) were included in this assay. A standard curve for calculating amplification efficiency and error was constructed by diluting *T. gondii* DNA serially and analysing some concentrations diluted. The sensitivity and accuracy of the assays with low sample concentrations of *T. gondii* DNA were also analysed. Melting curve analysis was used to verify that the correct product had been amplified.

Real-time PCR assay consisted of 10 µl GoTaq® qPCR Master Mix (2X) (Promega), 200nM–1µM of each primer, 4 µl of template and DNA/nuclease-free water up to a volume of 20 µl. All reactions were run in triplicates. DNA amplification was achieved by an initial denaturation time of 3 min to 95 °C, 40 cycles of 95 °C for 3 s and 56-60 °C for 30 s. A dissociation curve was performed after amplification by gradual rise in temperature from 65 to 95 °C with fluorescence signal measurement every 0.5 °C. All reactions were performed in a Bio-Rad CFX96 Connect Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany).

Calculation of the cycle threshold value (Ct) value or Cq indicates the number of cycles required for the fluorescent signal to pass the threshold. The ct value is inversely proportional to the number of target DNA in the sample (low Cq value indicates a large number of target DNA). Calculation of the Cq was based on automatic adaptive baseline settings, and a sample with Cq of <35 was considered positive. Samples with a Cq of ≥35 were analyzed again for confirmation, with the sample declared positive if the threshold was again reached in the second run (Schares *et al.,* 2021).

Results and Discussion

Toxoplasma gondii isolate

The final and definitive diagnosis of toxoplasmosis and precise differentiation of acute and chronic infections is critical nowadays (Hosseini-Safa *et al.,* 2020). The composition of the *T. gondii* excreted-secreted antigens (*ESAs*) is complex that *MICs, ROPs* and *GRAs*, have been identified (Wei *et al.,* 2018). Molecular tests can play a significant role in diagnosing of acute infections by detecting and tracking presence of parasite in the bloodstream or other clinical samples. Direct detection of DNA of *T. gondii* in the blood and other clinical samples can confirm the presence of the parasite in the patient's body resulted in development of acute primary infection, reactivation, or chronic infection especially in immunocompromised patients (Hosseini-Safa *et al.,* 2020). Furthermore, various molecular techniques

including conventional and qPCR have also been employed for diagnosis of toxoplasmosis.

Conventional PCR

The quantity of total DNA isolated can be tested by measuring the concentration using Nanodrop™ Spectrophotometers. The results of the measurement of the DNA concentration obtained were 2000 ng/ul with a ratio of A260/280 1,97. An A260/280 ratio of 1.8 to 2.1 at a pH of 7.5 indicates a very pure DNA content, with a ratio of more than 1.8 being considered a good indicator of DNA quality (Von Ahlfen and Schlumpberger, 2010). In this experiment, it was found that the A260/280 ratio was greater than 1.8, indicating that the RNA sample had a relatively high purity.

The DNA of *T. gondii* was extracted then amplified by PCR using genetic markers *GRA1* (#1, #2), *GRA7* (#1, #2), *BAG1, SAG1, MIC3*, and *ROP1.* The annealing temperature optimization was carried out using a temperature gradient on the Thermo Scientific™ Arctic™ Thermal Cycler (Thermo Fisher Scientific, Finland). The results of the optimization of the annealing temperature of the primers were 58°C, then could run simultaneously in one PCR reaction. DNA *T. gondii* amplified by several genetic markers. The specific primers for gene regions were designed and synthesized to amplified (Figure 1).

Conventional PCR product clarification by Macrogen sequencing. Primer specificity was clarified by sequencing, then nucleotide BLAST was performed to match the PCR product sequence with the DNA sequence database in Genbank. This clarification was carried out to ensure that the primer used for the qPCR reaction was properly amplified on the target DNA of *T. gondii*. The results of nucleotide BLAST primary PCR product sequences *GRA1* (#1, #2), *GRA7* (#1, #2), *BAG1, SAG1, MIC3*, and *ROP1* are briefly presented in Table 2.

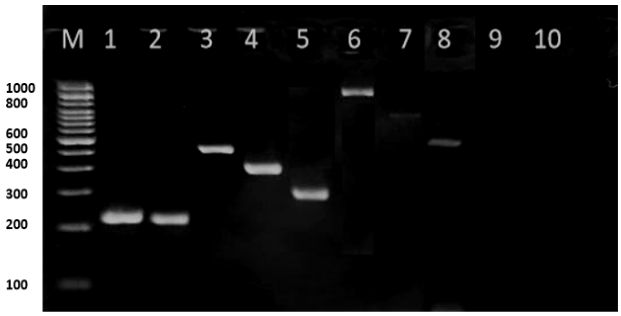

Figure 1. *Toxoplasma gondii* nucleic acid amplified by genetic markers. The PCR product was resolved on 1.5% agarose stained with SYBR™ safe staining. Lane M: Geneaid 100 bp DNA ladder, 1: GRA1#2 (226 bp), 2: GRA7#2 (217 bp), 3: BAG1 (460 bp), 4: SAG1 (340 bp), 5: MIC3 (300 bp), 6:ROP1 (1220 bp), 7: GRA1 #1 (802 bp), 8: GRA7#1 (616 bp), 9: negative control (NC), 10: no template control (NTC)

respective targets. Amplifications with the primers targeting the gene yielded products of the expected size. A successful conventional PCR assay requires efficient and specific amplification of the product. Both the primers and the target sequence can affect amplification efficiency and specificity and thus the accuracy of qPCR assays. Therefore, care must be taken when choosing a target sequence and designing primers. The use of PCR primers specifically designed and validated for qPCR assays with your target of interest is highly recommended. ne of the most important aspects of conventional PCR and qPCR testing is the design and use of sequence-specific primers (Ma *et al*., 2020).

Table 2. Primer specifity followed by BLAST nucleotide PCR product sequences against *T. gondii*

No.	Sample (isolate)	Gene target	Genetic marker	Percentage of similarity to *T. gondii* RH strain	Accession ID
1	gondii	GRA1	GRA1#1	99%	EU983103.1
2	RH strain	GRA1	GRA1#2	100%	EU983103.1
3		GRA7	GRA7#1	99%	MK250981
4		GRA7	GRA7#2	97%	MK250981
5		BAG1	BAG1	99%	Z48750.1
6		SAG1	SAG1	100%	HM776940.1
7		MIC3	MIC3	100%	KF153103.1
8		ROP1	ROP1	98%	AF350261.1

Quantitative Real Time Polymerase Chain Reaction (qPCR) Assay

Optimization of primer annealing temperature

Optimization of qPCR conditions for the 8 primers was carried out by gradient qPCR at five temperatures based on the primary melting temperature™ range and 10ng/uL DNA concentration as a procedure in master mix. Optimization was carried out using PCR with the annealing temperature setting used calculated based on (TM–5) C to (TM + 5) C (Nuryady *et al.,* 2020). In this study, the annealing temperature optimization was carried out by 5 gradients which ranged from temperatures of 56 °C to 62°C. RT-PCR cycling conditions for the qPCR CFX96 real-time system (Bio-Rad, USA) were as follows: Predenaturation/Hot Start: 95 °C for 3 minutes; PCR reaction in 40 cycles, consisting of: Denaturation: 95°C for 3 seconds; annealing: 56°C; 56,7°C; 58°C; 60°C; 62°C for 30 seconds; elongation: 72 for 10 seconds; the next reaction is melting curve analysis which is carried out at conditions: 95°C for 30s for denaturation and followed by 65°C for 30 seconds. Five gradients of annealing temperature were conducted to find the best result. The optimum product *GRA1* (#1, #2), *GRA7* (#1 and #2), *BAG1, SAG1* can be run predominantly at a temperature of 58°C, except for primers *ROP1* and *MIC3* which can be optimum at a temperature of 56°C. An annealing temperature gradient from 55 to 72°C was performed (Table 3).

The annealing temperature of a qPCR assay is one of the most critical parameters for reaction specificity. Determining the temperature of annealing is based on the lowest cycle threshold (Cq value) and a high fluorescent signal. The temperature of annealing in a real-time PCR reaction will affect the efficiency of the primer attachment. Setting the annealing temperature too low may lead to amplification of nonspecific PCR products. Conversely, setting the annealing temperature too high may reduce the yield of a desired PCR product (Puspita *et al.,* 2020; Nolan *et al.,* 2013).

Calculation of the melting temperature (Tm) of a primer is needed to determine the annealing temperature empirically by repeating the reaction at different temperatures. The optimal annealing temperature for an assay can be easily determined using a qPCR instrument that has a thermal gradient feature. The gradient feature can test a range of temperatures simultaneously, so the annealing temperature can be optimized in one experiment. In finding the optimal annealing temperature for your qPCR assay, test the temperature range above and below the calculated primer Tm (Bustin and Huggett, 2017).

Optimization of primer concentration

Optimization of the primer was carried out to find the optimum concentration of primer to be used in the qPCR reaction. The forward and reverse primer concentrations used for the

Table 3. The optimised annealing temperature of qPCR for detection *T. gondii* using several genetic markers

Primer	TM (°C)	Temperatur of Annealing (gradient)°C									
		62		60		**58**		56,7		**56**	
		Cq value	melting curve (°C)	Cq value	melting curve (°C)	Cq value	melting curve (°C)	Cq value	melting curve (°C)	Cq value	melting curve (°C)
GRA1#1	58	16,4	-	16,2	-	**16,2**	85,0	16,6	85,0	17,8	85,0
GRA1#2	60	16,6	84,5	**17,4**	**84,0**	**12,87**	**84,0**	18,8	84,5	16	84,0
GRA7#1	68	-	-	-	-	**24**	**87,0**	34,4	87,0	**26,9**	**87,0**
GRA7#2	60	18,3	-	**17,18**	**86,5**	**17,07**	**86,5**	19,6	86,5	**17,8**	86,5
BAG1	63	22,17	-	14,27	-	**14,05**	**75,0**	**14,07**	**75,0**	**13,9**	**75,0**
SAG1	63	-	-	**16,9**	**86,5**	**16,6**	**86,5**	22,0	86,5	**18,2**	**86,5**
MIC3	60	-	-	-	-	35,2	-	30,3	78,5	**27,5**	**78,5**
ROP1	57	-	-	-	-	23,5	-	31	72,5	**25,6**	**72,5**

Bold: annealing temperatur optimal for amplification, optimum temperature of dominant annealing that can be run imultaneously several primers at once

primer optimization test were 200nM–1µM (250nM, 500nM, and 1µM) with a total volume of 20 µl. We found that primer concentrations of 1µM per primer per reaction had the lowest Cq values. The primer concentration with the lowest Cq value for each primer pair was chosen as the optimal primer concentration for that primer pair. The Cq value obtained is between 12-23. The recommended Cq value for the study target gene is between 20-30 (Kuang *et al.,* 2018), while the Cq value for the primer is within the recommended range.

The difference in concentration is caused by differences in PCR machines, PCR methods, and enzymes used. In the previous study, the researcher used the primer concentration was 400nM (Puspita *et al.,* 2020). In the NC sample, the resulting Cq value of 40.02. This indicates that starting in the 40th cycle, dimer primer formation begins to occur so that the qPCR reaction should be carried out in cycles of less than 40 times (Data not shown). In the PCR reaction primer is important to initiate the reaction and determine the amount of amplified DNA. Primers play a role in the process of initiating DNA polymerization reactions and limiting the area to be amplified in the PCR reaction. Higher primer concentrations can cause primers to be non-specific (Puspita *et al.,* 2020).

Identification of the optimal DNA concentration

Identification of the optimal DNA concentration range and its associated best primer pair using the optimal annealing temperature and the optimal primer concentration. The DNA concentration obtained were 2000 ng/ul with a ratio of A260/280 1.97 high purity. We used DNA serial dilutions from 2000 ng/ul, then diluted to 200 ng/ul; 20 ng/ul; 10 ng/ul; 5 ng/ul; 2.5 ng/ul; 1 ng/ul as the templates to run qPCR. We found that different primer pairs had same optimal DNA concentration ranges for each gene. The optimal DNA concentration range means that the qPCR amplification is at the exponential stage under the conditions of the optimal primer annealing temperature, primer concentration, and DNA concentration range (Zhao *et al.,* 2021).

Based on the optimization results, the best Cq value were obtained at a concentration of 200 ng/ul; Cq 18,9 and decreased to 1 ng/ul; Cq 28,7. All series of DNA concentration of *T. gondii* was detected well because it was still below the range of Cq < 30 (Kuang *et al.,* 2018). Whereas at high concentration of 2000 ng/ul qPCR showed N/A in all primers. qPCR-based diagnostic assays may have low sensitivity or even false-negative results when PCR inhibitors are present in sample (Cai *et al.,* 2018). Several inhibitions of qPCR in a concentration-dependent manner, including effects on DNA melting temperature, and preferential binding to certain DNA sequences and the DNA concentration (Gudnason *et al.,* 2007). Some PCR inhibitors still exist even after DNA extraction (Schrader *et al.,* 2012). It can also result from the binding or degradation of low amounts of genomic DNA by inhibitory substances in high concentrations in the sample (Cai *et al.,* 2018). The highest concentration of DNA was inhibited therefore excluded from the standard curve (Gallup and Ackermann, 2006). it is very necessary to optimize the concentration of DNA in making standard curves in qPCR.

Table 4. Quantification of ampification of optimization DNA concentration by seven dilutions

No.	Sample (isolate)	DNA concentration (ng/ul)	Cq	Melting curve (°C)
1	*T.gondii* RH strain	2000	N/A	84,0
2		200	18,9	84,5
3		20	22	84,5
4		10	24,5	84,5
5		5	25,4	84,5
6		2,5	28	84,5
7		1	28,7	84,5
8		NC	40,02	-
9		NTC	N/A	-

N/A = No Amplification, optimum DNA concentration (200ng/ul) for RT PCR to detection *T. gondii*

Significant advantages of qPCR include its ability to measure DNA concentrations over a large range, its sensitivity, its ability to process multiple samples simultaneously, and its ability to provide immediate information. A graphic quantification of ampification showing the best result with optimal DNA concentration is shown in Table 4 and Figure 2.

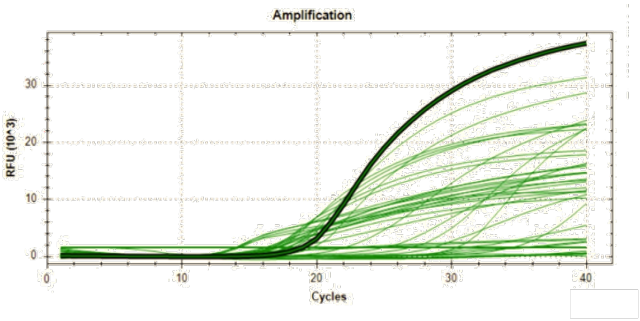

Figure 2. Optimal DNA concentration of *T. gondii* (200 ng/ul) at Cq 18.9 on RT PCR

Melting curve analysis

Melting curve on the PCR product showed a single peak which indicated that the reaction only produced one amplification product. The graph of melting curve analysis is presented in Table 3 and Figure 3.

The resulting curve shows the presence of one peak in each primer. This indicates that the primer reaction produces specific amplicons. The optimum qPCR reactions cycling conditions on Bio-Rad CFX96 real-time system were as follows: Predenaturation/Hot Start: 95 °C for 3 minutes; PCR reaction in 40 cycles, consisting of: Denaturation: 95°C for 3 seconds; annealing: 58°C (*GRA1* (#1, #2), *GRA7* (#1 and #2), *BAG1, SAG1*), and 56°C (*ROP1* and *MIC3)* for 30 seconds; elongation: 72 for 25 seconds; the next reaction is melting curve analysis which is carried out at conditions: 95°C for 5s for denaturation and followed by 65°C for 30 seconds for primary melting reaction. Fluorescence data collection was carried out at the elongation stage because SYBRgreen reacted at this stage. Melting curve analysis was done to confirm that the correct products had been amplified in positive samples and also to evaluate the specific primers of gene target. Selecting a suitable gene region and designing specific primers are critical for development and optimization of qPCR and other DNA-based methods (Azimpour-Ardakan *et al.,* 2021; Marino *et al.,* 2017; Chatzidimopoulos *et al.,* 2016).

qPCR with melting curve analysis is similar result to the studies used taqman probe qPCR for detecting the DNA of *T. gondii* in samples. Melting curve analysis developed to be high resolution Melting (HRM) technique in terms of diagnostic power, it is equivalent to taqman probe methods. qPCR with melting curve analysis is suitable, helpful, and in parallel with serological methods for early diagnosis of acute as well as active (Hosseini-Safa *et al.,* 2020; Lieveld *et al.,* 2017; Chatzidimopoulos *et al*., 2014). qPCR with melting curve analysis is a time-efficient, rapid, simple, cost-effective methodology that facilitates high throughput analysis (Słomka *et al.,* 2017; Chatzidimopoulos *et al.,* 2014). So far, few studies have been published for detecting and genotyping *T. gondii* using resolution melting curve analysis (Papini *et al*., 2017; Ivovic *et al.,* 2012).

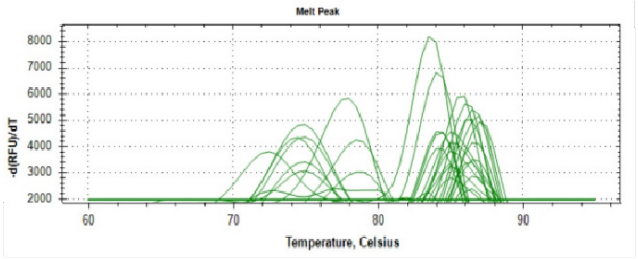

Figure 3. Melting curve analysis of the primer optimization reaction

The results of DNA amplification with conventional PCR, the presence of DNA was observed at the end of the reaction using agarose gel after the electrophoresis process was carried out. While the analysis using Real Time PCR allows for observations during the reaction, the presence of amplified DNA can be observed on the graph that appears as a result of fluorescence accumulation. In Real Time PCR, observation of the results no longer requires the electrophoresis step, so that agarose gel is no longer needed (Fatimi 2010). Real time PCR is also known as quantitative PCR (qPCR). The relatively small number of PCR products (DNA, cDNA or RNA) can be calculated quantitatively (Kralik and Ricchi, 2017). qPCR with melting curve analysis base identification is more sensitive and cheaper than conventional

PCR reaction followed by sanger sequencing for DNA sequencing (Azimpour-Ardakan *et al.,* 2021; Samarut *et al.,* 2016).

Conclusion

In this study, qPCR for the detection of *T. gondii* DNA, particularly *ESAs* gene (*GRA1#2, GRA7#2, ROP1,* and *MIC3*) demonstrated excellent agreement adapting from conventional PCR. qPCR also showed the opportunity to identify of different gene targets DNA. The optimal condition of the qPCR test to detect *T. gondii* is annealing temperature almost 58 °C, primer concentration 250 nM. The DNA template 1 ng/µl. Based on, melting curve analysis, the qPCR assay does not cross-react with other microorganisms. qPCR with melting curve analysis is a time-efficient, rapid, simple, cost-effective methodology that facilitates high throughput analysis to detect *T. gondii.* The optimal conditions obtained from the optimization results can facilitate further research to detect *T. gondii.* Researchers can further develop quantitative real time polymerase chain reaction - high resolution melting (qPCR-HRM) on other *T. gondii* genes to be more specific in genotyping.

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