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EVALUATION OF B1 GENE TO DETECT *Toxoplasma gondii*: COMPARISON OF THREE SETS of NESTED PCR PRIMER

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ABSTRACT

This study aimed to evaluate three sets of B1 gene DNA primer for the diagnosis of *Toxoplasma gondii*. The DNA of *Toxoplasma gondii* that stored on liquid nitrogen was isolated using DNAzol™ reagent. The first step of Polymerase Chain Reaction (PCRs) was performed using external and internal primer sets, respectively, and then nPCR. PCR products sequencing was performed by Apical Science. All sequences were analysed using CLC Sequence Viewer Version 8.0 software and compared to sequence database that deposited in ToxoDB (*Toxoplasma gondii* genome database) using BLAST (https://toxodb.org/toxo/app). Each B1 gene primer was evaluated by performing single PCR (forward and reverse) and nested PCR reactions. Three sets of B1 gene primer have different amplification precision. According to the results of amplicon sequencing, the primer set #2 has the best amplification precision of B1 gene.

Key words: B1 gene, nested PCR, sequence, *Toxoplasma gondii*, zoonosis

ABSTRAK

Penelitian ini bertujuan mengevaluasi tiga set DNA primer gen B1 untuk mendiagnosis Toxoplasma gondii. DNA Toxoplasma gondii yang tersimpan dalam nitrogen cair diisolasi menggunakan reagen DNAzol™. Langkah pertama Polymerase Chain Reaction (PCR) dilakukan dengan menggunakan set primer eksternal, internal, dan kemudian nPCR. Sekuensing produk PCR dilakukan oleh Apical Science. Semua sekuen dianalisis menggunakan software CLC Sequence Viewer Versi 8.0 dan dibandingkan dengan basis data sekuens yang disimpan dalam ToxoDB (database genom Toxoplasma gondii) menggunakan BLAST (https://toxodb.org/toxo/app). Setiap primer gen B1 dievaluasi dengan melakukan PCR tunggal (forward dan reverse) dan reaksi PCR nested. Tiga set primer gen B1 memiliki presisi amplifikasi yang berbeda. Berdasarkan hasil sekuensing amplikon, set primer #2 memiliki presisi amplifikasi gen B1 yang paling baik.

Kata kunci: gen B1, nested PCR, sekuen, Toxoplasma gondii, zoonosis

INTRODUCTION

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii* (*T. gondii*), an apicomplexan parasite. The protozoan *T. gondii* infects practically all warm-blooded species, including humans, livestock, and marine mammals. *T. gondii* infects over 30% of the world's human population on a long-term basis (Moncada and Montoya 2012; Luma *et al.* 2013). In Indonesia, there are many cases of toxoplasmosis. In West Bandung Regency, 7 women of reproductive age (14%) have toxoplasmosis. Frequent interaction with pets, particularly cats, was found to be a significant influence in illness transmission (P<0.005) (Naully and Supendi 2020). The frequency of toxoplasmosis in animals varies from 5-80% depending on the location and species of the animal (Subekti *et al.* 2005).

Toxoplasma gondii infection is asymptomatic in healthy people, but in immunocompromised people, a bradyzoite-containing cyst can burst, causing toxoplasma encephalitis (TE) (Munoz *et al.* 2011). Eating undercooked or raw meat with viable tissue cysts, or ingesting oocyst-contaminated food or drink, is the most common way for humans to become infected. Adults with primary toxoplasma infection are usually asymptomatic, although some patients develop ocular lymphadenopathy or toxoplasmosis (Neves *et al.* 2011; Abhilash *et al.* 2013; Cuomo *et al.* 2013).

In immunocompromised patients, reactivation of latent toxoplasmosis infection can result in deadly toxoplasmosis, encephalitis, myocarditis, and pneumonitis. Immunocompromised patients are at a higher risk of developing severe disease as a result of a primary infection or the reactivation of a chronic infection. Infections acquired during pregnancy can result in miscarriage, paralysis, stillbirth, or fetal death in the fetus. *T. gondii* is a single species of the genus Toxoplasma. *T. gondii* strains from North America and Europe identified limited genetic variation, which was divided into genetic categories I, II, and III in early research. (Tlamcani *et al.* 2013; Liu *et al.* 2015; Mattar *et al.* 2019). Severe toxoplasmic retinochoroiditis is more likely to be associated with biotype I or type I variations. In immunocompetent people, atypical *T. gondii* isolates frequently cause severe acute toxoplasmosis. Biotype I are uniformly lethal to outbred mice, while type II and III isolates are significantly less virulent. *T. gondii* infection symptoms are nonspecific and unreliable for diagnosis (Grigg *et al.* 2001).

The gold standard for toxoplasmosis diagnosis to detect *T. gondii* is using a microscope and bioassay; however, clinical diagnosis is more likely to be made using serological methods, and various serological tests have been established for the detection of specific *T. gondii* antibodies or circulating antigens. However, there

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are problems to identifying and distinguishing parasite strains. Currently, biotechnological approaches for detecting *T. gondii* infection and genotyping *T. gondii* isolates with a fast and accurate diagnosis are being developed. Molecular technology based on nucleic acid amplification can be used in addition to conventional serological methods for the diagnosis of toxoplasmosis. Polymerase Chain Reaction (PCR) method can characterize genetically or classifying *T. gondii* from biological samples (Wang *et al.* 2013; Liu *et al.* 2015).

The molecular methods are appealing due to their high sensitivity and specificity to determine the presence of parasites in clinical sample. Detection through molecular diagnostics based on a specific DNA sequence, mainly from highly conserved regions (Mattar *et al.* 2019). The molecular diagnosis by using PCR for various clinical specimens become a strong tool for *T. gondii* DNA detection. PCR has been used to identify *T. gondii* genotyping in a variety of mammals and birds, including humans (Shwab *et al.* 2014). Different primer sets of B1 gene for detecting *T. gondii* have been developed in the last few decades. Burg *et al.* (1989) and Grigg and Boothroyd (2001) introduced B1 gene as an efficient PCR target. *T. gondii* DNA can be detected by PCR using a primer for the B1 gene, which is commonly used and has high sequence conservation. DNA sequence analysis of several clonal strains has also validated it. According to several studies, using nested PCR to detect the B1 gene were improved the sensitivity and specificity of toxoplasmosis diagnosis (Teixeira *et al.* 2013; Vitale 2013; Halleyantoro *et al.* 2019).

The B1 gene was chosen as a target in this study because its amplification has advantages such as higher sensitivity than other targeting genes, did not amplify DNA from other microbes and fungal DNA, sensitivity was reliable, and had good gene conservation (Alfonso *et al.* 2013). However, the issue is that the accuracy of this technique has yet to be established. Although it has showed low specificity in some cases, B1 amplification has been used as a gold standard approach for *T. gondii* detection. It should be emphasized that PCR sensitivity was based on several criteria, including reaction chemical and physical conditions, target DNA concentration, primer selection, and DNA extraction method (Ajzenberg *et al.* 2016). The aim of this study is to evaluate the B1 gene primer sets for nested PCR to diagnose *T. gondii*.

MATERIALS AND METHODS

DNA isolates were obtained from *T. gondii* tachyzoites in Indonesian Research Center for Veterinary Science. *T. gondii* isolate originated from Dominique Soldati-Favre (Faculty of Medicine-University of Geneva CMU, Switzerland) as a gift to Didik T. Subekti, DVM, M.S. (Indonesian Research Center for Veterinary Science) in 2008.

Isolation of Genetic Material

The DNA of *T. gondii* was extracted from an isolate that stored on liquid nitrogen. The DNA template was successfully extracted using DNAzol™ reagent and quantified using nanodrop.

Primer Selection

The B1 genes are highly conserved in all *T. gondii* strains tested to date. The B1 gene is a multicopy gene found in the *T. gondii* genome, making it an ideal target for PCR amplification. The B1 gene PCR appears to be very selective during amplification, and it is the most specific and sensitive method for detecting *T. gondii* DNA (Burg *et al*. 1989; Grigg and Boothroyd 2001; Grigg *et al.* 2001; Adamska 2018).

T. gondii DNA was identified by amplification of the B1 gene in single PCR and nested PCR (nPCR) for molecular identification. PCR was carried out using B1 gene primers (forward and reverse) based on Burg *et al.* (1989), Grigg and Boothroyd (2001), and Grigg *et al.* (2001). Primer sequences are detailed in Table 1.

PCR was carried out in a Thermal Cycler (Bio-Rad). The steps of single PCR and nPCR assays were performed in 25 µL reactions containing 2 µL DNA template, 0.25 M primer, 1.5 mM $MgCl_2$, 0.01 U Taq DNA polymerase, and 0.2 mM dNTP. The first step of PCRs was performed by using external and internal primer sets, and then nPCR. Annealing temperature was 58º C and 35 cycles. PCR products were visualized in 1.5% agarose gels stained with flourosafe.

Calculation of the Amplicon Size (bp)

Amplicon size was calculated using retardation factor (Rf) according to the method used by Yuniarto *et al.* (2018).

DNA Sequencing of PCR Products

PCR products sequencing was performed by Apical Science (PT Genetika Science Indonesia). All sequences were analyzed using CLC Sequence Viewer Version 8.0 software and compared to sequence database that deposited in ToxoDB (*Toxoplasma gondii* genome database) using BLAST (https://toxodb.org/toxo/app).

RESULTS AND DISCUSSION

Amplification of B1 Gene from *T. gondii* DNA

A single amplicon with a predicted size was amplified by first-round single PCR (external and internal primers) and nPCR B1 gene primers (Figure 1). The PCR products in this study were found to be identical to those reported in earlier studies (Burg *et al.* 1989; Grigg and Boothroyd 2001).

The B1 gene was chosen as the marker for the following reasons: The largest collection of B1 gene sequences available, covering a diverse range of isolates; many studies use this gene as a toxoplasmosis identification marker; the B1 gene is precise and accurate for *T. gondii* detection as ribosomal DNA is

frequently repeated within eukaryotes; Because there are over 100 highly conserved copies of the and B1 genes in the *T. gondii* genome, they are highly conserved in all *T. gondii* strains studied to date. It has successfully recognized *T. gondii* DNA, but its PCR results have not been sequenced to confirm this (Burg *et al.* 1989; Grigg and Boothroyd 2001; Adamska 2018).

The most accurate form of diagnosis is based on the disease's characteristic clinical symptoms, however atypical presentations, particularly in immunocompromised patients, can present a diagnostic problem because they can be misinterpreted, leading to ineffective therapy (Ozgonul and Besirli 2017). For analyzing *T. gondii* genetics, combining molecular technology and bioinformatics is important (Wang *et al.* 2013). Mostly as consequence, it is important to do the sequencing analysis stage of the PCR results in order to obtain more accurate result. Sequencing is also an essential aspect in solving a problem.

Clinical diagnostics often use targeted sequencing. Regardless of the fact that whole-genome sequencing has become an important aspect of clinical molecular diagnostics, it is still problematic to apply in a clinical laboratory due to high sequencing costs and time-consuming processing, analysis, and data storage (Goswami, 2016).

Calculation of the Amplicon size (bp)

Calculated amplicon size using retardation factor (Rf) of each amplicon band is showed in Table 2. The amplicon size of B1 gene in the first step was standardized to amplify a fragment then the second step was standardized to ensure maximum sensitivity. Based on Table 2, the amplicon size of PCRs external primers has longer than internal primers, then the amplicon size

Table 1. The sets of B1 gene primers

Primer set of B1 gene	References	Nucleotides
#1	Grigg and Boothroyd 2001	F1 CGACAGAAAGGGAGCAAGAG R1 ACGGATGCAGTTCCTTTCTG F2 CCGGGCAAGAAAATGAGAT R2 CATGGTTTGCACTTTTGTGG
#2	Grigg *et al.* 2001	F1 TGTTCTGTCCTATCGCAACG R1 ACGGATGCAGTTCCTTTCTG F2 TCTTCCCAGACGTGGATTTC R2 CTCGACAATACGCTGCTTGA
#3	Burg *et al.* 1989	F1 GGAACTGCATCCGTTCATGAG R1 TCTTTAAAGCGTTCGTGGTC F2 TGCATAGGTTGCAGTCACTG R2 GGCGACCAATCTGCGAATACACC

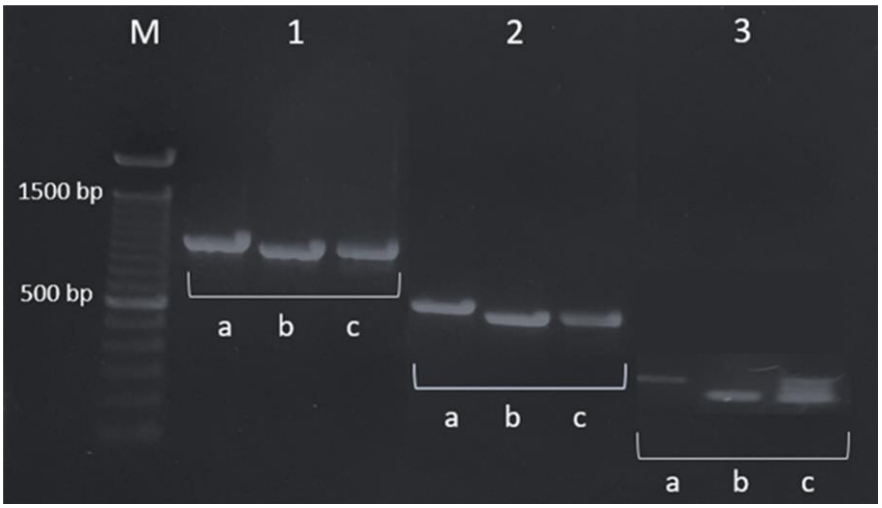

Figure 1. Detection of *Toxoplasma gondii* nucleic acid amplified by B1 gene (1. B1#1, 2. B1#2, 3. B1#3). A= Single PCR external primer; b= Single PCR internal primer; c= nPCR. The PCR product was resolved in 1.5% agarose stained with fluorosafe. Lane M: TrackIt™ 100 bp DNA molecular ladder (Thermo Scientific)

of nPCR longer than PCRs internal primer. Vitale *et al.* (2013) explained that the first pair of primers (external) amplifies the fragment which works similarly to single PCR in general. The second pair of primers (internal) are commonly referred to as nested primers (the pair of primers are located in the first fragment) because they bind to the fragment of the first PCR product and allow amplification of the second PCR product with a shorter than the first. Accordingly, the PCR product should be sequenced to check that the primers correctly amplify the target gene.

Nested PCR is a technique for multiplication (replication) of DNA samples that uses two sets of PCR primers to amplify fragments. If a fragment is incorrectly amplified in nPCR, the probability of that segment being amplified again by a second primer is very low. Then as a result, nPCR is a targeted PCR for amplification (Vitale *et al.* 2013).

DNA Sequence Results

The forward and reverse primer sequences were concatenated and verified using CLC Sequence Viewer Version 8.0 software. BLAST was performed in ToxoDB for whole sequence of each sample and it was selected for alignment (Table 3). Phylogenetic tree was constructed by CLC Sequence Viewer Version 8.0 software (Neighbor Joining, Jukes Cantor) as shown in Figure 2.

DNA sequencing is a method of determining the precise sequence of nucleic acids in the DNA molecule's basic components. DNA sequencing

Table 3. The homology sequences of B1 gene amplicon

Amplicon		Homology sequence (%)		Score	E
#1	External only	*Eimeria maxima*	90%	64.4	4e-08
	Internal only	*Toxoplasma gondii* (FOU strain)	99%	1676	0.0
	Nested	*Eimeria acervulina*	84%	113	2e-23
#2	External only	*Toxoplasma gondii* (p89 strain)	99%	962	0.0
	Internal only	*Toxoplasma gondii* (p89 strain)	95%	753	0.0
	Nested	*Toxoplasma gondii* (p89 strain)	93%	715	0.0
#3	External only	*Toxoplasma gondii* (FOU strain)	99%	282	3e-74
	Internal only	*Toxoplasma gondii* (FOU strain)	98%	89.7	1e-16
	Nested	*Toxoplasma gondii* (VEG strain)	96%	145	2e-33

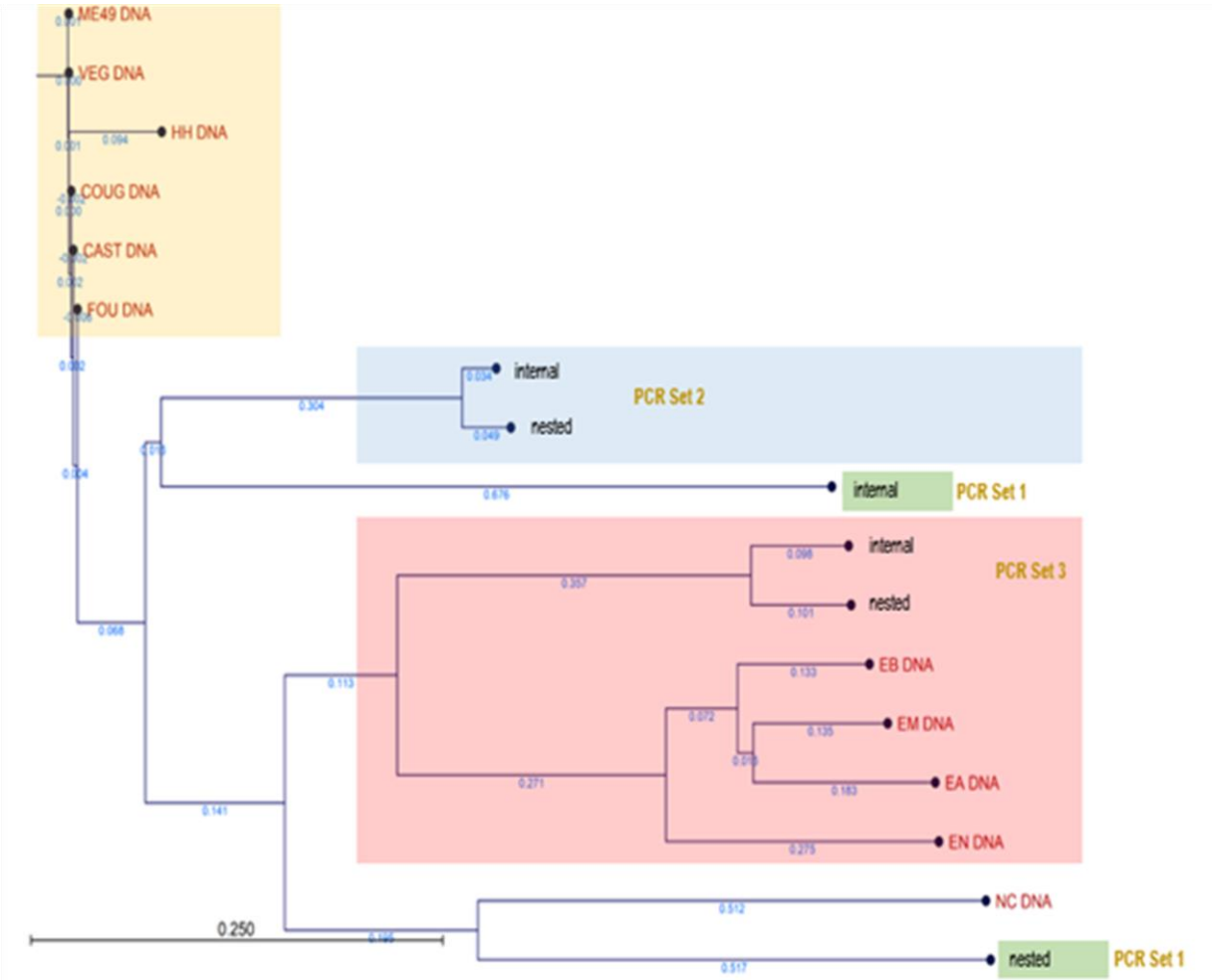

Figure 2. Phylogenetic tree of *Toxoplasma gondii* isolates. These *Toxoplasma gondii*, *Neospora caninum* (NC), *Hammondia* (HH), and *Eimeria* (EB, EM, EA, EN) references based on available information in ToxoDB (Toxoplasma Informatic Resources)

indicates how a DNA fragment's nucleotide bases is arranged. Every individual and organism has a specific nucleotide base sequence, resulting in a specific DNA sequence for every living organism. These sequences are responsible for encoding genetic information into particular DNA (Costa *et al.* 2016; Goodwin *et al.* 2017). PCR amplified marker gene sequencing is frequently used to identify organisms (Patwardhan *et al.* 2014).

All amplified PCR products were sequenced to confirm the identity of the amplified genes. Initially, entire or partial nucleotide sequences of genes which have been linked to *T. gondii* were selected from ToxoDB. Whole genomes or selected parts of the genome could be sequenced after PCR amplification.

All of the B1 gene targets were succesfully amplified, and their sequences homology to *T. gondii* ME49 were 84-99%. The nPCR amplicon of primer Set #1 has low homology (84%) and is closely related to *Eimeria acervulina*, whereas its single PCR amplicon for external primer has a low score (64.4) and is closely related to *Eimeria maxima*, according to Table 3 and Figure 2. On the contrary, its single PCR amplicon for internal primer has high score (1676) and homology (99%) to *T. gondii* FOU strain. The outcomes of primer Set #2 and #3 were different. Their nPCR amplicons have high score, high homology, and closely related to *T. gondii*. Whereas, set #3 amplicons have a score of error (E), indicating a potential error in detecting *T. gondii*.

Conversely, set #2 amplicons have no error score. It is possible that the primer annealing sites' high conservation allowed for a large boost in nested reaction sensitivity. They are unsuccessful in detecting a wide range of isolates due to the existence of polymorphisms in the annealing regions, increasing the incidence of false negative results. It should be emphasized that PCR sensitivity was depended on a number of criteria, including primer selection, target DNA concentration, annealing sites, sample volume limits, and DNA extraction process (Ajzenberg *et al.* 2016; Halleyantoro *et al.* 2019). Primary bias and chimeras can also influence amplicon sequencing (Guo *et al.* 2016). So, obtaining enough coverage depth, on the other hand, is a challenge (Zhou *et al.* 2015).

The DNA sequences allow for more accurate and faster species identification (Bourlat *et al.* 2013; Aylagas *et al.* 2016). Genome-based research is continually evolving with the application of DNA sequencing technologies (Cammen *et al.* 2016). The problem with DNA sequencing is determining a precise taxonomy classification, especially from short DNA sequences. This issue is aggravated when the reference genome is lacking (Roux *et al.* 2016).

In fact, the molecular method proved to be more efficient than morphological observations, and it was successfully applied to identify nonindigenous species that morphological examination had struggled to detect (Aylagas *et al.* 2016; Goodwin *et al.* 2017). The sequencing method can detect species with small concentrations of biological material (Quast *et al.* 2013) but requires increased coverage depth (Zhou *et al.* 2015). Although this sequencing technique is significantly more expensive than conventional method of species identification, it is a very efficient alternative to biodiversity monitoring (Bourlat *et al.* 2013) and helps long-term conservation biology research (Hancock-Hanser *et al.* 2013).

CONCLUSION

Three pairs of B1 gene primers for nested PCR have different amplification precision. According to the results of amplicon sequencing, Primer set #2 has the greatest performance for amplification of the *T. gondii* B1 gene.

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