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The Patterns of Restriction Fragment of Several Enzymes to Distinguish *Toxoplasma gondii* Isolates Virulent and Avirulent Strains using *GRA1* and *GRA7* Genetic Marker

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ABSTRACT

***Toxoplasma gondii* pathogenicity depends on the type derived from a clonal population. A genetic analysis of the locus has been carried out to determine the different genotypes of *T. gondii* (strain types I, II, and III) that are associated with human toxoplasmosis. The several genotypes of *T. gondii* (strain types I, II, and III) that are linked to human toxoplasmosis have been identified through genetic study of the locus. In this investigation, PCR-RFLP was found to be a useful, and simple method genotypic characterization. The objective of this study was to genotyping characterize *T. gondii* RH and BEV strains isolates by PCR-RFLP using several restriction enzymes. *T. gondii* tachyzoite DNA was extracted and amplified by PCR using dense granule genetic markers (*GRA1* and *GRA7*) designed with Primer3plus. The amplification were digested using the restriction enzymes. The PCR-RFLP amplified dense granule products was used to classify strains into two genotypes of *T. gondii* (virulent and avirulent). The results demonstrated that the RFLP patterns of the *GRA1* and *GRA7* gene area digested by DdeI, MvaI, HinfI, RsaI, and Sau96I enzymes can be used to identify virulent or avirulent strains of *T. gondii*. *Toxoplasma gondii* RH and BEV strain produced different digestion product which can be used to distinguished the strains.**

1. Introduction

Almost all warm-blooded species, including humans, are susceptible to infection by the opportunistic disease *Toxoplasma gondii* (Stelzer *et al.* 2019). Many factors, including host and parasite genetics, affect how quickly and severely the disease progresses in each patient. It is generally known that different *T. gondii* strains have varying degrees of pathogenicity in animals. Finding a potential relationship between strain genotyping and disease severity or type may be crucial for determining the best course of action and potential outcomes for each individual case (Liu *et al.* 2015).

Toxoplasma is thought to contain only one species, *T. gondii*. There are several strains of *T. gondii*, each with a different level of pathogenicity. The lethal dose of tachyzoites is usually used as a measure of the pathogenicity of a strain. Several strains of *T. gondii* have been classified into two categories based on lethal dose and tachyzoite virulence in mice: virulent strains (LD100 <10 tachyzoites), which can cause acute lethal infection with ascites in mice; and avirulent strains (LD100 >10^3 tachyzoites), which can cause chronic infection characterized by the development of tissue cysts in the mouse (Sibley *et al.* 2009). Using various techniques for characterizing *T. gondii* strains, such as isoenzyme electrophoresis, restriction fragment length polymorphism (RFLP), PCR, or random polymorphism amplified DNA (RPA), the strains have been grouped into two or three primary categories (Liu *et al.* 2015). According to Sibley *et al.* (2009), three distinct clonal lineages known as types I, II, and III were identified by PCR-RFLP analysis of six separate single-copy loci. The avirulent isolates were then allocated to clonal

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lineages of types II and III, while the virulent lineage strain was assigned to clonal lineage type I. There are several types of *T. gondii* strains, including RH (type I), Beverly (type II), Me49 (type II), and C56, VEG (type III) (Liu *et al.* 2015; Sibley *et al.* 2009). The strain of *T. gondii* can be identified using the restriction fragment length polymorphism (RFLP) pattern, which has been shown to be easy, quick, and reliable (Ekawasti *et al.* 2021).

A specialized organelle within the host cell called the parasitophorous vacuole (PV) is where parasites reproduce during acute infection. Proteins from secretory vesicles known as dense granules are released by the parasite to maintain the structure and stability of the PV (Fihiruddin *et al.* 2020; Gold *et al.* 2015; Heaslip *et al.* 2016; Subekti *et al.* 2012). Other dense granule proteins that are discharged control host cell gene expression (Bougdour *et al.* 2013; Braun *et al.* 2013; Mercier *et al.* 2015) and immune response (Pernas *et al.* 2014; Rosowski *et al.* 2011; Shastri *et al.* 2014), which is crucial for parasite survival and disease progression (Heaslip *et al.* 2016).

The *GRA* protein plays a role in modifying and perfecting the parasitophorous vacuole and enables the host cell to absorb nutrients from the cytoplasm to support the development of tachyzoites and bradyzoites during intracellular life until the final stage. *GRA* is a mitochondrial gene consisting of *GRA1* to *GRA15*. *GRA1* and *GRA7* were used as genetic markers in this study because they are the most well-known markers for identifying *T. gondii* genotypes. They can evoke cellular and humoral immune responses in mice (Mufasirin and Suprihati 2013). These proteins support structural integrity and maturation of parasitophorous vacuoles, and are also important for parasite growth and have been shown to be more thorough for comprehending host-parasite interactions and influencing *T. gondii* gene expression in the host, metabolism, or immune response during in vivo (Subekti *et al.* 2012).

The success of the polymerase chain reaction (PCR), particularly in the field of DNA sequencing, depends significantly on the choice of dense granule primers (*GRA1* and *GRA7*) that are acceptable and suitable for the PCR process. The part of the target DNA sequence to be amplified must be known before amplification is carried out by designing the optimum size primer for the PCR product (Ekawasti *et al.* 2021).

This study begins with the development of *in silico* *GRA1* and *GRA7* primer designs aimed at obtaining the best primers to be used in molecular analysis. Furthermore, RFLP PCR analysis was carried out using several restriction enzymes on the amplification of the *GRA1* and *GRA7* PCR products. In this investigation, profile fragments were produced from the digestion products using five enzymes. A more specialized molecular profile was anticipated to result from the usage of more enzymes. Also, the data would reveal which enzyme is best to employ for identifying species. Thus, a more precise classification of *T. gondii* strains is required. The goal of this work was to examine the molecular profiles of the *T. gondii* RH (virulent) and BEV (avirulent) strains based on the outcomes of the digestion of dense granule protein fragments by the restriction enzymes DdeI, MvaI, HinfI, RsaI, and Sau96I.

2. Materials and Methods

2.1. *Toxoplasma gondii* Isolate

The isolate of *T. gondii* RH strain was provided by the Indonesia Research Center for Veterinary Sciences, The Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, Indonesia. The *T. gondii* Beverly (BEV) strain isolate was provided by ATCC 50854TM. *T. gondii* isolates from liquid nitrogen were used as samples.

2.2. Isolation of Genomic DNA

T. gondii DNA was extracted from an isolate stored in liquid nitrogen. DNA template was extracted using DNAzol (Molecular Research Center, OH, USA). Purification and concentration of DNA extracts for DNA quantification were performed using Thermo Scientific NanoDrop products and extracts were stored at -20°C prior to PCR testing.

2.3. Genetic Markers

The *in silico* designed genetic markers *GRA1* and *GRA7* were used in this investigation. The software Primer3Plus on the website https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi was used to analyse the obtained FASTA format of dense granule genes (*GRA1* and *GRA7*) in order to produce primary candidates and Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast). After obtaining primary candidates, primer selection was performed according to the provisions of good primers (Hung and Weng 2016).

2.4. Polymerase Chain Reaction (PCR)

The PCR was performed in a Thermo Scientific™ Arktik™ Thermal Cycler using the dense granule genes *GRA1* and *GRA7* genetic markers (Thermo Fisher Scientific, Finland). The PCR and nPCR test procedures were carried out using 25 µL reactions containing 1 µL of DNA template, 0.25 M primer, 1.5 mM $MgCl_2$, 0.01 U Taq DNA polymerase, and 0.2 mM dNTP. 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min served as the amplification conditions. SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Finland) and 1.5% agarose were electrophoresed at 100 V for PCR products. An UV transilluminator was used to see the bands.

2.5. DNA Sequencing and Analysis

The PCR product were then sequenced and identified for the clarification. The sequencing was performed by Macrogen and the sequence result were evaluated with the use of the CLC Sequence viewer software version 8.0 (Qiagen, Denmark) (https://clcsequence-viewer.software.informer.com/8.0/).

2.6. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

The identification of diagnostic restriction fragments required the digestion of ten microliters of PCR product (10 µL DNA 10 µg/µL) by several restriction enzymes. Using the DdeI, MvaI, HinfI, RsaI, and Sau961 enzymes from Promega, Thermo Fisher Scientific, Inc., and Biolabs, respectively, restriction fragment length polymorphisms (RFLPs) for PCR products were carried out. According to each enzyme's manufacturer's protocol, the treatment process for enzymes was carried out. Agarose 1.5% and SYBRTM safe staining, loading dye, ran at voltage 100 Volt, were used for the electrophoresis of the PCR digested product. UV transilluminator was used to see the bands of the restriction patterns.

3. Results

3.1. Genetic Marker Design

Design of the primer was carried out to get primers that can be utilized in the polymerase chain reaction method of amplification of DNA. DNA amplification success depends on the precision of the primer used. Primer used in the PCR process must be able to limit area to be amplified.

I*n silico* revealed that 2 pairs primary designs of each gene *GRA1* and *GRA7* from *T. gondii* were close to the primary requirements Table 1. Similarity test was conducted to test whether the designed primer was able to amplify the *GRA1* and *GRA7* genes specifically. The primers from the online design were then carried out with nucleotide-BLAST.

3.2. Molecular Analysis

Proteins that are directly related to invasion of host cells include excretory-secretory antigen/ESA proteins which play a role in the process of penetration and formation of parasitoporous vacuoles as centers of parasite development. Excretory-secretory antigens (*ESA*) protein components, namely micronemes protein (*MIC*), rophtry protein (*ROP*), and dense granule protein (*GRA*).

The DNA of tachyzoite was extracted then amplified by PCR using genetic markers of dense granule protein (*GRA1* and *GRA7*). This primer designed can cut the target region (can limit the amplification area in the PCR process) precisely according to the *in silico* designed area range. This demonstrates that the selected primers are suitable for use in the PCR procedure and can give results consistent with the targeted regional range. 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 two primers were 58°C, so that

Table 1. Results of *in silico* primer design and BLAST primer analysis

Gene	Size (bp)	Oligo	Length (bp)	GC (%)	TM(°C)	Self/hairpin	Primer sequences
GRA1	226	Left primer	20	55	59.6	0	F 5'GGCAATGTTAACGTGGAGG 3'
		Right primer	20	55	60.2	0	R 5'CTGCACATCGTCGATCACCT 3'
GRA7	217	Left primer	20	55	60.0	0	F 5'TTGCCGC TGATAGACTT GTG 3'
		Right primer	20	55	60.0	0	R 5'GATTCAGGCACCTCTTGCTC 3'

the *GRA1* and *GRA7* primers could run simultaneously in one PCR reaction. Primer *GRA1* and *GRA7 T. gondii* with a sequence length of 20 oligonucleotides have been successfully designed in the best conditions with fragments of 226 bp and 217 bp, respectively.

Simulation of *GRA1* and *GRA7* primers on the template DNA of *T. gondii* in the PCR process can be seen in Figure 1.

Phylogenetic analyses of two strains of Toxoplasma gondii isolates were used for PCR reactions with each of the two indicated primer sets. In this study, primers *GRA1* and *GRA7* were designed *in silico* and simulated in PCR using samples of *T. gondii* RH and BEV isolates, showed results according to the target. The primer sets were able to amplify their respective targets. Amplifications with the primers targeting the *GRA1* and *GRA7* gene yielded products of the expected size (approximately 226 bp and 217 bp, respectively). BLAST searches of the GenBankdatabase with the sequences from Isolate No. 1 revealed nucleotide identities of (*GRA1*) 100% with *T. gondii* RH (EU983103.3; HM067753.1) and (*GRA7*) 95% with *T. gondii* ME49 (XM-002365660.2), 100% with *T. gondii* BEV (AY572709) and isolate No. 2 revealed nucleotide identities of (GRA1) 97% with *T. gondii* RH (HM916952; MK2509981; JX045573) and (GRA7) 95% with *T. gondii* ME49 (XM-002367572.1), 97% with *T. gondii* RMS (EF639059) 100% with *T. gondii* BEV (AY572750). The amplification specificity proved by *in silico* test and sequencing followed by nucleotide BLAST. The sequensing results from this product can clarificate the primary design used is already good.

We constructed the phylogenetic trees (cladograms) using the *GRA1* and *GRA7* gene sequence obtained in the present study and published the sequences of related *T. gondii* strain. The sequence from the current investigation was grouped with two closely related strains, one virulent (genotype I, RH), and the other avirulent (genotype II, BEV) (Figure 2).

The cladogram uses approach algorithms construction Neighbor Joining method and nucleotide distance measure jukes-cantor with 1,000 bootstrap replication in CLC sequence viewer 8.0.

3.3. Pattern of Restriction Fragment

After PCR for the dense granule fragment, restriction enzymes Dde I, Mva I, Hinf I, RsaI, and Sau96I were used to digest the results for a fuller molecular profile by *in silico*.

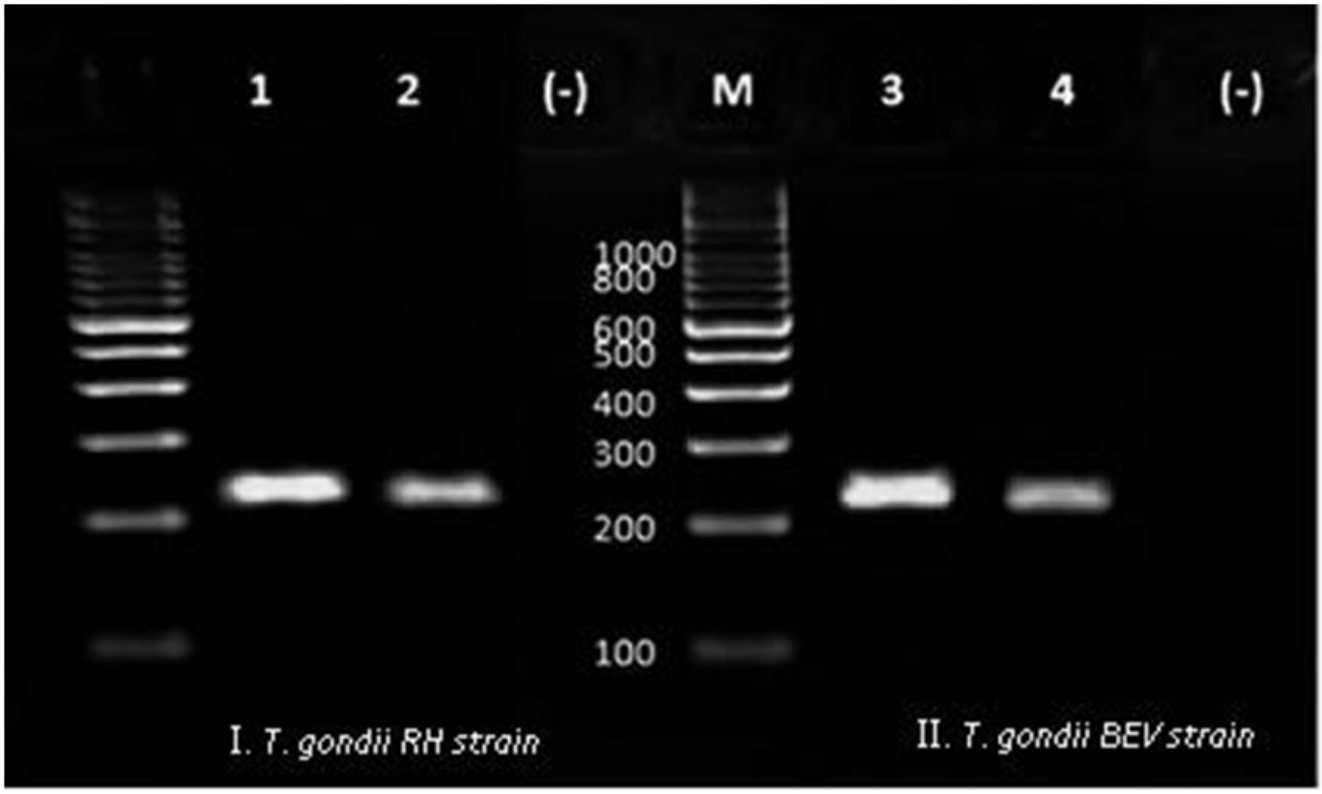

Figure 1. DNA from *Toxoplasma gondii* amplified by the genetic markers *GRA1* (226 bp) and *GRA7* (217 bp). The PCR product was resolved on 1.5% agarose stained with SYBR™ safe staining. Lane M: Geneaid 100 bp DNA ladder, 1: *GRA1* (RH strain), 2: *GRA7* (RH strain), 3: *GRA1* (BEV strain), 4: *GRA7* (BEV strain), (-): negative control

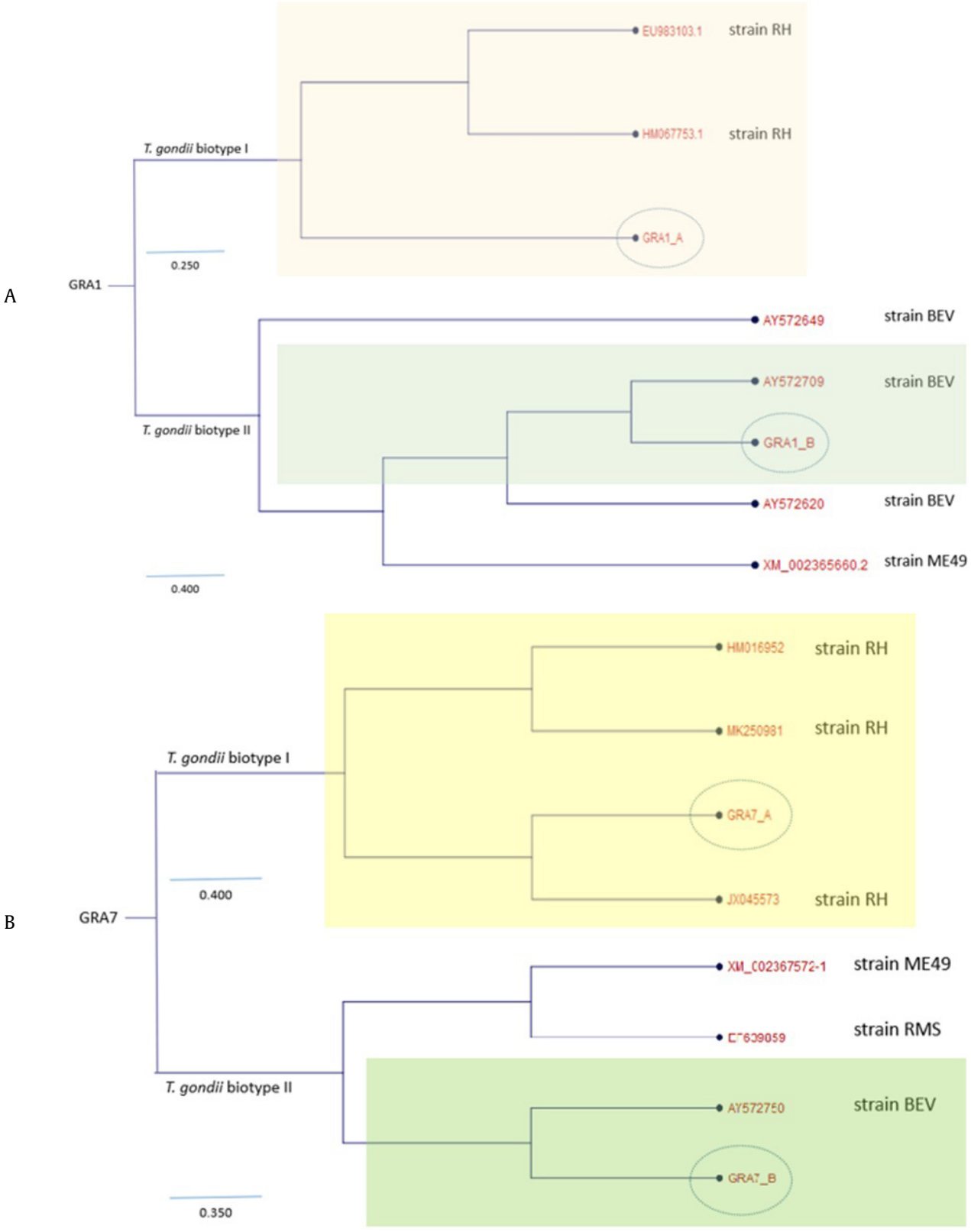

Figure 2. Cladogram of *Toxoplasma gondii* virulent (genotype I) and avirulent (genotype II) strains inferred by the neighbor-joining method using partial *GRA1* and *GRA7* gene sequences accession numbers and strains are shown. Nucleotide distance measure jukes-cantor with 1,000 bootstrap replication in CLC sequence viewer 8.0. A: primer GRA1, B: Primer *GRA7*

In RFLP analysis, we found patterns that were dissimilar from *in silico* restriction site analyses (Table 2 and Figure 3). We determined the RFLP pattern for the genotype that was verified by *in silico* analysis, and it turned out that some samples had a distinct fragment. Not all silico analysis fragments are visible on an electrophoresis gel, as illustrated in (Table 2 and Figure 3).

4. Discussion

A good primer is a primer that meets the criteria for primary parameters. Planning to acquire a primer that meets the criteria of good primer for amplification carried out *in-silico* (Hung and Weng 2016). In general, the ideal primer is between 18 and 30 oligonucleotides in length. This length is expected to be sufficient to

Table 2. *In silico* restriction site analysis of *GRA1* and *GRA7* genes sequences digested using restriction enzyme

T. gondii Strain	Primer	Size (bp)	Number of cut sites (cut position)									
			DdeI (CTNAG)		MvaI (CCWGG)		HinfI (GANTC)		RsaI (GTAC)		Sau96I (GGNCC)	
			PCR RFLP (bp)	Fragment *in silico* (bp)	PCR RFLP (bp)	Fragment *in silico* (bp)	PCR RFLP (bp)	Fragment *in silico* (bp)	PCR RFLP (bp)	Fragment *in silico* (bp)	PCR RFLP (bp)	Fragment *in silico* (bp)
RH	*GRA1*	226	0	1 (542)	1	2 (578,591)	1	1 (314)	1	2 (262,366)	0	2 (304,400)
	GRA7	217	-	-	0	1 (320)	0	1 (329)	1	2 (41, 122)	1	2 (354,364)
BEV	*GRA1*	226	1	2 (387,456)	1	1 (304) 2	1	1 (444)	-	-	1	1 (264)
	GRA7	217	-	-	0	(448,583)	0	1 (422)	0	2 (45, 122)	-	-

The size was determined from sequence between 5' forward and 3' reverse primer annealing position in this study. **Bold**: the fragments present in electrophoresis gel, Blue: *GRA1* gene, Brown: *GRA7* gene, --- *GRA1 GRA7* RH-BEV, --- *GRA1* RH-BEV, --- *GRA7* RH-BEV

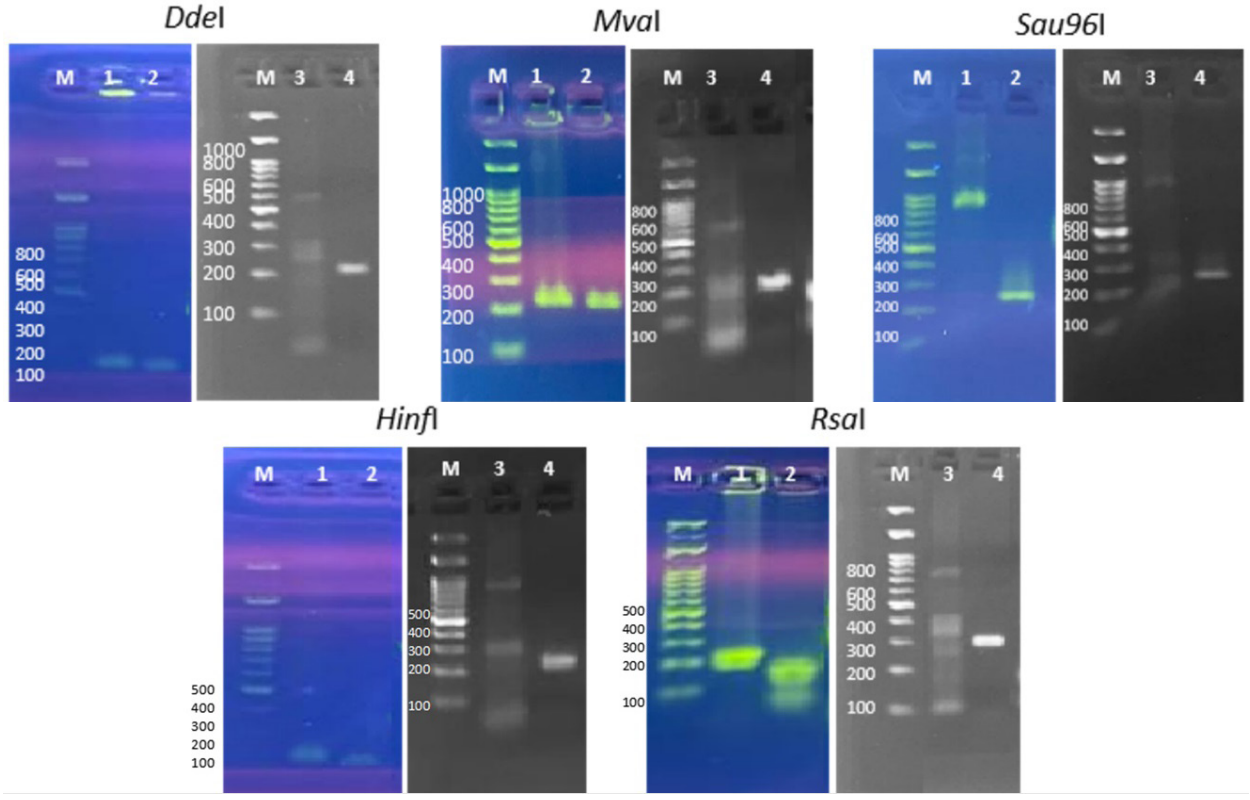

Figure 3. Restriction fragment PCR product after digestion with enzyme DdeI, MvaI, HinfI, RsaI and Sau96I. M: molecular marker, 1: *GRA1* (RH strain), 2: *GRA7* (RH strain), 3: *GRA1* (BEV strain), 4: *GRA7* (BEV strain)

be able to bind the template at annealing temperature and obtain a specific sequence (Hung and Weng 2016). Short primer length can lower the primer's specificity, making it more likely to adhere to the template at an unfavorable annealing temperature. The specificity is not considerably impacted by a long primer, nevertheless (Handoyo and Rudiretna 2000). Primer contains 40-60% base guanine (G) and cytosine (C) because the bond between G-C has a stronger bond than A-T. Primers must also pay attention to the Temperature Melting (TM) between the Forward and Reverse primers, not too much difference. Calculation of TM on each primer can be calculated using the formula 2(A+T) + 4(C+G) so that it will produce the temperature for TM. On the other hand, the base sequence in the primers must also be considered in order to avoid interactions between the primers themselves which are often referred to as self homology, cross homology, and dimer primers (Hung and Weng 2016).

The designed primer sets (Table 1) about the size, TM and GC percentage requirements (according to good primer requirements) in the *GRA1* and *GRA7 T. gondii* genes and were optimized in the laboratory (data not shown). The TM of the primer designed has met the criteria of 59-60°C. The TM of the primer can be used to determine the annealing temperature in PCR (Handoyo and Rudiretna 2000). Primers that melt at an excessively high temperature of TM can result in poor PCR results. TM that is too low, on the other hand, is more likely to persist elsewhere and produce generic goods. Optimization was carried out using PCR with the annealing temperature setting used calculated based on (TM–5) C to (TM + 5) C. The designed primer has a % GC of 55% which is still in the range of % GC criteria. Primers with low % GC can reduce the efficiency of the PCR process because the primers are not able to compete effectively for attachment to the template (Handoyo and Rudiretna 2000). Primers should not have 3 more bases G or C in the 3′dimer, because it can stabilize non-specific primer annealing and reduce primer specificity (Yustinadewi *et al.* 2018). The ability of a primer to adhere to the template is influenced by its stability. Repeats are nucleotides that are repeated in the primer, the presence of repeats can cause primer attachment in unwanted places (mispriming) (Ye *et al.* 2012; Yustinadewi *et al.* 2018).

In this primer designed there are no repeats, so the primer produced is in good condition. Hairpins are intramolecular interactions in primers. Hairpins in the primer can interfere with the primer attachment process to the template in the PCR process (Ye *et al.* 2012). In the primer designed there are no hairpins, so this primer performs well enough to be utilized in PCR.

The nucleotide primer sequence was amplified using specific primers that has been successfully designed was carried out using the sequences available in the NCBI GenBank database, *GRA1* (ID:HM067753.1; EU983103.1; M26007.1), and *GRA7* (ID: MK250981.1; MH352484.1; KY628191.1). Then, primers were obtained using the Primer3plus software and rechecked with PRIMER BLAST software (http://www.ncbi.nlm.nih.gov/tools/primerblast). BLAST primers resulting from the *in silico* design aim to find out which primers that have been designed *in silico* can bind to target genes in the gene bank (Bedwell and Goldberg 2020; Ye *et al.* 2012). Phylogenetic analysis was carried out to produce a cladogram that describes the kinship relationship (Shwab *et al.* 2013). Although they are different products, analysis of the cladogram can be used as a basis for generating a polyphasic taxonomy. Polyphasic taxonomic studies are reliable taxonomic studies to explain the phenomenon of biodiversity between organisms. This taxonomic study provides a very important role in understanding the holistic understanding of character variations between species (Gajurel *et al.* 2015). Consensus sequences were obtained from alignment of several sequences, with predicted amplicon sizes of *GRA1* (226 bp) and *GRA7* (217 bp) on the *T. gondii* strain sequencing.

Based on the result, the enzymes DdeI and Sau96I could be used on the *GRA1* gene alone to differentiate *T. gondii* types I and II. Meanwhile, the RsaI enzyme can only be used in the *GRA7* gene to differentiate *T. gondii* genotypes. Generally, enzymes that can be used by both genes simultaneously to differentiate genotypes namely MvaI and HinfI enzymes can be used to digest *GRA1* and *GRA7* genes to differentiate *T. gondii* genotypes I and II. The sequence influences the location of the cleavage site, resulting in a variation in the size of the digested products (Endrawati *et al.* 2021). The examination of the restriction fragment length distribution is conducted *in silico*, or on a computer. When *in silico* restriction gene programs search for restriction sites, it wraps around the DNA and breaks both strands of the DNA molecule when it encounters a DNA sequence with a shape that matches part of the enzyme, known as a recognition site (Ekawasti *et al.* 2021). Ekawasti *et al.* (2022) use Dde1, HinfI, and Sau961 to differentiate the genotype (strain) of *T. gondii*. This is in accordance with the current research where the three enzymes

can be used to differentiate between types I and II with restriction site analysis (Table 2).

Restrictions endonuclease digestion, a widely used technique for genome fragmentation, may seriously impair the resolution of genomic mapping and limit the useful annotation of specific chromosomal areas. To reduce mapping bias in genomic sequencing, *in silico* restriction enzyme digests are used. These digests produce correct DNA fragment size distributions and optimal resolution in genomic sequencing methods (Ekawasti *et al.* 2022). *In silico* analysis typically forecasts how they will appear in the experiment. Because the *T. gondii* genome sequence is publicly available, these methods may be quickly described and optimized using *in silico* modeling (Endrawati *et al.* 2021; Ekawasti *et al.* 2021, 2022). Due to insufficient digestion, *in silico* prediction analysis and electrophoresis produced different conclusions about the restriction sites. The most frequent response in PCR-RFLP is incomplete digestion. In a different instance, Ekawasti *et al.* (2021) also noted that less cutting was present in the *GRA1*, there was no cutting pattern, and *GRA7* fragments were digested by DdeI.

In this investigation, PCR was performed on *T. gondii* isolates using 10 distinct primers at the same time, at the same temperature, and with the same PCR cycle parameters. based on electrophoresis results (Figure 1). Both the virulent and nonvirulent isolates of *T. gondii* had their DNA amplified. The dense granule genetic markers in each *T. gondii* isolate strain RH and BEV were subjected to PCR-RFLP using the restriction enzymes Dde I, Mva I, Hinf I, RsaI, and Sau96I.

The concentration of the missing fragments may be quite low, and the electrophoresis gel did not show any signs of them. Moreover, it continued to leave uncut fragments in enzyme digestion that may have been brought on by the disease or an inefficient digestion process (Endrawati *et al.* 2021). As the concentration of the PCR result was not assessed, low-molecular-weight fragments, the optimization approach (the circumstances or digestion time being not optimal), and the ratio of enzyme and DNA may not be suitable (Endrawati *et al.* 2021; Ekawasti *et al.* 2021). a very low concentration that makes the electrophoresis gel undetectable (Endrawati *et al.* 2021). The digestive processes could have needed to be improved further. The loading buffer might have an impact. The use of four microliters of 6 loading buffer made it possible to see DNA overlaps clearly. Keep in mind that overnight digests are typically not essential and risk degrading DNA (Ekawasti *et al.* 2021).

The technique utilized to characterize the *T. gondii* strains implied that the fragments amplified would be digested using restriction enzymes. The products obtained are not the result of any contamination in the purification or amplification process because they were acquired from the same purification set along with nondigested products (Liu *et al.* 2015). Based on these findings, it is conceivable that the *GRA1* region used the DdeI and Sau96I and the *GRA7* region the RsaI enzyme to genotype the strain of *T. gondii*. To optimize and compare more isolated samples, however, more research is still required. In recent years, PCR RFLP has been a popular method for producing species-specific DNA for *T. gondii* identification (Endrawati *et al.* 2021; Ekawasti *et al.* 2021).

In general, the genetic markers *GRA1* and *GRA7* allows for identification *T. gondii.* However, to identify the *T. gondii* strains, RFLP PCR is necessary. Simultaneous digestion of the *GRA1* and *GRA7* regions using MvaI and HinfI was used as a specific marker successfully to differentiate virulent and avirulent *T. gondii* strains. In this study, analysis of digestion site fragments by restriction enzymes was found to be a useful, simple, and fast method compared to current molecular phenotypic techniques. However, further research is still needed to optimize and compare more isolated samples to get the right fragment pattern.

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