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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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...
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 Ddel, Mval, HinfI, Rsal, 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 DNsazol (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 MgCl₂, 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, Hinfl, Rsal, 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 SYBR® 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.

In 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>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Oligo</th>
<th>Length (bp)</th>
<th>GC (%)</th>
<th>TM(°C)</th>
<th>Self/hairpin</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRA1</td>
<td>226</td>
<td>Left</td>
<td>20</td>
<td>55</td>
<td>59.6</td>
<td>0</td>
<td>F 5’GGCAATGTTAACGTGGAGG 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Right</td>
<td>20</td>
<td>55</td>
<td>60.2</td>
<td>0</td>
<td>R 5’CTGCACATCGTCGATCACCT 3’</td>
</tr>
<tr>
<td>GRA7</td>
<td>217</td>
<td>Left</td>
<td>20</td>
<td>55</td>
<td>60.0</td>
<td>0</td>
<td>F 5’TTCGCCG TGATGACTT GTG 3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Right</td>
<td>20</td>
<td>55</td>
<td>60.0</td>
<td>0</td>
<td>R 5’GATTCAAGGCACCTCTTGCTC 3’</td>
</tr>
</tbody>
</table>
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 GenBank database 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-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 sequencing results from this product can clarify 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

<table>
<thead>
<tr>
<th>T. gondii Strain</th>
<th>Primer</th>
<th>Size (bp)</th>
<th>Number of cut sites (cut position)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ddel (CTNAG)</td>
</tr>
<tr>
<td>RH GRA1</td>
<td>226</td>
<td>0</td>
<td>1 (542)</td>
</tr>
<tr>
<td>GRA7</td>
<td>217</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BEV GRA1</td>
<td>226</td>
<td>1 (387,450)</td>
<td>1 (304)</td>
</tr>
<tr>
<td>BEV GRA7</td>
<td>217</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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, --- GRA7 RH–BEV

Figure 3. Restriction fragment PCR product after digestion with enzyme Ddel, Mval, Hinfl, Rsal 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, 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 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 Mval and Hinfl 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 DdeI, Hinfl, and Sau96I 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) noted that less cutting was present in the GRA1 region, there was no cutting pattern, and GRA7 fragments were digested by Ddel.

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 DdeI, MvaI, HinfI, 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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**References**


