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Research Article

VARIATIONS IN ANNEALING TEMPERATURE AND MAGNESIUM CHLORIDE CONCENTRATION FOR EIGHT RFLP MARKERS OF *TOXOPLASMA GONDII*

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ABSTRACT

Toxoplasma gondii is a protozoan parasite that infects one third of the world population and is capable of infecting any warm blooded animal. There are various methods to identify the genotype of *T. gondii* isolates. In our laboratory, PCR-RFLP method was elected, using eight RFLP markers i.e.3'SAG2, 5'SAG2, SAG3, BTUB, cB21-4, GRA6, SRS1 and GRA1. Before genotyping can be performed the PCR conditions need to be optimized. The critical conditions optimized were annealing temperature (T_a) and the magnesium chloride ($MgCl_2$) concentration which will highly influences the amplification process. The optimum annealing temperatures for markers 3'SAG2, 5'SAG2, BTUB, cB21-4, GRA1, SRS1, GRA6, and SAG3 were 63.0 °C,59.9 °C,58.7 °C, 53.7 °C, 61.1 °C, 64.2 °C,57.8 °C and 58.7 °C respectively. There were also differences in $MgCl_2$ concentration among the markers. Five markers were optimized at 1.5 mM of $MgCl_2$ namely 3'SAG2, BTUB, cB21-4, GRA6, and GRA1; 5'SAG2 and SAG3showed prominent bands at 2.0 mM of $MgCl_2$,and SRS1 marker was optimum at 2.5 mM$MgCl_2$. This study showed that re-optimizations of these two parameters need to be performed despite availability of optimized conditions in previous reports.

Keywords: *Toxoplasma gondi*, PCR, Restriction Fragment Length Polymorphism, Annealing temperature, Magnesium chloride.

INTRODUCTION

Toxoplasma gondii is a protozoan parasite capable of infecting all warm-blooded animals; including humans, avian, livestock and aquatic mammals(1-3).Felids play the role of definitive host for the parasite; whereby producing the environmentally resistant oocysts (4). Infection happens when a warm-blooded organism comes into direct contact with tainted feces or soil. An infection also takes place when one unwarily ingests water or undercooked meat laced with *T. gondii*(5). Another mode of transmission is through pregnant mothers passing on the infection to their fetus congenitally (6).

Although nearly one-third of the world human population is exposed to *T. gondii*, it is usually asymptomatic in healthy individuals (7-9). However, in an immune compromised individual, complications due to the infection can be fatal as it can cause cervical or occipital lymphadenopathy, ocular toxoplasmosis, congenital toxoplasmosis, encephalitis and schizophrenia (5, 10). It is also a proven cause of abortion, stillbirth, infertility and neonatal mortality in farm animals world-wide resulting in huge loss of revenue (11).

It is important to be aware of the dangers of the parasite and try to understand it further by studying it extensively. To identify the genotypes of *T. gondii* isolates, various methods are available such as polymerase chain reaction (PCR), DNA sequencing,allele-specific oligonucleotide (ASO)probes, DNA microarrays and hybridization. In our laboratory, the PCR-RFLP was selected.PCR is an effective technique to amplify a single or few copies of a DNA fragment into millions of copies, it has been proven to have great sensitivity, able to analyze multiple samples rapidly, relatively low cost and candiscriminate between species and strains given that suitable primers are selected for the analysis(12). The ability of PCR to detect small amounts of DNA without being affected by the viability of the parasite is important as well (13). Therefore, for this study, eight different restriction fragment length polymorphism (RFLP) markers have been identified and optimized for the detection of *T. gondii*genotypes. Genotyping can help in further studies of the parasite; including phylogenetic studies and even for studying antitoxoplasma activity of as mentioned by Singh and Raghav (14), against each *T. gondii* genotypes.

MATERIALS AND METHODS

DNA Fragment Template

The optimizations were performed using RH strain of *T. gondii.* Isolation of the DNA template was performed by using QIAamp DNA Mini Kit (QIAGEN, Germany), according to the protocol suggested by the manufacturer. The quality of the products was determined using NanoPhotometer (Implen, Germany) and 1% agarose gel electrophoresis stained with ethidium bromide.

Primer Selection

The eight selected markers for this study are as listed in Table 1. These have been commonly used for identifying and genotyping *T. gondii* from various isolates (15-19). The sequence of each primer was reconfirmed by referring to *Toxoplasma* Genome Map Database (http://toxomap.wustl.edu/Toxo_Genetic_Map_Table.html). The PCR primers were synthesized by a local scientific company(1st Base, Malaysia) and been purified by using HPLC purification method.

Optimization of PCR Conditions

The variables used for the optimization of the eight markers were magnesium chloride ($MgCl_2$) concentration and annealing temperature (T_a). The dNTP, primer and template concentrations were maintained at a constant value throughout the optimization. The temperature ranges were decided upon based on the reports of previous studies.

The basic content of the PCR tube was as follows: 1X *Taq*buffer, 400 µM each of the deoxyribonucleoside triphosphates (dNTPs), and 0.2 µM each of the forward and reverse primers as well as the *Taq* DNA polymerase. Each tube also contained 100 ng/µl DNA templates. The PCR grade Milli-Q water's (Millipore Corp., USA) was added to a final volume of 20 µl. The negative controls for this study were fish genomic DNA isolated using QIAamp DNA Mini Kit (QIAGEN, Germany) and water.

The PCR amplification process was carried out in the 96-well thermal cycler (My Cycler, Bio-Rad, USA). The reaction mixture was denatured at 95°C for 5 minutes; followed by denaturing step for 30 seconds, annealing for 30 seconds and extension at 72 °C for 1 minute for 35 cycles. Final extension was at 72 °C for 10 minutes.

PCR Product Analysis

The PCR products were observed with 1% agarose gel stained with ethidium bromide. The gels were electrophoresied at 100 V and 400 mA for an hour before being observed under a UV transilluminator (SyngeneIn Genius L, Synoptics, UK).The optimization results were repeated with *Pfu* DNA polymerase.

DNA Sequencing

The products amplified with *Pfu* DNA polymerase were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). The purified products were then sent for sequencing (1st Base, Malaysia). The results obtained were compared with the RH strain sequence from Toxoplasma Genome Map database by using blastn suite (http://www.ncbi.nlm.nih.gov).

Table 1: Markers and Primers used in this study

Markers	Primer
3'-SAG2 (15, 19)	Forward 5'-ATTCTCATGCCTCCGCTTC-3' Reverse 5'-AACGTTTCACGAAGGCACAC-3' PCR product size: 222 bp
5'-SAG2 (15, 19)	Forward 5'-GAAATGTTTCAGGTTGCTGC-3' Reverse 5'-GCAAGAGCGAACTTGAACAC-3' PCR product size: 242 bp
BTUB (18)	Forward 5'-GAGGTCATCTCGGACGAACA-3' Reverse 5'-TTGTAGGAACACCCGGACGC-3' PCR product size: 411 bp
cB21-4 (16, 17)	Forward 5'-CCAGGTGTTTCGATATTGAT-3' Reverse 5'-GCCTGTGTGGTGTTCGAATC-3' PCR product size: 502 bp
GRA1 (17)	Forward 5'-CGGTTTGCTTGTGTTGTTTG-3' Reverse 5'-CATGGGGTACGATCACAACA-3' PCR product size: 827 bp
GRA6 (16, 18)	Forward 5'-ATTTGTGTTTCCGAGCAGGT -3' Reverse 5'-TCGCCGAAGAGTTGACATAG-3' PCR product size: 351 bp
SAG3 (18)	Forward 5'-CAACTCTCACCATTCCACCC -3' Reverse 5'-GCGCGTTGTTAGACAAGACA-3' PCR product size: 311 bp
SRS1 (17)	Forward 5'-TTGTGCCTGAGTTCGTCTTG -3' Reverse 5'-TGTCCACTCACGTACCGAAA -3' PCR product size: 1386 bp

RESULTS

Purity of the Genomic DNA

The genomic DNA isolated from the pellets was estimated for purity by using the Nano Photometer and gel electrophoresis. The gel image obtained is as shown in Fig. 1, which showed no RNA smear. The ratio of $A_{260}/A_{280}$was 1.980 and the DNA concentration of the sample was 0.098µg/µl.

Optimization of $MgCl_2$ Concentration and T_a of Primers

The optimal $MgCl_2$ concentration and T_a results are shown in Table 2. The first marker optimized was 3' SAG2 whereby the marker was initially optimized between 55 – 65°C, and subsequently optimized between 63 – 68°C as shown in Fig.2 (A).The Fig. clearly indicates that the band was only present at about 63°C and it was best observed at 1.5 mM $MgCl_2$ concentration. The next marker, 5' SAG2, hasa sharp and obvious band with 2.0 mM$MgCl_2$ and 59.9°C as shown in Fig.2(B). BTUB marker amplification showed a prominent band with 1.5 mM $MgCl_2$and 58.7°CT_a as depicted in Fig.2(C). The PCR optimization for cB21-4, GRA1, and GRA6 showed optimal $MgCl_2$ concentration of 1.5 mM and optimal T_a of 53.7°C, 61.1°C and 57.8°C respectively. Meanwhile, SAG3 and SRS1 $MgCl_2$ concentration and T_a were optimizedand showed prominent bands with 2.0 mM$MgCl_2$ at 66.0°C and 2.5 mM$MgCl_2$ at 64.2°C respectively.

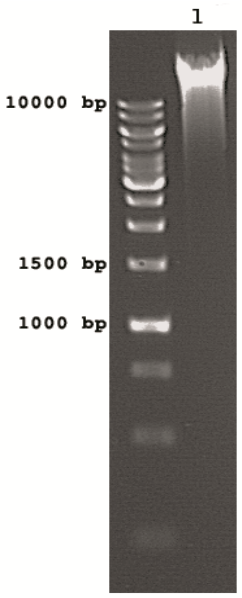

Fig. 1: Genomic DNA isolated from *Toxoplasma gondii* RH strain pellets

Table 2: Optimal and suggested MgCl2 Concentration and annealing temperature (°C) for each marker used in this study

Markers	Optimal $MgCl_2$ Concentration (mM)	Suggested $MgCl_2$ Concentration (mM)	Optimal T_a (°C)	Suggested T_a(°C)
3'-SAG2	1.5	3.0	63.0	63.0 (19)
5'-SAG2	2.0	3.0	59.9	65.0 (19)
BTUB	1.5	1.5	58.7	55.0 (18)
cB21-4	1.5	-	53.7	55.0 (17)
GRA1	1.5	-	61.1	55.0 (17)
GRA6	1.5	1.5	57.8	55.0 (18)
SAG3	2.0	1.5	58.7	55.0 (18)
SRS1	2.5	-	64.2	55.0 (17)

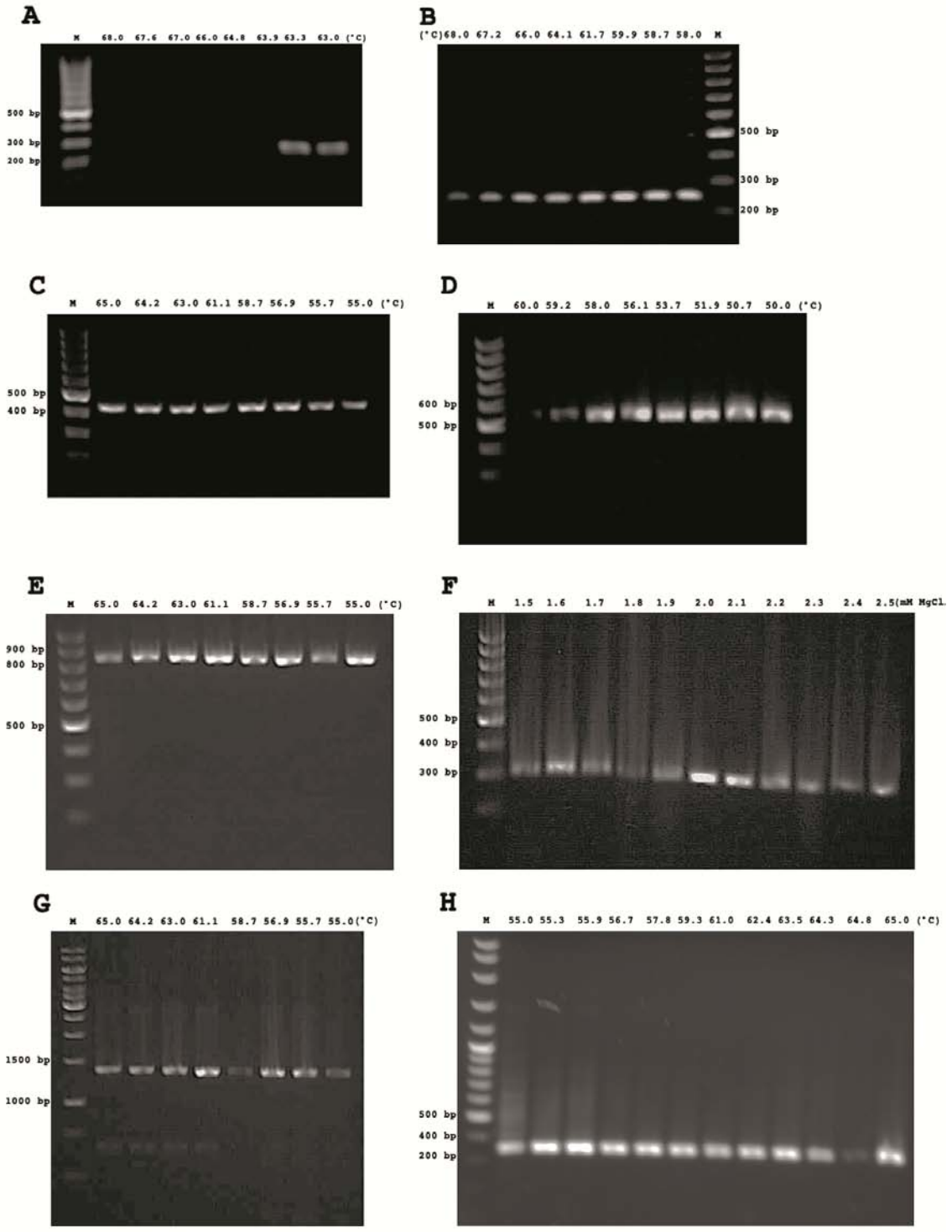

Fig. 2: Optimization of RFLP markers (A) 3' SAG2, (B) 5' SAG2, (C) BTUB, (D) CB21-4, (E) GRA1, (F) SAG3, (G) SRS1, (H) GRA6

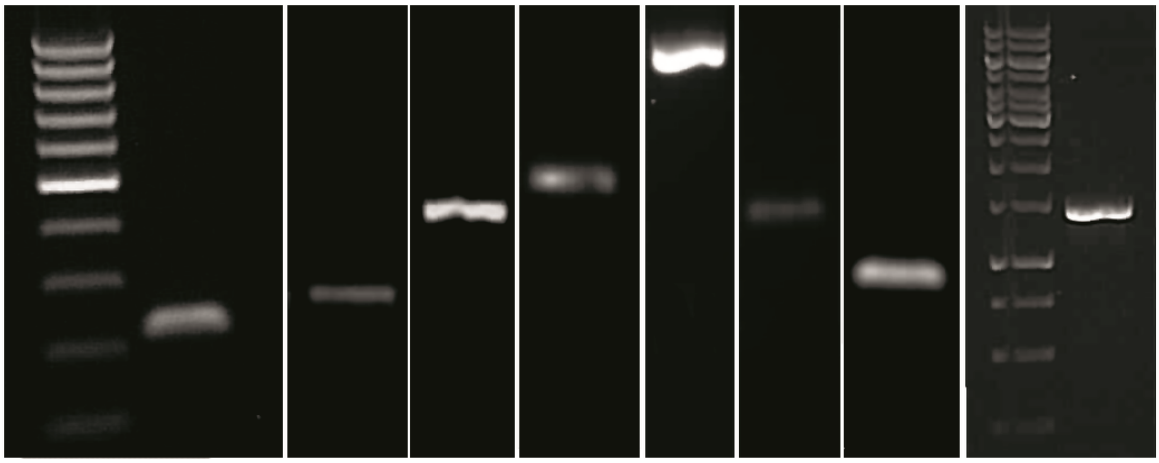

Fig. 3: Amplified products of 3' SAG2, 5' SAG2, BTUB, CB21-4, GRA1, GRA6, SAG3, SRS1 using Pfu DNA polymerase with optimized MgCl2 concentration and T_a.

Amplification of Optimized Conditions by Pfu Polymerase and Sequencing Results

The results obtained by using the *Taq* polymerase were replicated by using *Pfu* polymerase. The results are shown in Fig. 3; there were no differences in amplification conditions when the optimized conditions were replicated using *Pfu* polymerase. The sequencing results were aligned with original sequences from Toxoplasma Genome Map database using blastn suite (http://www.ncbi.nlm.nih.gov). All the sequences were found to be correct and without any mutations.

Sequencing results

After being aligned with original sequence from Toxoplasma Genome Map Database using blastn suite (http://www.ncbi.nlm.nih.gov/), the entire sequence products were >98% similar with the eight genetic markers; of which GRA1 showed 100% similar identities. For the percent of gaps, most of the sequence result has 0%, except for 5'SAG2 and SRS1 which has 1% and 2% gaps accordingly, however these gaps have not interrupted the restriction enzyme sites. Expect value of all the sequences were below 0 and this indicates that, all the sequence were correct and without any mutations. Thus results confirmed that the optimizations produced the correct PCR products.

DISCUSSION

The annealing temperatures (T_a) of the markers 5'-SAG2, GRA1 and SRS1 were different than those previously reported. The optimum temperature for markers 5'-SAG2, GRA1 and SRS1 were 6.0°C less, 6.1°C greater and 9.2°C greater respectively as compared to the previous reports(15, 17, 19). Many different factors can influence these results; including the performance of thermal cyclers (20, 21). Thermal cyclers are the programmable heating blocks that control and maintain the temperature of the sample. Most thermal cyclers presented some type of variation in their temperature profiles and differences in ability to maintain a consistent temperature both within and between experiments (21). The reaction temperature should change in a linear fashion until it reaches a specific step in the PCR and remain constant during the step. However, some thermal cyclers show slow changes in temperature at the beginning of a PCR step, resulting in "curved" temperature profiles, which substantially shorten the duration of the temperature level. In contrast, other instruments maintain rapid speed of temperature changes, but shows momentary fluctuation of temperature before stabilizing (21). If the optimum programmed temperatures are not met due to the block's overshooting and undershooting or due to the temperature across the thermal block is not uniform, it can have a significant impact on the optimal T_a and influence the PCR product (20).

As a rule, the $MgCl_2$ in the reaction mixture is generally 0.5 to 2.5 mM greater than the concentration of dNTPs(22). Mg^{2+} ions form a soluble complex with dNTPs which is important for dNTP incorporation; therefore, the concentration of dNTP was kept constant throughout the study.

Furthermore, if the cooling and heating rate is too fast or too slow, it will influence the concentration of Mg^{2+}; these ions are important factors for DNA stability in the PCR mixture (22). Besides, Mg^{2+} presence in PCR as an important cofactor for Taq DNA polymerase and strongly influences the entropy (ΔS) of PCR because ΔS are salt dependent (22). During annealing, the primers randomly moving; due to the Brownian motion (23), thus, short bondings are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little longer and on that piece of double stranded DNA, enabling the polymerase to attach and start copying the template(23). Brownian motion increases with temperature (24);therefore, the ions and molecule will also be moving around more and this will require more Mg^{2+} ions to stabilize the conditions (22).

A weaker interaction of the primer with the template, will result in low yield of PCR products as well as differences in optimal T_a and concentration of Mg^{2+}(25). Another factor to consider is the purity of the primers. Unpurified primer preparations may contain sequences that are truncated, oligonucleotides with base modifications that prevent hybridization, and fragments that are incomplete chains(25). In a PCR that is not operating with stringency, these oligonucleotides could lead to weak signals and irreproducible banding patterns. The best primer preparations are those purified by reversed-phase HPLC(25).

CONCLUSION

Optimization of annealing temperature and Mg^{2+} are vital for reproducibility of PCR results and to avoid false positive results. The results obtained and dissimilarities observed from the previous studies showed that combinations of various factors can affect the optimal results. Thusoptimizations must be carried out before performing genotyping by PCR-RLFP despite the availability of previous data.

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