Establishment and Application of a Loop-Mediated Isothermal Amplification Method for Simple, Specific, Sensitive, and Rapid Detection of *Toxoplasma gondii*

RUNNING HEAD: LAMP for Rapid Detection of *Toxoplasma gondii*

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Abstract: Loop-mediated isothermal amplification (LAMP) method amplifies DNA with high simply, specificity, sensitivity and rapidity. In this study, A LAMP assay with six primers targeting a highly conserved region of the GRA1 gene has been developed to diagnose Toxoplasma gondii. The reaction time of the LAMP assay was shortened to 30 min after optimizing the reaction system. The LAMP assay was found to be highly specific and stable. The detection limit of the LAMP assay was 10 copies, the same as that of the conventional PCR. We used the LAMP assay to develop a real-time fluorogenic protocol to quantitate T. gondii DNA, and generated a log-linear regression plot by plotting the time-to-threshold values against genomic equivalent copies. Furthermore, the LAMP assay was applied to detect T. gondii DNA in 423 blood samples and 380 lymph nodes samples from 10 pig farms. Positive results were 7.8% and 8.2% , anf the result shows LAMP method is some more sensitive than conventional PCR (6.1% and7.6%). Positive samples obtained from 6 pig farms. The LAMP assay established in this study resulted in simple, specific, sensitive and rapid detection of T. gondii DNA, and is expected to play an important role in the clinical detection.

Key words: detection; real-time fluorogenic LAMP; Toxoplasma gondii
1. Introduction

*Toxoplasma gondii* is a widespread zoonotic protozoan that infects humans and other warm-blooded animals [5]. Nearly one-third of humanity has been exposed to this parasite [9]. *T. gondii* mainly through peroral infections, bloodstream infections and congenital acquired infections [29]. The majority of horizontal transmissions are caused by the consumption of uncooked, infected meat. Pork is the main source of meat consumed by people in China, so *T. gondii* of pigs is considered an important source of *T. gondii* infection in humans [15]. Prevalences of *T. gondii* infection in fattening pigs had been found to vary from 3.32% to 66.39% in China [28]. The mortality rate can be as high as 60% in piglets when the acute infection outbreaks. In short, toxoplasmosis is a large threat to pork consumers and the economic benefits of pig industry.

Although serological testing is most widely used for the detection of *T. gondii* infections from humans and animals, it may fail to detect anti-*T. gondii* IgG or IgM antibodies in patients suffering from acute infection, organ transplant and AIDS [15, 25], and the target of serodiagnosis is antibody of pathogen, that will have the opportunity to result false positives. Several PCR-based techniques have been developed as alternative diagnostic measures for *T. gondii* infection [11, 19]. Even though these techniques are extremely sensitive and highly specific, diagnosis of *T. gondii* infection remains unsatisfactory because PCR methods are limited for the need of expensive equipment and reagents [16]. Loop-mediated isothermal amplification (LAMP) is the most recently developed molecular
detection method \cite{23}, which is known to be a sensitive, easy and fast detection method. LAMP amplifies DNA using a regular laboratory water bath under isothermal conditions, has been developed for the detection of many viral, bacterial, protozoan, and fungal diseases \cite{7, 14, 18, 22}.

*T. gondii* dense granule antigen GRA1 is a major excretory-secretory protein \cite{1}, which is highly conserved and recognized in chronically toxoplasma infected human \cite{24}. The recombinant GRA1 antigen has a great value in diagnosis and vaccine immunology of Toxoplasmosis \cite{13, 17, 8}. In this study, we used a conserved sequence in the GRA1 gene to design LAMP primers for detection of *T. gondii* and assessed its performance for diagnostic purposes. We investigated the detection sensitivity of the *T. gondii* LAMP assay in comparison with the conventional PCR on standard plasmid, and developed a real-time fluorogenic protocol to quantitate *T. gondii* DNA. Furthermore, the LAMP assay was applied to detect *T. gondii* DNA in pig samples from 10 pig farms.

2. Materials and methods

2.1. *T. gondii* strain and genomic DNA extraction

Tachyzoites of the highly virulent *T. gondii* (RH) strain were harvested from the peritoneal fluid of BALB/c mice after infected 5-6 days earlier \cite{4}. The genomic DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions.

2.2. Designing the LAMP primers

The LAMP primers were designed using Primer Explorer V4 software
based on a conserved region of the GRA1 gene identified by sequence alignment (Fig.1). All primers used in this study are listed in Table 1. The primers used in this study were synthesized by Takara (Dalian).

Fig.1 The target rejoin of GRA1 for the primers of LAMP assay

Table 1 Nucleotide sequences of LAMP primers designed in this study

2.3. Construction of a standard plasmid

A standard plasmid, pGEM-T-easy-GRA1, was constructed by insertion of a GRA1 gene fragment generated using the F3 and B3 primers into the pGEM-T Easy Vector (Promega, USA). After verification by sequencing, the concentration of plasmid was measured in a Bio Tek Epoch microplate spectrophotometer (Epoch, Bio Tek, USA). The copy number was calculated by the formula: amount(copies/μl) = 6×10^23 (copies/mol) × concentration (g/μl) / MW (g/mol).

2.4. LAMP and PCR

The LAMP reaction was carried out in a volume of 25 μl containing 1×ThermoPol buffer (NEB, USA), 8.0 mM MgCl2, 0.8 M betaine (Sigma, USA), 1.4 mM dNTPs, 8 U Bst DNA polymerase (NEB, USA), 0.2 μM of each of the F3 and B3 primers, 1.6 μM of each of the FIP and BIP primers, 0.8 μM of each of the LF and LB primers and 1 μl of extracted DNA as template. The mixture
was incubated at 65°C for 40 min and heated at 80°C for 10 min to terminate the reaction. A control containing no template was included in each test as the negative control. LAMP products were centrifuged at 12,000 rpm for 1 min to precipitate the white by-products of magnesium pyrophosphate. LAMP products were visualized with the naked eye after adding an intercalating dyes, SYBR Green I (Invitrogen, USA). They were also separated on a 2% agarose gel and visualized on a UV transilluminator.

The PCR reactions were performed in a 25 μl reaction mixture, which contained 1×PCR buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.2 μM of F3 and B3 primers, 2.5 U Taq DNA polymerase (Takara, China) and 1 μl of extracted DNA. PCRs had an initial denaturation step of 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min.

2.5. Optimization of the LAMP assay conditions

An evaluation of the effects of the reaction time (20-60 min), the amplification temperature (57-69°C), different concentrations of MgCl₂ (4-14 mM) and the ratio of outer and inner primers (1:1-1:14) were carried out to optimize the LAMP reaction.

2.6. Specificity and sensitivity of the LAMP reaction

The specificity of the LAMP assay was examined using DNA from T. gondii, Neospora caninum, Giardia lamblia, Cryptosporidium parvum, Eimeria tenella, and Leishmania. A control lacking template was included in each test as
a negative control. The sensitivity of the LAMP assay was tested and compared with PCR using serial 10-fold dilutions of the standard plasmid DNA \((10^0-10^7\) copies) as template. Both LAMP and PCR products were separated on a 2% agarose gel and visualized on a UV transilluminator. Moreover, each LAMP product was visualized after addition of an intercalating dye, (SYBR Green I).

2.7. Repeatability of the LAMP assay

A single technician analyzed a set of dilutions of the standard plasmid DNA \((10^0, 10^1, 10^5, 10^6, 10^9, 10^{10}\) copies) every three or four days, by using the same lot of reagents. The repeatability of LAMP assay were determined by comparing the results of ten replicates.

2.8. Real-time LAMP assay

Real-time LAMP was performed in a thermal cycler (7300, ABI, USA) using the same reaction mixture described above plus SYBR Green I as the intercalation dye. The reactions were subjected to 30 cycles of 65°C for 1 min and 80°C for 10 min.

2.9. Evaluation of the LAMP assay using clinical samples

To evaluate the LAMP assay, 423 blood samples and 380 lymph nodes were collected 10 pig farms in Jilin province of China, and DNA was extracted using the QIAamp DNA Mini Kit. All were tested by conventional PCR and LAMP assay in parallel. The rate for the positive detection of \(T. gondii\) in the samples was calculated.

3. Results
3.1. Optimizing the LAMP assay conditions

The LAMP reaction conditions were optimized by varying the concentration of MgCl₂, primers, amplification temperature and reaction time. The results indicated that a positive amplification could be detected as early as 20 min and negative control showed a smear after 50 min (Fig. 2A). Slightly different yields were observed when the reaction temperature was varied from 57 to 69°C, and the most clear pattern could be obtained from 61 to 65°C (Fig. 2B). The reaction could be carried out when the MgCl₂ concentration is higher than 6mM and the optimal amplification was got at 8mM. A smear was observed in negative controls when the concentration is higher than 10mM (Fig. 2C). Although positive reactions could be obtained using outer and inner primer ratios ranging from 1:1 to 1:12, a more distinct pattern was shown when the ratio was above 1:8 (Fig. 2D).

Considering all, the LAMP assay conditions were optimized in a 25 μl reaction volume as follows: 1×ThermoPol buffer, 8.0 mM MgCl₂, 0.8 M betaine, 1.4 mM dNTPs, 0.2 μM each of outer primer, 1.6 μM each of inner primer and 0.8 μM each of loop primer, 8U of Bst polymerase with 1 μl extracted DNA as template. The amplification was carried out at 65 °C for 30 min.

**Fig. 2 Optimization of the LAMP assay for Toxoplasma gondii**

A: The effect of reaction time; B: The effect of temperature; C: The effect of MgCl₂; D: The effect of ratio of outer and inner primers. Lane M, 100bp ladder marker; Lane N, negative
control; Lane 1, *T. gondii*; Lane 2, standard plasmid. All the results observed by Agarose gel electrophoresis and naked eyes inspection respectively.

3.2 Specificity and sensitivity of the LAMP assay

The LAMP method was found to be highly specific for the *T. gondii* template sequences in tests with the other protozoan genomic DNAs. The green and orange colored products could be visualized after SYBR Green I staining respectively (Fig. 3A), and the results were consistent with those obtained by gel electrophoresis (Fig. 3B). Based on the result of the specificity assay, the primers listed in Table 1 can accomplish a successful and specific amplification.

**Fig. 3 Specificity of the LAMP assay for Toxoplasma gondii**

A: Visual inspection with SYBR Green I staining; B: Agarose gel electrophoresis. Lane M, 100bp ladder marker; Lane N, negative control; Lane 1, *T. gondii*; Lane 2, standard plasmid; Lane 3, *Neospora caninum*.; Lane 4, *Giardia lamblia*; Lane 5, *Cryptosporidium parvum*; Lane 6, *Eimeria tenella*; Lane 7, *Leishmania*

The detection limits for the LAMP and the conventional PCR assay were both 10 copies of the standard plasmid. No amplified products were detected in the negative controls. Thus, the sensitivity of LAMP was the same as the PCR assay (Fig. 4).

**Fig. 4 Comparative sensitivities by PCR and LAMP for the specific detection of**
**Toxoplasma gondii**

A: Visual inspection of LAMP; B: Agarose gel electrophoresis of LAMP; C: The result obtained by PCR. Lane M, 100bp ladder marker; Lane N, negative control; Lanes 1–8, 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 copies, respectively.

### 3.3 Repetitability of the LAMP assay

The correspondence between the different time periods for dilutions of the standard plasmid DNA (10^0, 10^1, 10^5, 10^6, 10^9, 10^10 copies) was 100%, respectively.

### 3.4 Real-time LAMP assay

Setting the threshold at 2×10^4 (Fig.5), we generated a log-linear regression plot by plotting the time-to-threshold values against genomic equivalent copies.

For quantitative analysis, real-time LAMP was found to be satisfactory.

**Fig. 5 Fluorescence curve of the real-time LAMP assay**

N, negative control; 1–8, 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 copies, respectively.

### 3.5 Evaluation of the LAMP assay using clinical samples

The LAMP and conventional PCR assays were applied for detection of *T. gondii* DNA from 423 blood samples and 380 lymph nodes collected from pigs of 10 farms. Positive samples were obtained from 6 pig farms, and the positive rate were 7.8% (33/420) and 8.2% (32/380). However, 6.1%(26/420) and 7.6%...
(29/380) were positive by conventional PCR. All the PCR-positive samples were also positive when tested by LAMP.

4. Discussion

Initial experiments were performed to optimize the assay conditions by using different concentration of MgCl₂, primers, amplification temperature and reaction time. Mg²⁺ affects DNA polymerase activity and primer annealing[26]. The extremely high concentration may lead to false positives because of non-specific amplification, but the concentration of Mg²⁺ should be higher 0.5-3mM when use fluorescent PCR than use the conventional PCR according the SYBR Green I product manual. The optimum concentration of Mg²⁺ in this study is 8mM using the LAMP method which is higher than conventional PCR, that is because SYBR Green I was used in the reaction system. Very slightly different yields were observed when the reaction temperature was varied from 57°C to 69°C. Some paper use 63°C as the LAMP reaction temperature[30], but the LAMP system developed in this study worked well at 65°C, which would be the optimum temperature for Bst DNA polymerase. The results demonstrated that LAMP amplification products could be detected at 20-40 min, so 30min as the middle time point was choosen as the optimum amplification time, otherwise, Long reaction time may lead to the formation of primer dimmers, resulting in false positives.

Serial 10-fold dilutions of the standard plasmid DNA of T. gondii were used to evaluate the sensitivity of the newly established LAMP assay in comparison to
the conventional PCR method. The detection limit of the LAMP assay was 10 copies of the standard plasmid, which was the same as the conventional PCR, but the conventional PCR result is a very faint band on agarose gel. Furthermore, the LAMP result is visible either by agarose gel or visual inspection. It is high sensitivity that the DNA template start from 10 copies when detection the threshold of LAMP by performing LAMP on serial 10-fold dilutions of plasmid DNA. In addition, LAMP simply use a water bath for isothermal amplification and does not require special equipment compared to PCR, and isothermal amplification could greatly shorten the reaction time.

In present study, a LAMP method based on GRA1 gene was established. GRA1 gene is high conserved in T. gondii RH and other virulent strains and it presents in both T. gondii tachyzoite and bradyzoite. GRA1 antigen is not only a diagnostic marker but also as a value vaccine to Toxoplasmosis. The LAMP primers were designed based on a conserved region of the GRA1 gene identified by sequence alignment. Moreover, the LAMP assay employs a set of six primers that recognize a total of eight distinct sequences, These primers only target T. gondii DNA, whereas nontarget DNA of other protozoan (Neospora caninum, Giardia lamblia, Cryptosporidium parvum, Eimeria tenella and Leishmania), insuring high specificity for target amplification. Furthermore, high repeatability was demonstrated by ten replicates of dilutions of the standard plasmid DNA. We developed a real-time LAMP assay to quantitate T. gondii DNA referring to the real-time LAMP method for hepatitis B virus DNA
quantification\(^3\). Using SYBR Green I for real-time detection of the amplified product in a closed-tube environment, the assay can not only avoid false positive results because of contamination, but also apply the widely used real-time quantitative PCR detection system.

In a retrospective study of 131 mothers who had given birth to children infected with *T. gondii*, 50% recalled having eaten uncooked meat\(^2\). A single *T. gondii* infected pig can be a source of infection for many humans, since 1 market weight hog (100 kg or more) can yield over 600 individual servings of meat\(^6, 27\).

The positive rate of the 423 blood samples was 7.8% and of 380 lymph nodes taken from pigs was 8.2%, it is higher than the test using conventional PCR (6.1% and 7.6%), the results suggest the LAMP method is more sensitive than conventional PCR in this study. It also indicated the universality of prevalence of toxoplasmosis on pig farms in Jilin, China, the detection from blood samples suggests it could include the early diagnosis of Toxoplasmosis, But the further epidemiological investigation still needed in the future. Our findings provided valuable information on guiding people to form healthy eating habits.

In conclusion, the LAMP method based on GRA1 is established and optimized, the experiment protocol and the optimized conditions resulted in simple, specific, sensitive and rapid detection of *T. gondii* DNA. The current results indicated that the LAMP method could be used in detection of *T. gondii*. Therefore, the LAMP assay is expected to play an important role in the clinical detection.
Acknowledgments

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References


Fig. 1 The target rejoin of GRA1 for the primers of LAMP assay

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Fig. 4 Comparative sensitivities by PCR and LAMP for the specific detection of *Toxoplasma gondii*

A: Visual inspection of LAMP; B: Agarose gel electrophoresis of LAMP; C: The result obtained by PCR. Lane M, 100bp ladder marker; Lane N, negative control; Lanes 1–8, $10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6$ and $10^7$ copies, respectively

Fig. 5 Fluorescence curve of the real-time LAMP assay

N, negative control; 1–8, $10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6$ and $10^7$ copies, respectively
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>F3</td>
<td>CGGACTTGCTCAAGATCGC</td>
</tr>
<tr>
<td>B3</td>
<td>GCAGGGTTTGCTCCGAATT</td>
</tr>
<tr>
<td>FIP</td>
<td>TCGTCCCTCTGCGATGCTGTTTCA-TCAGATGGATCGTGACAGCGA</td>
</tr>
<tr>
<td>BIP</td>
<td>CTTCTGCTGTTGAAAGGCAG-CCCTCTGCTTGAGCCAC</td>
</tr>
<tr>
<td>LF</td>
<td>CCTCCACGTAACATTGCCGAC</td>
</tr>
<tr>
<td>LB</td>
<td>ACAGTAGAGGAAGCGATCGAGAC</td>
</tr>
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