

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

Lili CAO¹), Ronghua CHENG¹), Lin YAO²), Shuxian YUAN¹) and Xinhua YAO¹)*

¹)Jilin Academy of Animal Husbandry and Veterinary Medicine, Changchun, Jilin 130062, China

²)College of Agriculture, Yanbian University, Yanji, Jilin 133002, China

(Received 30 May 2013/Accepted 6 August 2013/Published online in J-STAGE 20 August 2013)

ABSTRACT. The Loop-mediated isothermal amplification (LAMP) method amplifies DNA with high simply, specificity, sensitivity and rapidity. In this study, A LAMP assay with 6 primers targeting a highly conserved region of the GRA1 gene was 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 node samples from 10 pig farms, and positive results were obtained for 7.8% and 8.2% of samples, respectively. The results showed that the LAMP method is slightly more sensitive than conventional PCR (6.1% and 7.6%). 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 clinical detection of *T. gondii*.

KEY WORDS: detection, real-time fluorogenic LAMP, *Toxoplasma gondii*.

doi: 10.1292/jvms.13-0275; *J. Vet. Med. Sci.* 76(1): 9–14, 2014

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], mainly through peroral infections, blood-stream 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* in pigs is considered an important source of *T. gondii* infection in humans [15]. Prevalences of *T. gondii* infection in fattening pigs have been found to vary from 3.32% to 66.39% in China [28]. The mortality rate can be as high as 60% in piglets in acute infection outbreaks. In short, toxoplasmosis is a large threat to pork consumers and the economic benefits of the pig industry.

Although serological testing is most widely used for the detection of *T. gondii* infections in humans and animals, it may fail to detect anti-*T. gondii* IgG or IgM antibodies in patients suffering from acute infection or who have had an organ transplant or have AIDS [15, 25], and the target of serodiagnosis is the antibody of the pathogen, which can potentially result in 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 due to the need for expensive equipment and reagents [16]. Loop-mediated isothermal amplification (LAMP) is the most recently developed molecular detection method [23] and is known to be a sensitive, easy and fast detection method. LAMP amplifies DNA using a regular laboratory water bath under isothermal conditions and has been developed for the detection of many viral, bacterial, protozoan and fungal diseases [7, 14, 18, 22].

T. gondii dense granule antigen GRA1 is a major excretory-secretory protein [1] that is highly conserved and recognized in humans with chronic toxoplasma infections [24]. The recombinant GRA1 antigen has great value in diagnosis of Toxoplasmosis and vaccine immunology [8, 13, 17]. 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 using a 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.

MATERIALS AND METHODS

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 infection 5–6 days earlier [4]. The genomic DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, Germany) according to the

*CORRESPONDENCE TO: YAO, X., Jilin Academy of Animal Husbandry and Veterinary Medicine, 4510 Xi'an Road, Changchun 130062, China.

e-mail: jlyxh168@163.com

©2014 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/3.0/>>.

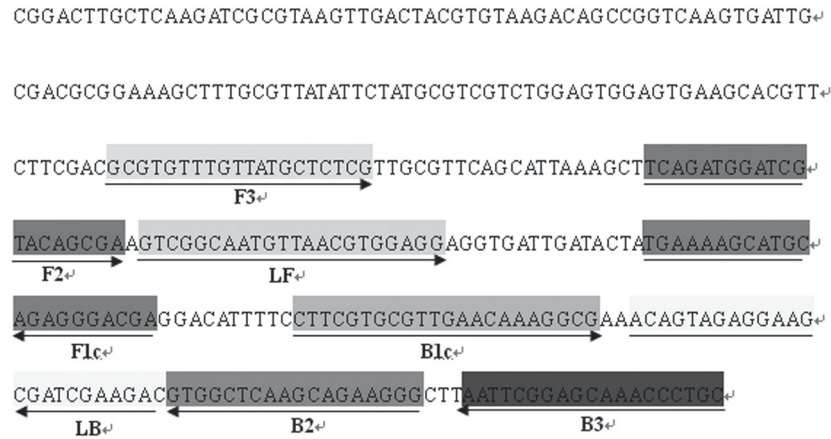


Fig. 1. The target rejoin of GRA1 for the primers of the LAMP assay.

Table 1. Nucleotide sequences of LAMP primers designed in this study

Primer	Sequence (5'–3')
F3	CGGACTTGCTCAAGATCGC
B3	GCAGGGTTTGCTCCGAATT
FIP	TCGTCCCTCTGCATGCTTTTCA-TCAGATGGATCGTACAGCGA
BIP	CTTCGTGCGTTGAACAAAGGCG-CCCTTCTGCTTGAGCCAC
LF	CCTCCACGTAAACATTGCCGAC
LB	ACAGTAGAGGAAGCGATCGAAGAC

manufacturer's instructions.

Designing the LAMP primers: The LAMP primers were designed using PrimerExplorer 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, China).

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, Madison, WI, U.S.A.). After verification by sequencing, the concentration of plasmid was measured in a BioTek Epoch Microplate Spectrophotometer (Epoch, BioTek, Winooski, VT, U.S.A.). The copy number was calculated by the formula: amount (copies/ μ l) = 6×10^{23} (copies/mol) \times concentration (g/ μ l) / MW (g/mol).

LAMP and PCR: The LAMP reaction was carried out in a volume of 25 μ l containing 1 \times ThermoPol buffer (NEB, Ipswich, MA, U.S.A.), 8.0 mM MgCl₂, 0.8 M betaine (Sigma-Aldrich, St. Louis, MO, U.S.A.), 1.4 mM dNTPs, 8 U *Bst* DNA polymerase (NEB), 0.2 μ M of each of the F3 and B3 primers, 1.6 μ M of each of the FIP and BIP primers, 0.8 μ M of each of the LF and LB primers and 1 μ l of extracted DNA as the 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 dye, SYBR Green I (Invitrogen, Carlsbad, CA, U.S.A.). They were also separated on a 2% agarose gel and visualized on a UV trans-illuminator.

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

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) was carried out to optimize the LAMP reaction.

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 a 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 (10⁰–10⁷ copies) as the

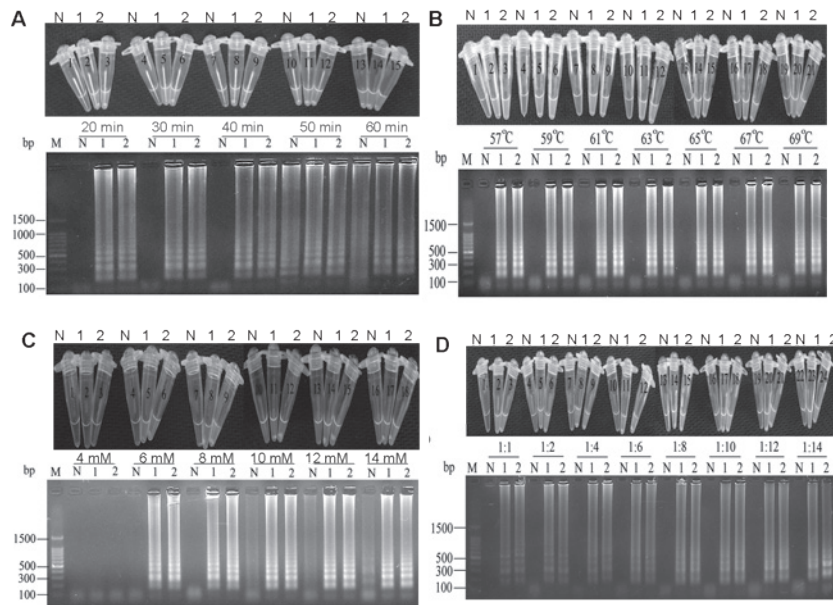


Fig. 2. Optimization of the LAMP assay for *Toxoplasma gondii*. A: The effect of reaction time. B: The effect of temperature. C: The effect of $MgCl_2$. D: The effect of ratio of outer and inner primers. Lane M, 100 bp ladder marker; lane N, negative control; lane 1, *T. gondii*; lane 2, standard plasmid. All the results were observed by agarose gel electrophoresis and naked eyes inspection, respectively.

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).

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 and 10^{10} copies) every 3 or 4 days, by using the same lot of reagents. The repeatability of the LAMP assay was determined by comparing the results of ten replicates.

Real-time LAMP assay: Real-time LAMP was performed in a thermal cycler (ABI 7300, Applied Biosystems, Foster City, CA, U.S.A.) using the same reaction mixture described above plus SYBR Green I as the intercalation dye. The reactions were subjected to 30 cycles of $65^\circ C$ for 1 min and $80^\circ C$ for 10 min.

Evaluation of the LAMP assay using clinical samples: To evaluate the LAMP assay, 423 blood samples and 380 lymph nodes were collected at 10 pig farms in Jilin Province of China, and DNA was extracted using a QIAamp DNA Mini Kit. All were tested by conventional PCR and LAMP assays in parallel. The rate for positive detection of *T. gondii* in the samples was calculated.

RESULTS

Optimizing the LAMP assay conditions: The LAMP reaction conditions were optimized by varying the concentration of $MgCl_2$, primers, amplification temperature and reaction time. The results indicated that positive amplification could

be detected with a reaction time of as little as 20 min and that the negative control showed a smear after 50 min (Fig. 2A). Slightly different yields were observed when the reaction temperature varied from 57 to $69^\circ C$, and the most clear pattern was obtained with a reaction temperature between 61 and $65^\circ C$ (Fig. 2B). The reaction could be carried out when the $MgCl_2$ concentration was higher than 6 mM, and the optimal amplification was obtained at 8 mM. A smear was observed in negative controls when the concentration was higher than 10 mM (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 above, the LAMP assay conditions were optimized in a $25 \mu l$ reaction volume as follows: $1 \times$ ThermoPol buffer, 8.0 mM $MgCl_2$, 0.8 M betaine, 1.4 mM dNTPs, $0.2 \mu M$ each of outer primer, $1.6 \mu M$ each of inner primer and $0.8 \mu M$ each of loop primer and 8U of *Bst* polymerase with $1 \mu l$ extracted DNA as the template. The amplification was carried out at $65^\circ C$ for 30 min.

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 results of the specificity assay, the primers listed in Table 1 can be used to perform a successful and specific amplification.

The detection limits for the LAMP and the conventional

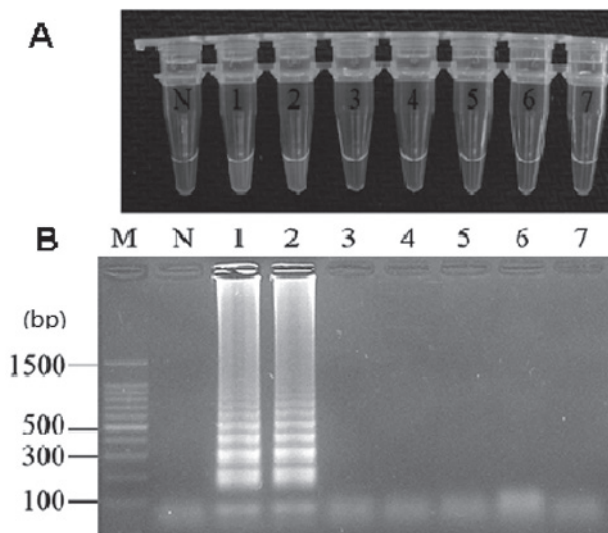


Fig. 3. Specificity of the LAMP assay for *Toxoplasma gondii*. A: Visual inspection with SYBR Green I staining. B: Agarose gel electrophoresis. Lane M, 100 bp ladder marker; lane N, negative control; lane 1, *T. gondii*; lane 2, standard plasmid; lane 3, *Neospora caninum*; lane 4, *Giardia lamblia*;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

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 that of the PCR assay (Fig. 4).

Repeatability 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 and 10^{10} copies) was 100%, respectively.

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.

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 rates were 7.8% (33/420) and 8.2% (32/380). However, 6.1% (26/420) and 7.6% (29/380) of samples were positive by conventional PCR. All the PCR-positive samples were also positive when tested by LAMP.

DISCUSSION

Initial experiments were performed to optimize the assay conditions by using different concentration of $MgCl_2$, primers, amplification temperature and reaction time. Mg^{2+} affects DNA polymerase activity and primer annealing [26]. An extremely high concentration may lead to false positives because of nonspecific amplification, but the concentration

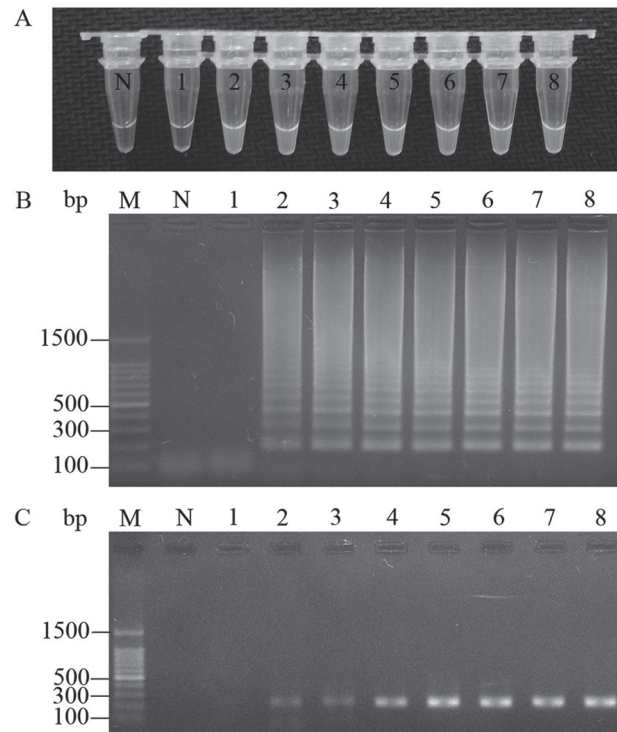


Fig. 4. Comparative sensitivities of PCR and LAMP for the specific detection of *Toxoplasma gondii*. A: Visual inspection of LAMP. B: Agarose gel electrophoresis of LAMP. C: The result obtained by PCR. Lane M, 100 bp ladder marker; lane N, negative control; lanes 1–8, 10^0 , 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 and 10^7 copies, respectively.

of Mg^{2+} should be higher than 0.5–3 mM when used for fluorescent PCR than when used for conventional PCR according to the SYBR Green I product manual. The optimum concentration of Mg^{2+} in this study is 8 mM for the LAMP method, which is higher than that for conventional PCR, and this is because SYBR Green I was used in the reaction system. Very slightly different yields were observed when the reaction temperature varied from 57°C to 69°C. Some papers have used 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 30 min, being the middle time point, was chosen as the optimum amplification time; a long reaction time may lead to the formation of primer dimers, 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 with 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 was a very faint band on agarose gel. Furthermore, the LAMP result is visible either by agarose gel or visual inspection. In addition, LAMP simply uses a water bath for

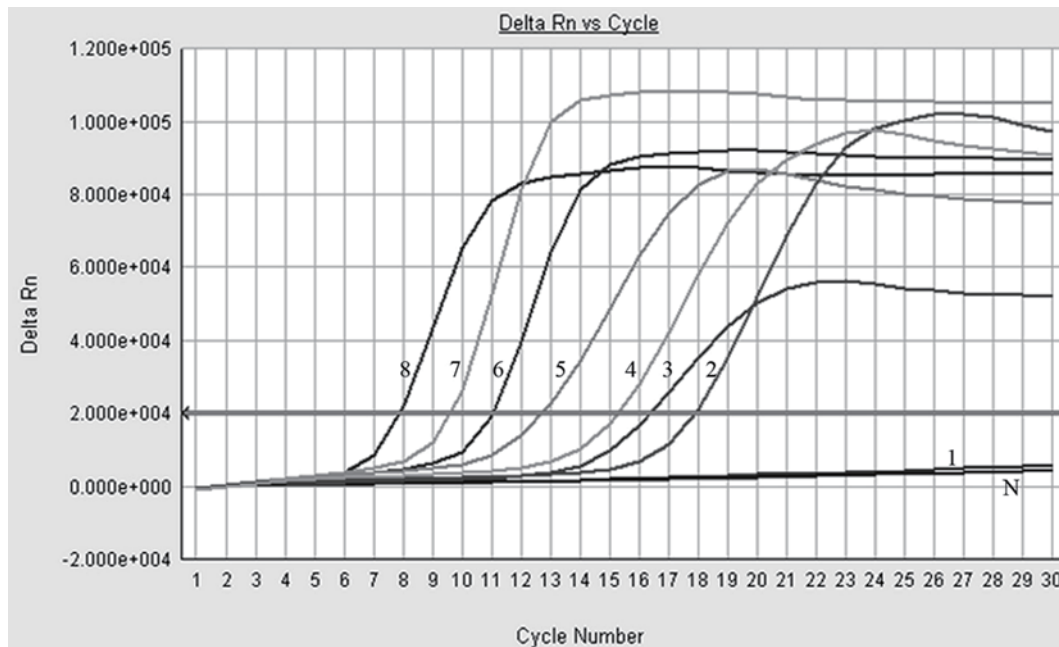


Fig. 5. Fluorescence curve of the real-time LAMP assay. N, negative control; 1–8, 10^0 , 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 and 10^7 copies, respectively.

isothermal amplification and does not require special equipment as compared with PCR, and isothermal amplification can greatly shorten the reaction time.

In present study, a LAMP method based on the GRA1 gene was established. The GRA1 gene is highly conserved in *T. gondii* RH and other virulent strains [12], and it presents in both the *T. gondii* tachyzoite and bradyzoite. GRA1 antigen is not only a diagnostic marker but also has value as a vaccine against 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 6 primers that recognize a total of eight distinct sequences. These primers only target *T. gondii* DNA, whereas nontarget DNA of other protozoa (*Neospora caninum*, *Giardia lamblia*, *Cryptosporidium parvum*, *Eimeria tenella* and *Leishmania*), insuring high specificity for target amplification [10, 20, 21]. 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 due to contamination but can also enable application of 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, it was 8.2%, both of which were higher than the rates when using conventional PCR (6.1% and 7.6%). The results suggest that the LAMP method was more sensitive than conventional PCR in this study. They also indicated the universality of the prevalence of toxoplasmosis on pig farms in Jilin, China, and detection from blood samples suggests that the LAMP method could be used for early diagnosis of Toxoplasmosis; however, further epidemiological investigation is still needed in the future. Our findings provided valuable information that can be used in guiding people towards healthy eating habits.

In conclusion, a LAMP method based on GRA1 was established and optimized, and the experiment protocol and 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 clinical detection of *T. gondii*.

ACKNOWLEDGMENTS. This work was supported by grants from the key projects of the Jilin Provincial Science and Technology Department (No. 20130206025NY) and the National Natural Science Foundation of China (No. 31302076).

REFERENCES

1. Assossou, O., Besson, F., Rouault, J. P., Persat, F., Ferrandiz, J., Mayençon, M., Peyron, F. and Picot, S. 2004. Characterization

- of an excreted/secreted antigen form of 14–3–3 protein in *Toxoplasma gondii* tachyzoites. *FEMS Microbiol. Lett.* **234**: 19–25. [[Medline](#)] [[CrossRef](#)]
2. Boyer, K. M., Holfels, E., Roizen, N., Swisher, C., Mack, D., Remington, J., Withers, S., Meier, P. and McLeod, R. 2005. Risk factors for *Toxoplasma gondii* infection in mothers of infants with congenital toxoplasmosis: Implications for prenatal management and screening. *Am. J. Obstet. Gynecol.* **192**: 564–571. [[Medline](#)] [[CrossRef](#)]
 3. Cai, T., Lou, G., Yang, J., Xu, D. and Meng, Z. 2008. Development and evaluation of real-time loop-mediated isothermal amplification for hepatitis B virus DNA quantification: a new tool for HBV management. *J. Clin. Virol.* **41**: 270–276. [[Medline](#)] [[CrossRef](#)]
 4. Diab, M. R. and El-Bahy, M. M. 2008. *Toxoplasma gondii*: virulence of tachyzoites in serum free media at different temperatures. *Exp. Parasitol.* **118**: 75–79. [[Medline](#)] [[CrossRef](#)]
 5. Du, F., Zhang, Q. L., Yu, Q., Hu, M., Zhou, Y. Q. and Zhao, J. L. 2012. Soil contamination of *Toxoplasma gondii* oocysts in pig farms in central China. *Vet. Parasitol.* **187**: 53–56. [[Medline](#)] [[CrossRef](#)]
 6. Dubey, J. P., Hill, D. E., Rozeboom, D. W., Rajendran, C., Choudhary, S., Ferreira, L. R., Kwok, O. C. H. and Su, C. 2012. High prevalence and genotypes of *Toxoplasma gondii* isolated from organic pigs in northern USA. *Vet. Parasitol.* **188**: 14–18. [[Medline](#)] [[CrossRef](#)]
 7. Endo, S., Komori, T., Ricci, G., Sano, A., Yokoyama, K., Ohori, A., Kamei, K., Franco, M., Miyaji, M. and Nishimura, K. 2004. Detection of gp43 of *Paracoccidioides brasiliensis* by the loop-mediated isothermal amplification (LAMP) method. *FEMS Microbiol. Lett.* **234**: 93–97. [[Medline](#)] [[CrossRef](#)]
 8. Gatkowska, J., Hiszczyńska-Sawicka, E., Kur, J., Holec, L. and Długowska, H. 2006. *Toxoplasma gondii*: an evaluation of diagnostic value of recombinant antigens in a murine model. *Exp. Parasitol.* **114**: 220–227. [[Medline](#)] [[CrossRef](#)]
 9. Hill, D. and Dubey, J. 2002. *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin. Microbiol. Infect.* **8**: 634–640. [[CrossRef](#)]
 10. Iwasaki, M., Yonekawa, T., Otsuka, K., Suzuki, W., Nagamine, K., Hase, T., Tatsumi, K., Horigome, T., Notomi, T. and Kanda, H. 2003. Validation of the loop-mediated isothermal amplification method for single nucleotide polymorphism genotyping with whole blood. *Genome Lett.* **2**: 119–126. [[CrossRef](#)]
 11. Jauregui, L. H., Higgins, J., Zarlenga, D., Dubey, J. P. and Lunney, J. K. 2001. Development of a real-time PCR assay for detection of *Toxoplasma gondii* in pig and mouse tissues. *J. Clin. Microbiol.* **39**: 2065–2071. [[Medline](#)] [[CrossRef](#)]
 12. Jia, X. M., Guo, H., Chen, G. J. and Zheng, H. Q. 1999. Comparison of GRA1 gene amplified *in vitro* from different geographical and heterologous *Toxoplasma gondii* isolates. *Chinese J. Zoonoses* **15**: 7–10.
 13. Kotresha, D. and Noordin, R. 2010. Recombinant proteins in the diagnosis of toxoplasmosis. *APMIS* **118**: 529–542. [[Medline](#)]
 14. Kuboki, N., Inoue, N., Sakurai, T., Cello, F. D., Grab, D. J., Suzuki, H., Sugimoto, C. and Igarashi, I. 2003. Loop-mediated isothermal amplification for detection of African trypanosomes. *J. Clin. Microbiol.* **41**: 5517–5524. [[Medline](#)] [[CrossRef](#)]
 15. Lin, M. H., Chen, T. C., Kuo, T. T., Tseng, C. C. and Tseng, C. P. 2000. Real-time PCR for quantitative detection of *Toxoplasma gondii*. *J. Clin. Microbiol.* **38**: 4121–4125. [[Medline](#)]
 16. Lin, Z. B., Zhang, Y. Y., Zhang, H. S., Zhou, Y. Z., Cao, J. and Zhou, J. L. 2012. Comparison of loop-mediated isothermal amplification (LAMP) and real-time PCR method targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Vet. Parasitol.* **185**: 296–300. [[Medline](#)] [[CrossRef](#)]
 17. Maksimov, P., Zerweck, J., Maksimov, A., Hotop, A., Gross, U., Pleyer, U., Spekker, K., Däubener, W., Werdermann, S., Niederstrasser, O., Petri, E., Mertens, M., Ulrich, R. G., Conraths, F. J. and Schares, G. 2012. Peptide microarray analysis of in silico-predicted epitopes for serological diagnosis of *Toxoplasma gondii* infection in humans. *Clin. Vaccine Immunol.* **19**: 865–874. [[Medline](#)] [[CrossRef](#)]
 18. Maruyama, F., Kenzaka, T., Yamaguchi, N., Tani, K. and Nasu, M. 2003. Detection of bacteria carrying the stx2 gene by *in situ* loop-mediated isothermal amplification. *Appl. Environ. Microbiol.* **69**: 5023–5028. [[Medline](#)] [[CrossRef](#)]
 19. Müller, N., Zimmermann, V., Hentrich, B. and Gottstein, B. 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* Infection by PCR and DNA Hybridization Immunoassay. *J. Clin. Microbiol.* **34**: 2850–2852. [[Medline](#)]
 20. Nagamine, K., Hase, T. and Notomi, T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell. Probes* **16**: 223–229. [[Medline](#)] [[CrossRef](#)]
 21. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* **28**: E63. [[Medline](#)] [[CrossRef](#)]
 22. Okafuji, T., Yoshida, N., Fujino, M., Motegi, Y., Ihara, T., Ota, Y., Notomi, T. and Nakayama, T. 2005. Rapid diagnostic method for detection of mumps virus genome by loop-mediated isothermal amplification. *J. Clin. Microbiol.* **43**: 1625–1631. [[Medline](#)] [[CrossRef](#)]
 23. Parida, M., Sannarangaiah, S., Dash, P. K., Rao, P. V. L. and Morita, K. 2008. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev. Med. Virol.* **18**: 407–421. [[Medline](#)] [[CrossRef](#)]
 24. Pietkiewicz, H., Hiszczyńska-Sawicka, E., Kur, J., Petersen, E., Nielsen, H. V., Paul, M., Stankiewicz, M. and Myjak, P. 2007. Usefulness of *Toxoplasma gondii* recombinant antigens (GRA1, GRA7 and SAG1) in an immunoglobulin G avidity test for the serodiagnosis of toxoplasmosis. *Parasitol. Res.* **100**: 333–337. [[Medline](#)] [[CrossRef](#)]
 25. Porter, S. B. and Sande, M. A. 1992. Toxoplasmosis of the central nervous system in the acquired immunodeficiency syndrome. *New Engl. J. Med.* **327**: 1643–1648. [[Medline](#)] [[CrossRef](#)]
 26. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487–491. [[Medline](#)] [[CrossRef](#)]
 27. Tao, Q., Wang, Z. S., Feng, H. H., Fang, R., Nie, H., Hu, M., Zhou, Y. Q. and Zhao, J. L. 2011. Seroprevalence and risk factors for *Toxoplasma gondii* infection on pig farms in Central China. *J. Parasitol.* **97**: 262–264. [[Medline](#)] [[CrossRef](#)]
 28. Yu, L., Zhu, X. Y., Hu, Y. S., Zhang, J. L., Luo, Q. L. and Shen, J. L. 2010. Establishment and application on a sensitive PCR assay for detection of *Toxoplasma gondii* in retail pork. *Chinese J. Zoonoses* **26**: 895–899.
 29. Zhang, H., Thekisoe, O. M., Aboge, G. O., Kyan, H., Yamagishi, J., Inoue, N., Nishikawa, Y., Zakimi, S. and Xuan, X. 2009. *Toxoplasma gondii*: sensitive and rapid detection of infection by loop-mediated isothermal amplification (LAMP) method. *Exp. Parasitol.* **122**: 47–50. [[Medline](#)] [[CrossRef](#)]
 30. Zhou, P., Chen, Z. G., Li, H. L., Zheng, H. H., He, S. Y., Lin, R. Q. and Zhu, X. Q. 2011. *Toxoplasma gondii* infection in humans in China. *Parasite. Vector* **4**: 165. [[CrossRef](#)]