

Detection of Acute Toxoplasmosis in Pigs Using Loop-Mediated Isothermal Amplification and Quantitative PCR

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Abstract: A loop-mediated isothermal amplification (LAMP) assay allows rapid diagnosis of *Toxoplasma gondii* infection. In the present study, the LAMP assay was evaluated using blood from both naturally and experimentally infected pigs. The sensitivity of the LAMP assay was compared with that of Q-PCR. Both assays detected *T. gondii* in the blood of experimentally infected pigs, with 100% agreement. In infected blood samples, the parasite was detected as early as 2 days post-infection and reached a peak in 3-5 days. In 216 field serum samples, the detection rates of LAMP and Q-PCR assays were 6.9% and 7.8%, respectively. This result indicates that the sensitivity of the LAMP assay was slightly lower than that of the Q-PCR assay. However, the LAMP may be an attractive diagnostic method in conditions where sophisticated and expensive equipment is unavailable. This assay could be a powerful supplement to current diagnostic methods.

Key words: *Toxoplasma gondii*, loop-mediated isothermal amplification (LAMP), Q-PCR, pig

Toxoplasma gondii is an obligate intracellular parasite that causes toxoplasmosis and is capable of infecting a variety of mammals and birds. *T. gondii* is an important foodborne parasite whose main route of transmission from animals to humans is through the consumption of infected meat [1]. In some countries, pork is the most common meat consumed, and some ethnic groups consume raw pork; thus, pigs are considered the primary source of human infections with *T. gondii* [2]. In addition, due to gross lesions in infected animals leading to their being condemned at slaughter, expenses associated with treatment, and weight loss associated with clinical diseases, toxoplasmosis is a source of significant economic loss for swine farmers. Therefore, the establishment of a rapid, highly specific, and accurate method for diagnosis of *T. gondii* infection is essential to administer appropriate treatment and reduce economic losses.

The diagnosis of toxoplasmosis is based on detection of specific antibodies in serum samples using serological assays, isolation of the parasite by mouse bioassay, or amplification of

parasite DNA from biological samples by PCR [3,4]. Classical serology methods cannot differentiate between vaccine-induced and infection-induced antibodies [5] and between past and present infections [6]. Although the definitive method of diagnosis of *T. gondii* infection is isolation of the pathogen from infected tissues, this method is labor-intensive, time-consuming and expensive, and it relies upon the submission of fresh material to the diagnostic laboratory. For these reasons, it is generally impractical in diagnostic situations. PCR (such as Q-PCR) is a useful diagnostic tool to detect the presence of infection indirectly [7]. Despite these advances, PCR methods are still limited in some regions where sophisticated and expensive equipment is unavailable.

Loop-mediated isothermal amplification (LAMP), which was originally developed by Notomi et al. [8], is a very sensitive, easy, and less time-consuming method. The advantage of this assay is that LAMP products can easily be detected by the naked eye due to the formation of magnesium pyrophosphate, a turbid white by-product of DNA amplification that accumulates as the reaction progresses [9]. LAMP products can also be detected by direct fluorescence [10]. Fluorescent dyes, such as ethidium bromide, SYBR green, and Evagreen, can also be used for visualization of LAMP products under UV light [11]. LAMP can amplify different types of samples, including purified DNA, heat-treated blood, and blood dried on filter paper [12,13]. In

• Received 28 April 2013, revised 10 July 2013, accepted 14 July 2013.

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addition, Thekisoe et al. [14] have reported that LAMP reagents are relatively stable even when stored at 25 or 37°C, which supports the use of LAMP in field conditions and resource poor laboratories.

Recently, this method was found to be a powerful diagnostic tool, and LAMP assays targeting the SAG1 gene (SAG1-LAMP), 529-bp repetitive element, B1 gene, SAG2 gene, and TgOWP of *T. gondii* were developed [6,15-19]. The LAMP based on the 529-bp repetitive element was shown to be useful for detection of *T. gondii* DNA extracted from veterinary samples [6,18]. Kong et al. [15] also reported that the LAMP based on the 529-bp repetitive element was able to detect *T. gondii* DNA in mouse blood samples. The SAG1-LAMP assay was applied to detect the presence of *T. gondii* in infected mouse organs. This indicates that the method is attractive for *T. gondii* detection in biopsy specimens [16]. LAMP assays based on SAG1, SAG2, and B1 genes were shown to be useful for detection of *T. gondii* DNA extracted from blood samples in humans [17]. Sotiriadou and Karanis [19] reported that a LAMP amplification method targeting the TgOWP and B1 genes is among the most accurate molecular methods as a specific, sensitive, simple, and rapid diagnostic tool for the detection of *T. gondii* in water samples.

Early diagnosis of toxoplasmosis is essential for a successful treatment. However, we know very little about the effectiveness of SAG1-LAMP for detection of acute toxoplasmosis in pigs. Therefore, the aim of this study was to utilize both LAMP and Q-PCR protocols to obtain data through amplification of *T. gondii* DNA from routine blood samples from the pig.

For this study, 15 healthy pigs (7-9-week-old), which were *Toxoplasma* seronegative as determined by ELISA, were purchased from a pig farm in Lanzhou, China. The pigs in this study were raised on a conventional farm or in experimental isolation units. The experimental protocol was approved by the Ethical Committee of the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China.

Each pig was challenged intraperitoneally with 10^7 tachyzoites of the highly virulent Gansu Jingtai strain (GJS) of *T. gon-*

dii. Blood samples were collected by armpit bleeding at days 2, 3, 4, 5, 6, 7, 10, 14, 21, 28, and 35 post-infection (PI) from 15 pigs. DNA from the blood samples for the LAMP and Q-PCR assays was extracted using the Universal Genomic DNA Extraction kit (TaKaRa Biotechnology Co., Ltd, Dalian, China) according to the manufacturer's protocol. DNA from healthy pig blood was extracted in parallel with the experimental samples and used as the negative control. The blood samples collected from each pig on day 5 PI were also used for mouse bioassay. Each sample was inoculated with a volume of 0.5 ml subcutaneously into 5 mice (25-30 g). Impression smears of the lung tissue from the mice that died were stained with Giemsa and examined microscopically. The brain of each mouse that survived 60 days after inoculation was examined microscopically for *T. gondii* tissue cysts. Parasites were successfully isolated in all of the blood samples collected on day 5 PI by mouse bioassay. The results were shown in Table 1.

For the LAMP assay targeting the SAG1 gene primers [17], B3 (GTAGCAGGACCTTTCGCG), F3 (GCGCAACGAAGACTGTTGA), BIP (TGGCAAGGAATGCACAGACTCGGACCTTAGCTGTCAAGACCG), and FIP (CGCCACTGGGACTGATGGTTAGTGCACCCTCCAGTGGTTC) were used. The LAMP assay was performed as previously described by Lau corrected as Lau et al. [17]. The LAMP reaction was performed in a total volume of 25 µl containing 2 µl of the extracted DNA, 40 pmol (each) of primers FIP and BIP, 5 pmol (each) of primers B3 and F3, 1 µl of Bst DNA polymerase (NEB) in 2.5 µl of buffer (20 mM Tris-HCl [pH 8.8], 10 mM KCl, 8 mM MgSO₄, 10 mM [NH₄]₂SO₄, 0.1% Tween 20), 0.8 M betaine (Sigma, St. Louis, Missouri, USA), and 1.4 mM deoxynucleoside triphosphate (dNTP). The LAMP reaction was incubated for 60 min at 65°C and inactivated for 2 min at 80°C. The resulting amplicons were detected by visual observations after addition of SYBR green I and analyzed by 1.5% agarose gel electrophoresis, followed by ethidium bromide staining and photography.

SYBR Green I-based Q-PCR targeting the SAG1 gene of *T. gondii* was carried out as previously described by Wang [20],

Table 1. Detection of *T. gondii* in blood from experimentally infected pigs by LAMP and Q-PCR

Assays	Blood samples collected from 15 pigs at different time points (day PI) ^a											Control samples ^b
	2	3	4	5 ^c	6	7	10	14	21	28	35	
LAMP	5/15	14/15	15/15	15/15	13/15	10/15	8/15	6/15	3/15	1/15	0/15	0/15
O-PCR	5/15	14/15	15/15	15/15	13/15	10/15	8/15	6/15	3/15	1/15	0/15	0/15

^aThe results are expressed as the number of positive samples/tested.

^bBlood samples collected before infection were used as control samples.

^cAll the blood samples collected on days 5 post infection were positive by mouse bioassay.

using the primers 5'-GCCTCATCGGTCGTC AATAA-3' and 5'-CTCCATCTTTCCCGCACAC-3'. The Q-PCR reactions were performed in a 25- μ l volume containing 2 \times SYBR Premix Ex Taq II supplemented with ROX II, primers (10 μ M) and 2 μ l of DNA template. The cycling parameters were to preheat at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 64°C for 20 sec. After amplification, the data were analyzed using the 7500 System software.

The sensitivity of the LAMP assay in comparison to the Q-PCR assay was determined using the genomic DNA of *T. gondii* GJS tachyzoites, which was diluted to contain an amount of DNA equivalent to a range of 0.01-10,000,000 tachyzoites per LAMP and Q-PCR reaction. As shown in Fig. 1, the detection limit for the LAMP assay was 100 fg, corresponding to 1 *T. gondii* tachyzoite. For the Q-PCR assay, the slope was -3.370, with an $R^2=0.995$ and a reaction efficiency of 98%. The standard curve generated using 10-fold dilutions of the *T. gondii* tachyzoite DNA was linear over 8 orders of magnitude (10 to 10⁹ fg), indicating that accurate measurement of target DNA over a large concentration range is possible with Q-PCR. The detection limit was found to be 10 fg, corresponding to 0.1 *T. gondii* tachyzoite (Fig. 2). These results indicated that the sensitivity of the LAMP assay was slightly lower than that of the Q-PCR method for detection of *T. gondii* DNA. Lin et al. [18] previously reported that the relative sensitivity of the LAMP assay

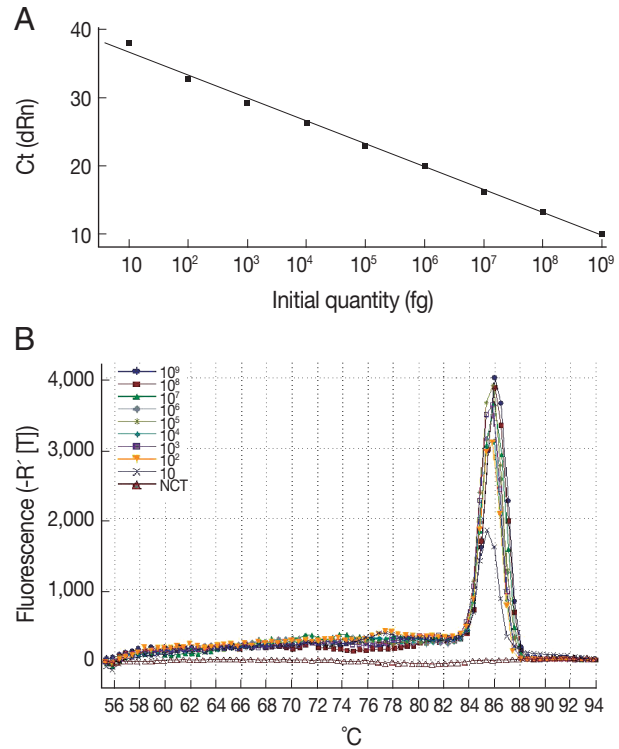


Fig. 2. Sensitivity and specificity of the Q-PCR assay for *T. gondii*. (A) Standard curve of Q-PCR for *T. gondii*. (B) Dissociation curve of Q-PCR for detecting *T. gondii*. The assay standard curve was generated from a 10-fold serial dilution of a known concentration of *T. gondii* tachyzoite genomic DNA (equivalent to a range of 0.1-10,000,000 tachyzoites per Q-PCR reaction).

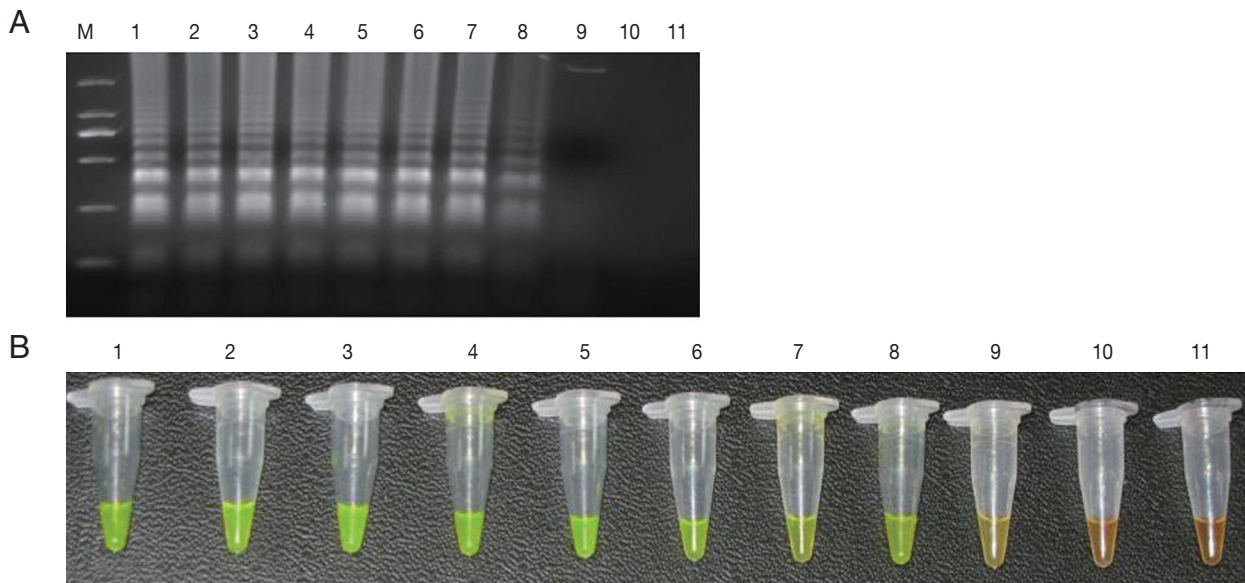


Fig. 1. Sensitivity of the LAMP assay using agarose gel electrophoresis (A) and SYBR Green I stain (B). M: DNA marker; 1-10 are the reaction results from a 10-fold serial dilution of *T. gondii* tachyzoite genomic DNA (equivalent to a range of 0.01-10,000,000 tachyzoites per LAMP reaction). 11: negative control.

targeting a 529-bp repetitive element was slightly lower than that of the Q-PCR method, which is consistent with our results.

To evaluate the efficiency of the LAMP assay for detecting *T. gondii* in blood, it was tested using the blood samples from experimentally infected pigs. All DNA samples were tested using both the LAMP and Q-PCR assays. Parasites were detected in most of the blood samples collected from days 2 to 14 PI, with a peak on days 3 to 5 PI (Table 1). Parasites could be detected in a few samples collected on days 21 and 35 PI. There was 100% agreement between the results of the LAMP and Q-PCR assays. In addition, the positive samples by mouse bioassay were also positive by LAMP, which suggested that LAMP is reliable for detection of *T. gondii* infection. In this study, the SAG1 gene was detected using these 2 assays in most of the blood samples from animals with clinical signs of disease. It is likely that tachyzoites express distinct sets of surface antigens, which may contribute to an immune evasion mechanism that allows *T. gondii* to persist [21]. The SAG1 gene may have potential for providing molecular information regarding the actual state of infection.

In addition, both the LAMP and Q-PCR assays were also used to test 216 field blood samples collected from Gansu province. Fifteen samples were positive by both the LAMP and Q-PCR assays. Two samples were negative according to LAMP but positive according to Q-PCR. This discrepancy was attributed to a lower level of *T. gondii* DNA, which fell below the limit of detection of the LAMP assay. The detection rates of the LAMP and Q-PCR assays in field blood samples were 6.9% (15/216) and 7.9% (17/216), respectively. This result indicated the sensitivity of the LAMP assay by visual inspection using SYBR Green I stain was slightly lower than that of the Q-PCR assay. It is important to take into consideration that Q-PCR is time-consuming and requires a thermal cycler with real-time monitoring and expensive data analysis systems. The LAMP assay has clear advantages over the Q-PCR assay, as it is a practical system that can be used in a standard diagnostic laboratory, particularly in developing countries where the disease is prevalent. In addition, previous reports have demonstrated that the sensitivity of the LAMP method is higher than the conventional PCR or nested PCR methods for detection of *T. gondii* [6,17].

In conclusion, although discrepancies between the LAMP and Q-PCR results occur in a minority of samples, the LAMP method can be applied for detection of *T. gondii* parasites for both experiment infected and field-infected blood samples from pig. Particularly, LAMP may be an attractive diagnostic

method in conditions where sophisticated and expensive equipment is not available.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Special Research Programs for Non-profit Trades (Agriculture) (200903036-02) and NBCITS, MOA (CARS-38). This investigation was also supported by National Non-profit Institute Research Grant (1610322012026). We thank Prof. Wanxiang Xv for providing the field samples.

Conflict of interest

We have no conflict of interest related with this study.

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