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Short Communication

Development and application of loop-mediated isothermal amplification assays based on ITS-1 for rapid detection of *Toxoplasma gondii* in pork

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ABSTRACT

The loop-mediated isothermal amplification (LAMP) assay is a novel method that rapidly amplifies DNA with high specificity and sensitivity under isothermal conditions. In this study, we established a LAMP assay with six primers targeting a highly conserved region of *Toxoplasma gondii* ITS-1 sequence. The amplification protocol completes within 30 min under isothermal condition in a 65 °C water bath while specificity tests confirmed no cross-reactivity with DNA templates of *Neospora caninum*, *Eimeria tenella*, *Cryptosporidium parvum*, *Listeria monocytogenes* and *Streptococcus suis*. The detection limit of the LAMP assay was 0.9 fg *T. gondii* genomic DNA, a sensitivity that was 10-fold higher than that of a conventional PCR assay. Both LAMP assay and conventional PCR were applied to detect *T. gondii* genomic DNA in 118 diaphragm samples obtained from pig farms in Zhejiang Province, China. Our results showed that the LAMP assay is more sensitive than conventional PCR (13.56% and 9.32%). The LAMP assay established in this study provides a simple, specific, sensitive and rapid method of *T. gondii* genomic DNA detection, hence is expected to plays an important role in the monitoring of *T. gondii* contamination in various food products.

1. Introduction

Toxoplasma gondii is an agent of toxoplasmosis, a lethal threat to immunocompromised individuals (Dubey, 2004). Among several transmission routes, peroral infectious is the major means caused by the intake of uncooked, infected meat (Zhang et al., 2009). As the main meat source of China, pork possesses an important source of *T. gondii* infection in humans (Tao et al., 2011). The prevalence of *T. gondii* infection in fatting pigs has been reported to vary from 24.5% in central (Tao et al., 2011) to 58.1% in southern China (Zhou et al., 2010), while that in small farms in Zhejiang Province can reach as high as 71.4% (Yu et al., 2011). Therefore, it is of crucial importance to devise means to guarantee pork quality for both consumers safety and farms profits.

Serological and molecular tests are conventional methods to detect *T. gondii* infections. For example, enzyme-linked immunosorbent assay (ELISA) has been widely used to detect toxoplasmosis among humans and domestic animals (Yu et al., 2011; Dubey et al., 2012). However, ELISA may fail to detect IgG or IgM during the active phase period of *T. gondii* infections (Lin et al., 2000) and to differentiate between previous and current infections (Yu et al., 2013). In recent years, polymerase chain reaction (PCR) and real-time PCR strategies have been developed successfully for *T. gondii* detection (Masala et al., 2007; Yu et al., 2013), however the requirement of expensive equipment for PCR methods hinders practical application in

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Table 1
Nucleotide sequence of LAMP and conventional PCR primers for ITS-1 of *Toxoplasma gondii* designed in this study.

Molecular assay	Target region	Sequence (5′–3′)	Length of primers	Amplification size
LAMP	ITS-1 (X75429.1) 6349–6547	F3: CTGAAGAAAGCCTCGCAGAA	20 bp	199 bp
		B3: ATTCGACGCAGTATGACCG	19 bp	
		FIP: GACGTGTACGACCCACCATGACTTTTAGCTTTTACTACCGCCTTGG	46 bp	
		BIP: TGAGGAATGTGACGCCAACGATTTTACAGCGAGGATAATCGCTCT	46 bp	
		LF: AACATGGCTGCGTCTCCCC	19 bp	
		LB: TGCACACTTTTAGATGGGCAC	21 bp	
PCR	ITS-1 (X75429.1) 6349–6547	F3: CTGAAGAAAGCCTCGCAGAA	20 bp	199 bp
		B3: ATTCGACGCAGTATGACCG	19 bp	

domestic pig farms. The loop-mediated isothermal amplification (LAMP) assay is a novel method that allows rapid DNA amplification with high specificity and sensitivity under isothermal conditions (Notomi et al., 2000) and has been reported to plays an important role for the diagnosis of many pathogens including *T. gondii* (Sotiriadou and Karanis, 2008), *Giardia lamblia* (Plutzer and Karanis, 2009) and *Shewanella putrefaciens* (Li et al., 2012). The LAMP reaction performs auto-cycling strand displacement DNA synthesis using *Bst* DNA polymerase and a set of six specific primers that recognize eight distinct regions of the target sequence (Parida et al., 2008). This method performs well in a simply regular laboratory water bath. These characteristics make LAMP a fast, concise and effective nucleic acid amplification methodology.

The internal transcribed spacer 1 (ITS-1) region of nuclear ribosomal DNA (rDNA) lies in between the 18S and 5.8S rRNA gene and has been developed as a genetic marker for species specific detection among various pathogens (Li et al., 2005, 2012). In this study we used LAMP assay targeting the ITS-1 region to detect *T. gondii* in diaphragm samples obtained from pig slaughterhouses. In addition, we compared the performance of the LAMP assay against conventional PCR assay for *T. gondii* detection in diaphragm samples.

2. Materials and methods

2.1. Origin of T. gondii genomic DNA

Tachyzoites of *T. gondii* RH strain were intraperitoneally inoculated into BALB/c mice and the peritoneal fluid was harvested after 3–5 days. Parasites were washed twice in phosphate-buffered saline (PBS) and centrifuged at 1000 × *g* for 5 min as previously described (McLeod et al., 1984). The genomic DNA of *T. gondii* was then extracted using a Universal Genomic DNA Extraction Kit (TaKaRa, China) according to manufacturer's instructions.

BALB/c mice used as experimental animals were treated according to the recommendations in the Guide for the Regulation for the Administration of Affairs concerning Experimental Animals of the People's Republic of China. Animal experiments were approved by Zhejiang University Experimental Animal Ethics Committee (Permit Number: ZJU201308-1-10-072).

2.2. Pork samples collection and DNA extraction

A total of 118 diaphragm samples were obtained from 118 individual pigs from a slaughterhouse in Jinhua, Zhejiang province, China. Genomic DNA was extracted from 1 g pork sample each using a Universal Genomic DNA Extraction Kit (TaKaRa, China) according to manufacturer's instructions. Purified DNA was dissolved in 50 μl of double-distilled water for subsequent PCR and LAMP assays.

2.3. Conventional PCR and LAMP reaction

LAMP primers targeted to a highly conserved sequence in the ITS-1 region (GenBank accession number X75429.1) were designed with the online software Primer explorer V4 (Eiken Chemical Co., 2011) to amplify a fragment about 200 bp. LAMP primers include a set of inner primers (FIP and BIP), outer primers (F3 and B3) and loop primers (LF and LB, to accelerate reaction) (Notomi et al., 2000). All primers used in this study were designed with T_m values for optimum efficiency and listed in Table 1. After optimization, the LAMP assay was carried out in a 25 μl reaction volume as follows: 8 U/μl Bst DNA polymerase (NEB, USA), 2× ThermoPol reaction buffer (NEB, USA), 0.8 M Betaine (Sigma, USA), 6.0 mM $MgCl_2$, 1.4 mM dNTP mixture, 40 pmol each of the primers FIP and BIP, 5 pmol each of primers F3 and B3, 20 pmol each of primers LF and LB, and l μl of template DNA. The reaction mixture was incubated at 65 °C for 30 min and then placed in an ice-bath for 10 min to terminate the reaction. According to Wang et al. (2012), the optimized condition of conventional PCR reaction was as follows: 2.5 μl 10× PCR buffer, 0.25 U/μl Taq DNA polymerase (TaKaRa, China), 2.5 mM dNTP mixture, 0.2 μM of each PCR primer and 1 μl of template DNA, 17.25 μl sterile distilled water. Amplification was performed at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, primer annealing at 61 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Sterile water was included in each test as the negative control.

2.4. Specificity and sensitivity of the LAMP reaction

The specificity of the LAMP assay was examined by using DNA templates from *T. gondii*, *Neospora caninum*, *Eimeria tenella*, *Cryptosporidium parvum*, *Listeria monocytogenes* and *Streptococcus suis* kept in our laboratory. Distilled

water was included as negative control in each test. Purified genomic DNA of *T. gondii* tachyzoites was 10-fold serially diluted with deionized water to concentrations ranging from 9 ng to 0.09 fg. Subsequently, the sensitivity of the LAMP assay was determined and compared with that of conventional PCR. Both LAMP and PCR products were separated on a 1.5% agarose gel and visualized on a UV transilluminator. In addition 1 μl SYBR green I dye (100×) (Invitrogen, USA) was added to each tube to allow visualization of LAMP amplifications by color change.

2.5. Application of LAMP assays for testing pork samples

The genomic DNA of 118 diaphragm samples obtained from a pig slaughterhouse was extracted as mentioned previously. All samples were subjected to LAMP assay and conventional PCR. The frequency of positive detection of *T. gondii* in the samples were then calculated and compared.

3. Results and discussion

When SYBR green I was added to the LAMP products, the reaction colors would turn into either green or orange representing positive or negative amplification, respectively. Results of color inspection of LAMP products were consistent with those obtained by gel electrophoresis where a fragment could be seen in green product while orange reaction yielded no band. DNA templates from *T. gondii*, *N. caninum*, *E. tenella*, *C. parvum*, *L. monocytogenes*, and *S. suis* were used to evaluate the specificity of the established LAMP assay. Our results (Fig. 1A and B) indicated that only the template from *T. gondii* was amplified suggesting that there are no cross-reactions and supporting that the assay we optimized provides accurate detection. The sensitivity of any detection method is a crucial factor especially when parasitic load is low. The detection limits for the LAMP and conventional PCR assay were determined to be 9 fg and 90 fg of *T. gondii* genomic DNA, respectively (Fig. 2A and B). These findings were in accordance with that reported by Kong et al. (2012) in targeting a 529 bp repeat element. We also found that the LAMP assay we established offers higher sensitivity than that based on SAG1 gene carried out by Wang et al. (2013), with a detection limit of 100 fg of *T. gondii* genomic DNA. In addition, compared against PCR method, LAMP can be assessed by either agarose gel electrophoresis or visual inspection after a short incubation in a water bath.

Table 2
Comparative results of conventional PCR and LAMP assay for the detection of *Toxoplasma gondii* from diaphragm samples of pigs collected from a slaughterhouse in Jinhua, Zhejiang Province.

Test method	Diaphragm samples (total no. 118)	
	Positive (%)	Negative (%)
Conventional PCR	11 (9.32)	107 (90.68)
LAMP	16 (13.56)	102 (86.44)

When using diaphragm samples of pigs, higher positive rates were detected by LAMP (13.56%, 16/118) than by PCR (9.32%, 11/118), as shown in Table 2. Our results suggest

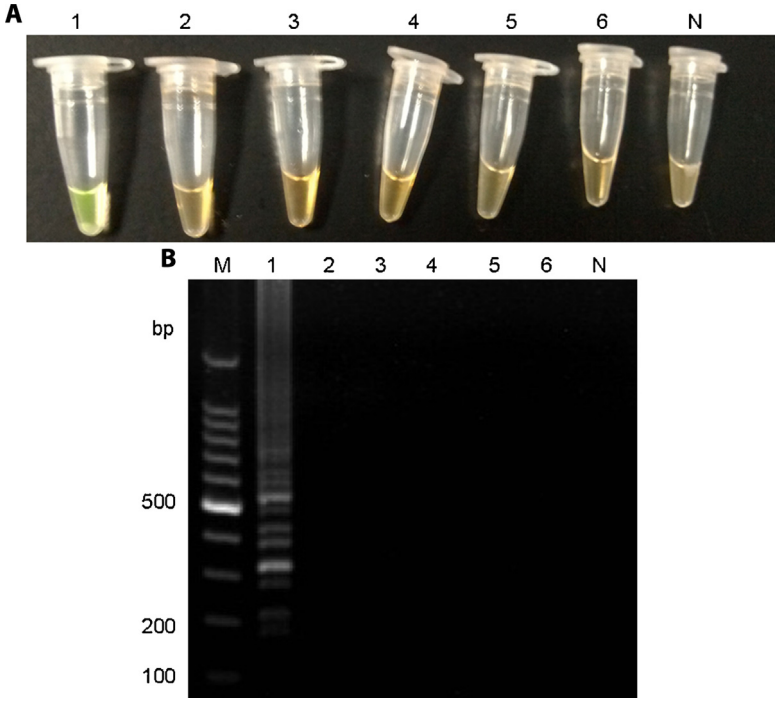

Fig. 1. Specificity of the LAMP assay for *Toxoplasma gondii*. (A) Visual inspection of the *Toxoplasma gondii* LAMP products with SYBR Green I staining. Tube 1, *Toxoplasma gondii*; tube 2, *Neospora caninum*; tube 3, *Eimeria tenella*; tube 4, *Cryptosporidium parvum*; tube 5, *Listeria monocytogenes*; tube 6, *Streptococcus suis*, tube N, negative control. (B) Agarose gel electrophoresis of the *Toxoplasma gondii* LAMP products. Lane M, DNA ladder marker; lane 1, *Toxoplasma gondii*; lane 2, *Neospora caninum*; lane 3, *Eimeria tenella*; lane 4, *Cryptosporidium parvum*; lane 5, *Listeria monocytogenes*; lane 6, *Streptococcus suis*; lane N, negative control.

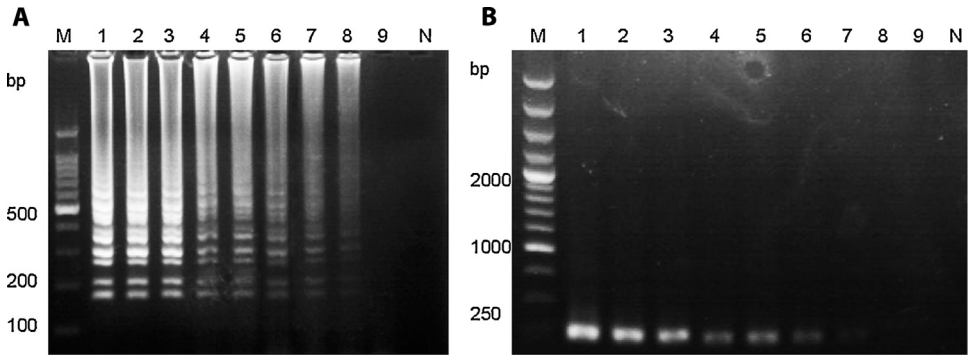

Fig. 2. Comparative sensitivities by LAMP (A) and conventional PCR (B) for the specific detection of *Toxoplasma gondii* tachyzoites DNA based on ITS-1 amplification. Lane M, DNA ladder marker; lanes 1–8 represents 9 ng, 900 pg, 90 pg, 9 pg, 900 fg, 90 fg, 9 fg, 0.9 fg, 0.09 fg of *Toxoplasma gondii* DNA, respectively; lane N, negative control.

that LAMP method exhibits higher sensitivity than conventional PCR where the latter may give rise to false negative results.

Our preliminary LAMP results pointed out the prevalence of toxoplasmosis in pig farms in Jinhua, Zhejiang Province, China. A large-scale epidemiological investigation should be followed up to control toxoplasmosis and ensure public health.

In conclusion, a rapid, simple, sensitive and specific LAMP method based on the ITS-1 sequence of *T. gondii* genomic DNA detection was established and optimized. Our current results show that this LAMP assay can be used as a reliable and portable diagnostic tool of *T. gondii* to enable inspection and control of infected livestock. Therefore, this assay will facilitate clinical and epidemiological investigations and play an important role in guaranteeing meat quality and safety.

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