Exploration of real-time fluorescence loop-mediated isothermal amplification (LAMP) assay for detection of *Isospora suis* oocysts

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Introduction

Swine coccidiosis is mainly caused by I. suis parasitizing in the jejunum and ileum. The delivery room contaminated by I. suis oocysts is the principal pathway for transmission from one generation to the next generation of piglets. The environmental conditions of pig farms, clinical treatment, and management level are strongly associated with the occurrence of swine coccidiosis. Swine coccidiosis often seriously affects the economic interests of farmers.

The most common method for detecting I. suis is a traditional microscopy-based examination of fecal oocysts. However, it is difficult for diagnosis because of the small size of coccidia and many impurities in feces. In 2000, loop-mediated isothermal amplification (LAMP) was invented by Notomi et al., who reported a simple, convenient, time-saving technology for in vitro nucleic acid amplification. It is highly sensitive and specific, amplifying at a constant temperature. Compared to conventional PCR, LAMP is faster (only 30-60 min for an entire amplification), more simple (DNA to be amplified at a single constant temperature), and more sensitive (greater than conventional PCR with 2-5 orders of magnitude). Coupled with the variety of LAMP detection methods, the technology is developing rapidly and has become one of the promising diagnostic methods. In this study, based on the 18S rRNA gene sequence of I. suis, a specific primer set was designed and the LAMP assay was established, which provides a more sensitive and specific approach for the detection of I. suis.

Materials and Methods

Fresh stool samples of piglets were taken. The oocysts were collected by the saturated brine flotation method, and then were identified using microscopy （400×）based on oocysts morphology and sporulation time. Oocysts morphologies were measured with Leica Microsystems (Leica DM2000). The oocysts were purified by Sheather's sugar gradient centrifugation.

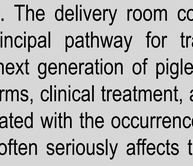


Fig.1 Sporulation and non sporulated oocysts（400×）

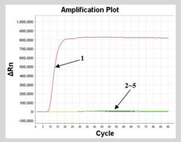
DNA extraction from I. suis oocysts was performed using repeated thawing and refreezing in combination with the Stool Genomic DNA Rapid Extraction Kit. A total of 6 primer sets for LAMP were designed from the 18S rRNA gene sequence of I. suis using the LAMP primer design software, Primer Explorer version 4 (Eiken Chemical Co., Ltd, Tokyo, Japan). Primers were synthesized and purified with PAGE by Sangon Biotech (Shanghai, China). After experimental verification, one primer set which was confirmed to be effective, was purified by HPLC for further use (Fig. 2).

Fig. 2 Inner primer FIP (F2 - F1c) and BIP (B2 - B1c), Outer Primer F3 and B3, loop primer LoopF and LoopB within the position where I.suis 18S rRNA

The real-time fluorescence LAMP assays were performed using a DNA amplification kit from Guangzhou DIAO according to the manufacturer's instructions. The reaction system (25 μL) contained 12.5 μL 2× reaction buffer, 8mM Mg2+, 1.6mM dNTPs, 2 μM inner primer (FIP/BIP), 0.2 μM outer primer (F3/B3), 0.8 μM loop primer (LoopF/ LoopB), 8U Bst DNA polymerase, 0.5 μL fluorescent dye, 1 M glycine betaine, and 1-100 ng DNA template. LAMP reactions were carried out at 63° C for 1 h using a real-time fluorescence quantification PCR instrument. Fluorescent signal was detected every 60 s in FAM channel, and the melting curve analysis was performed by ramping the temperature from 63° C to 95° C at 0.2° C/s transition rate.

Results

The results showed that the specific amplification of DNA from I. suis (Fig. 3) and the detection limit was 1 fg/μL (2.74×102 copies/μL) (Fig. 4). The detection limit of PCR was 3.37×104 copies/μL, converted to plasmid DNA concentration of 123 fg/μL (Fig. 5). The oocyst detection limits of LAMP assay and conventional PCR showed that the minimum detection limit was 5 oocysts (Fig. 6), lower than that of 13 oocysts of conventional PCR (Fig. 7).



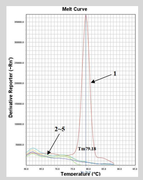


Fig.3 The specificity test and melt curve analysis of LAMP. DNA templates 1, I. suis; 2, E. mitis; 3, T. gondii ; 4, E. coli; 5, Negative control.

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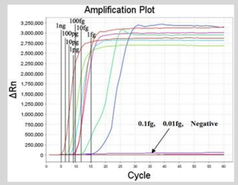

Fig.4 The sensitivity test of LAMP. LAMP assays were performed using 1 ng/μL, 100 pg/μL, 10 pg/μL, 1pg/μL, 100 fg/μL, 10 fg/μL, 1 fg/μL, 0.1fg/μL and 0.01 fg/μL of the template DNA. Sterile ultrapure water instead of the template DNA served as a negative control.

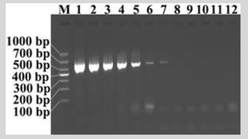

Fig.5 The sensitivity test of conventional PCR. Lanes 1-11, a 10-fold dilution series of the recombinant DNA from 3.37×1010 copies/μL to 3.37×100 copies/μL; lane 12, negative control; M, DL1000 DNA Marker.

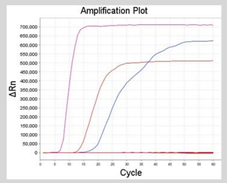

Fig.6 Determination of oocyst detection limits for LAMP assay. The LAMP assays were performed using the template DNA extracted from 1, 3, 5, 7 and 10 single oocysts from clinical stool samples, or sterile ultrapure water as a negative control.

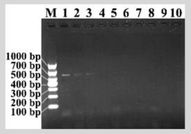

Fig.7 Determination of oocyst detection limits for conventional PCR. The conventional PCR was performed using the template DNA extracted from single clinical oocysts. Lanes 1-9 represent DNA from 20, 15, 13, 11, 9, 7, 5, 3 and 1 oocyst, respectively. Lane 10, negative control. M, DL1000 DNA Marker.

Conclusion

In this study, the LAMP technology in combination with real-time fluorescence quantification PCR instrument were used to establish a LAMP assay to detect the 18S rRNA gene sequence of *I. suis*. The fluorescent signals were continuously recorded and analyzed. The sensitivity tests were performed and detection limits were compared between the LAMP assay and conventional PCR. When using the recombinant plasmid DNA, the sensitivity of LAMP assay was about 100 times higher than that of conventional PCR, with a detection limit of 2.74×102 copies/μL (1 fg/μL) and 3.37×104 copies/μL (123fg /μL), respectively. When using DNA samples from a certain number of clinical oocysts, the sensitivity of LAMP assay was about 2 times higher than that of conventional PCR, with a detection limit of 5 oocysts and 13 oocysts, respectively. The study provides a valuable way for molecular detection of *I. suis*.

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Acknowledgements

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