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Research Brief

Loop-mediated isothermal amplification (LAMP) assays for the detection of *Theileria annulata* infection in China targeting the 18S rRNA and ITS sequences

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ABSTRACT

We have developed two loop-mediated isothermal amplification (LAMP) assays for the detection of *Theileria annulata*, an economically important cattle disease in China that occurs in subtropical and tropical areas. These assays target the ribosomal RNA (18S rRNA) and ITS LAMP sequences. The primer set for each gene target consists of four primers, and each set recognizes six distinct regions on the target gene to allow for the highly specific detection of *T. annulata*. The specific ladder bands were amplified from the autologous genomic DNA of four Chinese-laboratory-preserved standard *T. annulata* stocks, and there were no cross-reactions with the genomic DNA of normal bovine blood and other protozoan species. The LAMP assays were sufficiently sensitive to detect 0.1 pg/μl of genomic DNA. Furthermore, DNA extracted from blood collected from cattle experimentally infected with *T. annulata* (18–105 days post-infection) was amplified, demonstrating the high sensitivity of these primers. Of the 351 field samples collected from China, 24.5% were positively detected by two LAMP primers, and 18.2% were found to be positive for *T. annulata* infection by PCR. These results indicate that the LAMP assay could be a potential diagnostic tool for epidemiological studies of *T. annulata* infection in China.

1. Introduction

Theileria annulata, the causative agent of bovine tropical theileriosis, causes morbidity and loss in productivity in indigenous cattle. This disease especially affects imported high-grade cattle, in which theileriosis is a severe and often lethal disease. The disease is distributed over a wide geographical area, ranging from the Mediterranean littoral regions of Europe and Africa through the Middle East to India and China (Dolan, 1989). In China, it is the most virulent species of four reported bovine *Theileria* species and is mainly distributed in semi-dry and desert grasslands in Northern China, including 13 provinces (Luo and Lu, 1997).

Field diagnosis is normally achieved by observing clinical signs in infected animals. Theileriosis caused by *T. annulata* is indicated by a fever, enlarged lymph nodes, and is associated with tick vector infestation. In addition, acute disease with a high mortality rate on farms without effective tick control may also indicate theileriosis. The disease can be confirmed by finding *Theileria* parasites in Giemsa-stained blood smears and lymph node fine needle aspirate smears (OIE, 2008). This method is useful in the detection of acute cases but has limited value in carrier cases, where low numbers of erythrocytes remain infected with piroplasms. In addition, there have been numerous diagnostic assays developed for the detection of *T. annulata* infections, mainly intended for use in research, including vaccine and epidemiological studies. These assays include serological assays, such as the indirect fluorescence antibody test (IFAT; Billiouw et al., 2005), the indirect ELISA (Seitzer et al., 2007) and the cELISA (Renneker et al., 2008). IFAT is knowingly restricted due to a subjective interpretation, low throughput and inherent cross-reactivity problems (Billiouw et al., 2005). An ELISA that uses a defined recombinant *T. annulata* antigen appears to perform better than IFAT and shows good sensitivity and specificity (Seitzer et al., 2008; Renneker et al., 2008). However, serological assays are unable to differentiate between current and past infections due to the persistence of antibodies.

The PCR and reverse line blot (RLB) diagnostic assays have been developed for the sensitive and specific detection of *T. annulata*-infected cattle, including the detection of very low levels of the parasite in carrier animals (d'Oliveira et al., 1995; Jian et al., 2008). However, these techniques require laboratories equipped to perform tests, thus increasing application costs. In addition, well-trained personnel are needed to perform these tests; therefore, they are not widely used in laboratories with poor resources.

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Loop-mediated isothermal amplification (LAMP) is a simple technique that amplifies DNA with a high sensitivity and rapidity under isothermal conditions (Notomi et al., 2000). It can also amplify different types of templates, including purified genomic DNA, heat-treated blood and blood dried on filter paper (Kuboki et al., 2003; Poon et al., 2006). In addition, LAMP reagents are relatively stable even when stored at temperatures between 25 and 37 °C, which support their use in field conditions and resource-poor laboratories (Thekisoe et al., 2009).

Salih et al. (2008) reported a specific LAMP assay for the diagnosis of tropical theileriosis caused by *T. annulata*. In the present study, we developed two LAMP assays for the specific detection of *T. annulata* in China targeting the ribosomal RNA (18S rRNA) and (ITS) genes and evaluated their specificity and sensitivity in the blood samples collected from experimentally and naturally infected cattle. These assays are suitable to investigate the distribution of tropical theileriosis caused by *T. annulata* in China.

2. Materials and methods

2.1. Parasite isolates and DNA extraction

A total of 351 field samples were collected from cattle in six provinces in Northern China: Gansu, Henan, Hebei, Jilin, Heilongjiang and Xinjiang provinces. In addition, four Chinese-laboratory-preserved standard stocks, named *T. annulata* Inner Mongolia, *T. annulata* Xinjiang, *T. annulata* Ningxia and *T. annulata* Henan were used in this study. These stocks are maintained in our laboratory by blood passages in splenectomized calves and their classification status has been previously established biologically and phylogenetically (Dou et al., 1989; Liu et al., 2009). Genomic DNA from the above mentioned samples was prepared using a genomic DNA extraction kit (Gentra, Germany). DNA samples of *T. sergenti*, *T. sinensis*, *Babesia bovis*, *B. bigemina*, *B. ovate*, *B. major*, *B. U.* sp. and *A. marginale* collected from the infected cattle blood were used as heterologous negative controls. The amount of DNA was assessed by spectrophotometry and diluted to 100 ng/μl, which served as a template for the test.

2.2. Specific primers for LAMP and PCR

The sequences of 18S rRNA and ITS genes of *T. annulata* Inner mongolia (EU083801; EF547927), *T. annulata* Xinjiang (EU08379; EF547928), *T. sergenti* Liaoyang (EU083802; EF547929), *T. sergenti* Ningxian (EU083803; EF547930), *T. sinensis* Weiyuan (EU277003; EF547931), *T. sinensis* Lintan (EU274472; EF547931), *B. bovis* Shannxian (AY603398; EF547925), *B. bigemina* Kunming (AY603402; EF547924), *B. ovata* Wenchuan (AY603403; EF422218), *B. major* Yili (AY603399; EF422220) and *B. U.* sp. Kashi AY726556 were used for reference in this study. For each species listed, the first accession number is the 18S rRNA gene and the second is the ITS gene. To obtain the specific primer for the LAMP and PCR assays, multiple sequence alignments were performed using the Clustal V program algorithm in the MegAlign component of the DNASTAR program (Version 4.01 DNASTAR, Madision, Wis.). The specific primers were designed using PrimerExplorer V2 and Primer5.0 software for LAMP and conventional PCR, respectively, in the variable regions of the 18S rRNA and ITS gene sequences. The oligonucleotide sequences of the primers are shown in Table 1.

2.3. LAMP, conventional PCR procedures and PCR verification of target length

The LAMP reaction was conducted as described previously (Notomi et al., 2000; Guan et al., 2008). The reaction mixture was incubated at 60 °C for 50 min using a conventional heating block (Stuart Scientific, Staffordshire, UK) and then heated at 80 °C for 2 min to terminate the reaction. LAMP product was subjected to electrophoresis on a 2.0% agarose gel in a Tris–acetic acid-EDTA (TAE) buffer at 75 V for 1 h and visualized under UV light after staining with ethidium bromide.

A conventional PCR assay was developed based on the ITS sequence of *T. annulata* Inner Mongolia. The PCR was performed according to a previously described method (Liu et al., 2009). The primary PCR cycle parameters used were: an initial step at 96 °C for 3 min, 30 cycles at 96 °C for 1 min, 53 °C for 40 s and 72 °C for 1 min with a final extension step at 72 °C for 5 min. Finally, the PCR products were visualized by UV transillumination in a 1.5% agarose gel following electrophoresis and staining with ethidium bromide.

The outer LAMP primer pairs, designated F3 and B3, from 18S rRNA and ITS genes, respectively, were used for PCR amplification to verify whether the correct target was amplified. The primers were applied in a concentration of 10 pmol and the annealing temperature was set at 53 °C and 52 °C for 30 cycles, respectively. The PCR products were analyzed on a 1.5% agarose gel as described above. The PCR products were subjected to cloning and sequencing experiments.

2.4. Evaluation of LAMP using the samples from experimentally infected cattle

One intact (non-splenectomized) cow was infected with *T. annulata* Inner Mongolia and the animal experiments were performed according to the approved Institutional Animal Care and Use Committee guidelines. Disease monitoring lasting 5 months was accomplished by taking rectal temperatures and performing microscopic examination of Giemsa-stained blood smears for the presence of piroplasms. The monitoring was performed once every two days for 11 weeks and then once every week for a period of 22 weeks. Jugular vein blood was also collected in anticoagulant tubes for extracting parasite DNA.

3. Results and discussion

3.1. Detection and confirmation of LAMP product

To establish the standard protocol for the LAMP method, different reaction temperatures and incubation times were used. The reaction could successfully take place in temperatures ranging from 60 to 65 °C (data not shown), and 60 °C was chosen as the reaction temperature for all the following applications. The incubation time was varied from 30 to 60 min at 60 °C. Products were detectable after 30 min, and 50 min was chosen as the reaction time for the experiments that followed. Thus, the optimal temperature and time of 18S rRNA and ITS LAMP primer set were determined to be 60 °C and 50 min. The positive LAMP reaction produced a characteristic ladder of multiple bands on a 2% agarose gel. Similar results were observed in PCR with B3 and F3 primers, where single bands of 247 and 223 bp were identified for 18S rRNA and ITS sequences, respectively. The sequencing analysis of the PCR product confirmed that the specific gene segment was amplified (data not shown).

3.2. The specificity and sensitivity of LAMP and conventional PCR

In the present study, we used the DNA samples from *T. sergenti*, *T. sinensis*, *B. bovis*, *B. Bigemina*, *B. ovata*, *B. major*, *B. U.* sp. and *A. marginale* to evaluate the specificity of LAMP and PCR. DNA samples extracted from piroplasm-free cattle and distilled water was