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Loop-mediated isothermal amplification (LAMP) method based on two species-specific primer sets for the rapid identification of Chinese *Babesia bovis* and *B. bigemina*

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A B S T R A C T

Bovine babesiosis is a tick-transmitted hemoprotozoan disease that is mainly caused by *Babesia bovis* and/or *Babesia bigemina* and is characterized by significant morbidity and mortality worldwide. This disease is widespread in most parts of China. However, it is difficult to rapidly discriminate between the *B. bovis* and *B. bigemina* species. To detect and distinguish these species, a loop-mediated isothermal amplification (LAMP) platform that targets specific sequences of the internal transcribed spacer (ITS) genes was developed. Specificity testing revealed that there was no cross-reaction with the other tick-borne parasites *B. ovate*, *B. major*, unnamed bovine *Babesia*, *Theileria annulata*, *Theileria sinensis*, *Theileria sergenti*, and *Anaplasma marginale*, or with bovine white blood cells. The sensitivity of the LAMP method was 0.1 pg DNA for both *B. bovis* and *B. bigemina*, which was superior to that of the classical PCR methods. This assay was evaluated for its diagnostic utility using blood samples collected from experimentally and naturally infected cattle in China. These findings indicate that the *Babesia* species-specific LAMP assay may have potential clinical application in the detection and differentiation of *Babesia* species, particularly in countries in which babesiosis is endemic.

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1. Introduction

Bovine babesiosis is mainly caused by the tick-borne apicomplexan parasites *Babesia bovis*, *Babesia bigemina* and *Babesia divergens*. Historically, five bovine *Babesia* species have been reported in China, namely, *B. bovis*, *B. bigemina*, *B. ovate*, *B. major* and unnamed bovine *Babesia*; of these, *B. bovis* and *B. bigemina* are considered to be the most pathogenic [9]. The geographic distribution of the *Babesia* parasites corresponds with that of their tick vectors [6], mainly *Rhiphicephalus (Boophilus) microplus* and *Rhiphicephalus (Boophilus) annulatus*, in tropical and subtropical regions worldwide [4]. *B. bovis* and *B. bigemina* are also the most economically important species worldwide. The economic losses from *Babesia* infections are incurred not only through mortality, loss of milk/meat production, and the cost of control measures but also through their impact on the international cattle trade [2,17]. The most common clinical symptoms caused by the bovine intraerythrocytic protozoan parasites *B. bovis* and *B. bigemina* are associated with acute disease; in infected cattle, symptoms include fever, hemolytic anemia, anorexia, lethargy, hemoglobinuria, tachycardia and icterus. Generally, the disease caused by *B. bovis* is more severe than that caused by *B. bigemina* [2,19], and mixed infections typically occur in the same cattle. Cattle that have recovered from acute infection become asymptomatic carriers and serve as a reservoir for transmission [2]. Thus, the detection of carrier animals and differential diagnosis between *B. bovis* and *B. bigemina* infections are of fundamental importance and provide useful information for disease management and control strategies [5]. Acute infections are typically diagnosed by microscopic examination of blood smears, whereas subclinical infections should be identified serologically [21]. Differential diagnosis of *B. bovis* and *B. bigemina* infections can lead to a better understanding of the epidemiology of these parasites, and knowledge of their species-specific distribution in the field would provide useful information for controlling these diseases [5].

The efficiency of the polymerase chain reaction (PCR) method in the epidemiological study of babesiosis has been verified by several investigators in work that demonstrates its high sensitivity and specificity for this purpose [1,8,18]. However, for economic and practical reasons, PCR-based methods are not always available for laboratory diagnosis. In particular, automated and/or real-time quantitative thermocyclers are required, but these instruments are not always affordable. Furthermore, the cyclers function erratically when exposed to high ambient temperatures and humidity and/or dusty environments. Therefore, for the diagnosis of early and advanced bovine babesiosis, the development of cost-effective, simple, and rapid DNA

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amplification methods is needed. Loop-mediated isothermal amplification (LAMP) may provide the answer. This method is expected to amplify 10^9 copies in less than 1 h; it provides high specificity because four primers are used for the detection of six distinct sequences [16]. Most importantly, LAMP assays do not require expensive or complicated equipment; an ordinary water bath or heating block is sufficient. Direct visual identification can also be performed using SYBR Green I under an ultraviolet lamp.

In the present study, a species-specific LAMP method was developed for the detection and differentiation of *B. bovis* and *B. bigemina*, which are pathogenic to cattle in China. The specificity and sensitivity of the tests were evaluated with LAMP and PCR assays, and the feasibility of the use of the technology for experimentally infected cattle is discussed. In addition, we investigated the species-specific distribution of *B. bovis* and *B. bigemina* in cattle in different provinces of China using the LAMP method and discuss the utility of the LAMP method for large-scale epidemiological studies of bovine babesiosis.

2. Materials and methods

2.1. Collection of blood samples

Two 1-year-old cattle, free of *B. bovis* and *B. bigemina* infections as confirmed by microscopic examination and PCR [8], were used for experimental infection with *B. bovis* and *B. bigemina*. The *B. bovis* Shannxian strain and the *B. bigemina* Kunming strain, which were previously isolated from the Henan and Yunnan provinces of China, respectively, and preserved in liquid nitrogen as a ‘gold standard’ positive control [8], were used to infect the cattle. Each animal was subcutaneously injected with 10 ml of *B. bovis*- and *B. bigemina*-infected blood (percentage of parasitized erythrocytes, PPE, 5%). Blood samples were collected each day for 7 days and then every 2 days for 9 weeks post-infection and subjected to LAMP and PCR assays. Additionally, blood films were taken from the ear vein, fixed with methanol, stained with Giemsa and were examined for the presence of hemoprotozoan parasites.

Blood samples were also collected from 383 randomly selected cattle from eight provinces in China. The blood samples were collected in EDTA and were either used immediately or stored at −20 °C until use.

Blood samples from animals infected with *B. bovis* (Shannxian strain) and *B. bigemina* (Kunming strain) [8] were used as ‘gold standard’ positive controls. Blood samples from animals infected with *B. ovate*, *B. major*, unnamed bovine *Babesia*, [10–12], *Theileria annulata*, *Theileria sinensis*, *Theileria sergenti* and *Anaplasma marginale* were used as negative controls.

2.2. Purification of Babesia spp. merozoites

B. bovis (Shannxian) and *B. bigemina* (Kunming) merozoites were prepared from blood collected at peak parasitemia from experimentally infected splenectomized cattle. The infected blood was centrifuged at 1000 *g* for 10 min at 4 °C; the packed cells were washed three times with 10 mM Tris–HCl (pH 7.4), 150 mM NaCl (Tris–saline) by centrifugation as before, and the buffy coat was removed. A cellulose powder column (CF-11, Whatman) was used to remove the remaining leukocytes. The packed infected erythrocytes were suspended in 1 volume of Tris–saline and incubated in a 37 °C water bath for 5 min. AH-1 hemolysin (final concentration 300 HU/ml) was added to lyse the erythrocytes, and the sample was incubated in a 37 °C water bath for 10 min. Lysis was terminated by the addition of 0.01 volume of 500 mM EDTA followed by centrifugation at 1000 *g* for 10 min at 4 °C to remove cellular debris and intact erythrocytes. The supernatant was recovered and further centrifuged at 4000 *g* for 15 min at 4 °C to pellet the merozoites. The pelleted merozoites were washed with Tris–saline by further centrifugation until they are almost free of hemoglobin and stored at −70 °C.

2.3. Genomic DNA preparation

Total DNA of *B. bovis* (Shannxian) and *B. bigemina* (Kunming) were prepared from purified merozoites using the Puregene DNA Purification Kit (www.gentra.com) according to the manufacturer's instructions such that DNA samples could be considered free of cattle genomic DNA. Control piroplasm DNA of *B. ovate*, *B. major*, unnamed bovine *Babesia*, *T. annulata*, *T. sinensis* and *T. sergenti* were also prepared from purified merozoites and stored at −70 °C. Formerly *A. marginale* and experimentally infected intact bovine DNA were directly extracted from the anticoagulated blood of infected cattle. Three hundred and eighty-three blood samples were collected from cattle in different provinces between April and June 2011. For all of the above-mentioned samples, genomic DNA was extracted from 300 μl of blood using the QIAamp blood and tissue extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Aqueous DNA preparations were frozen at −70 °C until further analysis.

2.4. LAMP primer design

Primers specific for *B. bovis*, Lushi strain (GenBank EF547926) and *B. bigemina*, Kunming strain (GenBank EF547924) were designed. These primers are directed against the species-specific internal transcribed space (ITS) sequences that occur in variable regions of the ITS sequence and were designed using the PrimerExplorer V2 and Primer5.0 software for LAMP. The PCR primers were used as described [8]. The oligonucleotide sequences of the LAMP primers are shown in Table 1.

2.5. LAMP and PCR reaction conditions

The LAMP reaction was performed in a final volume of 25 μl. The reaction contained 12.5 μl of 2× LAMP reaction buffer [20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, and 0.2% Tween 20], 125 μM each deoxynucleoside triphosphate, 0.8 M betaine (Sigma, Deisenhofen, Germany), 8 U of Bst DNA polymerase large fragment (New England Biolabs, Frankfurt, Germany), 40 pmol each FIP and BIP primer, 5 pmol each F3 and B3 primer and 2 μl of target DNA. The mixture was incubated at 60 °C for 40 min (*B. bovis*) or 65 °C for 55 min (*B. bigemina*) using a conventional heating block (Stuart Scientific, Chelmsford, UK) and then heated at 80 °C for 2 min to terminate the reaction.

A conventional PCR assay was developed based on the ITS sequences of *B. bovis* (Shanxian) and *B. bigemina* (Kunming). PCR was performed according to a previously described method [8]. The primary PCR cycle parameters used were: heating at 96 °C for 3 min, 35 cycles of 96 °C for 1 min, 56 °C for 40 s (*B. bovis*) or 54 °C for 50 s (*B. bigemina*) and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The

Table 1
LAMP primers for *B. bovis* and *B. bigemina* detection.

Parasite	Primer	Sequence
B. bovis	FIP	GCGTTGCTAGTAGTGGCACCGGAATTCCA GCTTCCACCCAACGAG
	BIP	GCTACCCTAGTAGCCGGTTGGGGAATTCG AGCTTAACCCGGGTCGT
	F3	CACTAGCACCACACCAGTG
	B3	CAAAAGGGGGTGCATCTCG
B. bigemina	FIP	CAGGATTGGGGGCTCACTGAAAGAATTCGT AACAAACACACCGCCTCT
	BIP	GGCCCCGGCCCATTTATAACGGAATTCAGG AGCACGGACACATTCA
	F3	ACTTGCAGACTTCTGCGATT
	B3	AGAAATTGGGGCGACAAGG

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