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Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine *Babesia* parasites

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Abstract

A loop-mediated isothermal amplification (LAMP) technique has been used as a novel nucleic acid detection method, whereby the target DNA can be amplified with high specificity and sensitivity under an isothermal condition using a set of four specific primers. In this study, we designed two sets of the LAMP primers for rhoptry-associated protein-1 genes of *Babesia bovis* and *B. bigemina*, in which a restriction enzyme cleavage site was inserted into two pairs of species-specific primers to construct a multiplex LAMP (mLAMP) method by combining these two sets totaling eight primers. The mLAMP method was distinguishable between *B. bovis* and *B. bigemina*, simultaneously, due to the subsequent restriction enzyme analysis. The sensitivities of the mLAMP method were 10^3 and 10^5 times higher on the detection limits for *B. bovis* and *B. bigemina*, respectively, than those of the classical PCR methods. Of 40 blood samples collected from cattle living in Ghana, 12 and 27% were positively detected by the mLAMP for *B. bovis* and *B. bigemina*, respectively. Furthermore, 14 and 23% of 90 blood samples from cattle in Zambia showed mLAMP-positive reactions to *B. bovis* and *B. bigemina*, respectively. These findings indicate that this mLAMP method is a new convenient tool for simultaneous detection of the bovine *Babesia* parasites.
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Keywords: *Babesia bovis*; *Babesia bigemina*; LAMP; Multiplex; Restriction enzyme

1. Introduction

Bovine babesiosis is an economically important tick-borne disease of cattle in tropical and subtropical regions of the world (McCosker, 1981). The disease is mainly caused by two bovine intraerythrocytic protozoan parasites, *Babesia bovis* and *Babesia bigemina*. Although the clinical signs induced by these parasites are similar, as characterized by fever, anemia, and icterus in the infected cattle (de Vos and Potgieter, 1994), generally, the disease caused by *B. bovis* is more severe than that by *B. bigemina* (Ristic, 1981.). Acute infections are usually diagnosed by a microscopic examination of blood smears, whereas subclinical infections should be identified serologically (Weiland and Reiter, 1988.). Differential diagnosis between the *B. bovis* and *B. bigemina* infections leads to a better understanding of their epidemiology, and the species-specific distribution in the field would provide useful information to control the diseases (de Vos and Potgieter, 1994).

The efficiency of the polymerase chain reaction (PCR) method has been verified on the epidemiological study of babesiosis by several investigators because of its high sensitivity and specificity (Almeria et al., 2001; Fahrimal et al., 1992; Figueroa et al., 1993; Oliveira-Sequeira et al., 2005; Smeenk et al., 2000). However, this method has not always been adapted for laboratory diagnosis for economic and practical reasons. Especially, automated and/or real-time quantitative thermal

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cyclers are required for the PCR methods, but they are not always affordable. Furthermore, the cyclers work erratically due to high ambient temperatures and humidity and/or dusty environments. Therefore, the development of cost-effective, simple, and rapid DNA amplification methods is needed for the diagnosis of early and advanced bovine babesiosis.

A loop-mediated isothermal amplification (LAMP) method allows a whole reaction process at an isothermal condition and finally makes it possible to easily detect the LAMP-amplified product due to a visual confirmation of the reacted tube (Notomi et al., 2000). Furthermore, since four LAMP-specific primers are designed to recognize six distinct regions on the target gene, the reacted DNA can be amplified with high specificity (Notomi et al., 2000). Since the LAMP method does not require any complicated equipment, it may provide a cost-effective, simple, and rapid DNA amplification method.

The aim of this study was to develop a new diagnostic method based on the LAMP technique for the simultaneous detection of *B. bovis* and *B. bigemina*. In this study, we designed two sets of each of the four specific LAMP primers for *B. bovis* and *B. bigemina*, in which a restriction enzyme cleavage site was inserted into two pairs of the species-specific primers to construct a multiplex LAMP (mLAMP) method by combining these two sets of total eight primers. The mLAMP method was constructed to distinguish between *B. bovis* and *B. bigemina* after the subsequent restriction enzyme analysis. In addition, the sensitivity of the mLAMP method was compared to those of conventional PCR and nested PCR methods. Finally, we investigated the species-specific distribution in cattle and/or wild animals living in Ghana and Zambia by using the mLAMP method and discussed the utility of the mLAMP method for a large-scale epidemiological study of bovine babesiosis.

2. Materials and methods

2.1. Parasites

The Texas strain of *B. bovis* (Hines et al., 1992) and the Argentine strain of *B. bigemina* (Hotzel et al., 1997) were maintained in purified bovine red blood cells (RBC) with a microaerophilic stationary-phase culture system (Avarzed et al., 1997). Medium M199 (Sigma-Aldrich, Tokyo, Japan) was supplemented with 40% normal bovine serum to prepare the culture medium for the parasites.

2.2. DNA extraction

B. bovis- and *B. bigemina*-infected RBC were washed three times with cold phosphate-buffered saline (PBS) by centrifuging at 1000×g for 5 min at 4 °C and resuspended in PBS. The infected RBC were serially diluted 10-fold with normal RBC to adjust the parasite concentrations from 5×10^{0} (parasitemia: 0.0000001%) to 5×10^{7} (1%) infected RBC/200 µl of the total RBC, separately, and then subjected to a DNA extraction with a QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). The purified DNA samples were used as templates for the subsequent LAMP and PCR methods. DNA samples were extracted from blood-spotted filter papers (Abe and Konomi, 1998; da Silva et al., 2004). Briefly, the spotted filter papers were cut out with a 2-mm hole puncher (2.0-mm Harris Micro Punch; Whatman, Middlesex, UK). DNA samples were extracted from the cut portion containing the spotted blood by using a QIAamp DNA Mini Kit (QIAGEN). As negative controls, extracted DNA samples of normal bovine blood and other cultured parasites (*Theileria parva*, *Trypanosoma evansi*, and *Neospola caninum*) were prepared as described above.

2.3. LAMP reaction and restriction enzyme-digestion of the amplified DNA products

The non-multiplex LAMP method requires a set of four specific primers: a forward inner primer (FIP), a backward inner primer (BIP), and two outer primers (F3 and B3), which recognize a total of six distinct nucleotide sequences (B1, B2, B3, F1, F2, and F3) on the target gene (Notomi et al., 2000). The specific primers for *B. bovis* and *B. bigemina* were designed against the species-specific rhoptry-associated protein-1 (RAP-1) gene sequences (Gene Bank accession numbers: *B. bovis*, genbank: AF027149; *B. bigemina*, M60878). For the construction of a multiplex LAMP (mLAMP), we modified the FIPs and BIPs by inserting a restriction enzyme (*Eco*RI) cleavage site between the F1 complementary and F2 and between the B1 complementary and B2, respectively, as shown in Table 1. The LAMP reaction was conducted as described previously (Notomi et al., 2000). Briefly, the reaction was performed in 25 µl of a mixture containing 0.5 µl with different DNA weights (5×10^{0} ng to 5×10^{-7} ng/test) of the extracted DNA template, 40 pmol each of the FIP and BIP primers, 5 pmol each of the F3 and B3 primers, and 1 µl of *Bst* DNA polymerase (Eiken Chemical Co., Ltd.,

Table 1
LAMP primers for *Babesia bovis* and *Babesia bigemina* detection

Primer	Type	Sequence (5′–3′)[a]
Bovis-F3	F3	ACCAAAAACTATCTGAAAGCCAATG
Bovis-B3	B3c	GGAGCCTCCCTGAAGAACT
Bovis-FIP	F2-F1c	AGGTTCGGCTACATTCTCTTTCA*GAATTC*TGAGCCCACTAAAAAGTTTATGC
Bovis-BIP	B1-B2c	GCCAACCCACCAAGGAGTTTTTCA*GAATTC*TTGGTTGGTTGACCGATGTT
Bigemina-F3	F3	CGGCGGCTAAGTTCTTCAA
Bigemina-B3	B3c	GAACGAGGTCATCGCAGG
Bigemina-FIP	F2-F1c	CCTAACCAAACGCTTCAACGCC*GAATTC*TTGCTTTCACAACTCGCCTG
Bigemina-BIP	B1-B2c	AGCAACCTTCCCGTTGACCTT*GAATTC*CATCATGTACTCGCCGTAGC

[a] Underlining indicates a restriction enzyme site of *Eco*RI.

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