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Research paper

Rapid and sensitive detection of *Babesia bovis* and *Babesia bigemina* by loop-mediated isothermal amplification combined with a lateral flow dipstick

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ABSTRACT

Babesia spp. are apicomplexan protozoan parasites of the red blood cells of mammals and are transmitted by ticks. Bovine babesiosis mainly caused by *Babesia bovis* and *Babesia bigemina* occurs worldwide, which is a great threat to animal health. Microscopy examination is a gold standard for the diagnosis of babesiosis. However, its sensitivity is too low. This study was conducted to establish a simple, efficient and fast LAMP-LFP method used for early diagnosis of animal babesiosis. LAMP was developed with a set of four primers targeting and amplifying six distinct regions of cytochrome b gene of *Babesia* spp. under isothermal conditions. Afterwards, a chromatographic lateral-flow dipstick (LFD) was used to detect LAMP products that were labeled with FITC at the 5′ end, avoiding gel electrophoresis. The LAMP-LFD method was very specific, yielding no positive results with DNA templates of *Theileria sergenti*, *Thenileria ovis*, *Theileria equi* and *Toxoplasma gondii*. The LAMP-LFP was highly sensitive and could detect 0.85 fg *B. bigemina* DNA and 0.14 fg *B. bovis* DNA, 100-fold higher than a conventional PCR assay. This method could be adapted for quick and accurate diagnosis of bovine babesiosis in the fields in case the whole blood could be directly used, especially for identifying carrier animals with very low parasitaemia.

1. Introduction

Babesiosis is a parasite disease that spreads through tick vectors. It prevails in many parts of the world resulting in great economic losses to the livestock industry (Homer, 2000; Hunfeld et al., 2008). *Babesia bovis* and *Babesia bigemina*, members of Apicomplexa, are the main pathogens of bovine babesiosis in tropical and subtropical regions (Figueroa et al., 1998). Clinical signs of bovine babesiosis include fever, anemia, hemoglobinuria, ataxia (Bock et al., 2004) and even death in severely infected animals of which *Babesia* spp. may be found in erythrocytes by direct microscopic examination (Bose et al., 1995). On the other hand, some animals resistant to initial infection become carriers with low parasitemia that is beyond sensitivity of direct microscopic examination. These animals may serve as a long-term reservoir and are an important source of infection (Almeria et al., 2001).

At present, the methods of detection of *Babesia* spp. infections include blood smear (Morzaria et al., 1992), serological testing (Bose and Peymann, 1994), and conventional PCR detection (Fahrimal et al., 1992; Figueroa et al., 1992). Some methods were with low sensitivity; Yet others were with noticeable false-positive. Still, some other methods require sophisticated equipment, such as real-time or quantitative PCR (Buling et al., 2007). Therefore, to establish a simple, efficient, rapid and practical *Babesia* spp. detection method that can be used in diagnosis, especially in early diagnosis of animal babesiosis, has important practical significance.

Loop mediated isothermal amplification (LAMP) was invented by Notomi et al. (2000) as a novel nucleic acid amplification method under constant temperature. It can amplify target DNA from a few copies to 10^9 copies in a short period of time, and has been widely used in detection of various pathogens such as *Mycobacterium avium* (Enosawa et al., 2003), *Cryptosporidium* (Bakheit et al., 2008), *Giardia lamblia* (Plutzer and Karanis, 2009), *Toxoplasma gondii* (Gallas-Lindemann et al., 2013) and *Leishmania* (Sriworarat et al., 2015). LAMP has also been applied for the detection of *Babesia gibsoni* (Ikadai et al., 2004; Mandal et al., 2015), equine piroplasmosis (Alhassan et al., 2007) and *Babesia orientalis* (He et al., 2009).

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Table 1
LAMP primers (F3, B3, FIP, BIP) and probe used in this study.

Primer	Type	Length	Sequence (5′-3′)
F3	Forward-outer prime	20-mer	GTTGATTCTTTCGAATGTGT
B3	Backward-outer primer	20-mer	AATTATAACTGTTGCTCCCC
FIP	Forward-inner primer (F1c + TTTT + F2)	46-mer	CAAAAAGAAACACATTGAGC*TTTT* CTGAGGTTAATATGGGTTGGGC
BIP	Backward-inner primer (B1c+ TTTT + B2)	53-mer	GGTCCTGGTATTCAGGAATGGGGGC*TTTT* CCGGAAGAACATAACCTAAGAAAG
FITC-HP	Hybridization probe	18-mer	FITC- TTCATGATGTTACATATG

In 2008, the lateral flow dipstrip (LFD) was first used in combination with LAMP to establish the LFD-LAMP technology to detect shrimp Taura syndrome virus (Kiatpathomchai et al., 2008). The technique is based on the principle of LAMP amplification, 2 or 3 pairs of specific primers designed to detect 6 or 8 segments of target genes. The upstream inner primers are labeled by biotin and used to amplify products by Bst DNA polymerase under a constant temperature (60–65 °C) for 1 h. Afterwards the fluorecein isothiocyanate (FITC)-labeled DNA probe within the range of the inner primer amplification hybridizes with biotinylated LAMP products, and the hybridization products are detected by LFD. The technique requires only simple equipment such as a water bath or a heater to maintain reaction temperature as in LAMP, and greatly reduces cost and shortens time, yet with a sensitivity similar to, if not higher than, PCR. LAMP-LFP has been used in the detection of white spot syndrome virus (Jaroenram et al., 2009), spring viremia of carp virus (Puthawibool et al., 2009), infectious spleen and kidney necrosis virus (Ding et al., 2010), Yellow head virus (Khunthong et al., 2013), human African trypanosomiasis (HAT) (Njiru, 2011), *Plasmodium falciparum* and *Plasmodium vivax* (Yongkiettrakul et al., 2014) and citrus Huanglongbing (Rigano et al., 2014).

The aim of current study was to develop a simple, efficient, and practical LAMP-LFP method for the detection of *Babesia* spp. infection. Four specific primers and a FITC-labeled DNA probe were designed based on Cytochrome b gene of *B. bovis* and *B. bigemina*, taking into consideration of Cyt b gene of *Babesia canis* and *Babesia gibsoni* and the method can be used to detect babesiosis in the field with broad application prospects.

2. Materials and methods

2.1. Genomic DNA preparation

Total DNA of *B. bovis* and *B. bigemina* was kindly provided by Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province.

2.2. LAMP primer design

Primers specific for the species-specific Cytochrome b (cytb) sequences of *B. bovis* (GenBank GQ214235.1) and *B. bigemina* (GenBank GQ214234.1) were designed, taken the conserved regions into consideration. An online LAMP primer designing software (http://primerexplorer.jp/e/) was used. The oligonucleotide sequences of the LAMP primers are shown in Table 1. All primers including the biotin-labeled FIP primer were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., China.

2.3. Establishment and optimization of LAMP reaction system

The LAMP system was optimized based on the template of *B. bovis*. A initial volume of 25 μl LAMP mix included: 4 mM MgSO4, 1 mM dNTP, 0.8 mM betaine (Sigma, USA), 1 μM internal primers, 0.2 μM outer primers, 8 U of Bst DNA polymerase (NEB, USA), 1 × ThermoPol reaction buffer (NEB, USA) and 1 μl DNA template. The primer ratio and dNTP concentration were optimized as previous described (Deng et al., 2015). Effect of primer concentration ratio on LAMP reaction was measured by setting the primer concentration ratio gradient of outer primers to inner primers. The gradient primer concentration ratio was set to 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, and 1:9. At a ratio of 1:6, the LAMP products were the most abundant. Set gradients to study the effect of dNTPs concentration on LAMP and dNTPs concentrations were 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 mM. At 1.0 mM, the intensity of LAMP amplicons was the strongest. Therefore, the optimal LAMP mixture contained 1 × ThermoPol reaction buffer, 8 U of Bst DNA polymerase, 1.0 mM dNTPs, 0.8 mM betaine, 4 mM MgSO4, 0.2 μM each outer primer (F3 and B3), 1.2 μM each inner primer (FIP and BIP) and 1 μL of target DNA.

LAMP was also performed for different amplification time ranging from 30 to 90 min and different temperature ranging from 60 °C to 65 °C to determine the optimum conditions for amplification. According to sharpness and brightness of the LAMP products visualized under UV transilluminator on 2% agarose gel stained with ethidium bromide, the temperature at 64 °C and the reaction time of 60 min were chosen for the subsequent LAMP assays.

2.4. LAMP effectiveness and repeatability test

A *B. bovis* positive control sample and two negative control samples free of *Babesia* spp. were used to confirm LAMP effectiveness and repeatability. Genomic DNA of these samples was extracted by a Universal Genomic DNA Extraction Kit (TaKaRa, China) following manufacturer's instructions. These samples were tested three times by LAMP and the results of different times were compared in order to evaluate the effectiveness and repeatability of this method.

2.5. Lateral flow dipstick (LFD) assay

Generic LFD strips (Milenia® GenLine HybriDetect 2T by Milenia Biotec GmbH, Gießen, Germany, http://www.milenia-biotec.de/) were used in LFD assay. These strips detect the biotin-labeled DNA that hybridizes with a DNA probe (named FITC-HP) labeled with FITC at 5′ end, targeting to the sequence between the F1c and B1c regions of LAMP amplicons. Generic LFD strips were embedded with gold-labeled anti-FITC specific antibodies. The test lines were immobilized by biotin-specific molecules and the control line species-specific antibodies. If there is a positive reaction, hybridized products will be captured and generate a red test band over the time, and hybridized products not captured at the test line overflow the control band and generate a red control band. If there is a negative reaction, only the control line will appear. If the control line does not appear red, this means that the reaction is invalid. Preparation for LFD detection was carried out by hybridization with LAMP amplicons as previously reported (Jaroenram et al., 2009; Kiatpathomchai et al., 2008; Puthawibool et al., 2009). Briefly, at the end of the LAMP reaction without heat inactivation, 20 pmol DNA FITC probe was added into the LAMP reaction mix, and incubated at 64 °C for 5 min. Afterwards, 8 μL LAMP mix were combined with 100 μL of the assay buffer. The LFD strip was dipped into the mixture and the result was read in 5 min.

2.6. PCR assay

A 25-μl PCR mixture contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 μM each of F3 and B3 primers, 5 Units of rTaq DNA polymerase (Takara, China), and 1 μL template DNA. Amplification conditions included an initial incubation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 20 s and 72 °C for 30 s. A final extension step was conducted at 72 °C for 5 min. The products were then analyzed by 2% agarose. The 264 bp fragments were expected from the respective gene targets of *B. bovis* and *B. bigemina*.

2.7. Sensitivity by PCR, LAMP by gel electrophoresis (LAMP-AGE) and LFD

DNA concentrations were determined using a Nanodrop spectrophotometer (MaestroGen Inc., USA). The sensitivity of the LAMP assay was tested with *B. bovis* DNA and *B. bigemina* DNA samples, which were serially diluted at concentrations from approximately 14 pg/μL to 0.014 fg/μL and approximately 85 pg/μL to 0.085 fg/μL. One microliter of DNA was used as the template for biotin labeling LAMP according to the optimized conditions. The same amount of template was also used for PCR. DNA from *Babesia* spp. free cow was used as negative control. The products were examined by 2% agarose gel electrophoresis stained with ethidium bromide and by LFD as described above.

2.8. PCR, LAMP by gel electrophoresis and LFD specificity test

The specificities of the LAMP and PCR assay were determined with DNA templates from *B. bovis*, *B. bigemina*, *Theileria sergenti*, *Theileria ovis*, *Theileria equi* and *T. gondii* saved in our lab. DNA from *Babesia* spp. free cow was set as negative control. The amplified products were analyzed by 2% agarose gel electrophoresis and LFD.

3. Results

3.1. LAMP effectiveness and repeatability test

Three test results were completely consistent. The experimental results showed that the *B. bovis* control sample appeared positive and two control samples free of *Babesia* spp. negative. The experimental results were in accordance with setting targets. The above indicates this LAMP is repeatable and accurate.

3.2. Analytical sensitivity of the PCR, LAMP by gel electrophoresis and LFD

To evaluate the sensitivity of LAMP, compared with PCR, DNA samples from 10-fold serial dilutions of *B. bovis and B. bigemina* were subjected to LAMP and PCR (Figs. 1 and 2). Using a series dilution of DNA extracted from *B. bovis* and *B. bigemina*, LAMP-AGE gave positive results up to dilutions of 1×10^{-5} (*B. bovis* 0.14 fg and *B. bigemina* 0.85 fg) (Figs. 1 a and 2 a), while positive PCR amplications were only observed up to dilutions of 1×10^{-3} (*B. bovis* 14 fg and *B. bigemina* 85 fg) (Figs. 1 b and 2 b). Thus, LAMP-AGE was 100 times more sensitive than PCR. Similar to LAMP-AGE, LAMP-LFP was 100 times more sensitive than PCR (Figs. 1 c and 2 c). Although LAMP-LFP and LAMP were equally sensitive, the former could not only save time but also is more convenient and accurate to judgment the results.

3.3. Analytical specificity of the PCR, LAMP-AGE and LAMP-LFD

In present study, we used the DNA samples from *B. bovis*, *B. bigemina*, *T. sergenti*, *T. ovis*, *T. equi* and *T. gondii* to evaluate the specificity of the LAMP and PCR. Primers F3 and B3 successfully amplified a 264 bp region of the Cytb gene of *B. bovis* and *B. bigemina* when used in PCR (Fig. 3). *B. bovis* DNA and *B. bigemina* DNA generated a red test band and a red control band when subjected to the LAMP-LFP assay, while just a red control band was seen with any other species (Fig. 3c). *B. bovis* and *B. bigemina* DNA also demonstrated a typical ladder pattern when subjected to the LAMP assay (Fig. 3a). No amplification was seen with any of the other species. The negative samples also tested negative as expected. PCR showed the same result (Fig. 3b). In addition, sequencing confirmed that they were cytb gene products of *B. bovis* and *B. bigemina* (data not shown). These results indicate that none of the specificity controls could be detected by the PCR, LAMP-AGE and LAMP-LFD assay.

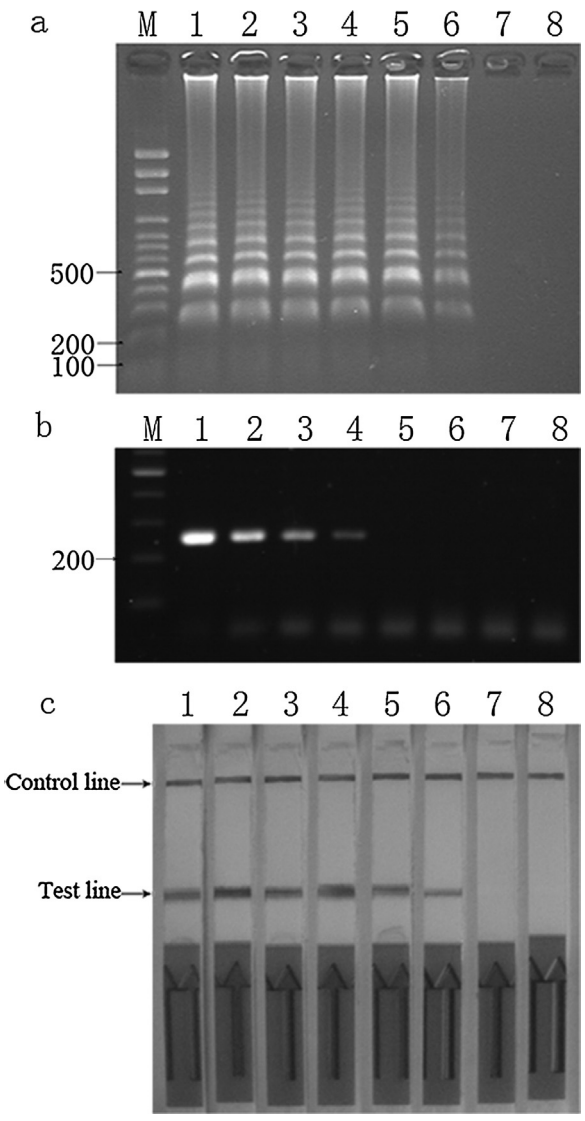

Fig. 1. Comparative sensitivities by (a) LAMP-AGE, (b) PCR-AGE, and (c) LAMP-LFD for the detection of *Babesia bovis* based on Cyt b amplification.
M:100 bp DNA Marker;Lane 1–7:amounts of DNA template were, in order, 14 pg, 1.4 pg, 140 fg, 14 fg, 1.4 fg, 0.14 fg and 0.014 fg *B. bovis* DNA;Lane 8:negative control. LAMP-AGE: LAMP combined with agarose gel electrophoresis; PCR-AGE: PCR combined with agarose gel electrophoresis; LAMP-LFP: LAMP combined with LFP. The sensitivity of LAMP-AGE and LAMP-LFD was 0.14 fg, while that of PCR-AGE was 14 fg.

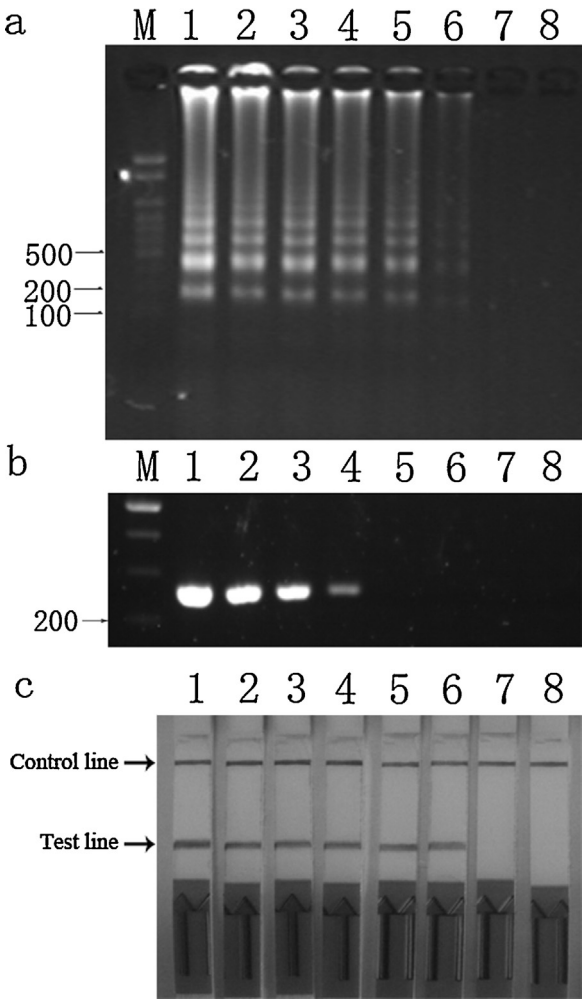

Fig. 2. Comparative sensitivities by (a) LAMP-AGE, (b) PCR-AGE, and (c) LAMP-LFD for the detection of *Babesia bigemina* based on Cyt b amplification.
M:100 bp DNA Marker;Lane 1–7:amounts of DNA template were, in order, 85 pg, 8.5 pg, 850 fg, 85 fg, 8.5 fg, 0.85 fg and 0.085 fg *B. bigemina* DNA;Lane 8:negative control. LAMP-AGE: LAMP combined with agarose gel electrophoresis; PCR-AGE: PCR combined with agarose gel electrophoresis; LAMP-LFP: LAMP combined with LFP. The sensitivity of LAMP-AGE and LAMP-LFD was 0.8 fg, while that of PCR-AGE was 85 fg.

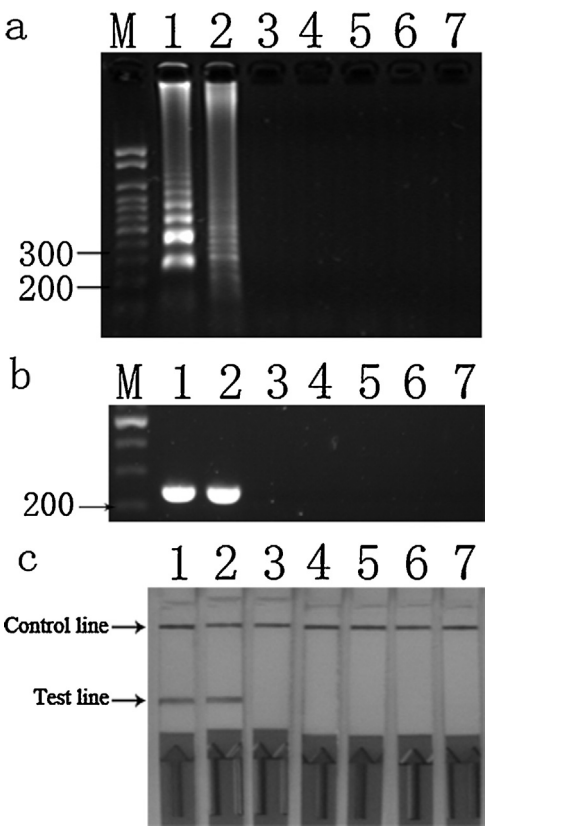

Fig. 3. Specificity of (a) LAMP-AGE, (b) PCR-AGE, and (c) LAMP-LFD based on Cyt b amplification.
M:100 bp DNA Marker; Lane 1: *Babesia bigemina* DNA; Lane 2: *Babesia bovis* DNA; Lane 3: T*heileria sergenti* DNA; Lane 4: *Theileria ovis* DNA; Lane 5:*Theileria equi* DNA; Lane 6: *Toxoplasma gondii* DNA; Lane 7: negative control.

4. Discussion

Babesiosis, caused by *Babesia* spp. protozoa, is a common parasitic disease. The protozoan mainly invades red blood cells of bovine, horse (donkey, mule) and other animals (Penzhorn, 2006). *Babesia* spp. include *B. bovis*, *B. bigemina*, *Babesia divergens*, *B. canis*, *Babesia caballi* and *Babesia ovis*, with different degrees of host specificity (Penzhorn, 2006). The disease mainly occurs in tropical and subtropical areas, bringing huge losses to the livestock industry and the global economy, increasingly necessary to detect in the livestock industry and other fields (Perez de Leon et al., 2010).

Currently, the gold standard for diagnosis of babesiosis is direct microscopy of blood smear. This method is simple and fast. Disadvantages include low sensitivity and demanding highly trained personnel. Different species of parasites may be similar in morphology that they are indistinguishable. In addition, this method is not suitable for detection of animals with low parasite burden in the peripheral blood and carrier carrier animals with very low parasitaemia (Almeria et al., 2001). Serological method is an effective means of epidemiological research, but it cannot distinguish infected animals from resistant ones after infection, neither determine cross infection, nor accurately tell carrying host from storage host (Wagner et al., 1992).

18S rRNA gene is usually chosen as a target in diagnosis of bovine babesiosis because the information relevant to the convenient gene can be easily obtained from molecular database. However, the copy number of this gene in the *Babesia* genomes is low. There are three copies in *B. bovis* genome (Dalrymple, 1990), two copies in *T. parva* genome (Kibe et al., 1994). The same situations of *P. falciparum* (Corredor and Enea, 1994; Dame and McCutchan, 1983) and *Cryptosporidium parvum* (Le Blancq et al., 1997) exist, each with 4–5 copies. Cytochrome b (Cyt b) gene of *Babesia* spp. exists in extachromosomal mitochondrial DNA, compared to 18S rRNA gene, with higher copy number (Salem et al., 1999). PCR sensitivity is closely related to the number of target gene. Selecting genes of high copy number as target gene will greatly increase the sensitivity of the existing PCR methods. Salem et al. (1999) used Cyt b gene as target gene to detect *B. bovis* and *B. bigemina* by PCR amplification and discovered it was 20% more sensitive than 18SrRNA gene as a target. Regarding Cyt b gene as a target gene, a quantitative PCR method was able to detect 0.1 ng–0.1 fg of DNA *B. bovis* and *B. bigemina* (Buling et al., 2007). Therefore, Cyt b gene was chosen in the current study of developing a LAMP-LFP met hod, which is more convenient and applicable than the conventional PCR.

The LAMP established by Notomi et al. (2000) is very suitable for clinic detection and has been applied to detection of various pathogens, because of high specificity, sensitivity and no need for expensive equipment like thermal cycler. Application of LAMP to monitor microbial pathogens has been reviewed by Karanis and Ongerth (2009). LAMP was performed previously for detection of *B. gibsoni* by Ikadai et al. (2004), with the detection limit 0.0005%

parasitaemia and a equal sensitivity of LAMP and PCR. To confirm the LAMP reaction products some poisonous substances may be used such as ethidium bromide in some labs (Mandal et al., 2015). In addition, false-positive results occasionally occur when the turbidity and fluorescence detection methods are performed. LAMP-LFD is based on LAMP and depends on the specific hybridization of the reaction products with DNA probes. Using *B. bigemina* Cyt b and *B. bovis* Cyt b conserved sequence to design primers, the LAMP-LFP established can accurately identify *B. bigemina* and *B. bovis*, while the other genera of protozoa *T. sergenti*, *T. ovis*, *T. equi* and *T. gondii* were not detected, suggesting that the detection method is highly specificity, in accordance with previous studies (Njiru, 2011; Yongkiettrakul et al., 2014). Sensitivity of the LAMP-LFP method was further confirmed. By optimizing the primer concentration ratio, dNTPs and other conditions, the sensitivity of the LAMP-LFP is 100 times higher than gel PCR, with a detect threshold of 0.85 fg *B. bigemina* DNA and 0.14 fg *B. bovis* DNA, with a comparable sensitivity of the quantitative PCR established before (Buling et al., 2007). LAMP assays in previous studies showed different sensitivity. LAMP method for the identification of Chinese *B. bovis* and *B. bigemina* (Liu et al., 2012) was with a limit detection of 0.1 pg DNA, 1000-fold more sensitive than the conventional PCR assay. Moreover, the LAMP assay could detect 12 pg DNA of *B. gibsoni*, 10 times greater than nested PCR (Mandal et al., 2015). In contrast, LAMP were found less sensitive than PCR (Muller et al., 2010). Relatively, LAMP-LFP in present study was more sensitive. LAMP-LFD was first applied to the detection of *B. bigemina* and *B. bovis*, with high specificity and sensitivity, short time-consuming and simple result judgement, which fits the need for practitioners and has a wide application future.

5. Conclusion

The LAMP-LFD method established in the current study can specifically detect *B. bigemina* and *B. bovis* in just 70 min excluding DNA extraction and preparation time. This method, with high specificity and sensitivity and without requirement of professional equipment, is very suitable for field application and pretty hopeful to be commonly applied in fast detection.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

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