Experimental Parasitology 120 (2008) 39–44

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

The development and evaluation of a loop-mediated isothermal amplification (LAMP) method for detection of *Babesia* spp. infective to sheep and goats in China

Guiquan Guan [a,b], Alain Chauvin [b], Jianxun Luo [a], Noboru Inoue [c], Emmanuelle Moreau [b], Zhijie Liu [a], Jinliang Gao [a], Oriel M.M. Thekisoe [c], Miling Ma [a], Aihong Liu [a], Zhisheng Dang [a], Junlong Liu [a], Qiaoyun Ren [a], Yurong Jin [a], Chihiro Sugimoto [c], Hong Yin [a,*]

[a] *Key Laboratory of Veterinary Parasitology of Gansu Province, State Key Laboratory of Veterinary Etiological Biology, Department of Veterinary Parasitology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science, Xujiaping 1, Lanzhou Gansu 730046, PR China*
[b] *UMR ENVN/INRA 1300, BIOEPAR, École Nationale, Vétérinaire de Nantes, Atlanpole-La Chantrerie, BP 40706, 44307 Nantes Cedex 03, France*
[c] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan*

ARTICLE INFO

Article history:
Received 21 September 2007
Received in revised form 9 April 2008
Accepted 10 April 2008
Available online 18 April 2008

Index Descriptors and Abbreviations:
Loop-mediated isothermal amplification (LAMP)
Babesia sp.
18S rRNA gene

ABSTRACT

The loop-mediated isothermal amplification (LAMP) reaction is a method that amplifies with high sensitivity, efficiency, and rapidity, deoxyribonucleic acid (DNA) under isothermal condition in simple incubators. Two primer sets for the LAMP method were designed using the nucleotide sequences of 18S rRNA gene of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 isolated in China. The primers were used to detect parasite DNA extracted from infected blood and purified parasites by LAMP. The specific ladder bands were amplified from the autologous genomic DNA of two *Babesia* species, respectively, and did not cross-react with the genomic DNA of *Theileria* sp. China 1, *Theileria* sp. China 2, *B. bovis*, *Theileria* sp. (Japan) and sheep. The LAMP was sensitive enough to detect 0.02 pg and 0.2 pg genomic DNA of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005, respectively, from 10-fold serially diluted samples corresponding to the amount of DNA present in 50 μl of 0.000002% and 0.00002% parasitemic erythrocytes. Furthermore, DNA extracted from blood of intact (non-splenectomized) sheep experimentally infected with *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 was amplified by the LAMP from week 1 to 9 and week 2 and 3 post-infection, respectively, demonstrating the high sensitivity of these primers. Of 365 samples collected from Gansu province, 14.3% (52/365) were positively detected by the LAMP. Of 145 samples collected on filter papers (Whatman) from the grazing sheep in Xinjiang province, 3.5% (5/145) were positive. These results show that the LAMP could be an alternative diagnostic tool for the detection of babesial infection in sheep and goats.

1. Introduction

The piroplasms of small ruminants are common parasites in the north of China, and they cause serious problems for the livestock industry. The rate of infection is 15–80% and the mortality of lambs is more than 50% in some regions (Luo and Yin, 1997; Yin et al., 2002). In the past two decades, the pathogen was considered to be an unidentified *Theileria* transmitted by *Haemaphysalis qinghaiensis*. However, recently, several strains of *Babesia* spp. infective to sheep and goats were also isolated from different places in China, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. BQ1 (Ningxian), *Babesia* sp. (Tianzhu), *Babesia* sp. (Hebei), *Babesia* sp. (Madang), *Babesia* sp. (Liaoning) and *Babesia* sp. Xinjiang-2005. The symptoms of babesiosis; fever, hemolytic anaemia, icterus, and sometimes death could be observed in sheep parasitized by *Babesia* in the field (Bai et al., 2002; Guan et al., 2001, 2002). These strains can be divided into two groups based on the sequences of 18S rRNA gene, one including, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. (Ningxian), *Babesia* sp. (Tianzhu), *Babesia* sp. (Hebei), *Babesia* sp. (Madang), and *Babesia* sp. (Liaoning) and the other just containing *Babesia* sp. Xinjiang-2005 (Liu et al., 2007a; Schnittger et al., 2003, 2004). This suggests that ovine babesiosis, together with ovine theileriosis, may be wide-spread in the north of China causing enormous economic loss in the small ruminant industry. To control the disease, it is important that an accurate, highly efficient, simple method should be developed for diagnosis and epidemiological surveillance of the disease in China.

Recently, Notomi et al. (2000) reported a novel nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP) that has the advantages of high specificity, sensitivity and efficiency, simple operation and equipment but also amplifies under isothermal conditions. This makes it possible to diagnose disease quickly and accurately. The original method has been improved on and used to diagnose viral, bacterial and

* Corresponding author. Fax: +86 931 8340977.
E-mail addresses: yinhong@public.lz.gs.cn, ttbdcn@public.lz.gs.cn (H. Yin).

0014-4894/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2008.04.012

protozoan diseases of humans, animals and even plants (Alhassan et al., 2007; Fukuta et al., 2003; Kuboki et al., 2003; Thekisoe et al., 2005; Toriniwa and Komiya, 2006;), and has potential for development as a the routine diagnostic method.

In this study, we developed a LAMP method for diagnosing ovine babesiosis in China based on the sequences of 18S rRNA gene of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 and evaluated its specificity and sensitivity in the blood samples collected from experimentally infected and naturally infected sheep.

2. Materials and methods

2.1. Animals

Eight sheep, 5–7 months of age, were purchased from a *Babesia*- and *Theileria*-free area. Four sheep were splenectomized 2 months before the study. Blood films taken from the ears of the sheep were fixed with methanol, stained with Giemsa and examined every week for the presence of haemoprotozoan parasites. Only negative sheep were used in the experiment.

2.2. Infection of animals

Four piroplasm strains were used in the study, *Babesia* sp. BQ1 (Lintan), *Babesia* sp. Xinjiang-2005, *Theileria* sp. China 1 and *Theileria* sp. China 2. Four splenectomized sheep were infected with cryopreserved parasites. The rectal temperature was measured and blood smears were examined every day post-infection for presence of parasites. When the parasitemia was 5–20%, blood was collected from the jugular vein for purifying merozoites and extracting genomic parasite DNA of parasites using Alsever's solution as anticoagulant. Four intact (non-splenectomized) sheep were divided into two groups and infected with the *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005, respectively. Disease monitoring was performed by taking rectal temperatures and microscopic examination of Giemsa-stained blood smears for the presence of piroplasms. Jugular vein blood was also collected in anticoagulant for extracting parasite DNA every week for a period of 12 weeks.

2.3. Purification of Babesia spp. merozoites

Babesia sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 merozoites were prepared from the blood collected at peak parasitemia from experimentally infected splenectomized sheep. Infected blood was centrifuged at 1000*g* for 10 min at 4 °C and 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 removed. A cellulose powder column (CF-11, Whatman) was used to remove the remaining leukocytes. The packed infected erythrocytes were suspended with 1 volume Tris–saline and incubated in 37 °C water bath for 5 min. AH-1 hemolysin (final concentration: 300 HU/ml; provided by Professor Chihiro Sugimoto) was added for lysing erythrocytes in 37 °C water bath for 10 min. Lysis was stopped by addition of 0.01 volumes of 500 mM EDTA and 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 pellet of merozoites was washed with Tris–saline by further centrifugation until it was almost free of hemoglobin and then stored at −70 °C.

2.4. Genomic DNA preparation

Total DNA of *Babesia* sp. BQ1 (Lintan) and *Babesia* sp. Xinjiang-2005 were prepared from purified merozoites with the Puregene DNA Purification Kit (www.gentra.com) according to the manufacturers instructions. So that DNA samples could be considered free of sheep genomic DNA, *Theileria* sp. China 1, *Theileria* sp. China 2 and experimentally infected intact sheep DNA were directly extracted from the anticoagulated blood of infected sheep. The DNA samples of *Theileria* sp. (Japan) isolated from serow, and *B. bovis* were kindly provided by the Research Unit of Advanced Preventive Medicine, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan. Three hundred and sixty-five blood samples were collected from the sheep on pastureland in Zhuoni, Biandu, Lintan and Xiahe counties, Gansu province from April to June 2004. One hundred and forty-five blood samples were collected on filter paper (FTA card; Whatman, WB120205) from the grazing sheep in Bayituohai, Kashi, Yingtamu, Quluhai counties, Xinjiang province in April 2005. The DNA samples were extracted from the blood. Aqueous DNA preparations were frozen at −70 °C until use. The DNA samples on FTA cards were prepared according to manufacture's instructions before use.

2.5. Specific primers of LAMP and PCR

The sequences of 18S rRNA gene of *B. ovis* (AY260178), *B. motasi* (AY260180), *Babesia* sp. BQ1 (Lintan) (AY260181), *Babesia* sp. Xinjiang- (DQ159073), *Theileria* sp. China 1 (AY262115), *Theileria* sp. China 2 (AY262120) were used for reference in this study. To obtain the specific primer for the LAMP and PCR, multiple sequence alignments were performed using the program algorithm of Clustal V in the MegAlign component of the DNASTAR programme (Version 4.01 DNASTAR, Madision, Wis.) (Figs. 1 and 2). The specific primers were designed using the PrimerExplorer V2 and Primer5.0 software for LAMP and nested PCR, respectively, in variable region of 18S rRNA gene sequence. The oligonucleotide sequences of primers are shown in Table 1.

2.6. LAMP procedures

The reaction was performed in 25.0 μl mixture containing 12.5 μl LAMP buffer (40 mM Tris–HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M betaine and 2.8 mM concentration of each deoxynucleoside triphosphate (dNTP)), 0.9 μl primers mix (FIP and BIP at 40 pmol each and F3 and B3 at 5 pmol each), 2.0 μl extracted DNA or a piece of FTA card, 1.0 μl (8 U) *Bst* DNA polymerase (New England Biolabs, M0275L) and 8.6 μl distilled water. The reaction mixture was incubated in a heat block, at 63 °C for 30 min (for *Babesia* sp. BQ1 (Lintan)) or 60 °C for 45 min (for *Babesia* sp. Xinjiang-2005) and then inactivation was carried out at 80 °C for 2 min. The LAMP products were loaded on 1.5% agarose gel in a Tris–acetic acid–EDTA (TAE) buffer for electrophoresis and visualized and photographed under UV light after staining with ethidium bromide.

2.7. Nested PCR amplification

The PCR was performed in an automatic thermocycler in a total reaction volume of 50 μl containing 5.0 μl 10× PCR buffer (Mg^{2+} Plus), 4.0 μl dNTPs of 2.5 mM each of the four deoxynucleotide triphosphates, 1.25 U Taq polymerase, 2.0 μl template DNA or a piece of FTA, 1.0 μl each primers (20 pmol) and 36.75 μl distilled water. For the primary PCR cycle parameters used an initial were followed by initial step at 94 °C 3 min, 40 cycles at 94 °C for 30 s, 55 °C for 40 s and 72 °C for 1 min with a final extension step at 72 °C for 10 min. Subsequently, the nested PCR was performed using 1.0 μl of the primary product as template and the primary PCR mixture. The condition of reaction was as follows: an initial step at 94 °C for 3 min, 40 cycles at 94 °C for 30 s, 55 °C (*Babesia* sp. Xinjiang-