Rapid Detection of *Babesia motasi* Responsible for Human Babesiosis by Cross-priming Amplification Combined with a Vertical Flow

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Research

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Abstract

**Background:** *Babesia motasi* is known as an etiological agent of human and ovine babesiosis. Diagnosis of babesiosis is traditionally performed by microscopy, examining Giemsa-stained thin peripheral blood smears. Rapid detection and accurate identification of species are desirable for clinical care and epidemiological studies.

**Methods:** An easy to operate molecular method, which requires less capital equipment and incorporates cross priming amplification combined with a vertical ow (CPA-VF) visualization strip for rapid detection and identification of *B. motasi*.

**Results:** The CPA-VF targets the 18S rRNA gene and has a detection limit of 50 fg per reaction; no cross reaction was observed with other piroplasms infective to sheep or *Babesia* infective to humans. CPA-VF and real-time (RT)-PCR had sensitivities of 95.2% (95% confidence interval [CI], 78.1-99.4%) and 90.5% (72-97.6%) and specificities of 95.8 (80.5-99.5%) and 97.9 (83.5-99.9%), respectively, versus microscopy and nested (n) PCR combined with gene sequencing. The clinical performance of the CPA-VF assay was evaluated with field blood samples from sheep (*n* = 340) in Jintai county, Gansu Province, and clinical specimens (*n* = 492) obtained from patients bitten by ticks.

**Conclusions:** Our results indicate that the CPA-VF is a rapid, accurate, nearly instrument-free molecular diagnostic approach for identification of *B. motasi*. Therefore, it could be an alternative technique for epidemiological investigations and diagnoses of ovine and/or human babesiosis caused by *B. motasi*, especially in resource-limited regions.

**Background**

Babesiosis, caused by protozoan pathogens of the genus *Babesia* infective to human, domestic and wild animals, is one of the emerging and re-emerging tick-borne disease in the tropical and subtropical regions of the world [1]. A wide spectrum of clinical signs ranges from mild fever to serve anemia, haemoglobinuria and even death. Given the increasing reports of human babesiosis, great attention has been paid to this emerging human disease [2, 3]. Predominately, three *Babesia* spp., *Babesia microti*, *B. divergens*, and *B. duncani*, have been described to be involved in human infections in the United States, Europe, and Asia [4, 5]. Recently, two newly emerging *Babesia* species, named as *B. motasi* and *B. crassa*, which were previously reported as causative agents of ovine babesiaosis, have been sporadically reported in cases of human babesiosis in Asia [6, 7, 8, 9, 10]. As a causative agent responsible for human babesiosis, the first case caused by *B. motasi*-like was reported in Korea in 2005 [11]. Recently, infected with *B. motasi* was diagnosed in a 70-year-old man in Korea [12].

In China, four strains of *B. motasi*, *B. motasi* Lintan, *B. motasi* Tianzhu, *B. motasi* Ningxian, and *B. motasi* Hebei, which are responsible for ovine babesiosis, have been isolated from different endemic areas by the Vector and Vector-Borne Diseases (VVBD) laboratory, Lanzhou Veterinary Research Institute (LVRI) [13, 14, 15]. Epidemiological studies have revealed that *B. motasi* infections have a wide distribution in sheep,
goats, and vector ticks across China, according to molecular detection and serological assay [16, 17, 18]. Given that it poses a severe threat to public health, rapid and accurate detection of *B. motasi* infection is important for performing epidemiological studies and providing appropriate clinical management. Several methods, based on molecular techniques that detect the presence of *B. motasi* genomic DNA, have been extensively accepted as the usual strategies for diagnosis of *B. motasi* infection. These methods, including polymerase chain reaction (PCR), RT-PCR, reverse line blot (RLB), and loop-mediated isothermal amplification (LAMP), require costly instruments and skilled personnel to perform the procedures, which has restricted their wide application in clinical care, infection control, and epidemiological studies [17, 19, 20].

Cross priming amplification (CPA), a novel isothermal amplification technique, was developed as an alternative methodology for disease diagnosis in endemic areas where limited resources were available [21]. This approach has been applied to detection of a number of animal and plant pathogens, such as bacteria, viruses, and herbal products, with high specificity and sensitivity [22, 23, 24, 25]. Given that it is an effective detection technique for reliable diagnosis of pathogen infection, in the present study a novel CPA targeting the 18S rRNA gene was established for on-site detection of *B. motasi* infection. The labeled products from the CPA can be detected with VF strip to visualize the specific amplicon of *B. motasi*.

**Methods**

**Primer design**

*Babesia motasi* specific primers for CPA were designed using the sequence alignments of the 18S rRNA gene of *Babesia* spp. and *Theileria* spp. infective for sheep and humans (Table 1). A region that is conserved intra-*B. motasi* and variable among species was used as the target sequence for primer location. Two sets of primers and probes were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA); each set of primers and probes was composed of two displacement primers (BLT-5s and BLT-4a), one cross primer (BLT-2s1a), and two detector primers (BLT-2s and BLT-3s). The detector primer (BLT-2s) was labeled with biotin at the 5’ end and the BLT-3s was labeled with fluorescein isothiocyanate (FITC) at the 5’ end. The cross primer was composed of the BLT-2s at the 5’ end and 1a at the 3’ end. These primers were synthesized by TsingKe Biotech Co., Ltd (Beijing, China).

**Table 1.** The sequences of *B. motasi* CPA-VF primers and probes.
| Primer name  | Sequence (5’-3’)                                      |
|-------------|-------------------------------------------------------|
| **Set one** |                                                       |
| BLT-5s      | GCTAATTGTAGGGCTAATACAAG                               |
| BLT-2s      | FITC-CGATGCCTTTTGGCGGCG                              |
| BLT-3s      | Biotin-GCTTTTAACCAATTGTGG                            |
| BLT-2s1a    | CGATGCCTTTTGGCGGCGGCGATTCGCAAGTTTATTATG              |
| BLT-4a      | CTTGAATGGAACATCGCTAA                                 |
| **Set two** |                                                       |
| BLT-5s      | GGADWWDGTCCGKTITTG                                   |
| BLT-2s      | FITC-CTTAGAGGGACTCCTGC                               |
| BLT-3s      | Biotin-GCTTGAAGCGTGGG                                |
| BLT-2s1a    | CTTAGAGGGACTCCTGCAGACCTGTTATGCGCTT                  |
| BLT-4a      | CGCCTGCGGTTCGACGATT                                 |

**Blood samples**

Standard positive samples were obtained from sheep experimentally infected with *B. motasi*. Briefly, 16 6-month-old sheep that were divided into four groups (group 1-4) containing the same number of experimental animals were purchased from Jingtai county, Gansu Province and confirmed to be free of piroplasm infection by microscopy, RT-PCR, nPCR and ELISA assay [16, 20, 26, 27]. Group 1, 2, 3 and 4 were inoculated intravenously 10 mL of cryopreserved blood infected with *B. motasi* Lintan, *B. motasi* Tianzhu, *B. motasi* Ningxian and *B. motasi* Hebei, respectively. When the parasitemia reached 8%–10%, blood samples were collected into EDTA-coated tubes. Each of three intact sheep was inoculated with 50 ml blood infected with *B. motasi* Lintan/*B. motasi* Tianzhu/*B. motasi* Ningxian/*B. motasi* Hebei via the jugular vein. Jugular blood was collected every two days after inoculating *Babesia* species. Negative blood samples were collected into EDTA coated tubes from randomly selected sheep in Jintai county, Gansu province, where *B. motasi* is not endemic. All blood samples were transported to the VVBD laboratory, LVRI in iceboxes and stored at ~ 20 °C before DNA extraction.

Genomic DNA was extracted from 200 μl of above-mentioned blood samples using commercial DNA extractions kits according to the manufacturer’s instruction (QIAamp DNA Blood Mini Kit, Germany).

**Optimization of the CPA-VF assay for *B. motasi* detection**
Initially, we designed two sets of primers to develop a highly sensitive and specific method. The CPA amplification was performed in a 20 μl volume. Optimization of reaction composition led to the following: 1.25 μM each of displacement primers (BLT-5s and BLT-4a), 7.5 μM each of detector primers (BLT-2s and BLT-3s), 12.5 μM of cross primer (BLT-2s1a), 6 mM MgSO$_4$, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 1 M betaine, 8U Bst DNA polymerase (New England BioLabs), 8 mM deoxynucleotides triphosphates (dNTPs), 0.1% Triton X-100, and 2 μl genomic DNA. The CPA reaction tubes were incubated at 63 °C for 60 min, followed by 80 °C for 2 min to terminate the reaction. Finally, VF strips, purchased from Hangzhou Ustar Company (Hangzhou, China), were used to detect CPA products: 5 μl of CPA products and 90 μl of PBS were added to the sample pad. A reaction was identified as positive when both the test line and the control line were developed, whereas it was considered as negative when only the control line was developed.

Furthermore, the CPA reaction were performed at different temperatures, ranging from 55 to 65°C and various time settings, ranging from 40 to 100 min. Subsequently, the amplified products were detected using VF strips.

**Specificity and sensitivity of the CPA assays**

Genomic DNAs of *Theileria luwenshuni*, *T. uilenbergi*, *T. ovis*, Babesia sp. Xinjiang and Babesia sp. Dunhuang were provided by VVBD. The specificity of the assay was evaluated using genomic DNA from *B. motasi* Lintan, *B. motasi* Tianzhu, *B. motasi* Ningxian, *B. motasi* Hebei, Babesia sp. Xinjiang, Babesia sp. Dunhuang, *T. luwenshuni*, *T. uilenbergi*, *T. ovis*, *B. diergens*, *B. duncani*, and plasmid DNAs bearing the 18S rRNA gene of *B. microti* (KF410825) and *B. crassa* (AY260176). To evaluate the assay’s sensitivity, serial dilutions of genomic DNA from purified *B. motasi* Lintan merozoites were used as the template for CPA amplification in concentrations: 4 ng/μl, 800 pg/μl, 160 pg/μl, 32 pg/μl, 6.4 pg/μl, 1.28 pg/μl, 0.256 pg/μl, 50 fg/μl, 10 fg/μl. Each concentration of genomic DNA was tested in three independent experiments to ensure reproducibility of the CPA assay.

To evaluate the performance of the CPA-VF assay, its sensitivity and specificity were determined using standard positive samples (experimentally infected animals) and field collected negative samples, versus microscopy, RT-PCR, and nPCR targeting the 18S rRNA combined with gene sequencing [20, 26, 28].

**Clinical performance of CPA-VF assay for clinical specimens**

Field blood samples were randomly collected from 340 sheep in Gansu province, transported to VVBD, LVRI in iceboxes and stored at –20°C before DNA extraction. The collection and manipulation of blood samples were approved by the Animal Ethics Committee of the LVRI, Chinese Academy of Agricultural Sciences. All sampling procedures were handled in accordance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China.
A total of 492 patients who lived in the Gannan Tibetan Autonomous Prefecture, Gansu Province and visited the Second Hospital of Lanzhou University for a tick bite in the past few months, between May 2017 and July 2019, were recruited. Blood samples collected from patients were tested using the CPA-VF assay to determine the infection status of \textit{B. motasi}. All participants agreed to participate in this study and signed an informed consent form.

The clinical performance of the CPA-VF approach was evaluated with these field blood samples collected from sheep and clinical specimens from patients with a history of tick bite.

**Results**

**Optimization of the CPA primers, reaction temperature and time.**

Sequence alignment of the \textit{18S} rRNA genes of piroplasms infective to sheep and goats available in NCBI showed that two regions are conserved intra-species and variable among species. The sequences and locations of the primers are presented in Table 1. The primers and probes of set one showed high specificity. Therefore, set one was used for the subsequent experiments. To determine a suitable amplification temperature, the CPA reactions were incubated at 55 to 65°C for 60 min. The results showed that the assay could be performed at a wide range of temperatures, from 58 to 63°C. Changes in amplification temperature had a slight impact on brightness of the bands, indicating that incubation temperature is significant for the CPA reaction. Optimal brightness of red–purple band was observed in the VF strip at the temperature 61°C (Fig. 1A).

The CPA amplification was conducted at 61°C for 40–100 min. The results revealed that positive signs could be developed as early as 40 min amplification; however, the brightness of the positive band was not strong as at 60 min and 80 min. To provide a high sensitivity and time efficiency of the CPA assay, an amplification time of 60 min was used in \textit{B. motasi} detection (Fig. 1B).

**Cross reaction of the developed CPA approach.**

The CPA technique was evaluated by testing piroplasms infective for sheep, goats and humans. As shown in Fig. 2, no cross reaction was observed with other \textit{Babesia} spp. and \textit{Theileria} spp. These available results demonstrated that the CPA assay was specific for identification of \textit{B. motasi} (Fig. 2).

**Limit of detection of the CPA-VF assay**

The limit of detection of the CPA-VF assay was evaluated using 5-fold serially diluted DNA from purified merozoites of \textit{B. motasi} in three independent reactions from 4 ng/μl to 10 fg/μl. The assay could detect as few as 50 fg/μl DNA of \textit{B. motasi} (Fig. 1C). As shown, two bands on the VF strips were developed using from 4 ng to 50 fg of genomic DNA, while only one band was observed in the 10 fg and negative control reactions.

**Sensitivity and specificity of CPA-VF**
A total of 42 samples of standard positive genomic DNA from experimentally infected sheep and 48 negative field samples were studied using both CPA-VF and RT-PCR (Table 2). The performance of CPA-VF and RT-PCR is presented in Table 3. The CPA-VF and RT-PCR had sensitivities of 95.2% (95% confidence interval [CI], 78.1–99.4%) and 90.5% (72–97.6%) and specificities of 95.8% (80.5–99.5%) and 97.9% (83.5–99.9%) (Table 3). There was no significance difference between the performance of the CPA-VF and RT-PCR methods.

**Table 2.** Standard positive and negative samples, confirmed by thin blood smears microscopy and nested PCR combined with gene sequencing.

| Result | No. of samples |
|--------|----------------|
| B. motasi Lintan | 10 |
| B. motasi Tianzhu | 10 |
| B. motasi Hebei | 12 |
| B. motasi Ningxian | 10 |
| Positive | 10 |
| Negative | |
| Total | 10 |

**Table 3** The performance of CPA-VF assay was compared with that of RT-PCR.

| Primer name | Sequence (5’-3’) |
|-------------|------------------|
| BLT-5 s     | GCTAATTGTAGGCTAATAACAAG |
| BLT-2 s     | FITC-CGATGCGTTTTTGCGCG |
| BLT-3 s     | Biotin-GCTTTTTAACCAATTGTTG |
| BLT-2s1a    | CGATGCCCTTTTGCGGCGGCGATTGCAAGTTTATTATG |
| BLT-4a      | CTTGAATGGAACATCGCTAA |
| BLT-5 s     | GGADWWDGTCCGKTGTTT |
| BLT-2 s     | FITC-CTTAGAGGGACTCCTGC |
| BLT-3 s     | Biotin-GCTTGAGCGGCTG |
| BLT-2s1a    | CTTAGAGGGACTCCTGCCAGACCTGTTATGCTTT |
| BLT-4a      | CGCCTGCGGCTCGACGATT |

**Evaluation of the CPA using samples from the field and clinical samples**

To evaluate the feasibility of using CPA-VF as an alternative approach for *B. motasi* detection, 340 whole blood samples from sheep and patients were subjected to CPA-VF. The results of the CPA-VF assay showed that 3.8% (13/340) of the samples collected from sheep in Gansu province were positive and the remaining samples were negative for *B. motasi* infection.

A total of 492 blood samples collected from patients bitten by ticks, who visited the hospital, were investigated for the presence of *B. motasi*. From the results of CPA-VF, three samples were positive for *B.
motasi infection. Furthermore, to validate the presence of *B. motasi* in these samples, RT-PCR and nPCR were also employed and the results showed that all samples were negative for *B. motasi* infection.

**Discussion**

To provide an effective diagnostic tool, a CPA method targeting the 18S rRNA sequences of *B. motasi* was successfully developed for rapidly detecting and discriminating *B. motasi* infection. The CPA assay could detect four strains of *B. motasi*, *B. motasi* Lintan, *B. motasi* Tianzhu, *B. motasi* Hebei, and *B. motasi* Ningxian. In addition, cross reaction was not observed with piroplasms infective for sheep (*Babesia* sp. Xinjiang, *T. uilenbergi*, *T. luwenshuni*, *T. ovis*, *A. ovis*) and humans (*B. duncani*, *B. divergens*, *B. microti*, and *B. crassa*). Further studies should be performed to investigate potential cross reaction with other pathogens infective for humans using the CPA-VF approach developed herein.

In our present study, it could detect as few as 50 fg of genomic DNA from *B. motasi* per reaction, which was equal to approximately 50 μl of 0.000005% parasitized erythrocytes. The CPA reaction does not require expensive equipment and can be performed in a constant temperature block to maintain a reaction temperature of 61°C for 60 min. Furthermore, products generated by the CPA amplification can be detected using a VF strip, which only needs 2–5 min and is visible to the naked eye. Thus, the CPA assays are suitable for rapid, simple, and sensitive detection of *B. motasi* infection in limited-resource settings in endemic regions.

To assess its suitability for clinical use, we conducted the first diagnostic study in clinical specimens and host animals with CPA-VF, comparing it with microscopy, RT-PCR, and nPCR combined with PCR product sequencing. The results from studies of a positive and negative panel revealed that CPA-VF has better sensitivity than that of RT-PCR. Because of its sensitivity, CPA-VF could be useful for the preliminary screening of low-level parasitemia. Our results demonstrate that excellent sensitivity was observed for the CPA-VF approach in comparison with that of RT-PCR. False positives that needed to be confirmed by microscopy should be noted with CPA-VF assay. Three samples determined to be negative for piroplasm infection by nPCR were shown to present *B. motasi* infections by CPA-VF analysis. According to the clinical records, these three people presented virus-like or flu-like symptoms, clinical lab data showed they might be infected with bacteria (Additional file 1: Table 1). They were treated with azithromycin and cephalosporin and did not come back to hospital again. since they were outpatients so we didn't obtain enough information about the outcomes of these patients. One limitation of this study was the small number of positive specimens, which were used to evaluate clinical performance. Further studies are needed regarding the implementation of this approach into clinical practice.

**Conclusions**

We successfully developed a CPA-VF analysis for rapid, specific, high sensitivity detection of *B. motasi*, with high sensitivity (95.2%) and specificity (95.8%). The developed CPA-VF assay does not require sophisticated equipment and has an easy nucleic acid detection system. The study provided a practical,
easy-to-operate and alternative method for performing epidemiological study and point-of-care diagnosis in B. motasi infection. Although, false positive results should be cautious when the use of CPA for clinical screening.

**Abbreviations**

CPA-VF: cross priming amplification combined with a vertical flow; LAMP: loop-mediated isothermal amplification; RT-PCR: Real-time PCR; VVBD: Vectors and Vector-Borne Diseases Laboratory; LVRI: Lanzhou Veterinary Research Institute; nPCR: nested PCR; RLB: reverse line blot

**Declarations**

**Acknowledgements**

Not applicable.

**Ethics approval and consent to participate**

The collection and manipulation of sheep blood samples were approved by the Animal Ethics Committee of the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All sampling procedures were handled in accordance with the Animal Ethics Procedures and Guidelines of the People's Republic of China (Permit No. LVRIAEC-2018-001).

The study of clinical specimens were approved by the Ethics Committee of The Second Hospital of Lanzhou University (reference 2018A-046). All the procedures conducted were according to the Ethical Procedures and Guidelines of the People's Republic of China.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.

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**Competing interests**
The authors declare that they have no competing interests.

Authors' contributions

Designed the study: HY, JLu and GG. Performed the experiments, analyzed the results and wrote the manuscript: JW. Contributed reagents/materials/equipment: SG, SZ, JLi, XH, YL, GL, AL. All authors read and approved the final manuscript.

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Additional File
Additional file 1: Table 1. Clinical information of 3 patients.

Figures

Figure 1

Optimization of CPA reaction temperature and time and limit of detection evaluation of the CPA-VF assay with serial dilution of B. motasi DNA. a reaction temperature of CPA-VF assay; b reaction time of CPA-VF assay; c detection limit of the CPA-VF.

Figure 2
Evaluation of specificity of the CPA-VF with genomic DNA (B. motasi Lintan, B. motasi Tianzhu, B. motasi Hebei, B. motasi Ningxian, Babesia sp. Xinjiang, Babesia sp. Dunhuang, T. uilenbergi, T. luwenshuni, T. ovis, A. ovis, B. duncani, B. divergens) and plasmids (B. microti, and B. crassa).

**Supplementary Files**

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