Rapid Detection of *Babesia motasi* Responsible for Human Babesiosis by Cross-priming Amplification combined with a vertical flow

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Research

Keywords: Ovine babesiosis, human babesiosis, Babesia motasi, cross priming amplification, vertical flow visualization strip, detection, identification

Posted Date: January 23rd, 2020

DOI: https://doi.org/10.21203/rs.2.21776/v1

Version of Record: A version of this preprint was published at Parasites & Vectors on July 29th, 2020. See the published version at https://doi.org/10.1186/s13071-020-04246-4.

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 flow (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* = 240) 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.

Keywords: Ovine babesiosis, human babesiosis, *Babesia motasi*, cross priming amplification, vertical flow visualization strip, detection, identification

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. Due to 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, termed 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].

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 Vector and Vector-Borne Diseases (VVBD) laboratory, Lanzhou Veterinary Research Institute (LVRI) [11,

12, 13]. Epidemiological studies have revealed that B. motasi infections are wide distribution in sheep, goats, and vector ticks across China, according to molecular detection and serological assay. Given that it poses 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 usual strategies for diagnosis of B. motasi infection. Those 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 procedures, which restricted their wide application in clinical care, infection control, and epidemiological studies [14, 15, 16].

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 [17]. This approach has been applied to detection numbers of animal and plant pathogens, such as bacteria, virus, and herbal products, with high specificity and sensitivity [18, 19, 20, 21]. 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 to 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 to sheep and humans. A region that is conserved intra-B. motasi and variable among species was used as 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-5 s and BLT-4a), one cross primer (BLT-2s1a), and two detector primers (BLT-2 s and BLT-3 s). The detector primer (BLT-2 s) was labeled with biotin at the 5' end and the BLT-3 s was labeled with fluorescein isothiocyanate (FITC) at the 5' end. The cross primer was composed of the BLT-2 s at the 5' end and 1a at the 3' end. These primers were synthesized by TsingKe Biotech Co., Ltd (Beijing, China).

Blood samples

Standard positive samples were obtained from experimentally infected sheep with B. motasi. Briefly, 16 of 6-month old sheep were purchased from Jingtai county, Gansu Province and confirmed to be free of piroplasm infection by microscopy, RT-PCR, nPCR and ELISA assay [16, 22, 23, 24]. Four splenectomized sheep 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 50 ml blood infected with B. motasi Lintan/B. motasi Tianzhu/B. motasi Ningxian/B. motasi Hebei via 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-5 s and BLT-4a), 7.5 µM each of detector primers (BLT-2 s and BLT-3 s), 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 deoxynucleoside 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 the PBS were added to the samples pad. A reaction was identified as positive when both of the test line and 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 by 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. divergens, B. duncani, and plasmid DNAs bearing the 18S rRNA gene of B. microti (KF410825) and B. crassa (AY260176). To evaluate the assay's sensitivity, serials dilutions of genomic DNA from purified B. motasi Lintan merozoites were used as the template for CPA amplification, ranging from 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 CPA-VF assay, its sensitivity and specificity were determined using experimentally infected positive and field collected negative samples, versus microscopy, RT-PCR, nPCR targeting the 18S rRNA combined with gene sequencing [16, 22, 25].

Clinical performance of CPA-VF assay for clinical specimens

Field blood samples were randomly collected from 340 sheep in Gansu province and transported to VVBD, LVRI in iceboxes and stored at -20℃ 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 B. motasi. All participants agreed to participate in this study and signed an informed consent form.

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

Results

Optimization of the CPA primers, reaction temperature and time

Sequence alignment of the 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 were presented in Table 1. The primers and probes of set one showed the high specificity (Fig. 1). 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 CPA reaction. Optimal brightness of red-purple band was observed in the VF strip at the temperature 61 °C (Fig. 2A).

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-GCTTTTAAACCAATTGTTGG
	BLT-2s1a	CGATGCCTTTTGGCGGCGCGATTCGCAAGTTTATTATG
	BLT-4a	CTTGAATGGAACATCGCTAA
Set two	BLT-5s	GGADWWDGTCCGKTTTTG
	BLT-2s	FITC-CTTAGAGGGACTCCTGC
	BLT-3s	Biotin-GCTTGAAGCGTGGGGT
	BLT-2s1a	CTTAGAGGGACTCCTGCCAGACCTGTTATTGCCTT
	BLT-4a	CGCCTGCCGTTCGACGATT

The CPA amplification was conducted at 61 °C for 40–100 min. The results revealed that positive sign could be developed as early as 40 min amplification; however, the brightness of positive band was not strong as that of 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 B. motasi detection (Fig. 2B).

Cross reaction of developed CPA approach

The CPA technique was evaluated by testing piroplasms infective to sheep, goats and humans, including B. motasi Lintan, B. motasi Tianzhu, B. motasi Hebei, B. motasi Ningxian, Babesia sp. Xinjiang, Babesia sp. Dunhuang, T. luwenshuni, T. uilenbergi, T. ovis, B. divergens, B. microti, B. duncani, and B. crassa. As shown in Fig. 3, only four strains of B. motasi isolated from different regions of China could be detected as positive. No cross reaction was observed with other Babesia spp. and Theileria spp. These available results demonstrated that the CPA assay was specific for identification of B. motasi (Fig. 3).

Limit of detection of the CPA-VF assay

The limit of detection of CPA-VF assay was evaluated using 5-fold serially diluted DNA from purified merozoites of B. motasi in duplicated reactions from 4 ng/μl to 10 fg/μl. The assay could detect as few as 50 fp/μl DNA of B. motasi (Fig. 4), which corresponds to about 50 μl of 0.000005% parasitemic erythrocytes (McLaughlin et al., 1986). 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 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 was presented in Table 2. Compared with that of microscopy and nPCR combined with gene sequencing, the CPA-VF and RT-PCR had a sensitivities of 95.2% (95% confidence interval [CI], 78.2–99.4%) 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 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	*B. motasi* Tianzhu	*B. motasi* Hebei	*B. motasi* Ningxian	Negative	Total
Positive	10	10	12	10		42
Negative					48	48
Total	10	10	12	10	48	90

Table 3

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

	Primer name	**Sequence (5'-3')**
Set one	BLT-5 s	GCTAATTGTAGGGCTAATACAAG
	BLT-2 s	FITC-CGATGCCTTTTGGCGGCG
	BLT-3 s	Biotin-GCTTTTAAACCAATTGTTGG
	BLT-2s1a	CGATGCCTTTTGGCGGCGCGATTCGCAAGTTTATTATG
	BLT-4a	CTTGAATGGAACATCGCTAA
Set two	BLT-5 s	GGADWWDGTCCGKTTTTG
	BLT-2 s	FITC-CTTAGAGGGACTCCTGC
	BLT-3 s	Biotin-GCTTGAAGCGTGGGGT
	BLT-2s1a	CTTAGAGGGACTCCTGCCAGACCTGTTATTGCCTT
	BLT-4a	CGCCTGCCGTTCGACGATT

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

To evaluate the feasible of using CPA-VF as an alternative approach for B. motasi detection, 240 and 492 whole blood samples from sheep and patients were subjected to CPA-VF, RT-PCR and nPCR combined with gene sequencing. The results of CPA-VF assay showed that 3.8% (13/340) of the samples collected from sheep in Gansu provinces 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 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 combined with gene sequencing were also employed and the results showed that all samples were negative for B. motasi infection.

Discussion

Babesiosis, caused by bites from ticks infected with the genus Babesia or transfusion of blood products, is one of the most prevalent protozoan disease in human and animals worldwide [26]. The first case of babesiosis was described in Rameses in 1888, when a Hungarian pathologist investigated the febrile hemoglobinuria in cattle [27]. It was caused by infection with B. bovis. Shortly afterwards, an organism that was similar to B. bovis was also described in cattle in Texas. Currently, more than 100 Babesia spp. have been identified in humans and other animals across the world [28]. However, only few Babesia spp. have been reported to be responsible for human babesiosis, including B. microti, B. divergens, B. duncani, B. venatorum, B. crass, and Babesia sp. XXB/HangZhou [29, 30, 31, 32, 33]. As a causative agent responsible for human babesiosis, the first case caused by B. motasi-like was reported in Korea in 2005 [31]. Recently, infected with B. motasi was diagnosed in a 70-year-old man in Korea [34]. Moreover, it is the most prevalent pathogen among vector ticks and host animals on the basis of serological and molecular epidemiological investigations conducted in China[24]. However, till now there was no report of human babesiosis caused by B. motasi in China.

As clinical signs and symptoms of human babesiosis are viral-like and often overlap with those of several other illnesses, it can be challenge to discriminate human babesiosis. However, the increasing cases of human babesiosis has led to an urgent need to identify Babesia species. The rapid and accurate diagnosis of Babesia infection can facilitate clinical managements and provide probable treatment. Several molecular methods have been developed with high specificity and sensitivity for detection of the B. motasi infection, including nPCR, RT-PCR, RLB [14, 15, 25, 35]. However, those approaches have intrinsic disadvantages, such as time-consuming, and requiring expensive thermal cycle instruments and/or manipulation of amplified products, which have limited their application in field-site and low-resource laboratories. When compared with PCR, RT-PCR and RLB, isothermal amplification technique is a powerful tool to overcome these drawbacks. Furthermore, CPA-VF is a novel isothermal amplification technique developed in recent years and the results can be visualized by naked eye, needing no additional instruments. To provide an effective diagnostic tool, a CPA method targeting the 18S rRNA sequences of B. motasi was successfully developed for rapid 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 piroplasm infective to sheep (Babesia sp. Xinjiang, T. uilenbergi, T. luwenshuni, T. ovis, A. ovis) and humans (B. duncani, B. divergens, B. microti, and B. crassa). These results indicated that this CPA assay has good specificity. Further studies should be performed to investigate potential cross reaction with other pathogens infective to humans using the CPA-VF approach developed herein.

The CPA assay was high sensitivity and 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% parasitic erythrocytes. The process of CPA reaction does not require expensive equipment and can be performed in a constant temperature block to maintain reaction temperature of 61 °C for 60 min. Furthermore, generated products by the CPA amplification can be detected using a VF strip, which only need 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 setting in endemic regions.

To access 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 gene 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. Furthermore, 340 field blood samples from animals and 492 clinical specimens from patients were used to evaluate analytic performance regarding B. motasi identification. Our results demonstrate that excellent sensitivity was observed in CPA-VF approach in comparison to that of RT-PCR. False positives that were needed to be confirmed by microscopy should be noted with CPA-VF assay. To avoid a risk of contamination, genomic DNA extraction, CPA amplification and its products analysis with VF strips were performed in separated rooms, so contamination was not observed in our study. Three samples determined to be negative of piroplsam infection by nPCR were shown to present B. motasi infections by CPA-VF analysis. This could be explained by false positive or more sensitivity of CPA analysis, compared with nPCR. A proposed algorithm for B. motasi identification in patients suspected babesiosis, caused by B. motasi, is presented in Fig. 5. However, further studies are needed to explore this issue. 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 sensitive detection of B. motasi, with high sensitivity (95.2%) and specificity (95.8%). Due to the small number of positive samples, 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.

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.

The studys 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.

Funding

This study was financially supported by the National Key Research and Development Program of China (2017YFD0501200), 973 Program (2015CB150300), ASTIP (CAAS-ASTIP-2016-LVRI), NBCIS (CARS-37) and Jiangsu Co-innovation Center programme for Prevention and Control of Important Animal Infectious Disease and Zoonose. Cuiying Scientific and Technological Innovation Program of Lanzhou University Second Hospital.

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, QM, XH, YL, GL. All authors read and approved the final manuscript.

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References

1. Uilenberg G. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. Vet Parasitol. 1995;57 1-3:19-41. https://www.ncbi.nlm.nih.gov/pubmed/7597784.
2. Friedhoff KT. Tick-borne diseases of sheep and goats caused by *Babesia*, *Theileria* or *Anaplasma* spp. Parassitologia. 1997;39 2:99-109. https://www.ncbi.nlm.nih.gov/pubmed/9530692.
3. Yin H, Luo J. Ticks of small ruminants in China. Parasitol Res. 2007;101 Suppl 2:S187-9; doi: 10.1007/s00436-007-0688-3. https://www.ncbi.nlm.nih.gov/pubmed/17823826.
4. Vannier E, Krause PJ. Human babesiosis. N Engl J Med. 2012;366 25:2397-407; doi: 10.1056/NEJMra1202018. https://www.ncbi.nlm.nih.gov/pubmed/22716978.
5. Mueller I, Shakri AR, Chitnis CE. Development of vaccines for *Plasmodium vivax* malaria. Vaccine. 2015;33 52:7489-95; doi: 10.1016/j.vaccine.2015.09.060. https://www.ncbi.nlm.nih.gov/pubmed/26428453.
6. Kim JY, Cho SH, Joo HN, Tsuji M, Cho SR, Park IJ, et al. First case of human babesiosis in Korea: detection and characterization of a novel type of *Babesia* sp. (KO1) similar to ovine babesia. J Clin Microbiol. 2007;45 6:2084-7; doi: 10.1128/JCM.01334-06. https://www.ncbi.nlm.nih.gov/pubmed/17392446.
7. Hong SH, Kim SY, Song BG, Rho JR, Cho CR, Kim CN, et al. Detection and characterization of an emerging type of *Babesia* sp. similar to *Babesia motasi* for the first case of human babesiosis and ticks in Korea. Emerg Microbes Infect. 2019;8 1:869-78; doi: 10.1080/22221751.2019.1622997. https://www.ncbi.nlm.nih.gov/pubmed/31179860.
8. Man SQ, Qiao K, Cui J, Feng M, Fu YF, Cheng XJ. A case of human infection with a novel *Babesia* species in China. Infect Dis Poverty. 2016;5:28; doi: 10.1186/s40249-016-0121-1.

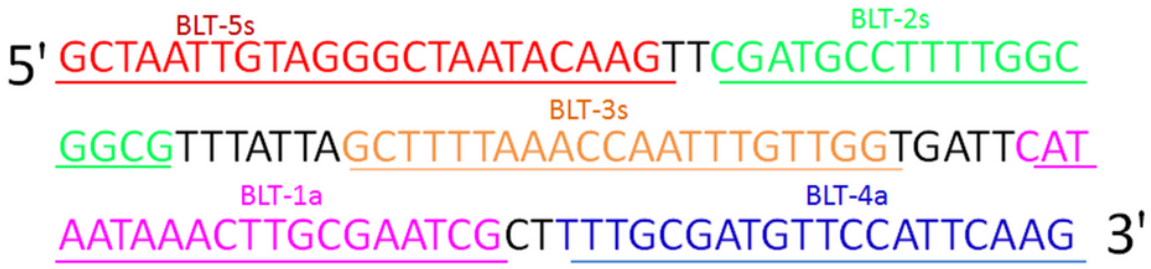
https://www.ncbi.nlm.nih.gov/pubmed/27025290.

9. Jiang JF, Zheng YC, Jiang RR, Li H, Huo QB, Jiang BG, et al. Epidemiological, clinical, and laboratory characteristics of 48 cases of "*Babesia venatorum*" infection in China: a descriptive study. Lancet Infect Dis. 2015;15 2:196-203; doi: 10.1016/S1473-3099(14)71046-1. https://www.ncbi.nlm.nih.gov/pubmed/25539588.
10. Wang J, Zhang S, Yang J, Liu J, Zhang D, Li Y, et al. *Babesia divergens* in human in Gansu province, China. Emerg Microbes Infect. 2019;8 1:959-61; doi: 10.1080/22221751.2019.1635431. https://www.ncbi.nlm.nih.gov/pubmed/31244397.
11. Bai Q, Liu G, Liu D, Ren J, Li X. Isolation and preliminary characterization of a large *Babesia* sp. from sheep and goats in the eastern part of Gansu Province, China. Parasitol Res. 2002;88 13 Suppl 1:S16-21. https://www.ncbi.nlm.nih.gov/pubmed/12051600.
12. Guan GQ, Yin H, Luo JX, Lu WS, Zhang QC, Gao YL, et al. Transmission of *Babesia* sp. to sheep with field-collected Haemaphysalis qinghaiensis. Parasitol Res. 2002;88 13 Suppl 1:S22-4. https://www.ncbi.nlm.nih.gov/pubmed/12051601.
13. Liu AH, Yin H, Guan GQ, Schnittger L, Liu ZJ, Ma ML, et al. At least two genetically distinct large *Babesia* species infective to sheep and goats in China. Veterinary Parasitology. 2007;147 3-4:246-51; doi: 10.1016/j.vetpar.2007.03.032. <Go to ISI>://WOS:000248130700006.
14. Guan GQ, Chauvin A, Luo JX, Inoue N, Moreau E, Liu ZJ, et al. The development and evaluation of a loop-mediated isothermal amplification (LAMP) method for detection of *Babesia* spp. infective to sheep and goats in China. Experimental Parasitology. 2008;120 1:39-44; doi: 10.1016/j.exppara.2008.04.012. <Go to ISI>://WOS:000259119800006.
15. Niu QL, Luo JX, Guan GQ, Ma ML, Liu ZJ, Liu AH, et al. Detection and differentiation of ovine *Theileria* and *Babesia* by reverse line blotting in China. Parasitology Research. 2009;104 6:1417-23; doi: 10.1007/s00436-009-1344-x. <Go to ISI>://WOS:000265442600023.
16. Yang. Q, Liu. A, Liu. J, Jifei. Y, Youquan. L, Zhijie. L, et al. Molecular epidemiological investigation of ovine *Babesia* spp. in 10 provinces of China. Chinese Veterinary Science. 2016;46 5:597-601; doi: 10.3201/eid1406.061513. https://www.ncbi.nlm.nih.gov/pubmed/18507918.
17. Xu G, Hu L, Zhong H, Wang H, Yusa S, Weiss TC, et al. Cross priming amplification: mechanism and optimization for isothermal DNA amplification. Sci Rep. 2012;2:246; doi: 10.1038/srep00246. https://www.ncbi.nlm.nih.gov/pubmed/22355758.
18. Gao Y, Meng XY, Zhang HW, Luo YZ, Sun Y, Li YF, et al. Cross-priming amplification combined with immunochromatographic strip for rapid on-site detection of African swine fever virus. Sensors and Actuators B-Chemical. 2018;274:304-9; doi: 10.1016/j.snb.2018.07.164. <Go to ISI>://WOS:000443960000033.
19. Huo YY, Li GF, Qiu YH, Li WM, Zhang YJ. Rapid Detection of Prunus Necrotic Ringspot Virus by Reverse Transcription-cross-priming Amplification Coupled with Nucleic Acid Test Strip Cassette. Sci Rep. 2017;7 1:16175; doi: 10.1038/s41598-017-16536-6. https://www.ncbi.nlm.nih.gov/pubmed/29170535.

20. Wang Y, Wang Y, Ma A, Li D, Ye C. Rapid and sensitive detection of Listeria monocytogenes by cross-priming amplification of lmo0733 gene. FEMS Microbiol Lett. 2014;361 1:43-51; doi: 10.1111/1574-6968.12610. https://www.ncbi.nlm.nih.gov/pubmed/25273275.
21. Su ZD, Shi CY, Huang J, Shen GM, Li J, Wang SQ, et al. Establishment and application of cross-priming isothermal amplification coupled with lateral flow dipstick (CPA-LFD) for rapid and specific detection of red-spotted grouper nervous necrosis virus. Virol J. 2015;12:149; doi: 10.1186/s12985-015-0374-5. https://www.ncbi.nlm.nih.gov/pubmed/26409445.
22. Yang JF, Li YQ, Liu ZJ, Liu JL, Guan GQ, Chen Z, et al. Molecular evidence for piroplasms in wild *Reeves' muntjac* (*Muntiacus reevesi*) in China. Parasitol Int. 2014;63 5:713-6; doi: 10.1016/j.parint.2014.06.002. https://www.ncbi.nlm.nih.gov/pubmed/24970769.
23. Guan G, Ma M, Liu A, Ren Q, Wang J, Yang J, et al. A recently identified ovine *Babesia* in China: serology and sero-epidemiology. Parasitol Int. 2012;61 4:532-7; doi: 10.1016/j.parint.2012.04.004. https://www.ncbi.nlm.nih.gov/pubmed/22579523.
24. Wang JM, Ma ML, Liu AH, Ren QY, Li AY, Liu ZJ, et al. A sero-epidemiological survey of Chinese *Babesia motasi* for small ruminants in China. Parasitol Res. 2013;112 6:2387-91; doi: 10.1007/s00436-013-3310-x. https://www.ncbi.nlm.nih.gov/pubmed/23371500.
25. Olmeda AS, Armstrong PM, Rosenthal BM, Valladares B, delCastillo A, deArmas F, et al. A subtropical case of human babesiosis. Acta Tropica. 1997;67 3:229-34; doi: Doi 10.1016/S0001-706x(97)00045-4. <Go to ISI>://WOS:A1997XK20400007.
26. Vannier EG, Diuk-Wasser MA, Ben Mamoun C, Krause PJ. Babesiosis. Infect Dis Clin North Am. 2015;29 2:357-70; doi: 10.1016/j.idc.2015.02.008. https://www.ncbi.nlm.nih.gov/pubmed/25999229.
27. V B. Sur l' hémoglobinurie bactérienne du boeuf. C R Acad Sci 1888;107:692-4; doi: 10.1007/s00436-018-6026-0. <Go to ISI>://WOS:000445159500023.
28. Levine ND. Progress in taxonomy of the Apicomplexan protozoa. J Protozool. 1988;35 4:518-20. https://www.ncbi.nlm.nih.gov/pubmed/3143826.
29. Meer-Scherrer L, Adelson M, Mordechai E, Lottaz B, Tilton R. *Babesia microti* infection in Europe. Curr Microbiol. 2004;48 6:435-7; doi: 10.1007/s00284-003-4238-7. <Go to ISI>://WOS:000221478700009.
30. Hunfeld KP, Lambert A, Kampen H, Albert S, Epe C, Brade V, et al. Seroprevalence of Babesia infections in humans exposed to ticks in midwestern Germany. Journal of Clinical Microbiology. 2002;40 7:2431-6; doi: 10.1128/Jcm.40.7.2431-2436.2002. <Go to ISI>://WOS:000176605800018.
31. Kim JY, Cho SH, Joo HN, Tsuji M, Cho SR, Park IJ, et al. First case of human babesiosis in Korea: Detection and characterization of a novel type of *Babesia* sp (KO1) similar to ovine *Babesia*. J Clin Microbiol. 2007;45 6:2084-7; doi: 10.1128/Jcm.01334-06. <Go to ISI>://WOS:000247286500081.
32. Jia N, Zheng YC, Jiang JF, Jiang RR, Jiang BG, Wei R, et al. Human Babesiosis Caused by a *Babesia crassa*-Like Pathogen: A Case Series. Clin Infect Dis. 2018;67 7:1110-9; doi: 10.1093/cid/ciy212. <Go to ISI>://WOS:000445387500017.

33. Wang JM, Zhang SD, Yang JQ, Liu JL, Zhang DK, Li YQ, et al. *Babesia divergens* in human in Gansu province, China. Emerg Microbes Infec. 2019;8 1:959-61; doi: 10.1080/22221751.2019.1635431. <Go to ISI>://WOS:000473023400001.
34. Hong SH, Kim SY, Song BG, Rho JY, Cho CR, Kim CN, et al. Detection and characterization of an emerging type of *Babesia* sp. similar to Babesia motasi for the first case of human babesiosis and ticks in Korea. Emerg Microbes Infec. 2019;8 1:869-78; doi: 10.1080/22221751.2019.1622997. <Go to ISI>://WOS:000472571500001.
35. Yang JF, Li YQ, Liu ZJ, Liu JL, Guan GQ, Chen Z, et al. Molecular evidence for piroplasms in wild Reeves' muntjac (Muntiacus reevesi) in China. Parasitology International. 2014;63 5:713-6; doi: 10.1016/j.parint.2014.06.002. <Go to ISI>://WOS:000340513800012.

Figures



BLT-5s: 5'-GCTAATTGTAGGGCTAATACAAG-3'

BLT-2s: 5'-CGATGCCTTTTGGCGGCG-3'

BLT-3s: 5'-GCTTTTAAACCAATTTGTTGG-3'

BLT-2s1a: 5'-CGATGCCTTTTGGCGGCGCGATTCGCAAGTTTATTATG-3'

BLT-4a: 5'-CTTGAATGGAACATCGCAAA-3'

Figure 1

Primer sequences and locations. The underlined sequences indicate the position of the primers used for the CPA-VF assay.

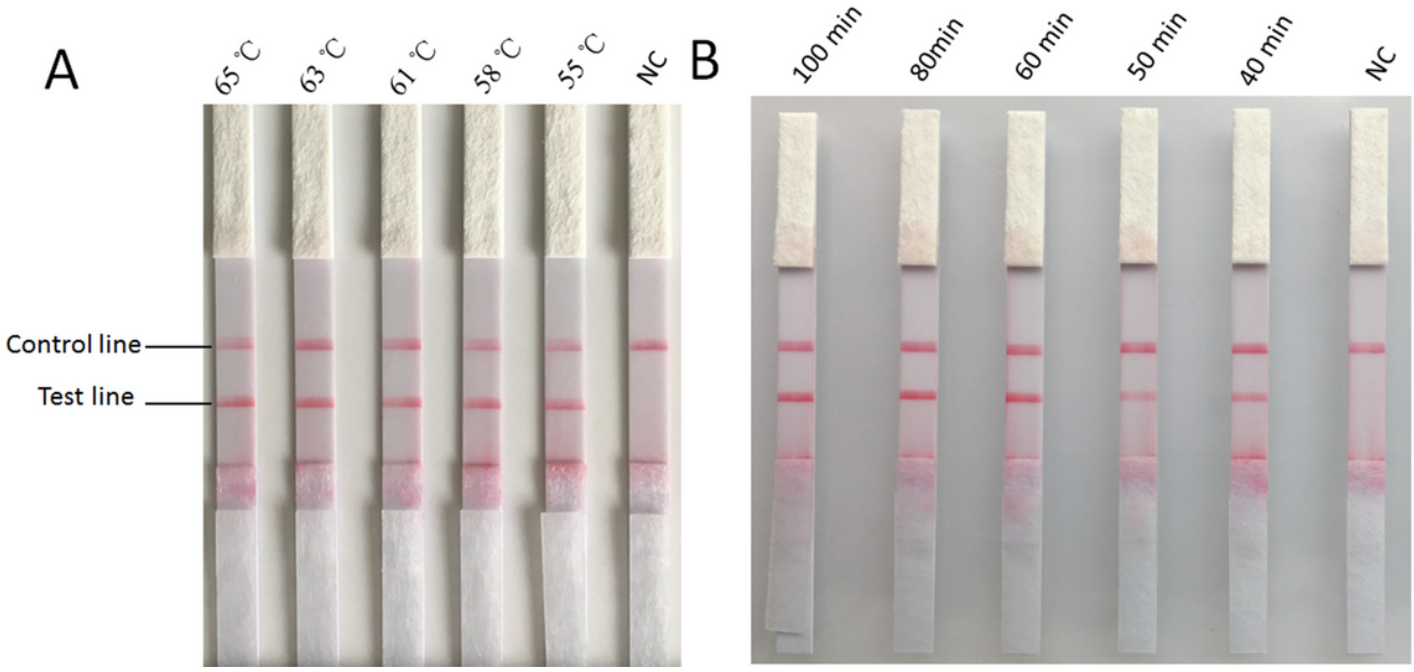


Figure 2

Optimization of CPA reaction temperature and time: a reaction temperature of CPA-VF assay; b reaction time of CPA-VF assay.

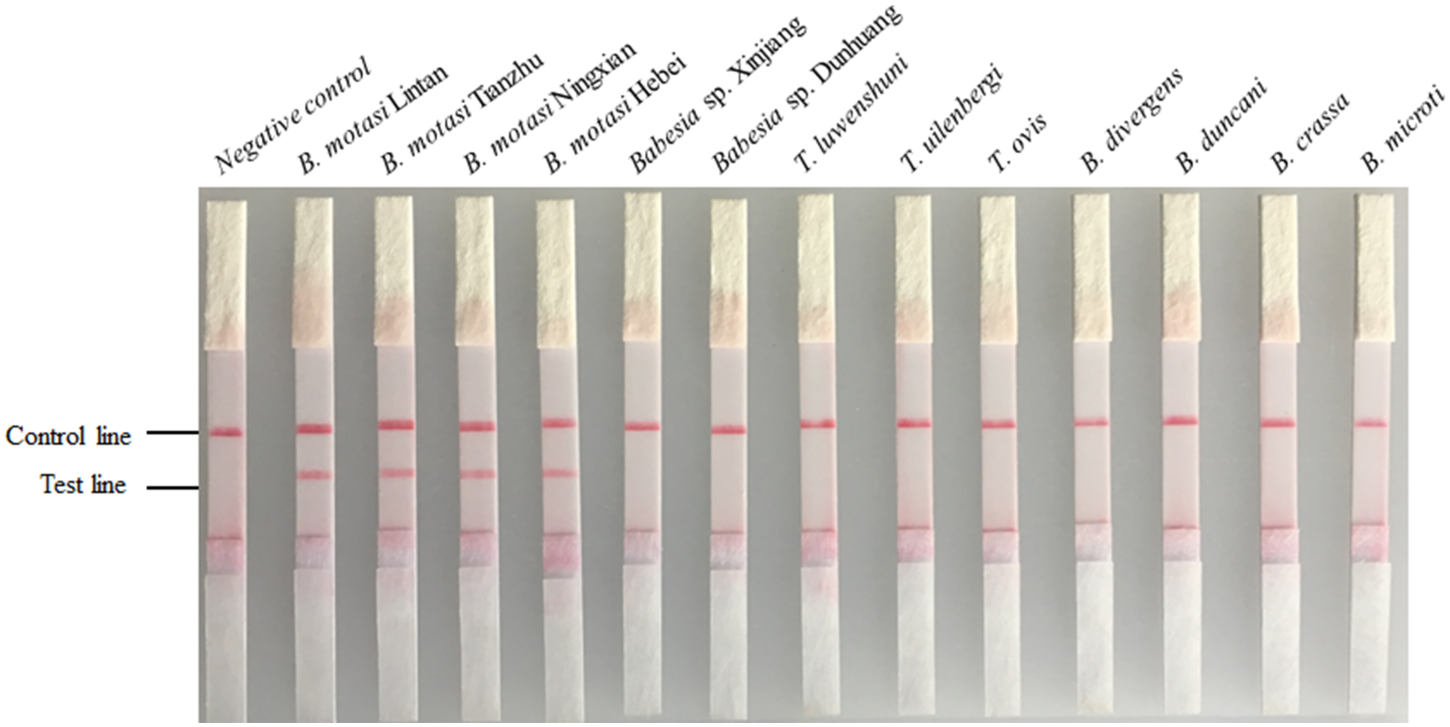


Figure 3

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).

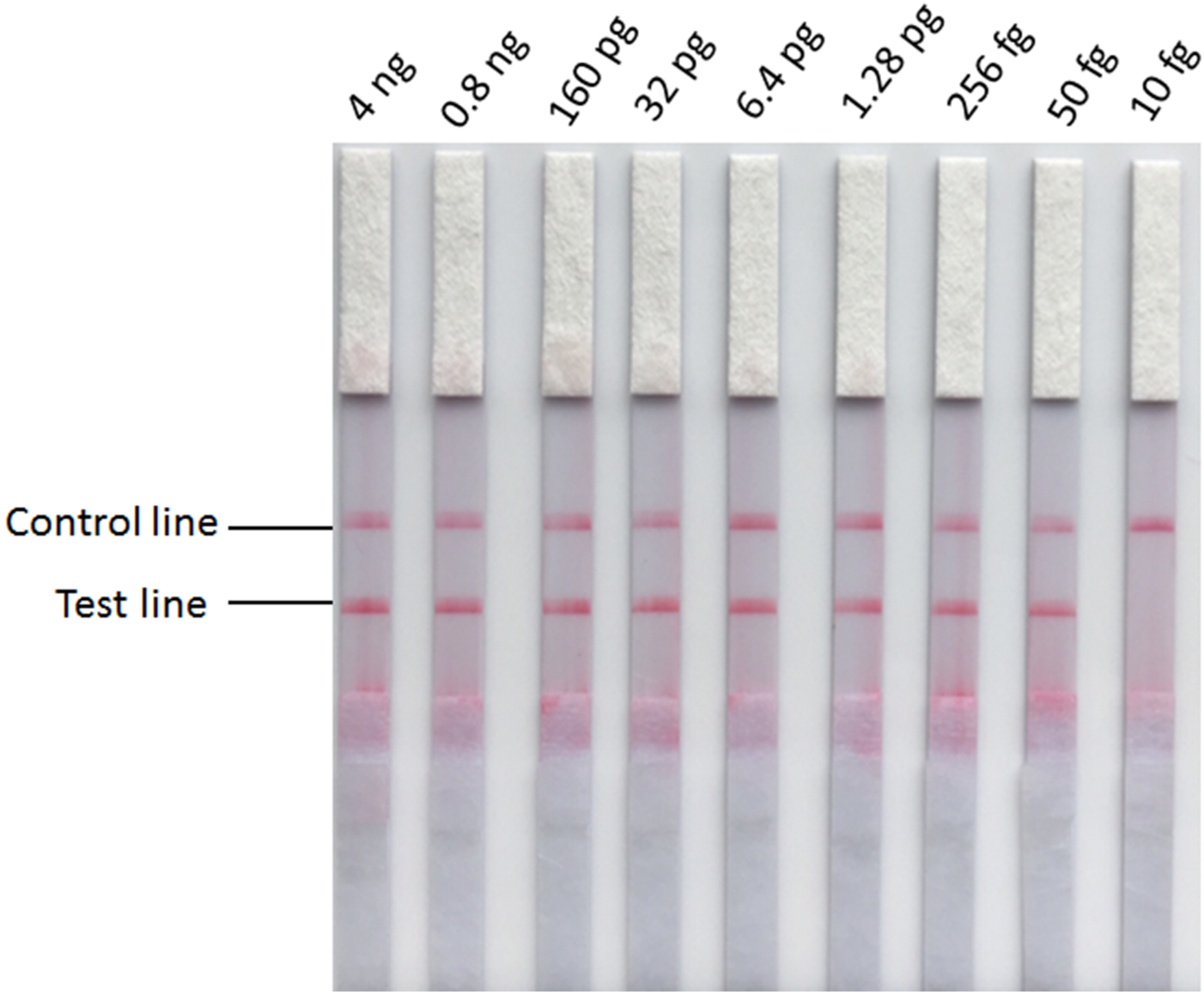


Figure 4

Limit of detection evaluation of the CPA-VF assay with serial dilution of B. motasi DNA.

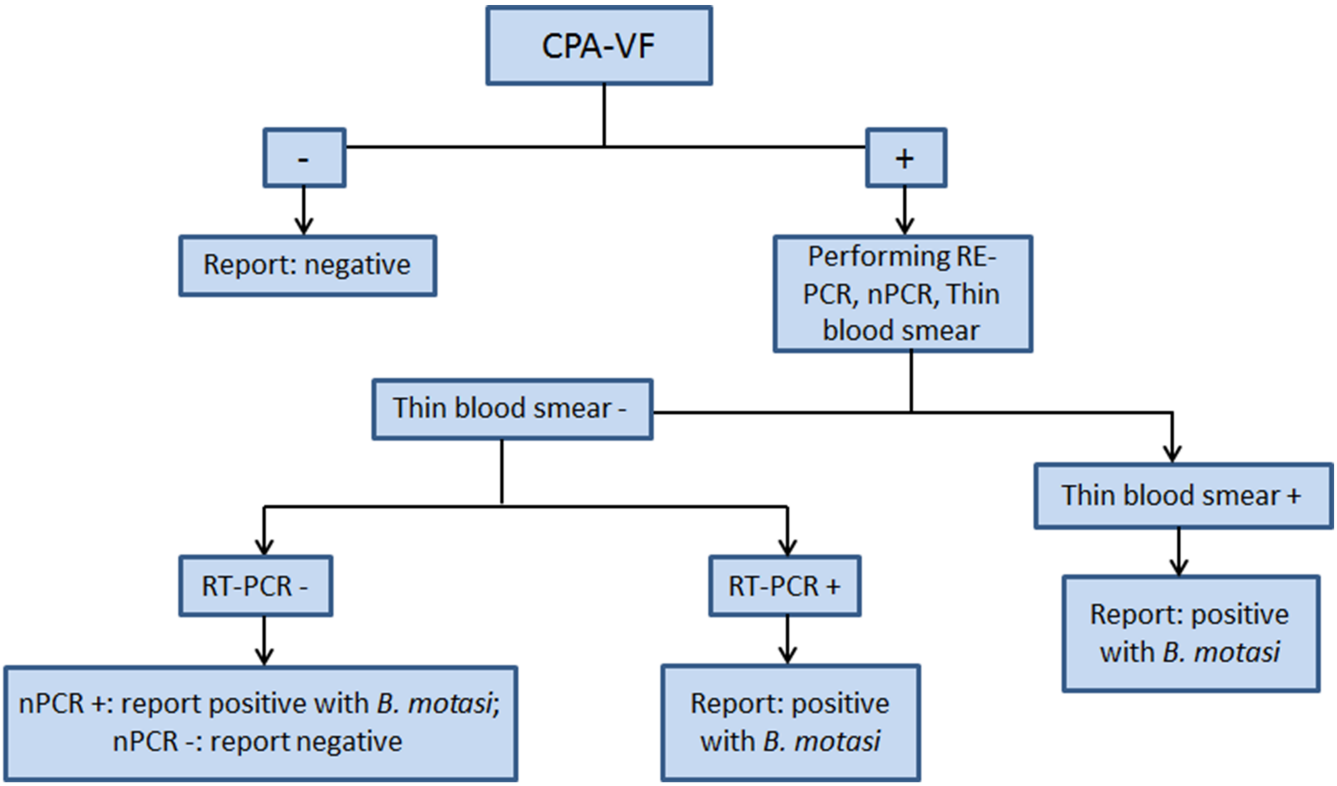


Figure 5

A strategy for human babesiosis diagnosis caused by B. motasi using CPA-VF assay as a screen.

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