A novel nested-qPCR assay for detection of Babesia duncani infection in hamsters and humans

Yanbo Wang
The Second Hospital of Lanzhou University

Shangdi Zhang
The Second Hospital of Lanzhou University

Jinming Wang
Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science

Muhammad Rashid
The Islamia University of Bahawalpur

Xiaorong Wang
The Second Hospital of Lanzhou University

Xinyue Liu (liuxy@lzu.edu.cn)
The Second Hospital of Lanzhou University

Hong Yin
Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science

Guiquan Guan
Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science

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Abstract

Background

*Babesia duncanii* is a causative pathogen for human babesiosis, transmitted by tick bite, blood transfusion, and pregnancy. As an important haemo protozoan disease, diagnostic assay of this pathogen is not available and its detection is neglected during transfusion and utilization of blood products.

Methods

In this study, primers and probes were designed in variable regions of *B. duncanii* 18S rRNA gene. A novel molecular approach, nested quantitative polymerase chain reaction (nested-qPCR), was developed to specifically detect DNA of *B. duncanii* in blood samples from hamster and human. Comparative analyses of our established technique with previously reported nested-PCR and microscopy were conducted using experimentally infected and clinical samples.

Results

The newly optimized diagnostic technique showed no cross reaction with other zoonotically important *Babesia* spp. such as *Babesia microti*, *Babesia divergens*, *Babesia crassa*, and *Babesia motasi* Hebei. The detection limit of nested-qPCR was approximately 1 copy of plasmid in a total of 20 µl volume or 1 infected red blood cells in 200 µl of whole blood. The specificity and sensitivity of this method are 100% and 98.6%, respectively. Comparative analyses revealed that nested-qPCR showed relatively higher efficacy and specificity for detecting *B. duncanii* than microscopic examination and nested-PCR. Investigation of 492 specimens from patients of tick bite from Gansu Province, China presents no *B. duncanii* infection.

Conclusions

In this study, we provide a novel diagnostic assay for determining *B. duncanii* infection and investigating its prevalence in specific areas.

Introduction

Human babesiosis is a vector-borne disease caused by protozoan parasites of genus *Babesia*, such as *B. duncanii*, *B. microti*, *B. divergens*, and *B. venatorum* (Lobo, Singh, & Rodriguez, 2020; Schnittger, Rodriguez, Florin-Christensen, & Morrison, 2012). The main transmission routes of *Babesia* include infected tick (ixodid) bites (Vannier, Diuk-Wasser, Ben Mamoun, & Krause, 2015; Young et al., 2019b), blood transfusion and transplacental transmission (Fox et al., 2006; Joseph et al., 2012). In recent years,
increasing number of cases of human babesiosis have been reported in Asia, Africa, South America, North America, and Europe (Chen et al., 2019; Fang et al., 2015; Karshima, Karshima, & Ahmed, 2022; Krause, 2019; Man et al., 2016; Scott & Scott, 2018). B. duncani was first isolated from a patient in Washington State in 1991 (Conrad et al., 2006a). At first, human cases of B. duncani were documented in the Western United States, mainly in Washington, Oregon and California. Nonetheless, till 2017, 1119 human cases of B. duncani infection have been identified across Canada (Scott & Scott, 2018).

It is reported that B. duncani present stronger pathogenicity and reproductive ability than B. microti (Wozniak, Lowenstine, Hemmer, Robinson, & Conrad, 1996), and even has strong resistance to the currently recommended antibabesia drugs (Abraham et al., 2018). So, the accurate diagnosis of B. duncani infection is important for making treatment regimen in patients (Conrad et al., 2006b; Young et al., 2019b) (O'Connor, Kjemtrup, Conrad, & Swei, 2018). The current molecular diagnostic methods for detecting B. duncani include nested-PCR (O'Connor et al., 2018), droplet digital PCR (Wilson et al., 2015), the Roche Molecular Systems the cobas Babesia, 2021 (Stanley et al., 2021), and Nucleic Acid Testing NAT in use in the United States (Tonnetti, Dodd, Foster, & Stramer, 2022). These methods either need expensive equipment or have limited detection performance, which is not conducive to wide application. Due to the lack of effective diagnostic methods and the no symptoms in immune competent patients, the true infection status and distribution of the B. duncani is still unknown.

The number of reported cases of transmission through blood transfusions and naturally-acquired Babesia infections has increased in the last decade. In the United States, parasitic infections transmitted through blood transfusions are almost entirely caused by Babesia (Leiby, 2011a). However, blood screening for B. duncani is still lacking or only available in limited blood collection centers (Busch, Bloch, & Kleinman, 2019). Therefore, blood screening in blood donors is required to prevent TTB caused by B. duncani in areas where B. duncani is endemic, such as the Canada and United States (Wagner, Leiby, & Roback, 2019).

Human babesiosis caused by B. duncani has gradually become a public health problem and has the potential to spread continuously in new areas, so regular monitoring and effective intervention are required to prevent further spreading and development of this pathogen. Therefore, there is an urgent need to develop and validate a rapid, effective and reliable diagnostic method for the specific identification of B. duncani. The aim of this study was to develop a reliable diagnostic technique to identify B. duncani infection in humans and animals.

Materials And Methods

Sample preparation and DNA extraction

One-month old LVG Golden Syrian Hamster were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. B. duncani strain was purchased from ATCC (American Type Culture Collection) and preserved at the Vectors and Vector-Borne Diseases Laboratory in the Lanzhou Veterinary Research
Institute, Chinese Academy Of Agricultural Sciences. After the *B. duncani* suspension preserved in liquid nitrogen was thawed rapidly at 37°C in water bath, each hamster was inoculated intraperitoneally with 200 µl of *B. duncani* suspension (About 2.0×10^6 infected red blood cells). Three days post-infection, blood smear stained with Giemsa were examined. About 3,000 red blood cells (RBCs) were counted per blood smear to calculate the percentage of parasitized erythrocytes (PPE). All parasitized RBCs were counted regardless of the stage of the parasite. Whole blood was collected into EDTA-coated tubes at different time post-infection. In addition, artificial positive blood samples with various PPE were prepared as follow. Briefly, hamster whole blood containing *B. duncani* was diluted with healthy human blood, ranged from 10^-1 infected RBCs (iRBCs)/200 µl to 1 × 10^7 infected erythrocytes/200 µl whole blood. These positive samples were used to evaluate the specificity and sensitivity of our developed method and comparative analyses with microscopic examination and nested-PCR.

Total genomic DNA was extracted from 200 µl of blood sample using a QIAamp DNA Blood Mini-Kit, 250 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of DNA was determined using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Genomic DNA was also extracted from the whole blood of uninfected hamster as a negative control. The extracted DNA was stored at −20°C for following experiments.

The whole sequence of the 18S rRNA of *Babesia* was amplified with the designed primers. The PCR products were purified by the Zymo™ Gel DNA Recovery Kit (ZYMO, Los Angeles, USA) and connected with pGEM-T Easy vector (Promega, USA). The recombinant expression vector was transformed into the Escherichia coli DH5α competent cells (TaKaRa Biotech Co., Ltd., Dalian). After amplification culture, the monoclonal strain of each plasmid was selected and contained 100 µg/ml ampicillin (Germany Darmstadt Merck) was cultured in 3 ml LB medium for 12 hours. Then, the positive plasmid was extracted with a Plasmid Miniprep Kit (Axygen, USA). Plasmids were sequenced by Sangon Biotechnology (Shanghai, China) and the sequencing results were compared with reference sequences by DNA Star software. Each of these plasmids was diluted into 1 copy/ul to 10^7 copy/ul, which were used to evaluated the detection limit and specificity of established nested-qPCR.

### Primers and Probes

The 18S rRNA sequences of *B. duncani* (HQ285838), *B. divergens* (FJ944826), *B. crassa* (AY260176), *B. microti* (KF410825), *B. motasi* Hebei (DQ159074.1) were derived from NCBI (https://www.ncbi.nlm.nih.gov/). Sequence alignment of these genes were performed using MAGE 10.0 to identify variable regions. Three pairs of primers and corresponding probes were designed targeting the variable region of 18S rRNA gene of *B. duncani* to specifically determine *B. duncani* infection in blood samples of host animals and human (Table 1). A set of primers, PIRO-F (5´-GAAACTGCGAATGGCTCATTAC-3´) and PIRO-R (5´-CACCAGATCCTCGATCGGTAGG-3´) (Wang et al., 2019), was used in the first-round of PCR to amplify 1,561 bp fragment of *B. duncani*, which could be further amplified by qPCR. The nested-PCR primers targeted β-tubulin were designed by Cacció et al.
Primers and probe mentioned above were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd.

Table 1
Primer and probe sequences used in this study for the identification of *B. duncani*.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIRO-F</td>
<td>5´-GAAACTGCGAATGGCTCATTAC-3´</td>
<td>Wang et al.(2019)</td>
</tr>
<tr>
<td>PIRO-R</td>
<td>5´-CACCAGTCACTCGATCGGTAGG-3´</td>
<td>Wang et al.(2019)</td>
</tr>
<tr>
<td>BWA-1F</td>
<td>5´-GCCTTGGCTTCTGTCTTG-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-1P</td>
<td>5´-ACTCGAAACCTTCCGCTTGCCTG-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-1R</td>
<td>5´-CCATCGCCGCAAAAGCCA-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-2F</td>
<td>5´-TCTGCGCTTGCCTTCG-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-2P</td>
<td>5´-CGCTTGGGTGCTATTACCCTCTGGCCTG-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-2R</td>
<td>5´-AAGTAAAATCTGGCAAATGG-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-3F</td>
<td>5´-CCTTGGGTTTCTCTTTCAGGT-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-3P</td>
<td>5´-CGGTGGTTCTCCATTTGCAGTT-3´</td>
<td>Original</td>
</tr>
<tr>
<td>BWA-3R</td>
<td>5´-CTGCTTGAACACTCT-3´</td>
<td>Original</td>
</tr>
<tr>
<td>Initial forward primer-F34</td>
<td>5´-TGCTGATACAGATGCTGCGG-3´</td>
<td>Cacció et al.</td>
</tr>
<tr>
<td>Initial reverse primer-R323</td>
<td>5´-TCNGTRTARTGNCCYTRGCCCA-3´</td>
<td>Cacció et al.</td>
</tr>
<tr>
<td>Nested forward primer-BtubFn</td>
<td>5´-TCWGACGAGCAGCCGATYGA-3´</td>
<td>K. E. O'Connor et al.</td>
</tr>
<tr>
<td>Nested forward primer-BtubFn</td>
<td>5´-CCAGGCTCAGACTCCATYAA-3´</td>
<td>K. E. O'Connor et al.</td>
</tr>
</tbody>
</table>

The FAM channel and ROX channel were selected as reference dye to detect 18S rRNA. Each run contained a positive control (Plasmids of *B. duncani*) and a negative control (Genomic DNA of infection free hamster). All FAM and ROX channels showed a typical S-shaped amplification curve, and a cycle threshold (CT) ≤ 35 was considered to be positive. The qPCR conditions with the lowest cycle threshold and exhibiting the best specificity and a typical S-shaped amplification curve was selected. A 20 µl PCR reaction mixture was prepared containing 10 µl of Premix Taq DNA polymerase (TaKaRa, Dalian, China), 1 µl of template DNA, 1 µl of each primer (10 µmol/L) and 7 µl of RNase-free water. Cyclic conditions for PCR reactions were as follows: initial denaturation at 95°C for 3 min; 15 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 2 min with a final extension step at 72°C for 5 min. The success of the qPCR depends on the amplified product of PCR. The qPCR reaction components consist of 1 µl of the PCR product, 10 µl of Premix Ex Taq™ (Probe qPCR) (TaKaRa, Dalian, China), 0.6 µl of upstream and downstream primers (10 µ mol / L), 0.2 µl of probe (10 µ mol / L), 0.2 µl of
ROX and 7.4 µl of easy-dilution. After mixing and centrifugation, the reaction tubes were transferred into the detector Agilent Mx3005P (Agilent Technologies) with cyclic conditions of 3 minute at 95°C, followed by 40 cycles of 10 seconds at 95°C, and 34 seconds at 60°C. The nested PCR completely follows the system and procedures developed by O’Connor et al (O’Connor et al., 2018), But using duncani’s merozoite DNA as a positive control.

**Specificity of the nested-qPCR**

Specificity of the established method was evaluated with the plasmids of four other zoonotically important *Babesia* species including *B. divergens*, *B. motasi* Hebei (Wang et al., 2020), *B. crassa*, and *B. microti*. The plasmid of each *Babesia* spp. was quantified to 20 ng/µl as template. The specificity test was carried out according to the previously determined optimal reaction conditions, while DNA from uninfected hamster were used as negative control, the plasmid of *B. duncani* was used as positive control.

**Sensitivity of the nested-qPCR**

The analytical limit of detection (LOD) of nested-qPCR is evaluated by using genomes extracted from whole blood containing iRBCs ($10^7$-$10^{-1}$) as prepared previously. After extracting the genome for quantification, it is used as a template for nested-qPCR and tested in triplicate for each concentration in 3 independent runs.

In order to compare the sensitivity of the established detection method with the standard plasmid concentration. The plasmid was quantified to 4.9ng/µl (1.0×10⁹ gene copies) followed by a 10-fold serial dilution. Final sensitivity tests ranged from 1.0×10⁷ to 1.0×10⁻¹ gene copies of template DNA in the initial PCR reaction. All samples were screened in triplicate.

**Clinical appliance of nested-qPCR assay**

The clinical performance of the nested-qPCR method was evaluated by blood specimens from patients with a history of tick bites and experimentally infected hamsters (*B. duncani*). The clinical samples were collected from 492 tick-bited patients of Gannan Tibetan Autonomous Prefecture of Gansu Province who visited the Second Hospital of Lanzhou University for their diagnosis and treatment. All participants agreed to contribute in this study and signed an informed consent form. Nested-qPCR was used to test blood samples collected from those patients to determine the status of *B. duncani* infection.

A total of 70 experimentally infected specimens of hamster blood were divided into four groups according to different parasitemia (0.05%~1%, 1%~10%, 10%~30% and 30%~67%), each with 17~18 samples. Some of the hypoparasitemia specimens were prepared by diluting with healthy human blood. Genomic DNA from 70 blood samples was extracted and quantified. And adding different concentrations of plasmids of *B. microti*, *B. divergens*, *B. crassa*, and *B. motasi* Hebei to the genome of healthy human to make a total of 40 specimens.
The above mentioned 602 clinical specimens were randomized, relabeled, and blindly detected by nested-qPCR and nested-PCR. In addition, 562 blood samples were subjected to microscopic examination. Finally, the nested-PCR and nested-qPCR products are sequenced so as to verify the accuracy of the results.

Results

Primers and probes

Initially, three sets of primers and corresponding probes were generated in variable regions of targeted gene. Among these, only one set of primer and probe was specific for *B. duncani* with high performance of amplification. In the second-round qPCR, the pair of primer BWA-3F(5´-CCTTGGGGTTT CGTTCG-3´), BWA-3R(5´-CTGCTTGAAACACTCT-3´) and BWA-3P(probe)(5´-CGGTGGTTCTCCATTGGCAGTT-3´) were used to amplify approximately 91bp fragment of desired gene.

Specificity of nested-qPCR

The results of nested-qPCR revealed that it could specifically detect *B. duncani*, whereas no amplification signals were observed in *B. divergens*, *B. motasi* Hebei, *B. crassa*, and *B. microti* (Fig. 1). We determined that 18S rRNA is one of the most conserved genes in *Babesia* genome, so nested-qPCR will not show false positive when detecting other pathogens. These results showed that nested-qPCR did not cross-react with several other common zoonotic *Babesia*, and was specific for the identification of *B. duncani* infection.

Sensitivity of nested-qPCR

We performed sensitivity experiments using both criteria: infected RBCs and standard plasmids. The results of multiple repetitive experiments revealed that the limit of detection of our nested-qPCR developed in this study was as few as 1 infected red blood cells/200µl with Cycle Threshold (Ct) 35. The standard curve showed that the linear relationship was good (Fig. 2A).

On the other hand, the lower detection limit of nested-qPCR for amplification of *B. duncani* was as few as approximately 1 plasmid copy that was reliably detectable in 100% of replicates (3/3) (Fig. 2B). The results of nested-qPCR, microscopic examination and nested-PCR demonstrated that none of 492 patients bitten by ticks were infected by *B. duncani*. Among 70 positive samples, 69 were detected by nested-qPCR, 67 were detected by nested-PCR, and 59 were detected by ME. The results of nested-qPCR detection of clinical samples indicate that there is a correlation between the CT value and the level of parasitemia (Fig. 3).

No false positive results were detected by ME, but two false positive results were detected by nested-PCR after sequencing verification of clinical samples. The specificity and sensitivity of nested-qPCR are 100% and 98.6%. ME and nested-PCR had sensitivities of 84.3% and 95.7% and specificities of 100% and 99.2%, respectively (Table 2).
Table 2
The detection results of clinical samples were analyzed by nested-qPCR, nested-PCR and ME.

<table>
<thead>
<tr>
<th>Result</th>
<th>Detection method</th>
<th>Microscopy</th>
<th>Nested PCR</th>
<th>Nested-qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>True positive</td>
<td></td>
<td>59/70</td>
<td>67/70</td>
<td>69/70</td>
</tr>
<tr>
<td>False positive</td>
<td></td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>True negative</td>
<td></td>
<td>492/492</td>
<td>530/532</td>
<td>532/532</td>
</tr>
<tr>
<td>False negative</td>
<td></td>
<td>11</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td></td>
<td>100.0</td>
<td>99.2</td>
<td>100.0</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td></td>
<td>84.3</td>
<td>95.7</td>
<td>98.6</td>
</tr>
</tbody>
</table>

Discussion

Mostly, microscopic examination of blood smears stained with Giemsa is considered the gold standard test for the diagnosis of Babesia infection. (Baltrunaite, Kitryte, & Krizanauskiene, 2020; Krause et al., 2021). However, the morphological characteristics of B. duncani trophozoites and merozoites in blood smear stained with Giemsa are indistinguishable from B. microti (Conrad et al., 2006a). It is even difficult to distinguish Babesia from piroplasms (Young et al., 2019a). Additionally, parasite is barely detectable when parasitemia is less than 0.1% in patient’s blood which require highly skillful person to perform. Therefore, classification and diagnosis of Babesia relying on morphology is a huge challenge for doctors.

Compared with the current gold standard tools (ME) and nested-PCR, the specificity and sensitivity of nested-qPCR is relatively higher. It has been examination no cross-reactivity with other zoonotic Babesia subspecies such as B. divergens, B. microti, B. crassa and B. motasi Hebei. The sensitivity of the current diagnostic method is compared with the previously established PCR-based detection assay. This assay can detect parasitemia as low as 1 infected erythrocytes/200µl in the actual infected host or 1 copy of plasmid in a total volume of 20µl. The nested-PCR method targeted β-tubulin gene was established by K. E. O’Connor can detect 1 copy of template DNA (O’Connor et al., 2018). However, this method requires time-consuming agarose gel electrophoresis analysis, and the target sequence length of B. microti is only about 15 bp shorter than that of B. duncani and other related Babesia species, which cannot be clearly distinguished by electrophoresis analysis. Wilson et al. used droplet digital PCR to discriminate B. microti and B. duncani which has ability to detect 10 gene copies in a reaction volume (Wilson et al., 2015). Stanley et al. used cobas Babesia (Roche Molecular Systems, Inc.) to detect B. duncani and claimed that the detection limit of B. duncani is 50.2 iRBCs/ml (Stanley et al., 2021). Therefore, the sensitivity of these two methods is relatively lower than that of nested qPCR developed in this study.
The nested-qPCR was established to overcome the disadvantage of the qPCR, which exhibits relatively less sensitivity for detecting *B. duncani* infection in blood samples from infected hamster. Traditional PCR has poor specificity and sensitivity in detecting blood protozoa (Tu et al., 2021), and there is interference from host DNA (Flaherty, Barratt, Lane, Talundzic, & Bradbury, 2021). Therefore, qPCR is performed on the PCR amplified products to amplify specific target genes with improved accuracy and sensitivity of detection (Flaherty et al., 2021). The optimized method has several advantages such as no need of gel electrophoresis, parasite morphology, no time-consuming and expensive sequencing, and no need for noble instruments. The high-performance nested-qPCR can identify *B. duncani* during the window period of the infected person. This makes up for the inability of IFA and FISH to distinguish between past and current infections (Shah, Caoili, et al., 2020; Shah, Mark, et al., 2020). Furthermore, the qPCR can be used for high-throughput screening (Leiby, 2011b). We also encountered some challenges when evaluating the method for clinical application. The primary challenge is the inability to quantitatively detect the infective dose of the parasite. Secondly, we have only specifically detected *B. duncani* in this assay. Still there need to develop a method that can detect multiple *Babesia* sp. in a single reaction.

One of the main functions of the newly established nested-qPCR is the precise clinical diagnosis of *B. duncani*. Since the symptoms and course of *B. duncani* infection are much more severe than *B. microti* infection. Moreover, *B. duncani* is highly resistant to the currently recommended treatment regimen for *Babesia*, and even the recommended therapy may lead to serious adverse reactions in patients (Abraham et al., 2018; Krause et al., 2000; Simon et al., 2017; Smith, Hunfeld, & Krause, 2020; Thomford et al., 1994; Wozniak et al., 1996). Therefore, the accurate diagnosis of babesiosis is whether the disease is caused by *B. microti* or *B. duncani* is crucial.

Additionally, the high-performance of nested-qPCR method will help to facilitate urgently needed investigations into the spatial distribution and prevalence of *B. duncani* in potential tick vectors and hosts. The identification of *Babesia* in ticks and mammalian hosts is necessary to further study about host-*Babesia*-vector interactions (Bajer & Dwuznik-Szarek, 2021). Detection of *B. duncani* in different tick species and their stages to investigate its prevalence is helpful to understand the spatial distribution and transmission ability of *B. duncani* (Martinez-Garcia, Santamaria-Espinosa, Lira-Amaya, & Figueroa, 2021). Due to the limited sensitivity and specificity of the detection methods, the incidence of babesiosis caused by *B. duncani* may be higher than reported in previous studies. Planning and conducting epidemiological investigations to determine the possibility of babesiosis outbreak will help to prevent and control pathogens.

**Conclusions**

We have developed a highly sensitive and specific assay (nested-qPCR) for detection of *B. duncani*, Nested-qPCR assay targeting the conserved region of 18S rRNA gene showed comparable specificity with ME and even higher sensitivity than ME and nested-PCR assay. The study also confirmed that *B. duncani* infection does not exist in some tick bite patients in Gansu Province. In conclusion, nested-qPCR assay
can specifically diagnose *B. duncani* in areas with great genetic diversity of parasites and can be used to monitor the epidemiological status. This will be a very useful diagnostic tool, especially in areas where *B. duncani* is prevalent and medical conditions are underdeveloped so that it cannot be sequenced.

**Abbreviations**

PCR  
polymerase chain reaction  
qPCR  
Real-time PCR  
ME  
microscopic examination  
VVBD  
Vectors and Vector-Borne Diseases Laboratory  
LVRI  
Lanzhou Veterinary Research Institute  
TTB  
transfusion transmitted babesiosis  
iRBCs  
injected red blood cells  
PPE  
percentage of parasitized erythrocytes  
IFA  
Immunofluorescence  
FISH  
Fluorescence In Situ Hybridization

**Declarations**

**Funding**

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**Conflict of interests** The authors declare that they have no conflict of interest.

**Availability of data and material**

All data generated or analysed during this study are included in this published article.

**Authors' contributions**
Wang Yanbo wrote the main manuscript text, and Zhang Shangdi and Wang Xiaorong participated in the specimen collection. Wang Jinming and Muhammad Rashid revised the article, and all authors reviewed the manuscript.

**Ethics approval**

The collection and manipulation of hamster 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-2020–001).

The study of clinical specimens was 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 to participate** All authors consent to be participated.

**Consent for publication** All authors consent to be published.

**References**


differentiation from other piroplasms. *Int J Parasitol*, 36(7), 779-789.
doi:10.1016/j.ijpara.2006.03.008


Figures

Figure 1

Evaluation of specificity of the nested-qPCR with plasmids of B. duncani, B. divergens, B. motasi Hebei, B. microti, and B. crassa.

Figure 2

Sensitivity of nested-qPCR.

a, The standard curve was drawn with X-axis as iRBCs in 200 μl whole blood and y-axis as CT value. The range of standard spans 9 concentrations, from 10⁷ iRBCs/200μl to 0.1 iRBCs/200μl. The standard curve equation was y = -1.153ln(x) + 34.64, and the correlation coefficient was R² = 0.9988.
b. The Ct is plotted on the y-axis and the copies of plasmid per reaction on the x-axis. The standard curve equation was \( y = -1.182\ln(x) + 32.96 \), and the correlation coefficient was \( R^2 = 0.9978 \).

**Figure 3**

**Nested-qPCR test results of different parasitemia.** The Ct is plotted on the y-axis and the PPE on the x-axis.