A PCR method targeting internal transcribed spacers: the simultaneous detection of Babesia bigemina and Babesia bovis in cattle

Junlong Liu, Guiquan Guan, Aihong Liu, Youquan Li, Hong Yin and Jianxun Luo*

State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Science, Xujiaping 1, Lanzhou, Gansu, 730046, P. R. China

Abstract
In this study, two pairs of oligonucleotide primers were designed according to the nucleotide sequence of the internal transcribed spacers (ITSs) of Babesia bigemina and B. bovis isolates from China. The primers were used in a multiplex PCR to detect parasite DNA in blood samples from cattle. There was no cross reactions with B. ovata, B. major, B. sp. Kashi, Theileria annulata, T. sergenti, T. sinensis or normal bovine DNA. The sensitivity of multiplex PCR assay was 1 pg and 10 pg DNA for B. bigemina and B. bovis, respectively. A total of 260 field blood samples collected from cattle in five provinces of China were analyzed by multiplex PCR and light microscopy. PCR testing revealed that 7.3% (19/260) and 5.8% (15/260) of cattle were positive for B. bigemina and B. bovis and 1.2% (3/260) of cattle were co-infected with B. bigemina and B. bovis. Using light microscopy, 2.3% (6/260) and 1.5% (4/260) of cattle were infected by B. bigemina and B. bovis, respectively, and no co-infection was found. The results showed that the multiplex PCR developed in the present study could be an alternative diagnostic tool for the detection of B. bovis and B. bigemina infection in cattle.

Keywords
Babesia bigemina, Babesia bovis, Multiplex PCR, ITS, ribosomal DNA

Introduction
Bovine babesiosis is an economically important tick-born disease of cattle in tropical and subtropical regions of the world (McCosker 1981). B. bigemina and B. bovis are the most common species. In the field, B. bigemina and B. bovis often result in co-infection because they share the same vectors (Rhipicephalus (Boophilus) microplus, R. annulatus, and R. geigyi) (Kim et al. 2008). Although the clinical symptoms caused by these two parasites are very similar, the disease caused by B. bovis is more serious than that of B. bigemina. Light microscopy is often used to detect infection, but the low sensitivity of this method does not permit its use in epidemiological studies where it is necessary to identify carrier animals (Almeria et al. 2001, Barros et al. 2005). A serological method was used to analyze the epidemiology of the disease and evaluate the vaccine, but cross reactions occurred between different Babesia species, making too difficult to determine the cattle carrier. Identification of the animal carrier is important for the assessment of the infection risk and provides a clinical trajectory for treatment. Several sensitive methods, such as reverse line blot, PCR-ELISA, and loop-mediated isothermal amplification, have been developed, but they are not cost-effective or require special equipment (García-Sanmartín et al. 2006, Gubbels et al. 1999, Notomi et al. 2000).

The small subunit ribosomal RNA (SSU rRNA) gene is widely used to discriminate ruminant piroplasms, but it is highly conserved, which restricts its use between closely related species. Because internal transcribed spacers (ITSs) have great variability in both nucleotide and length, ITS sequences were used for discriminating different geographic isolates of piroplasms, identifying new species, and differentiating between piroplasm species and subspecies (Holman et al. 2003, Baneth et al. 2004, Conrad et al. 2006, Aktas et al. 2007, Liu et al. 2008).

This study aims to develop a multiplex PCR for the simultaneous detection of B. bovis and B. bigemina. We designed two sets of specific primers for B. bovis and B. bigemina based on ITSs. A multiplex PCR was developed to distinguish B. bovis and B. bigemina and was used to detect
these parasites from experimentally infected calves and field-collected samples from cattle. The results of PCR were compared with those of light microscopic examinations.

**Materials and Methods**

**Animal infection**

Calves of 6–12 months of age were purchased from an area where babesiosis has not been reported. Thirty days before the study began, all calves were splenectomized. Ten days prior to the pathogen experiments infection, thin blood films smears obtained were prepared from the ear bloods of the calves for the were fixed with methanol, stained with Giemsa, and examination of for the presence of hemoparasites (Salih et al. 2007). Only those calves negative for hemoparasites were used in the experiment. One calf was infected with *B. bigemina* Kunming strain, and another calf was infected with *B. bovis* Shanxian strain. Both babesia strain were previously collected from Henan and Yunnan province, respectively, and preserved in liquid nitrogen as a gold standard positive control (Liu et al. 2005). Daily blood smears were obtained post-infection for the presence of parasites. When the parasitemia was 5%–20%, blood was collected from the jugular vein for purifying parasites and extracting the genomic DNA of parasites.

Blood samples collected from animals infected with *B. bigemina* Kunming and *B. bovis* Shanxian were used as positive control. The normal blood samples collected from the same animals before infection were used as negative control.

The animal experiments reported in this study were approved by the Gansu Provincial Science and Technology Department and in accordance with the Animal House of Lanzhou, Veterinary Research Institute Instructions.

**Microscopic examination**

Thin blood smears were prepared from the peripheral blood and air dried. After fixation with methanol for 5 minutes, slides were stained with 10% Giemsa for 45 minutes and then washed with distilled water. One hundred microscopic filed were examined under oil-immersion lens (100× magnification). The presence of piroplasm greater or equal to 1 was considered positive (Salih et al. 2007).

Microscopic differentiation of *Babesia* species depends on the shape and size of the parasite. *B. bigemina* is a large pleomorphic *Babesia* and identified by the pear-shaped bodies joined at an acute angle within the erythrocyte. Compared to *B. bigemina*, *B. bovis* is smaller, and identified by the pear-shaped bodies joined at an obtuse angle. For the identification of *Babesia* from the single bodies which are in round forms or pear-shaped was mainly depends on the size. The size of the round forms for *B. bigemina* is around 2 µm, for *B. bovis* is 1–1.5 µm. The size of pear-shaped for *B. bigemina* and *B. bovis* is 4–5 µm and 1.5–2.4 µm, respectively (Taylor et al. 2007).

Isolation of *Babesia* spp. parasites

*Babesia bigemina* and *B. bovis* merozoites were prepared from the blood collected at peak parasitemia from experimentally infected calves. Leukocytes were removed with Transfusion Sets with Leukocyte Reduction Blood Processing Systems (Nanjing, China). The infected red blood cells were washed three times with 10 mM Tris-HCL (pH 7.4) and 150 mM NaCl (Tris-saline) by centrifuging at 1000 × g for 10 min at 4°C (Alhassan et al. 2005). The packed erythrocytes were suspended with Tris-saline with 7% glycerol at a volume ration 1:5, and then incubated at room temperature for 30 min. After centrifugation as described above, the supernatant was discarded and Tris-saline was added with the same volume of Tris-saline contained glycerol for lysing erythrocytes. Samples were then centrifuged at 1000 × g for 10 min at 4°C to remove cellular debris and intact erythrocytes. The supernatant was recovered and centrifuged at 4000 × g for 20 min at 4°C to pellet the parasites. The pellets of parasites were washed with Tris-saline and centrifuged until most of the hemoglobin was removed and then stored at −70°C.

**Field samples collection**

In order to evaluate the multiplex PCR assay for the diagnosis of field samples, a total of 260 blood samples were collected from 5 provinces of China (Table II). Blood from the jugular veins of cattle was collected in tubes containing EDTA for DNA extraction, and samples from ear vessels were obtained for blood smears and subsequently stained with Giemsa for the detection of the parasite.

**DNA extraction and primer designation and PCR amplification**

The total DNA of *B. bigemina* and *B. bovis* was prepared from purified parasites using the Genomic DNA Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For all of the blood samples including field samples, genomic DNA was extracted from 300 µl of anticoagulated blood. The DNA preparations were stored at −70°C until further analysis.

The nucleotide sequences of *B. bovis* (EF547925) and *B. bigemina* (EF547924) were used to design suitable diagnostic primers by aligning sequences with the DNAStar software program.

The nucleotide of the primers BbS and BbR for *B. bigemina* and BoS and BoR for *B. bovis* are shown in the Table I. The 50 µl amplification reactions contained 5 µl 10× Buffer (TakaRa, China), 200 µM of each dNTP, 50 pmol of each primer (BbS, BbR, BoS and BoR), 1.5 U of Taq DNA polymerase (TakaRa, China), and 10.5 µl different DNA weights (10 ng to 10⁻³ ng/test) of extracted DNA template. The reaction was performed under the following conditions: 5 min at 96° to activate the Taq DNA polymerase at 35 cycles (1 min
of denaturation at 92°C, 1 min of annealing at 56°C, 1 min of extension at 72°C), and 10 min of final extension at 72°C. Negative and positive controls were included in all runs. PCR products were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide and then visualized under ultraviolet light.

Sequencing of PCR products

The PCR products were extracted from the 2% agarose gel by Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa, China), ligated into pGEM-T Easy vectors (Promega, USA), and transformed into Escherichia coli JM 109. Plasmid DNA from the selected clones was identified using PCR using primers as above (program and reaction mixtures were used as same as PCR amplification described above) and EcoR1 (Fermentas, EU) restriction enzyme digestion were applied to verify the presence of correct inserts in selected clones before for the sequencing process by the Big Dye Terminator Mix of TaKaRa Company (China).

Sensitivity and specificity of the single-round and multiplex PCR

To determine the specificities of the primers for the multiplex PCR, other parasite DNA (B. ovata, B. major, B. sp. Kashi, T. annulata, T. sergenti, T. sinensis) stored by our group (Liu et al. 2008) was used applied to for the evaluate evaluation of specificity.

The B. bigemina and B. bovis DNA was initially diluted to10 ng/µl and then serially diluted 10-fold. This diluted DNA was used as a template in the PCR to evaluate the sensitivity of each pair of primers. Additionally, the sensitivity of the multiplex PCR for co-infection was also evaluated using a mixture of B. bigemina and B. bovis DNA.

Results

Development of the multiplex PCR method

The A multiplex PCR was developed for the simultaneous detection of B. bigemina and B. bovis infection. Two pairs of species-specific primers either (Table I) for B. bigemina and B. bovis were applied in one system. These primers could specifically amplify fragments from the target gene of the respected parasites in single-round PCR (Fig. 1) of 646 bp and 408 bp for B. bigemina and B. bovis, respectively (Fig. 1). No amplicons from the DNA of normal bovine blood and other protozoans (B. ovata, B. major, B. sp. Kashi, T. annulata, T. sergenti, and T. sinensis) DNA was used as controls to identify the specificities of the primers; only the positive controls of

Table I. Primers sets of ITS developed for B. bigemina and B. bovis amplification in the present study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>BbS</td>
<td>5’-GCGTTGTTCGTCGCTCTTG-3’</td>
</tr>
<tr>
<td>BbR</td>
<td>5’-CTTTAAATTCGGCGGATGGG-3’</td>
</tr>
<tr>
<td>BoS</td>
<td>5’-CTTGCGGCGATTTGGC-3’</td>
</tr>
<tr>
<td>BoR</td>
<td>5’-CGTGAAGGAGGTCGGTAGAG-3’</td>
</tr>
</tbody>
</table>

Fig. 1. PCR detection of B. bigemina and B. bovis with a set of primer combinations (BbS, BbR and BoS, BoR). Lane M: DNA Marker DL2000; Lane 1: B. bigemina DNA; Lane 2: B. bovis DNA; Lane 3: the mix DNA of B. bigemina and B. bovis. The size of the positive bands is indicated on the right.
B. bigemina and B. bovis had specific amplification (Fig. 2A, B). The size of the single-round PCR products is consistent with the predicted size that PCR results were sequenced. As respected, 646 bp and 408 bp fragments for B. bigemina and 408 bp fragments were obtained from the specific primers for B. bigemina and for B. bovis, respectively, shown in Figure 1. Additionally, the multiplex PCR method with both set of primers simultaneously produce two bands from B. bigemina and B. bovis mixture (Fig. 1, lane 3), which corresponded to the same size as from the single-round PCR, which verified in order to confirm the nucleotide sequence of the PCR products, the PCR amplified products from the positive controls were purified and cloned into the vector. The sequencing results of all the DNA fragments were consistence to the ITS target sequence of B. bovis (EF547925) and B. bigemina (EF547924), respectively.

Comparison of the sensitivity of single-round and multiplex PCR

The DNA samples of B. bigemina and B. bovis were initially diluted to 10 ng/µl and serially diluted 10-fold. The sensitivity of the B. bigemina or B. bovis primers in single PCR amplification was evaluated using 10-fold serially diluted DNA.
Fig. 3 (A, B, C). Sensitivities of the single-round PCR and the multiplex PCR to 10-fold serial dilutions of *B. bigemina* DNA (A), *B. bovis* DNA (B), and the mixed DNA of both parasites (C). In all panels, Lane M: DNA Marker DL2000; Lane1: 10 ng/test; Lane10: 0.01 fg/test. The size of the positive bands is indicated on the right.
Table II. Comparison of the positive numbers among microscopic method and multiplex PCR for the detection of *B. bigemina* and *B. bovis* from field bovine blood samples from five provinces of China

<table>
<thead>
<tr>
<th>Region</th>
<th>Number</th>
<th>Microscopy</th>
<th>PCR</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td><em>B. bigemina</em></td>
<td><em>B. bovis</em></td>
</tr>
<tr>
<td>Jilin</td>
<td>20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Gansu</td>
<td>30</td>
<td>0/30</td>
<td>0/30</td>
</tr>
<tr>
<td>Hebei</td>
<td>30</td>
<td>1/30</td>
<td>0/30</td>
</tr>
<tr>
<td>Guangxi</td>
<td>100</td>
<td>1/100</td>
<td>1/100</td>
</tr>
</tbody>
</table>

The sensitivity of the multiplex PCR was also evaluated using a mixture of *B. bigemina* and *B. bovis* DNA. The results showed that the single-round PCR of *B. bigemina* and *B. bovis* primers could detect 0.1 pg and 1 pg of DNA in the samples, respectively. The multiplex PCR could detect 1 pg and 10 pg of DNA of *B. bigemina* and *B. bovis*, respectively, which corresponds to the approximate amount of DNA in 50 μL of 0.0001% and 0.001% parasitemic erythrocytes (Mclaughlin et al. 1986) (Fig. 3A, B and C).

**PCR analysis of the field blood samples**

A total of 260 blood samples from domestic cattle from the 5 provinces of China were examined for the infection of *B. bigemina* and *B. bovis* with the multiplex PCR method; the blood smears corresponding to the blood samples were examined using light microscopy (Table II). With the multiplex PCR method, 19 (7.3%) and 15 (5.7%) positive samples showed species-specific reactions for *B. bigemina* and *B. bovis*, respectively, and 3 (1.2%) samples were co-infected by both parasites. Using the microscopy, 6 (2.3%) and 4 (1.5%) blood smears were found to contain *B. bigemina* and *B. bovis*, respectively. But and no co-infection was observed with microscopy detection.

**Discussion**

To diagnose piroplasms infections, microscopic examination remains a useful method, especially in acute cases, because it is easy and can be rapidly applied (Bishop and Adams 1973). However, expertise is required when using this method because the morphological features of babesias are very similar, and it is difficult to differentiate these parasites, especially when co-infection has occurred. Additionally, in chronic infections, it is difficult to detect parasites in the blood smear due to low levels of parasitemia. Serological methods have been developed for the diagnosis of babesiosis, but cross-reaction between closely related *Babesia* species often occurs (Tenter and Friedhoff 1986, Passos et al. 1998, Allred 2003). To resolve these problems with sensitivity and specificity, PCR and associated PCR methods have been used to detect various *Babesia* infections (Oliveira et al. 2008). However, these methods require complex procedures, such as reverse line blot (RLB) and nested PCR. These methods are time consuming and not cost-effective, which restricts their use as routine diagnostic methods. MA multiplex PCR method is a variant PCR in which several target sequences can be amplified by using more than one pair of primers in one reaction. Considering the potent saving of time and effort, was used with different primer pairs in one reaction system to differentiate multiplex PCR has been successfully used in the identification of virus, bacteria and parasites from field samples several parasites. (Figuerola et al. 1993; Markoulatos et al. 2002, Kho et al. 2003, Alhassan et al. 2005); previous studies have reported that this is a rapid and relatively easy method to diagnose the parasite.

We established a multiplex PCR method for the detection and differentiation of *B. bigemina* and *B. bovis* using two pairs of primers to amplify a portion of the internal transcribed spacer sequences of these two parasites. The evaluations showed that the two pairs of primers were specific for *B. bigemina* and *B. bovis*. This PCR assay could detect 1 pg and 10 pg of DNA for *B. bigemina* and *B. bovis* at level. This finding indicates that the primer sets can be used to detect and differentiate the two *Babesia* species. The field samples detection showed that PCR method had higher detection rates than blood film method. With the multiplex PCR method, 7.7% (19/20) and 5.8% (15/260) of samples were positive for *B. bigemina* and *B. bovis*, these results are 3 times higher than that of microscopic method.

A total of 260 bovine blood samples were collected from 5 provinces located in northern, central, southern, and western China and were examined using the multiplex PCR assay. Consistent with the earlier report (Yang 1984, Fu et al. 1997, Qin, 2005), our study confirms that cattle infected with *B. bigemina* exist in the central and southern China (Henan, Hebei and Guangxi); the infection of cattle with *B. bovis* exist in Henan (central), Guangxi (south) provinces. But to our knowledge, the positive identification of *B. bovis* from the Lintao County of the Gansu (west) province was the first report in this district.

In this study, we used molecular methods for the epidemiological study of *B. bigemina* and *B. bovis* in China. Considering the geographical size of China, additional samples from more districts would be critical in assessing the distribution and endemicity of these diseases.
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References


