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Short communication

Discrimination between ovine *Babesia* and *Theileria* species in China based on the ribosomal protein S8 (RPS8) gene

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ABSTRACT

Ovine babesiosis and theileriosis are important hemoprotozoal diseases of sheep and goats in tropical and subtropical regions that lead to economic losses in these animals. PCR-restriction fragment length polymorphism (PCR-RFLP) is a reliable molecular diagnostic tool for discriminating *Theileria* or *Babesia* species in the same host. In this study, the DNA sequences of a ribosomal protein S8 (RPS8) gene from four species of piroplasms in China were used to develop a species-specific PCR-RFLP diagnostic tool. The sensitivity of the PCR assays was 0.1 pg DNA for *B. motasi* and 1 pg DNA for *T. uilenbergi* and 10 pg DNA for *Babesia* sp. Xinjiang-2005 and *T. luwenshuni*. The clear size difference of the PCR products allowed for a direct discrimination for *B. motasi*, *Babesia* sp. Xinjiang-2005 and ovine *Theileria* species (*T. uilenbergi* and *T. luwenshuni*), except that the mixed infection between *T. uilenbergi* and *T. luwenshuni* may be difficult to distinguish, simply after the electrophoretic separation of the amplification products. Further *T. uilenbergi* and *T. luwenshuni* diagnoses were made by digesting the PCR product with *Sac*I. The established method could be applicable for the survey of parasite dynamics, and epidemiological studies as well as prevention and control of the disease.

1. Introduction

Theileria and *Babesia*, the causative agents of theileriosis and babesiosis, respectively, are tick-borne parasitic protozoa, and a number of them are highly pathogenic for cattle, sheep, and goats. The economic losses are enormous in tropical and subtropical areas because of theileriosis and babesiosis (Kuttler, 1988). Theileriosis of small ruminants caused by *T. luwenshuni* and *T. uilenbergi* (Yin et al., 2007) is a severe and often lethal disease, constituting a severe restriction for the development of the small ruminant livestock industry in the northwest of China, especially regarding the use of exotic animals (Luo and Yin, 1997). Ixodid ticks, *Haemaphysalis qinghaiensis* and *Haemaphysalis longicornis* have been demonstrated to be responsible for transmission of the disease (Yin et al., 2002; Li et al., 2007).

In China, ovine babesiosis is endemic in the Sichuan and Heilongjiang provinces and is believed to be caused by *B. motasi* due to the severe clinical symptoms observed (Yin et al., 1997a,b). Later, several ovine *Babesia* isolates were collected from distinct geographical areas (Bai et al., 2002; Yin et al., 1997a,b; Guan et al., 2001). The biological vector of *B. motasi* Lintan isolate has been shown to be *H. qinghaiensis* and *H. longicornis* but no evidence was found for the vector for *B. motasi* Tianzhu and Ningxian isolates (Guan et al., 2002, 2010). *B. motasi* Lintan isolate, *B. motasi* Tianzhu

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iaolate and *B. motasi* Ningxian isolate were not clustered into the same group in the phylogenetic tree based on ribosomal RNA internal transcribed sequences (rRNA ITS) and the cytochrome b (COB) gene (Niu et al., 2009a,b; Tian et al., 2013).

A *Babesia* sp. Xinjiang-2005 was isolated from a splenectomised sheep infested by *Rhipicephalus sanguineus* and *Hylomma anatolicum anatolicum* in Xinjiang province (Guan et al., 2009). It was considered to be a novel ovine *Babesia* species on the basis of its morphology, pathogenicity, vector tick species and alignments of 18 S ribosomal RNA (18 S rRNA) gene and rRNA ITS sequences (Niu et al., 2009a; Guan et al., 2002, 2010; Liu et al., 2007). Recently, a *Babesia* sp. Xinjiang-like parasite was isolated using an in vitro culture system from 1 of 19 sheep blood samples collected from western Gansu province, China (Guan et al., 2012).

In the field, the risk of co-infection between the four ovine *Babesia* and *Theileria* species is very high. So it is necessary to develop a simple, reliable and cost-effective method that is suitable for large-scale epidemic investigation, particular for co-infections in field (Figueroa and Buening, 1995).

Babesia and *Theileria* spp. infections are traditionally examined by observation of piroplasms under the microscope (Luo and Yin, 1997). However, the method do not always detect carrier status, are time consuming, not very sensitive and require operators with high expertise. To improve diagnostic methods, reverse line blot (RLB) (Schnittger et al., 2004; Niu et al., 2009b) and Loop mediated isothermal amplification (LAMP) with highly sensitive and specific characteristics (Guan et al., 2008) have been developed. However, compared with single PCR, RLB and LAMP are laborious and expensive. More generally, it would be desirable to have a 'universal' PCR-based test for the simultaneous detection and identification of these parasites. This requires the analysis of a molecular target conserved among piroplasms, which contains enough genetic variation to design a reliable species identification protocol.

In the study described here, an informative molecular target has been identified in the ribosomal protein S8 (RPS8) gene from ovine *Babesia* and *Theileria* species endemic in China. The amplified gene with non-coding regions that varied extensively both in length and in sequence, allowed the development of an assay to differentiate the species directly on the basis of the specific size of the PCR products combined with a simple PCR-restriction fragment length polymorphism (RFLP) protocol.

2. Materials and methods

2.1. Animals

Seven sheep, 5–7 months of age, were purchased from a *Babesia* and *Theileria*-free area and splenectomized 2 months before the study. During this period, blood films were taken weekly from the ears of the sheep to be examined by Giemsa stain for the presence of haemoprotozoan parasites. The experimental animals were tested by PCR with the universal primers for *Babesia* and *Theileria* species based on the 18 S rDNA sequences prior to use to ensure that they were free of hemoparasites (Ano et al., 2001).

2.2. Parasite isolates

The *B. motasi* Tianzhu and Ningxian isolates were obtained by inoculating field-collected blood of asymptomatic sheep into splenectomised sheep (Yin et al., 1997a,b). The *B. motasi* Lintan isolate was isolated from a sheep infested with adult *H. qinghaiensis* ticks from Lintan, Gansu Province (Bai et al., 2002). The *Babesia* sp. Xinjiang-2005 isolate was obtained from a sheep infested with *H. anatolicum anatolicum* from Kashi, Xinjiang Region (Guan et al., 2009), *T. luwenshuni* Ningxian isolate and Mangdang isolate and *T. uilenbergi* Longde isolate were obtained from sheep infested with adult *H. qinghaiensis* ticks (Yin et al., 2007).

2.3. Infection of animals

Seven piroplasm isolates were used in the study, three *B. motasi* isolates, *Babesia* sp. Xinjiang-2005, two *T. luwenshuni* isolates and one *T. uilenbergi* isolate. Seven splenectomized sheep were infected with cryopreserved parasites. The rectal temperature was measured and blood smears were examined daily post-infection for presence of parasites. When the parasitemia was 5–20%, blood, for genomic parasite DNA extraction, was collected from the jugular vein into Alsever's anticoagulant solution (Guan et al., 2008). Daily post-infection the rectal temperature was measured to monitor for disease and blood smears were examined to monitor for presence of piroplasms.

2.4. Genomic DNA preparation

Total DNA was extracted from anticoagulated blood collected from sheep infected with seven isolates using the Puregene DNA Purification Kit (www. gentra. com) according to the manufacturers instructions. DNA concentration was determined by spectrophotometric estimation. Aqueous DNA preparations were frozen at −70 °C until use.

2.5. PCR amplification and sequencing of RPS8 gene

The full-length RPS8 gene was amplified using the forward primer: 5′-ATGGGTATTTCACGTGACAG-3′ and the reverse primer: 5′-GCGTTTCTTCTTATCCATACG-3′. Each PCR mixture (total volume, 50 μl) contained 5 μl of 10 × PCR buffer (Mg^{2+} free), each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 200 nM, 2.5 U of rTaq DNA polymerase (TaKaRa), and 20 ng of DNA template. A total of 35 cycles, each consisting of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 1 min, were performed; an initial hot start at 94 °C for 3 min and a final extension step at 72 °C for 7 min were also included. The purified PCR products were ligated into pGEM-T Easy vector (Promega) according to the manufacturer's recommendations, The plasmids with inserts were extracted by using MiniBEST Plasmid Purification Kit Ver.