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At least two genetically distinct large *Babesia* species infective to sheep and goats in China

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Abstract

A fatal disease of sheep and goats in the northern part of China has been reported to be due to *Babesia ovis*. However, some characteristics of the causative agent in recent reports are not in accordance with the original attributes ascribed to this parasite. Therefore, the 18S small subunit ribosomal RNA (18S rRNA) genes of a number of *Babesia* isolates in China were sequenced and compared with that of other *Babesia* and *Theileria* species in an attempt to clarify their taxonomic position. In the present study, seven *Babesia* isolates were collected from distinct areas of northern China, and the 18S rRNA genes were amplified and sequenced. The phylogenetic trees were inferred based on 18S rRNA gene sequences of the Chinese ovine *Babesia* isolates and some of ovine *Babesia* and *Theileria* species available in GenBank. In the phylogenetic tree, *Babesia* sp. isolates from Madang, Tianzhu, Lintan, Ningxian, Hebei and Liaoning all grouped with *B. motasi* with 88.2–99.9% identity, while *Babesia* sp. Xinjiang grouped in a separate clade between *B. ovis* and *B. crassa* with 79.7–81.2% identity. The results indicated that there are at least two distinct *Babesia* species groups—*B. motasi* and *Babesia* sp. Xinjiang, the latter was dinstinctly different from other ovine *Babesia* isolates from China with less than 86.6% identity.
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1. Introduction

Ovine babesiosis is the most important hemoparasitic tick-borne disease of small ruminants caused by *Babesia ovis*, *B. motasi* and *B. crassa*, which is characterized by fever, anemia, icterus and hemoglobinuria. These parasites are widespread in tropical and subtropical areas of the world (Uilenberg, 2001). In China, ovine babesiosis was first recordered in 1982 (Chen, 1982), where it is responsible for considerable economic losses in sheep production (Zhao et al., 1986; Yin et al., 1997). To date several distinct geographic isolates have been collected in our laboratory (Yin et al., 1997; Guan et al., 2001; Guan et al., 2002), and marked differences between *Babesia* sp. Xinjiang isolate and others in vector specificity and pathogenicity have been described. *Babesia* sp. Xinjiang isolate, by *Hyalomma anatolicum anatolicum*, often led to clinically inapparent infection, *Babesia* sp. from Lintan and Ningxian, transmitted by *Haemaphysalis* spp., could lead to a moderate or severe clinically disease. However, the taxonomic relationship

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between these geographic isolates of *Babesia* infective to sheep or goats still remains uncertain.

The 18S small-subunit ribosomal RNA (18S rRNA) genes have been used for evolutionary studies which have led to a more accurate classification of some of the unknown *Babesia* species infective to cattle in China (Luo et al., 2005a,b), and phylogeny between two Chinese *Babesia* sp. isolates and other ovine piroplasma has been also studied by sequencing the 18S small-subunit ribosomal RNA gene sequences (Schnittger et al., 2003). In this paper, new information based on the 18S rRNA gene sequences was provided in an attempt to determine the taxonomic relationship among seven different geographic isolates in China.

2. Materials and methods

2.1. Parasite isolates

Seven Chinese isolates of *Babesia* sp., infective to sheep, were used in this study. Five *Babesia* isolates, from Chengde, Hebei Province; Liaoyang, Liaoning Province; Tianzhu, Ningxian and Madang, Gansu Province, were obtained by inoculating the fieldly collected blood of the asymptomatic sheep to the splenectomised animals. The sixth *Babesia* isolate was isolated from adult *Haemaphysalis qinghaiensis* ticks from Lintan, Gansu Province. The seventh isolate was obtained from a batch of mixed *Rhipicephalus sanguineus* and *H. a. anatolicum* from Kashi, Xinjiang province. These isolates were designated as *Babesia* sp. Hebei, *Babesia* sp. Liaoning, *Babesia* sp. Tianzhu, *Babesia* sp. Ningxian, *Babesia* sp. Madang, *Babesia* sp. Lintan and *Babesia* sp. Xinjiang, respectively.

2.2. Sheep

Sheep, 0.5–1 years of age, were purchased from a haemoprotozoa-free area and maintained in isolation stables. Thirty days before the study began, all of sheep used for these experiments were splenectomised. Ten days prior to the experiment, blood films were taken from the ears of the sheep, fixed with methanol, stained with Giemsa and examined for the presence of haemoparsites. Only those sheep negative for haemoparasites were used.

2.3. DNA extraction

Seven splenectomized sheep were inoculated intravenously with 10 ml of cryopreseved infected blood of the different *Babesia* isolates. When the parasitemia was more than 5%, erythrocytes were isolated from venous blood collected into heparin as anticoaglutant. Parasite DNA was isolated with a genomic DNA Purification Kit (Gentra) according to the manufacturers' instructions, and the amount of isolated DNA was assessed photometrically. Control DNA was isolated from venous blood taken from uninfected sheep.

2.4. PCR amplification

The 18S rRNA gene sequences were amplified from *Babesia* genomic DNA samples by PCR. Two sets of primers specific for *Babesia* were designed. They were: primer set 1-rDNA-S_3/920AS (5′-aacctggttgatcctgccagt-3′, 5′-gggattgaaagcaagaactaatt-3′), and primer set 2-370S/rDNA-AS (5′-ggcctctccctcgga ctctt-3′, 5′-catccacttggacatcttcct-3′).

The PCR amplification was performed in a final volume of 50 μl reaction conditions follows: 1.0 μM of each primer, 5 μl PCR buffer, 4 μl dNTP, respectively (TaKaRa Biotechnology), 0.25 μl TaKaRa Taq (5 U/μl) and 2 μl of purified DNA sample. The reactions were performed on an automated DNA Thermal Cycler (Biometra) using a three phase program. The first phase consisting of 5 min incubation at 94 °C, to degrade any contamination template from previous PCR amplification by the uracil DNA glycocylase, the reaction mix was incubated at 94 °C for 5 min. This was followed by 35 incubation cycles of 94 °C for 1 min, annealing temperature (T_A, value for the different primers used see below) for 1 min and 72 °C for 2 min with a final extension step of 72 °C for 7 min. The T_A, for primer pairs was set as follows: rDNA-S_3/rDNA920AS: (T_A = 55 °C, specific for a region of the *Babesia* ssrRNA gene) and rDNA370S:/rDNA-AS (T_A = 60 °C, specific for a region of the *B.* ssrRNA gene), amplifying DNA fragments covering the ssrRNA gene region of *Babesia* species. At the end of cycling the reaction was held at 72 °C for 7 min and cooled to 4 °C. A final extension step of the test DNA samples, a positive control (DNA from ovine infected blood), a negative sample control (containing DNA from the cleaned sheep) and a no DNA negative control (5 μl of Milli-Q water substituted for DNA) were included in the PCR amplification. The PCR products (5 μl) were separated by electrophoresis on a 1.0% agarose gel containing 0.5 μg/ml ethidium bromides according to standard methods and visualized under UV illumination.

2.5. Cloning and sequencing of amplified DNA fragments

The PCR products were extracted from 1% TAE agarose gel by DNA purification with glass-milk kits

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