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Continuous *in vitro* cultivation of a recently identified *Babesia* that infects small ruminants in China

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ABSTRACT

Babesia sp. Xinjiang was isolated from a splenectomised sheep infested by *Rhipicephalus sanguineus* and *Hylomma anatolicum anatolicum*, collected from sheep and cattle in Xinjiang province. It was considered to be a novel ovine *Babesia* species on the basis of its morphology, pathogenicity, vector tick species and alignments of 18S ribosomal RNA (18S rRNA) and internal transcribed spacers (ITS) gene sequences. Continuous *in vitro* cultures of the ovine parasite were established using infected sheep blood. In RPMI 1640 medium with 7.5% sheep red blood cells (RBCs) maintained in an incubator at 37 °C and 5% CO_2, the percentage of parasitized erythrocytes (PPE) peaked at 10% in 24- and 6-well plates. It increased to 20–50% with the same culture medium but with 2.5% RBC in 75 cm^2 flasks. Two clonal lines of *Babesia* sp. Xinjiang were screened using the limiting dilution method. Growth characteristics of these lines *in vitro* were measured by a microtiter-based spectrophotometric method and from the PPE. The generation time in sheep erythrocytes was between 15.20 h and 16.27 h. Furthermore, the host range of parasite was identified with *in vitro* culture and *in vivo* infection. Erythrocytes of sheep, cattle, sika deer and humans could be invaded into by lines *in vitro*, but the parasites could not propagate in human erythrocytes. The parasites could not enter erythrocytes from goats *in vitro*. However, *in vivo*, only sheep could be infected by lines. Finally, a *Babesia* sp. Xinjiang-like parasite (which shared 99.5% identity with the original strain of *Babesia* sp. Xinjiang) was isolated using this *in vitro* culture system from 1 of 19 sheep blood samples collected from western Gansu province, China.

1. Introduction

The tick-transmitted parasites of genus *Babesia*, which belong to the phylum *Apicomplexa*, are the second most common protozoan hemoparasites of mammals after the trypanosomes (Telford et al., 1993). To date, five species of *Babesia* have been described, *B. ovis*, *B. motasi*, *B. crassa*, *B. foliata* and *B. taylori*, in sheep and goats (Hashemi-Fesharki and Uilenberg, 1981; Uilenberg, 2006). In China, babesiosis is a common hemoprotozoan disease of cattle, buffalo, Pianniu (a hybrid between cattle and yaks), sheep, goats, horses, donkeys, dogs and yaks (Yin et al., 1997b; Liu et al., 1997). From the economic point of view, bovine, equine and small ruminant (sheep and goats) cases of babesiosis are certainly the most important. However, the *Babesia* infectious of small ruminants has not been clarified owing to a lack of systematic research in China. Chen (1982) and Zhao et al. (1986) first reported that ovine babesiosis occurred in Sichuan and Heilongjiang provinces, respectively. They demonstrated that the pathogens were *B. ovis* on the basis

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of the morphology of the pathogens and the clinical appearance of ill animals. Unfortunately, they did not isolate the parasite and no further work was published on the two cases of ovine babesiosis.

In 1996, an unexpected outbreak of ovine babesiosis occurred in herds of small-tailed sheep imported from Shandong province in Ningxian county, eastern Gansu province. Pathogens considered to be *B. ovis*, *B. motasi* or an unrecognized large *Babesia* were isolated from blood collected from diseased sheep (Lian et al., 1997; Yin et al., 1997a; Bai et al., 2002). Subsequently, several large *Babesia* strains were isolated from field-collected blood or ticks that had infested splenectomised sheep from different regions of China (Guan et al., 2001, 2002; Liu et al., 2007). The abovementioned strains could be divided into two groups on the basis of their 18S ribosomal RNA (18S rRNA) and internal transcribed spacers (ITS) gene sequences, a *B. motasi*-like group and the *Babesia* sp. Xinjiang group (Liu et al., 2007; Niu et al., 2009).

Babesia sp. Xinjiang was isolated from a sheep infested with the tick *Rhipicephalus sanguineus* and *Hyalomma anatolicum anatolicum* in 2001, and was thought to represent an asymptomatic benign infection in this species (Guan et al., 2001, 2009). *Hy. a. anatolicum* has been shown to be its vector tick through experimental transmission. The morphological features of *Babesia* sp. Xinjiang are very different from those of the Chinese *B. motasi*-like strains, and the European *B. motasi* and *B. crassa*. It has been considered to be a novel *Babesia* species infectious for small ruminants (Guan et al., 2001, 2009).

The microaerophilic stationary phase culture (MASP) technique for *Babesia* was developed by Levy and Ristic (1980), and, together with the cloning techniques used with *in vitro* culture for *B. bovis* and *B. bigemina* (Rodriguez et al., 1983; Vega et al., 1986), it provides a new approach to the isolation of babesial pathogens and the diagnosis of *Babesia* infection. Since then, several *Babesia* strains have been isolated using this technique (Goff et al., 1993; Thomford et al., 1993; Holman et al., 1994a,b, 2005). Cultures provide a source of parasites for *in vivo* inoculation for use in the establishment of host range, *in vitro* determination of erythrocyte specificity, comparative morphology, and for the development of vaccines and diagnostic tests (Moreau et al., 1988; Montenegro-James, 1989; Zhao et al., 2002). However, there are few studies that involve *in vitro* culture of *Babesia* infectious for sheep and goats.

In the present study, we developed an *in vitro* culture system for *Babesia* sp. Xinjiang, and two monoclonal lines were screened. Their generation time, infectivity for splenectomised sheep, goats and cattle, invasion of erythrocytes from different hosts, and the isolation of the parasite from field-collected samples were evaluated using this *in vitro* culture system.

2. Materials and methods

2.1. Parasite

Blood infected with *Babesia* sp. Xinjiang, collected from sheep nos. 2204 and 1216 which were infested with adult ticks of *Hyalomma anatolicum anatolicum* (Guan et al., 2009), was cryopreserved in liquid nitrogen with 8% dimethyl sulphoxide in 5 ml aliquots as previously described by Dou et al. (1989). Fifteen milliliters of the abovementioned blood samples was inoculated into a splenectomised sheep (no. 08102) that had been immunosuppressed by injection of dexamethasone (10 mg daily for 3 days). When parasitemia reached 2.5%, the animal was bled and samples of the infected blood were either cryopreserved in liquid nitrogen or used to initiate *in vitro* culture.

2.2. Animals and blood

All the blood employed during in the experiments were demonstrated free of piroplasms, on the basis of microscopic examination and PCR. Blood was collected in 9 ml sterile K_3EDTA vacutainers from sheep (Tan mutton breed), goats (Inner Mongolia Cashmere goat) and cattle (Qinchuan breed), which were between 0.5 and 1.5 years old, sika deer (*Cervus Nippon*) (10 animals) and man. The animals were kept in tick-free folds, with food and water available *ad libitum*.

2.3. Preparation of RBC suspension

Peripheral blood collection and preparation of the red blood cell (RBC) suspension were performed as described previously by Guan et al. (2010) with some modifications. Briefly, blood was collected from the jugular vein of animals by venipuncture into 9 ml sterile K_3EDTA vacutainers (Greiner bio-one, Austria) and similarly from a volunteer. The blood was centrifuged at $800 \times g$ for 10 min at room temperature. The plasma and buffy coat were discarded. The RBC were washed once with 10 ml RPMI 1640 medium (Lonza, USA) containing 0.5 μg/ml amphotericin B (Lonza, USA) and 50 μg/ml gentamicin (Lonza, USA) (RPMI 1640g), and centrifuged as above. The packed RBCs were suspended in 2 volumes of RPMI 1640 and kept at 4 °C until used.

2.4. Initiation of culture

Peripheral blood was collected from sheep infected with *Babesia* sp. Xinjiang and the RBC suspension was prepared as described above. Initiation of the culture was performed in 24-well plates as described previously by Guan et al. (2010), except that fetal bovine sera (FBS) were purchased from Gibco (C2027050, Uruguay) and the cultures were incubated in an atmosphere containing 5% CO_2.

2.5. Screening of donor sheep for Babesia sp. Xinjiang

The initiation culture of *Babesia* sp. Xinjiang used RBCs from a donor sheep (Tan mutton breed) that could sustain and propagate *Babesia* sp. BQ1 (Lintan) with success *in vitro*. Nine spleen-intact sheep were used to screen for the most suitable donor of normal RBC as described previously by Guan et al. (2010). The RBCs of sheep nos. 02 and 91 were chosen as donor sheep.