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Rhoptry-associated protein (*rap-1*) genes in the sheep pathogen *Babesia* sp. Xinjiang: Multiple transcribed copies differing by 3′ end repeated sequences

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ABSTRACT

Sheep babesiosis occurs mainly in tropical and subtropical areas. The sheep parasite *Babesia* sp. Xinjiang is widespread in China, and our goal is to characterize *rap-1* (rhoptry-associated protein 1) gene diversity and expression as a first step of a long term goal aiming at developing a recombinant subunit vaccine. Seven different *rap-1a* genes were amplified in *Babesia* sp. Xinjiang, using degenerate primers designed from conserved motifs. *Rap-1b* and *rap-1c* gene types could not be identified. In all seven *rap-1a* genes, the 5′ regions exhibited identical sequences over 936 nt, and the 3′ regions differed at 28 positions over 147 nt, defining two types of genes designated α and β. The remaining 3′ part varied from 72 to 360 nt in length, depending on the gene. This region consists of a succession of two to ten 36 nt repeats, which explains the size differences. Even if the nucleotide sequences varied, 6 repeats encoded the same stretch of amino acids. Transcription of at least four α and two β genes was demonstrated by standard RT-PCR.

1. Introduction

Babesia is a tick-transmitted obligate intraerythrocytic protozoan parasite, belonging to the phylum Apicomplexa, which infects a broad range of vertebrate hosts (Telford et al., 1993). Babesiosis, the hemolytic disease caused by *Babesia,* is responsible for significant mortality and morbidity rates and enormous economic losses to the international trade of animals and the livestock industry in tropical and subtropical regions worldwide (De Waal, 2000; Brown and Palmer, 1999). Several *Babesia* species are known to infect small ruminants, e.g., *Babesia ovis*, *B. motasi* and *B. crassa* (Uilenberg, 2006). Recently, many more *Babesia* isolates from small ruminants have been described in China, most of them closely related to *B. motasi* (*Babesia* sp. BQ1 (Lintan and Ningxian), *Babesia* sp. Tianzhu, *Babesia* sp. Madang, *Babesia* sp. Liaoning and *Babesia* sp. Hebei) (Liu et al., 2007). One of them, however, *Babesia* sp. Xinjiang, belongs to a separate and new phylogenetic clade, together with other recently described *Babesia* from wild ruminants in South Africa and *B. pecorum* isolated from red deer in Spain (Liu et al., 2007; Niu et al., 2009; Oosthuizen et al., 2009; Jouglin et al., 2014). This *Babesia* was isolated from a splenectomized sheep that had been experimentally infested with adult *Rhipicephalus sanguineus* and *Hyalomma anatolicum anatolicum* collected from farmed sheep and goats in the Xinjiang autonomous region (Guan et al., 2001). This isolate exhibited different morphological characteristics to the above-mentioned *B. motasi*-like isolates: i.e., typical pyriform but more slender pairs than the other Chinese isolates with an average size of 2.42 (±0.35) μm × 1.06 (±0.22) μm (Guan et al., 2009). The widespread distribution of *Babesia* sp. Xinjiang-related parasites in China (over 50 prefectures in 22 provinces) was demonstrated in a sero-epidemiological survey (Guan et al., 2012).

Clinical symptoms of babesiosis become apparent when the parasite invades and replicates within the host erythrocytes and attains detectable parasitemia. These symptoms are characterized by fever and mild hemolytic anemia, whereas severe cases exhibit depression and hemoglobinuria with subsequent organ and systemic failure resulting in death (Wright and Goodger, 1988). Control strategies for babesiosis, introduced in recent years, are still based on treatment with toxic chemotherapeutic agents and/or

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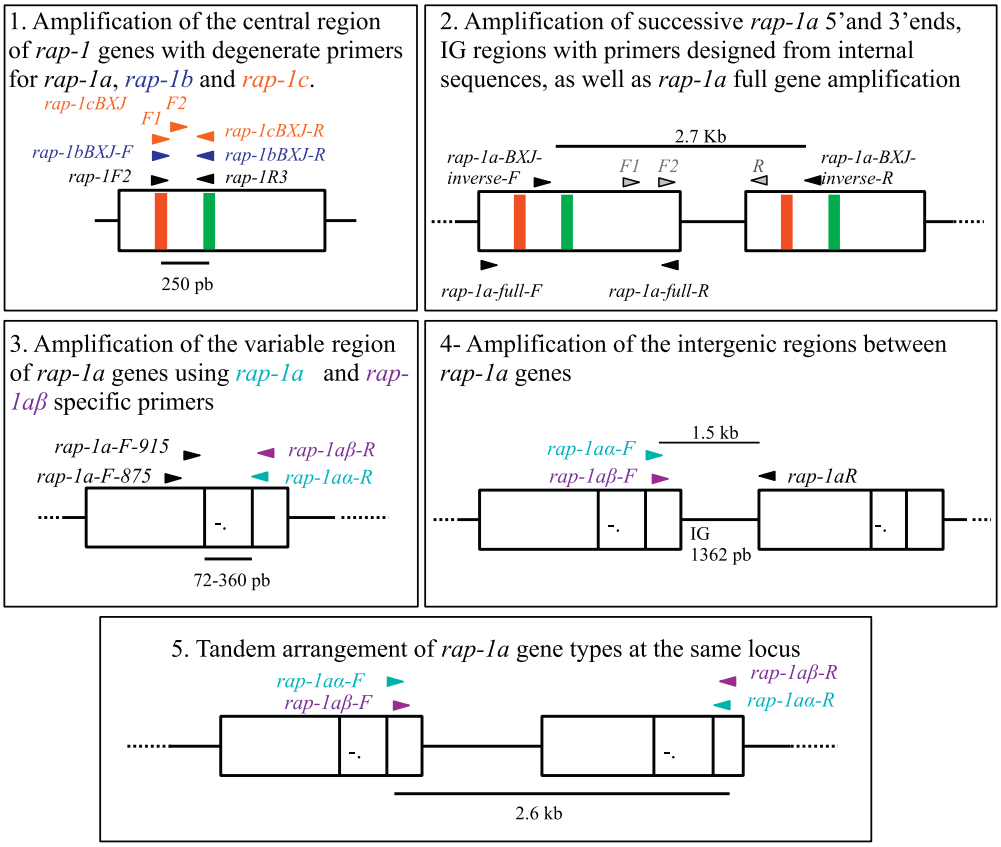

Fig. 1. *Rap-1a* locus amplification strategy and primers location. Two highly conserved regions used to design degenerated primers are indicated (DAAF and YKTYL). Primers used for amplification are indicated with solid-line arrows, primers used for sequencing are indicated with grey arrows.

vaccination with live attenuated strains of the parasite (Wright, 1990; Kuttler, 1990). However, drawbacks associated with drug residues, poor induced protection against challenge with virulent parasites, and reversion of live attenuated strains to virulence, have prompted a search for more effective ways of dealing with babesiosis (De Waal and Combrink, 2006). An effort is now being made to develop multivalent recombinant subunit vaccines that target parasite multiplication within the host and, more precisely, the steps involved in erythrocyte invasion (Gohil et al., 2013). Potential vaccine candidates include rhoptry -associated protein-1 (RAP-1), apical membrane antigen-1 (AMA-1), and spherical body proteins (SBP-1, 2, 3), all of which are secreted by the apical complex organelles (rhoptries, micronemes and spherical bodies or dense granules) to achieve erythrocyte invasion. These candidates have been identified and characterized mainly in *B. bigemina* and *B. bovis* (Yokoyama et al., 2006; Lobo et al., 2012). One such protein, the rhoptry protein *RAP-1*, is an immunogenic protein whose role in red blood cell invasion was inferred from a series of erythrocyte adhesion or invasion trials and a test of *Babesia* growth inhibition in vitro using *RAP-1* antibodies, although the precise function of *RAP-1* is still unknown (Figueroa and Buening, 1991; Brown et al., 1998; Brown and Palmer, 1999; Brown et al., 1999; Mosqueda et al., 2002; Yokoyama et al., 2002; Norimine et al., 2003).

Rap-1 genes have been identified in all *Babesia* species examined to date (Goff et al., 1988; Wright et al., 1992; Dalrymple et al., 1993; Skuce et al., 1996; Suarez et al., 1998a, 2003; Kappmeyer et al., 1999; Zhou et al., 2007; Niu et al., 2013, 2014). The organization of these *rap-1* genes is characterized by the presence of a tandem arrangement of multiple gene copies. A simple arrangement with only two almost identical copies of *rap-1a* is found in *B. bovis* (Suarez et al., 1998a). In contrast, the *rap-1* locus in *B. bigemina* and in the sheep parasites *Babesia* sp. BQ1 Lintan, *Babesia* sp. BQ1 Ningxian, *Babesia* sp. Tianzhu and *Babesia* sp. Hebei contains three types of *rap-1* genes, (*rap-1a*, *rap-1b* and *rap-1c*), with multiple copies of polymorphic *rap-1a* and conserved *rap-1b* arranged in tandem (Suarez et al., 2003; Niu et al., 2013, 2014). All babesial *RAP-1a* proteins exhibit well-defined molecular features such as strict conservation of 4 cysteine residues at the N-terminus, a 14 amino acid motif and several shorter conserved motifs, and the presence of a signal peptide (Suarez et al., 1991a,b, 1994; Dalrymple et al., 1996).

The aim of our study was to amplify and sequence the *rap-1* genes present in the sheep parasite *Babesia* sp. Xinjiang. Even if this sheep parasite is widespread in China (Guan et al., 2012), it has been only recently described (Guan et al., 2009) and only one isolate is cultivated *in vitro* from which one clone has been isolated by limiting dilution and used in our study. As multiple genes were detected, their transcription was also analyzed by standard reverse transcription PCR. Implications in terms of gene evolution, diagnostics and vaccine development are discussed.

2. Materials and methods

2.1. Babesia *sp. Xinjiang in vitro culture*

A monoclonal line of *Babesia* sp. in Xinjiang was derived from *in vitro* culture by limiting dilution, as described previously (Malandrin et al., 2009). Briefly, infected blood samples cryopreserved in liquid nitrogen were thawed in a water bath at 37 °C, washed with RPMI 1640 (Lonza, Belgium) and centrifuged (1200 × g, 10 min). Initiation of culture was performed in 24-well culture plates containing RPMI 1640 medium supplemented with