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Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa

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ABSTRACT

A competitive-inhibition enzyme-linked immunosorbent assay (cELISA) developed for the detection of antibody specific for *Babesia caballi* was used to test sera collected from 1237 South African horses. None of these samples tested positive using the cELISA, although 63 samples tested positive for *B. caballi* antibody using the indirect fluorescent antibody test (IFAT). We therefore characterized the *rap-1* gene that codes for the antigen (rhoptry-associated protein, RAP-1) used in the cELISA, from South African *B. caballi* isolates. Three sets of primers were designed to amplify the complete gene and flanking regions (~1800 bp), but only one set of primers yielded PCR products, and we were only able to amplify a region at the 5′ end of the gene (615 bp) from ten South African *B. caballi in vitro*-cultured isolates. Sequence data from seven of these were obtained. The sequences showed between 79% and 81% identity to *B. caballi rap-1* gene sequences that have been reported in the literature (accession numbers: AF092736 and AB017700). The GenomeWalker Universal kit (Clonetech) was used to amplify the regions flanking the 615 bp *B. caballi rap-1* fragment from two South African isolates. Amplified products were cloned into the pGEM-T Easy vector and sequenced. The complete *rap-1* gene sequence, comprising a single open reading frame of 1479 bp that encodes a protein consisting of 493 amino acids, was obtained from the two South African isolates. This sequence data was used to redesign the amplification primers and *rap-1* homologues were obtained from a further eight isolates. BLASTP analysis indicated an amino acid identity of between 57.9% and 65.1% to the two RAP-1 protein sequences, AF092736 and AB017700, with most differences occurring at the carboxy-terminus. The amino acid sequence differences probably explain why it was not possible to detect *B. caballi* antibody in IFAT positive sera from South Africa using the cELISA. Redesigning the current cELISA using a conserved epitope of the RAP-1 antigen, or a more conserved protein as the target antigen, may overcome this problem.

1. Introduction

Babesia caballi is a tick-borne haemoprotozoan parasite, and is one of the causes of equine piroplasmosis. The clinical manifestations of the disease are often variable, making it easy to confuse with *Theileria equi* infections. *B. caballi* infections, characterized by fever and anaemia, are considered less severe than *T. equi* infections, which are more commonly associated with haemoglobinuria and death (de

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Waal, 1992; Camacho et al., 2005). Many infections caused by either parasite are subclinical and, in addition, animals that have recovered from the infections often remain carriers of the parasites for long periods of time.

The international movement of horses has led to the spread of equine piroplasmosis from its endemic tropical and subtropical regions to more temperate non-endemic regions. Many countries have introduced stringent import restrictions to prevent the introduction of these parasites into disease-free areas (Friedhoff et al., 1990). Serological methods of determining the carrier status of horses and other equine species are currently the prescribed methods for certifying animals to be free of these parasites. These include the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) (Donnelly et al., 1980; Weiland, 1986; Brüning et al., 1997). Although it is possible to distinguish between *T. equi* and *B. caballi* infections using IFAT, differentiation between weak positive and negative reactions can be difficult. Cross-reactions that occur between *B. caballi* and *T. equi* in the indirect ELISA prevent this test from being recognized as a differential diagnostic assay (Weiland, 1986). Recently, however, *T. equi* and *B. caballi* recombinant merozoite proteins and monoclonal antibodies to immunogenic epitopes on these proteins have been used in competitive-inhibition ELISAs (cELISAs) with promising results. These cELISAs have been shown to have higher specificities for *T. equi* and *B. caballi*, when compared to those of the IFAT and indirect ELISA (Knowles et al., 1991, 1992; Shkap et al., 1998; Kappmeyer et al., 1999; Katz et al., 2000; Xuan et al., 2002).

Secreted proteins from the apical organelles of apicomplexan parasites are thought to play pivotal roles in parasite attachment to, invasion of and expansion and maintenance within the host cell (Sam-Yellowe, 1996). Among these proteins is the rhoptry-associated protein-1 (RAP-1), which was initially described in *Babesia bovis* and *Babesia bigemina*, but has subsequently been described in other *Babesia* parasites (Dalrymple et al., 1993; Skuce et al., 1996; Suarez et al., 1998; Kappmeyer et al., 1999; Ikadai et al., 1999). The RAP-1 family of proteins contains several immunogenic epitopes and antibodies directed against these proteins have been shown to inhibit merozoite invasion (Ikadai et al., 1999; Machado et al., 1999; Yokoyama et al., 2006). This phenomenon suggests that RAP-1 proteins are important targets of the protective immune response (Suarez et al., 2003).

A monoclonal antibody to recombinant RAP-1 was used in the development of a cELISA for the detection of *B. caballi* antibody in infected horses (Kappmeyer et al., 1999). This assay has been successfully used for the detection of *B. caballi* antibody in the sera of infected horses in North and South America and several European countries (Kappmeyer et al., 1999; Sevinc et al., 2008). However, in preliminary studies in South Africa, the commercially available cELISA was not able to detect antibody in horses infected with *B. caballi*. This result led to the hypothesis that differences in the RAP-1 antigen within South African *B. caballi* isolates could prevent the detection of *B. caballi* antibody. This study was therefore focused on the characterization of *rap-1* gene homologues in South African *B. caballi* isolates in an attempt to determine the cause of the failure of the commercial cELISA in South Africa.

2. Materials and methods

2.1. Field samples and in vitro-cultured isolates

A total of 1237 whole blood samples were collected from horses at the National Sale of two-year-old thoroughbred horses in 2005 ($n = 273$) and the National Yearling Sales in 2005 ($n = 455$) and 2006 ($n = 509$). Ten *in vitro*-cultured *B. caballi* isolates, designated Bcab5, Bcab9, Bcab13, Bcab19, Bcab105, Bcab167, Bcab418, Bcab443, Bcab502 and BcabE7 (Zweygarth et al., 2002), were also used in this study.

2.2. cELISA and IFAT

Sera obtained from the blood samples were examined for the presence of antibody against *T. equi* and *B. caballi* using the commercially available cELISA kit, as described by the manufacturer (VMRD Inc., Pullman, WA, USA).

The IFAT was conducted at the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI). A standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Anonymous, 2008) was used, with the exception that cultured antigen, produced locally in South Africa, was used.

2.3. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 200 μl of each of the *in vitro*-cultured isolates using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Primers were designed to amplify complete *rap-1* homologues and flanking sequences (~1800 bp) from the South African isolates based on two *B. caballi rap-1* DNA sequences available in the public sequence databases [accession numbers: AF092736 (Kappmeyer et al., 1999) and AB017700 (Ikadai et al., 1999)]. Initially, primers BC-RAP1F and BC-RAP1R (Table 1, Fig. 1) were designed to amplify the full-length gene and flanking sequences. Subsequently, primer pairs BC-RAP1F and BC-RAP3R, BC-RAP2F and BC-RAP2R, and BC-RAP3F and BC-RAP1R (Table 1) were designed to amplify overlapping fragments, respectively, at the 5′ end, in the middle and at the 3′ end of the ~1800 bp *rap-1* fragment (Fig. 1). The FastPCR software program (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) was used to design all primers used in this study, and to check for the formation of secondary structures and primer dimers. All PCR primers were obtained from Integrated DNA Technologies (IDT). Reactions were performed in a final volume of 25 μl, containing High Fidelity PCR Master mix (Roche Diagnostics, Mannheim, Germany), 0.2 μM of each primer and 30 ng of genomic DNA. The cycling conditions were: an initial denaturation of 5 min at 95 °C, followed by 40 cycles