Analysis of the culture-derived soluble 
**Babesia canis canis** antigens derived from 
the Polish strains of the parasites

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Key words

*Babesia canis canis*, Western blotting, SPA, dogs

Summary

**Objective, material and methods:** The aim of this study was to analyse the protein fractions of the soluble parasitic antigen (SPA) from in vitro cultures of the native Polish strains of *Babesia canis canis* and to determine their immunogenicity through Western blotting using the sera of dogs vaccinated with this antigen. **Results:** Polyacrylamide gel electrophoresis revealed 21 protein fractions with molecular weights from 12 to 205 kDa. The most intense reaction in Western blotting was observed between the serum antibodies of the SPA-vaccinated dogs and the fraction with the molecular weight of 52 kDa. **Conclusion:** Detailed studies on the composition of SPA of *Babesia canis canis* and reactivity of its individual protein fractions can be a starting point for the development of subunit vaccines against babesiosis. Using a preparation with only some electrophoretic fractions of SPA in the production of vaccines would allow to avoid putting an unnecessary protein burden in the vaccine which could cause side effects.

Schlüsselwörter

*Babesia canis canis*, Western Blot, SPA, Hund

Zusammenfassung

**Ziel, Material und Methoden:** Ziel dieser Studie war, aus In-vitro-Kulturen der endemischen polnischen Stämme von *Babesia canis canis* einzelne Proteinfraktionen des löslichen parasitären Antigens (soluble parasitic antigen, SPA) zu analysieren und deren Immunogenität im Western Blot mit den Seren der mit diesem Antigen geimpften Hunde zu bestimmen. **Ergebnisse:** Durch Polyacrylamid-Gelelektrophorese ließen sich 21 Proteinfraktionen mit den Molekulargewichten von 12 bis 205 kDa isolieren. Die intensivste Reaktion in Western Blot ergab sich zwischen den Serumantikörpern der SPA-geimpften Hunde und der Fraktion mit einem Molekulargewicht von 52 kDa. **Schlussfolgerung:** Detaillierte Untersuchungen zur Zusammensetzung des SPA von *Babesia canis canis* und zur Reaktivität der einzelnen Proteinfraktionen können als Grundlage für die Entwicklung von Subunit-Impfstoffen gegen die Babesiose des Hundes dienen. Bei Verwendung einzelner SPA-Fraktionen lassen sich belastende Fremdproteine im Impfstoff und daraus möglicherweise resultierende Nebenwirkungen bei geimpften Tieren reduzieren.

Introduction

Canine babesiosis is a common and clinically significant tick-borne disease caused by hematozoan parasites of the genus *Babesia* (1, 11). The classification of *Babesia* spp. places them in the order Pirosplasmida within the phylum Apicomplexa. Two morphologically distinct forms of the erythrocytic stage in the canine host were recognized in early studies that led to the naming of the larger form, measuring approximately 3–5 μm as *B. canis*, and the smaller form (1–3 μm) as *B. gibsoni*.

*Babesia canis* has been reclassified into three separate species: *B. canis canis, B. canis vogeli* and *B. canis rossi*. *B. canis canis*, which is transmitted by *Dermacentor reticulatus*, is endemic in Europe. The occurrence of *B. canis vogeli* correlates with the distribution of the vector *Rhipicephalus sanguineus* and is mainly localized in southern European countries. *B. canis rossi* is transmitted by *Hae maphysalis leachi* and is localized in the South of Africa (8, 31). *B. canis canis* is probably “the most common etiologic agent of babesiosis in dogs in Europe” (2, 3, 5, 7, 16, 28, 32). Clinically, these pathogens cause remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly and hepatomegaly in dogs and, in some cases, the death of infected animals (3, 19).

Prevention of the disease consists in avoiding sites where ticks live and using effective prophylaxis against ectoparasites. Attempts...
to obtain a vaccine aimed at protecting dogs against the development of babesiosis have been ongoing for several years. For this purpose the soluble parasitic antigen – SPA, derived from the in vitro culture of the *Babesia canis* protozoans is used. The attempts have resulted in the development of a commercial preparation, Nobivac Piro® (saponin adjuvanted bivalent vaccine containing SPA produced by *B. canis canis* and *B. canis rossi*) and Pirodog® (saponin-adjuvanted vaccine containing SPA produced by *B. canis canis*), which admittedly does not prevent the protozoan infection in dogs, but protects them from the developing disease (9, 25, 26).

It is assumed that SPA are toxic substances released in the host’s body by *Babesia* protozoa, responsible for clinical signs of the disease (25, 26). No detailed study on the composition of this antigen were conducted so far. For this purpose the Western blotting technique may be used (12, 13). The Western blotting is a widely used analytical technique used to detect specific proteins in the given sample of blood, tissue homogenate or extract. It uses gel electrophoresis to separate native proteins. The proteins are then transferred to a membrane, where they are detected using antibodies specific to the target protein (17, 29).

Our earlier studies attempted to obtain the SPA antigen from the native Polish strains of *Babesia canis canis*, which would then serve for vaccination of dogs against babesiosis (4).

The present study was concerned with the identification of soluble babesial antigens derived from the in vitro culture system. In order to do that, the in vitro culture of the *Babesia canis canis* protozoans was set up, and the obtained SPA antigen was analyzed electrophoretically. To determine the reactivity of individual SPA protein fractions, Western blotting analysis using the sera of dogs vaccinated with this antigen was conducted.

### Material and methods

#### Determination of the Babesia canis strain

Parasite DNA was purified from infected dog blood following the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or the Blood Mini DNA isolation kit (A&A Biotechnology, Gdańsk, Poland) protocol.

The amplification of *B. canis canis* DNA through PCR was performed using the forward primer BAB GF2 (5′-GTC TTT TGA GAA TGA TGC-3′), and the reverse primer BAB GR2 (5′-CCA AAG ACT TTG ATT TCT CTC-3′), which amplify a 559-bp region of the 18S rRNA gene of *B. canis canis*. Briefly, each reaction mixture (50 μL) contained 100 μM of each dNTP, 1.6 mM of MgCl₂, 0.25 μM of each primer, 2.5 U of Taq DNA polymerase, and 5 μL of DNA template. PCR amplification was performed using a programmable thermal cycler (Biometra, Goettingen, Germany) with the following program: an initial denaturation at 92°C for 2 minutes, 50 cycles of denaturation at 92°C for 60 seconds, annealing at 52°C for 60 seconds, and extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. Positive and negative controls were included in all amplifications. PCR results were evaluated by agarose gel (1%) electrophoresis stained with ethidium bromide in parallel with a 100-bp DNA ladder (Gibco/BRL, Gaithersburg, MD, USA). The final identification of the *Babesia canis canis* parasites was performed based on the results of the sequences analysis of the obtained PCR products. PCR products were purified using the QIAquick spin columns (Qiagen) and eluted in 50 μL of Tris 10 mM, pH 7.6. DNA sequence was determined on both strands using the same primers employed for PCR at a DNA sequencing core facility (Research Institute, Polish Academy of Sciences, Warsaw, Poland). DNA sequences were assembled and edited using SeqMan (DNASTar, Lasergene, Madison, USA), and ClustalW alignments to the published *B. canis canis* 18S rRNA gene EU622792 and EU622793 from a previous study (2).

#### In vitro cultivation of Babesia canis canis

Red blood cells (RBCs) from the infected blood sample were prepared ex tempore for culture as follows. The blood was centrifuged at 500 × g for 20 minutes to pellet the cells, and the plasma and buffy layer were removed and discarded. A 0.2 ml aliquot of packed RBC was mixed with 1 ml of 0.15 M phosphate buffered saline (PBS) and washed by centrifugation at 885 × g for 5 minutes. The supernatant and buffy layer were removed, and the cell pellet washed two more times as above in 1 ml of PBS. After the final wash, the supernatant was removed and the RBC used as packed cells. The cultures were initiated in duplicate wells of a 96-well plate with 50 μl washed packed infected RBC and 350 μl of either RPMI-1640 medium (Biomed Lublin, Poland) supplemented with 40% canine serum (Pel-Freez Biologicals, Rogers, AZ), and buffered with 20 mM HEPES. Antibiotics were added to all media at a final concentration of 200 μg/ml streptomycin and 200 U/ml penicillin. The cultures were incubated at 37 °C in a humidified incubator chamber in air atmosphere with a 5% carbon dioxide.

Cultures were fed daily by removing about 300 μl without disturbing the settled RBC layer, and replacing this with 300 μl fresh medium, as appropriate. Non-infected donor packed RBC (20 μl), prepared as described below, were added every 7 days. At subculture, the culture medium was replenished as above and RBC resuspended in the fresh medium. Subcultures were performed at a split ratio of 1 : 4, with transfer of 100 μl of the RBC suspension to a new well. The volumes were brought to 400 μl with fresh medium and 10% donor RBC (v/v) prepared as below. The cultured parasites were subcultured into HL-1 and EMEM media with 40% dog or fetal bovine sera respectively, to determine the optimal formulation to support the in vitro growth of the parasite.

Non-infected donor RBC were obtained from two 2-year-old female mixed breed dogs. The donors did not show any signs of illness and were negative for the presence of canine *Babesia* spp. by microscopy and PCR. The blood was drawn via venipuncture into a EDTA tube and then centrifuged at 500 × g for 20 minutes to pellet the cells. The plasma and buffy layer were removed and the RBC pellet was used in the study.
Parasite growth was monitored by examination of Diff-Quick stained (POCH Lublin, Poland) thin blood films at 1-day intervals. The blood smears were made on a degreased slide and stained. Upon drying, they were observed through an Olympus CH 20 (Olympus Optical CO. Ltd., Japan) under an immersion objective (1000× magnification) (4).

Preparation of the SPA antigen

The supernatant media from the in vitro culture were collected and mixed when parasitemias reached the level of 0.6%. The obtained supernatant media mixture was subjected to centrifugation at 700 g for eliminating the red globules and possible parasites as well as their remains. The centrifugation lasted for 30 minutes.

The centrifugated medium was subjected to sterilizing filtration on a 0.22 micron membrane and concentrated by ultra-filtration on a membrane of 20 000 Daltons. The concentration was treated with formol in a proportion of 0.12 mg/ml for a night at a temperature of 4 °C, and than lyophilized and divided into doses. Before administration to the dogs the lyophilized antigen was dissolved in a solution of saponin at 0.5 mg/ml in apyrogenic sterile water (18).

Animals and vaccination schedule

The study included 15 mixed dogs (seven males and eight females), aged 20–27 months, divided into two groups. Dogs from group 1 (n = 5, no. 1–5) were used as negative controls and did not receive SPA Babesia canis canis antigen. Dogs from group 2 (n = 10, no. 6–15) were the study group, where SPA was administered subcutaneously. All animals used in the experiment were kept for experimental purposes in the Clinic of Infectious Diseases at the Faculty of Veterinary Medicine in Lublin and had no contact with the Babesia protozoans (they were kept in closed spaces without contact with ticks; the results of an immunofluorescence assay and a PCR for Babesia performed in these dogs before the start of the study were negative).

The antigen was administered subcutaneously in the subscapular region. The vaccination comprised two injections spaced apart by 2 weeks on day 0 and 14 of the study.

Two weeks after the administration of the second dose of the vaccine, blood was collected from all 15 animals both from the test group and the control group in order to obtain their sera for the immunoblotting analysis.

Electrophoresis of the SPA and Western blotting

Electrophoresis was performed on 3–20% polyacrylamide gradient gel as described by Laemmli (17) and was carried out at a constant voltage of 100 V. Protein samples were heated at 99 °C for 5 minutes in SDS-PAGE sample buffer containing 50 mM 2-mercaptoethanol. Approximately 4 μg of total protein was applied to each lane. PageRuler™ Prestained Protein Ladder (Fermentas) with a mass range of 11–250 kDa was used as a molecular weight standard.

Following SDS-PAGE, gels were equilibrated in blotting buffer (12 mM Tris, 96 mM glycine, 20% methanol, pH 8.3) and then the proteins were electroblotted (Mini Trans-Blot cell, Bio-Rad) onto polyvinylidene difluoride (PVDF) membranes as described by Towbin et al. (29). Transfer was conducted at 100 volts for 2 hours. After blocking with low-fat milk protein, the membranes were incubated overnight with canine serum diluted 1:100 in Tris-Buffered Saline Tween-20 (TBST). Polyclonal peroxidase-conjugated rabbit anti-dog IgG (Jackson ImmunoResearch) diluted 1:10000 in TBST (time of incubation 2 hours) was used as a second antibody. To induce a colour reaction, 1.4 chloronaphthol (Bio-Rad) was used as a substrate.

Densitometric analysis of the obtained protein bands was carried out using the Gel Doc 2000 gel documentation system (Bio-Rad) and the computer program Quantity One (Bio-Rad).

Results

Electrophoresis and Western blotting

The electrophoresis image of the SPA protein profile is presented in Figure 1. Densitometric analysis showed that the SPA of the

Fig. 1  Electrophoresis image of the Babesia canis canis SPA protein profile from the in vitro culture system. s = weight standards; a = protein profile of Babesia canis canis SPA from in vitro culture.

Abb. 1  Elektrophoretische Auffüllung der Proteinfraktionen des löslichen parasitären Antigens (SPA) aus In-vitro-Kulturen von Babesia canis canis. s = Molekülmasstenstandards, a = SPA-Proteinprofil von Babesia canis canis
Polish strain *Babesia canis canis* had a protein profile with 21 fractions with molecular weights from 12 to 205 kDa. The highest intensity was observed for the fractions of proteins with the masses of 15 kDa, 48 kDa, 52 kDa, 62 kDa, 73 kDa, and 83 kDa.

Antibodies for particular SPA protein fractions of *Babesia canis canis* were found in all 10 samples of the analyzed sera obtained from the dogs vaccinated with this antigen. Densitometric analysis of the images obtained on the nitrocellulose membranes showed 11 specific reactions between antibodies contained in the examined sera and the SPA protein fractions of 12, 25, 33, 38, 52, 62, 83, 98, 121, 144 and 198 kDa, with the strongest reaction observed for the protein with the molecular weight of 52 kDa (Fig. 2).

No antibodies against any fraction of SPA were found in the serum of any dog belonging to group 1 (control).

**Clinical observations of dogs after vaccination**

In 2 out of 10 dogs from group 2, vaccinated with the *Babesia canis canis* SPA antigen, a painful swelling appeared at the injection site 24 hours after the administration of the second dose of the antigen. The swelling receded spontaneously within 7 days. In addition, one of these dogs developed flu-like symptoms such as fever 39.8 °C, apathy, lack of appetite, and serous nasal discharge, which also receded spontaneously within a week.

**Discussion**

SPA plays an important role in the etiology of canine babesiosis. It appears that SPA activates the coagulation system. Earlier results of Schetters et al. (24) have shown that in canine *Babesia* infections, that are characterized by erythrocyte retention, disease is correlated with effects on the coagulation system, in contrast to infections characterized by exponentially developing parasitaemia, where disease is correlated with peripheral parasitaemia. This antigen appears to prime Th-cells that assist in the generation of antibodies against SPA, which interfere with the trigger of this pathological process (23). Therefore, the research on the use of SPA for the immunization of dogs against babesiosis appears to be definitely expedient.

Although the mechanism of immunity stimulation in dogs by SPA of *Babesia canis* has been relatively well described to date (21, 22, 25–27), little attention has been paid to the research on the structure of the antigen. Azzar et al. (6) showed the presence of three glycoprotein fractions with the masses of 12.5, 40 and 100 kDa in the supernatant from the *Babesia canis* culture. The preparative gel electrofocusing suggested that these antigens focus in the pH range of 3–5. This finding is consistent with the existence of known acidic protein antigens derived from detergent extracts of *B. bovis* (15) and from *Plasmodium vivax* sporozoites (20). One or more soluble *Babesia canis* antigens are thought to be in the merozoite surface coat and facilitate the immune destruction of the latter by macrophages (12). The presence of such antigen with the mass of 12 kDa on the surface of merozoites of *Babesia canis* was shown by Hauschild et al. (13). This protein band appears in all pathogenic, but not in apathogenic, isolates of *Babesia canis* and may determine the virulence of the parasite.

In our own studies, all sera derived from the vaccinated dogs reacted with the SPA fraction of 12 kDa. An additional factor confirming the hypothesis that this fraction originates from the surface of merozoites may be the fact that the SPA antigen for the immunization was obtained from the cultures of pathogenic strains of *Babesia canis* isolated from dogs with clinical babesiosis (4).
Polyacrylamide gel electrophoresis of SPA obtained in our own studies revealed 21 protein fractions with the masses ranging from 12 to 205 kDa. These results correlate well with those reported for certain soluble antigens of *Plasmodium* and *Babesia* species (10, 14, 30). In the supernatant from the in vitro culture of these pathogens, antigens with the masses of 44–55 kDa have been the most common. Our Western blot results, where the strongest reactions for all 10 sera of the SPA-immunized dogs were observed for the protein with the mass of 52 kDa, indicate that fractions with such a mass may be crucial for stimulating immunity against babesiosis.

**Conflict of interest**

The authors confirm not to have any conflict of interest.

**Ethical content statement, animal welfare**

The study was conducted in accordance with the EU-Convention on the protection of animals used for scientific purpose (Revised Directive 86/609/EEC). The use of animals for research was approved by the ethical committee (Directive 18/2009).

**References**

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