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Development of a new diagnostic tool for detecting *Babesia canis* infections in canine babesiosis

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Summary

The aim of this study was to develop a rapid and sensitive diagnostic test for canine babesiosis caused by *Babesia canis*, based on the detection of parasite antigens in the blood of infected dogs using specific monoclonal antibodies. Three *B. canis* antigens (SPA, BcMSA1, Bc28.1) were used for *in vitro* immunization of murine B lymphocytes, which were subsequently fused with SP2/0-Ag14 myeloma cells. Hybridoma clones producing antigen-specific antibodies were selected, purified, and characterized using ELISA and Dot-Blot assays. The most promising clone was sequenced, and variable regions (VH and VL) were cloned into expression vectors and expressed in CHO-K1 cells. Recombinant antibodies were purified and incorporated into a prototype lateral flow immunoassay (LFIA). Diagnostic performance was evaluated using 100 blood samples from PCR-confirmed *B. canis*-infected dogs and 100 samples from healthy controls. Monoclonal antibodies specific to BcMSA1, Bc28.1, and SPA antigens were generated. Recombinant expression in CHO-K1 confirmed strong specificity to BcMSA1. In LFIA format, the assay demonstrated 90% accuracy with purified recombinant antigen and 70% accuracy with clinical samples (compared to PCR). The test showed complete specificity, with no false-positive results. The developed prototype of a rapid immunochromatographic test enables the detection of *B. canis* antigens in canine blood and may significantly improve clinical diagnosis of canine babesiosis. The test is rapid, simple, cost-effective, and suitable for routine veterinary practice.

Keywords: *Babesia canis*, canine babesiosis, rapid immunochromatographic test, monoclonal antibodies, veterinary diagnostics

Canine babesiosis is a common and clinically significant tick-borne disease caused by hematozoan parasites of the genus *Babesia* (5). *Babesia* spp. are classified in the order Piroplasmida in the phylum Apicomplexa. Early studies identified two distinct morphological forms of the erythrocytic stage in the canine host. The larger form, measuring approximately 3-5 µm, was named *Babesia canis*, while the smaller form measuring 1-3 µm, was designated as *Babesia gibsoni*.

B. canis is the protozoan species prevailing in dogs in Poland (5). Clinically, these parasites cause remittent fever, progressive anemia, hemoglobinuria, and marked splenomegaly and hepatomegaly in dogs and, in some cases, the death of infected animals.

Although demonstrating parasites in the blood collected from a carrier animal by blood smear examination is a method of diagnosing canine babesiosis, this test has low sensitivity. Molecular methods, such as polymerase chain reaction (PCR), offer higher sensitivity and specificity for detecting babesial infection in peripheral blood than peripheral blood smear evaluation (14). Furthermore, the PCR may differentiate species that cannot be morphologically distinguished by the smear method. However, this method is quite expensive and can sometimes produce false negative results, especially when the parasite infestation is accompanied by severe hemolysis (7).

The aim of this study was to develop a rapid and sensitive method for diagnosing canine babesiosis caused by *B. canis* infections, based on the detection of protozoan antigens in dog blood using specific antibodies obtained from various research systems.

Material and methods

To develop rapid tests, it was necessary to obtain the *B. canis* antigen, which would subsequently enable the production of monoclonal antibodies for use in diagnostic tests. The test technology was developed based on monoclonal antibodies produced using hybridoma technology (16). During the research, mouse B lymphocytes were immunized with *B. canis* antigens (SPA, BcMSA, and Bc28.1), which, after fusion with myeloma cells, produced stable hybridoma lines that produced specific antibodies. These antibodies were purified by affinity chromatography and then conjugated with fluorescent dyes or gold nanoparticles, enabling their use in a simple, easy-to-read strip test (10, 20).

Antigens used in the study. SPA (soluble parasitic antigen) was obtained from a cultivation of *B. canis* on canine erythrocytes according to the procedure described by Adaszek and Winiarczyk (4) and Adaszek et al. (2). The antigens BcMSA and Bc28.1 originated from a commercial source (RecomBioTech).

Purification and culture of murine splenic B lymphocytes. Spleens were collected from 8-12-week-old mice, and B cells were isolated using the EasySep™ Mouse B Cell Isolation Kit (StemCell Technologies). Spleens were disrupted in PBS/HBSS buffer (without Ca^{2+}/Mg^{2+}) supplemented with 2% FBS and 1 mM EDTA. The resulting suspension was filtered through a 70 µm nylon strainer to remove debris. Cells were centrifuged (300 × g, 10 min), resuspended at 1×10^8 cells/mL, and incubated with Rat Serum (50 µL/mL). The sample was transferred to a 5 mL polystyrene tube, and Isolation Cocktail (50 µL/mL) was added. The mixture was then incubated for 10 min at room temperature. RapidSpheres™ were vortexed and added (75 µL/mL), followed by a 2.5 min incubation. The sample was topped up to 2.5 mL with a buffer and mixed gently. The tube was placed in the EasySep™ magnet for 2.5 min. The enriched B cell suspension was collected by inverting the magnet and transferring the supernatant to a new tube for immediate use.

Purified B cells were cultured in 24-well plates at a density of 10^6 cells/well, using 1 mL RPMI 1640 Medium (ATCC modification, Gibco). This complete medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and 50 µM 2-mercaptoethanol (Merck) and maintained at 37°C in a humidified incubator with 5% CO_2.

Immunization *in vitro*. Naïve B lymphocytes were stimulated with a 10 µg/mL of an antigen model. Various combinations of stimuli were added to the culture, including 2 µg/mL rat anti-mouse CD40 antibody, 2 ng/mL interleukin-4 (IL-4) and/or 1 µg/mL lipopolysaccharide (LPS): see Table 1. The control cultures remained untreated, and the initial stimulation was conducted over five days, followed by a second stimulation period of five additional days.

Tab. 1. *In vitro* immunization conditions

No.	Antigen	IL-2	IL-4	CD40	LPS
1	BcMSA1	✓	✓	✓	✓
2	Bc28.1	✓	✓	✓	✓
3	SPA	✓	✓	✓	✓
4	BcMSA1	✓		✓	✓
5	Bc28.1	✓		✓	✓
6	SPA	✓		✓	✓

Explanations: Naïve B lymphocytes were stimulated with 10 µg/mL of a model antigen (BcMSA, Bc28.1, or SPA). Various combinations of stimuli were added to the cultures, including 2 µg/mL rat anti-mouse CD40 antibody, 2 ng/mL interleukin-4 (IL-4), and/or 1 µg/mL lipopolysaccharide (LPS), as indicated. Control cultures remained untreated. Stimulation was carried out for five days, followed by another five-day stimulation period. ✓ indicates the presence of a specific antigen or stimulus in each condition

Detection of antigen-specific antibodies (ELISA assay) BcMSA and Bc28.1. Reagents were brought to room temperature (18-25°C) before use. A 96-well protein binding plate (Genoplast) was coated with 50 µL/well of 5 µg/mL antigen BcMSA1 (RecomBioTech) or Bc28.1 (RecomBioTech) in PBS and incubated overnight at 4°C. Plates were washed three times with tap water, then blocked with 100 µL/well of PBS containing 1% BSA (Sigma Aldrich) and 0.1% cold water fish skin gelatin (Sigma-Aldrich) for 60 minutes at room temperature. After another washing step, 50 µL of undiluted culture supernatants were added to each well and incubated for 60 minutes. A secondary antibody solution was prepared using goat anti-mouse IgG-HRP (Cell Signaling) at a 1 : 1000 dilution in 0.1% gelatin-PBS. After three additional washes, 50 µL/well of the HRP-conjugated secondary antibody (anti-mouse IgG or IgM) was added (Cell Signaling) and incubated for 45 minutes at room temperature. A fresh substrate solution containing 0.12 mg/mL tetramethylbenzidine (TMB) was prepared. The plate was then washed 10 times with tap water. A substrate solution (50 µL/well) was added and incubated for 15 minutes in the dark, with timing initiated upon filling the first well. The reaction was stopped by adding 50 µL of 1 M sulfuric acid per well. Finally, absorbance was measured at 450 nm with a reference wavelength of 620 nm (13, 16).

Commercial Babesia-ELISA Dog for the detection of SPA-specific antibodies. The test was performed using the Afosa Babesia Dog ELISA Kit (Afosa GmbH, Germany) according to the manufacturer's instructions. Briefly, 96-well plates pre-coated with recombinant *B. canis* antigen were incubated with dog serum samples, as well as positive and negative controls, for 60 minutes at room temperature. After washing, HRP-conjugated anti-dog IgG antibody was added and incubated for 30 minutes. Plates were washed again, and tetramethylbenzidine (TMB) substrate was added for 15 minutes in the dark. The reaction was stopped by adding a sulfuric acid stop solution. Absorbance was measured at 450 nm with a reference wavelength of 620-650 nm using a microplate reader. Results were interpreted based on the comparison with the provided controls. All reagents were brought to room temperature before use, and washing steps were performed as specified by the kit protocol.

Cell fusion of murine B lymphocytes and HAT selection. SP2/0-Ag14 myeloma cells were used as the fusion partner. The cells were thawed in a 37°C water bath and transferred into 10 mL of complete culture medium consisting of RPMI 1640 Medium ATCC modification (Gibco) supplemented with 10% fetal bovine serum (FBS) from Sigma Aldrich, 50 µM 2-mercaptoethanol (Merck) and 1% Penicillin–Streptomycin Solution Hybri-Max™ (Gibco). Cells were centrifuged at 200 × g for 10 minutes, resuspended in fresh medium, and then seeded into a 25 cm^2 flask. Cultures were maintained at 37°C and 5% CO_2 and expanded to two 75 cm^2 flasks prior to fusion. Glutamine was replaced weekly.

Fusion was carried out by electroporation. A 1 g/mL PEG8000 (Sigma Aldrich) solution was prepared in HBSS buffer. HAT selection medium was prepared by supplementing complete RPMI 1640 with 100 µM hypoxanthine, 5.8 µM azaserine, and 16 µM thymidine. Splenocytes and myeloma cells were mixed at a 3 : 1 ratio in BSS buffer (125 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2.5 mM $MgCl_2$, 5 mM Tris-HCl, pH 7.4) to a final volume of 1 mL. A 200 µL aliquot of this suspension was combined with 200 µL of PEG8000 and transferred into a 2 mm gap electroporation cuvette. Electrofusion was performed using a single pulse at 600-650 V for 25 ms. Following a 3-minute incubation at room temperature, the cells were transferred dropwise into 100 mL of HAT medium in an Erlenmeyer flask. This fusion procedure was repeated four times to utilize the entire 1 mL cell mixture. After three hours of incubation at 37°C and 5% CO_2, the cells were supplemented again with HAT and then plated at 100 µL/well onto pre-seeded feeder layers in 96-well plates. The final concentrations of HAT in the wells were 100 µM hypoxanthine, 5.8 µM azaserine, and 16 µM thymidine. Plates were incubated at 37°C and 5% CO_2 for hybridoma selection. The cells were analyzed under the microscope after 7 days post-fusion (16).

Single clones isolation, propagation, and analysis. After selection, in each well of a 96-well plate, the medium was replaced with freshly prepared cell culture medium RPMI 1640 (ATCC modification, Gibco) containing 10% FBS, 50 µM 2-mercaptoethanol (Merck), and 1% Penicillin–Streptomycin Solution Hybri-Max™ (Gibco). The cells were cultured for an additional seven days. The microscopic observation of each well was performed, and the supernatant was collected for an ELISA to verify whether the production of the antibody of interest occurred in the fused hybridoma cell combinations. The ELISA procedure was carried out according to previously described protocols. Clones of hybridoma cells that produced specific antibodies were selected and expanded to 24-well plates. After reconfirmation of antibody production, the selected positive clones were expanded to T25, T75, and T150 flasks as follows. The cells were cultured in the medium mentioned above.

Antibody concentration and purification. The medium from the cell culture of each clone was collected into Falcon-type vials and centrifuged twice: first at 100 × g to separate the medium from the cells and at maximum speed to clear out cell debris. The medium was filtered using a 0.2 µm syringe filter. The filtered medium was concentrated using a Pierce Protein Concentrator with a 30 MWCO (Thermo Fisher) to concentrate the antibody solution. The concentrated antibody was purified using Mini Proteus Protein G (BIO-RAD) according to the manufacturer's protocol. The resulting concentrated and purified antibody samples were analyzed again by ELISA (using previously described protocols) and SDS-PAGE under both non-denaturing and denaturing conditions.

Dot-Blot analysis. The total protein concentration of each obtained antibody sample was determined using a Pierce™Rapid Gold BCA Protein Assay Kit (Thermo Fisher) according to the manufacturer's instructions. An equal amount of total protein (0.82 µg (2 ×) and 0.41 µg (1 ×)) from each sample was loaded onto a PVDF activated membrane (BIO-RAD) to measure the efficacy of the produced antigen-specific antibodies. The samples were incubated for 30 minutes to allow binding. After that, the membrane was blocked for 1 hour with a 3% skim milk powder (Sigma Aldrich) solution in TBST (Tris-Buffered Saline with Tween-20) buffer. The membrane was washed with TBST and incubated for 1 hour with secondary anti-mouse antibody conjugated to a detectable substrate (HRP-conjugated) (cat. ab6728, Abcam), and diluted 1 : 2000 with a 3% Skim Milk Powder solution in TBST buffer. Subsequently, the membrane was visualized by chemiluminescence using Westar Supernova (Cyanagen) with the Azure c400 imaging system from Azure Biosystems, Dublin.

Antibody sequencing and cloning. Total RNA was extracted from hybridoma cells using an RNA Isolation Kit, following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from total RNA using isotype-specific antisense primers or universal primers and SMARTScribe™ Reverse Transcriptase, according to the manufacturer's protocol. Variable regions of the heavy (VH) and light (VL) chains were amplified using the rapid amplification of cDNA ends (RACE) method. The amplified antibody fragments were individually cloned into a standard cloning vector. Colony PCR was performed to identify clones containing inserts of the expected size. For each fragment, at least five positive clones were sequenced. The resulting sequences were aligned, and consensus sequences were determined from the aligned clones. The expression plasmids were then constructed using the Gibson assembly protocol. Plasmids were sequence-verified and introduced into CHO-K1 (RRID:CVCL_0214) cells (ATCC Company, USA) using Lipofectamine 3000. Antibodies were purified using a Protein G column, and their concentration was determined using a BCA assay (Thermo Fisher) according to the manufacturer's instructions.

Lateral-flow assay preparation. The lateral flow test strip was assembled from three main components: a cellulose membrane (Merck) serving as the sample receiving material, a glass fiber membrane (Glass Fiber Diagnostic Pad 10 mm × 300 mm, GFDX103000, Merck) acting as the conjugate pad, and a Hi-Flow™ Plus membrane (Merck) as the detection membrane.

The anti-BcMSA1 antibody was conjugated either with gold nanoparticles using the Gold Conjugation Kit (Abcam) or with the green fluorescent dye CF488A (Merck). The resulting conjugate was diluted to a final concentration of 0.2 µg/mL using a 2 mM borate buffer containing 10% sucrose. The conjugate solution was applied to the glass fiber membrane, which had been pre-treated by soaking in a buffer composed of commercial DPBS (pH 7.4) supplemented with 2.5% sucrose, 2% BSA, and 1% Tween 20. The treated membrane was air-dried at room temperature and stored in a desiccator until use.

Subsequently, the test line and control line were dispensed onto the Hi-Flow™ Plus 120 membrane and allowed to dry at room temperature. Finally, the sample pad, conjugate pad, Hi-Flow™ Plus membrane, and absorbent pad were assembled into a complete strip, cut into 5 mm-wide segments, and placed into lateral flow assay cassettes (Drummond Scientific Company) (10, 20).

Clinical evaluation of the test. The clinical evaluation of the test was performed by examining the antibody-antigen reaction on the surface of the strips. The antigen source was blood obtained from 100 dogs infected with *B. canis* protozoa, in which the infection was confirmed by PCR (5) and purified recombinant BcMSA1 antigen (100 samples).

At the same time, to exclude false-positive results, the tests were also performed using 100 blood samples from healthy dogs that were not infected with protozoa.

The previously diluted blood and antigen suspension were applied to strips in a volume of 50 µL. The results were read after 15 minutes. The test result was considered positive if a test line (T) appeared, or both a test line (T) and a control line (C) were visible on the test strip in a competitive test (LFIA).

Results and discussion

An *in vitro* immunization protocol (16) was used to generate hybridomas as part of the study. The initial objective was to determine the most effective composition of the immunization mixture. For each antigen tested, three stimulation conditions were applied: (i) antigen alone, (ii) antigen with CD40L, LPS, and IL-4, and (iii) antigen with CD40L, IL-2. The antigens used included recombinant BcMSA1 and Bc28.1 proteins (RecomBioTech), as well as soluble parasite antigen (SPA) isolated from *Babesia canis*-infected erythrocyte cultures.

All stimulated cultures exhibited proliferation of murine B lymphocytes, indicating effective activation of these cells. Early-stage ELISA screening confirmed immunization efficacy, with the highest optical density (OD) values observed in wells stimulated with BcMSA1 and SPA in combination with CD40L, IL-2, and IL-4 (Fig. 1 A-C).

Following confirmation of successful immunization, SP2/0-Ag14 myeloma cells were fused with the activated B lymphocytes. After the first round of HAT selection, the most prolific outgrowth was observed

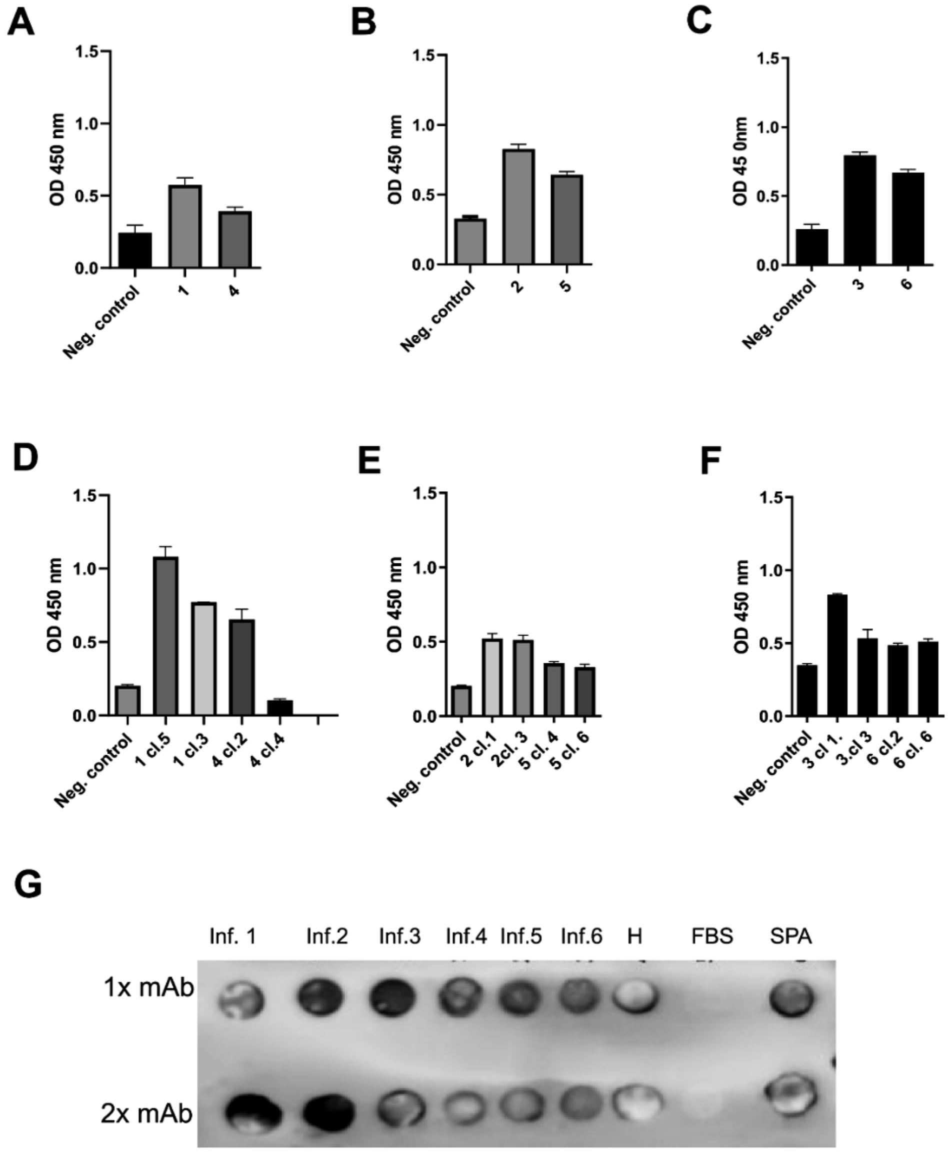

Fig. 1. Representative results of analyses performed before clonal selection (A-C) and after clonal selection using selected clones (D-F)

Explanations: Antibody specificity following immunization with BcMSA1 and Bc28.1 antigens was assessed using in-house assays developed in the laboratory (A, B, D, E). Antibody specificity following immunization with SPA was evaluated using a commercial assay (C, F). Panel G shows Dot-Blot results, where Inf. denotes a dog infected with *B. canis*, H indicates a healthy control dog, and FBS refers to fetal bovine serum used as an additional control in the fusion culture immunized with BcMSA1. Subsequent ELISA assays confirmed antibody production in cultures derived from BcMSA1, Bc28.1, and SPA-stimulated lymphocytes.

Clonal selection of hybridomas yielded several unique clones. Based on preliminary screening, three candidates were selected for further optimization: clone 1 cl. 5 (BcMSA1-specific), clone 2 cl. 1 (Bc28.1-specific), and clone 3 cl. 4 (SPA-specific).

To evaluate the productivity and antigen specificity of these clones, multiple assays were conducted. These included custom-designed ELISA assays for the respective recombinant proteins (Fig. 1 A-B and D-E), and a commercial diagnostic test to validate antibodies derived from SPA-immunized cells (Fig. 1 C and F). In the next step, Dot-Blot analysis was prepared using antibodies purified from the 1 cl. 5 culture medium. Six blood samples from *B. canis*-infected dogs and one from a healthy control dog were used for this test (Fig. 1 G).

The results obtained from the Dot-Blot analysis were consistent with expectations, confirming the reactivity of the purified antibodies with *B. canis*-positive samples and the absence of signal in the healthy control.

ELISA assays confirmed the presence of antibodies in the culture supernatants of all selected hybridoma clones. Based on these results, antibody concentration and purification were attempted using protein A/G affinity chromatography. Preliminary post-purification analyses, including protein concentration measurements, confirmed the presence of immunoglobulins in the eluates. However, the eluate contained approximately 0.15 mg/mL of protein, significantly below the expected yield, indicating possible issues related to immunoglobulin aggregation during the purification process.

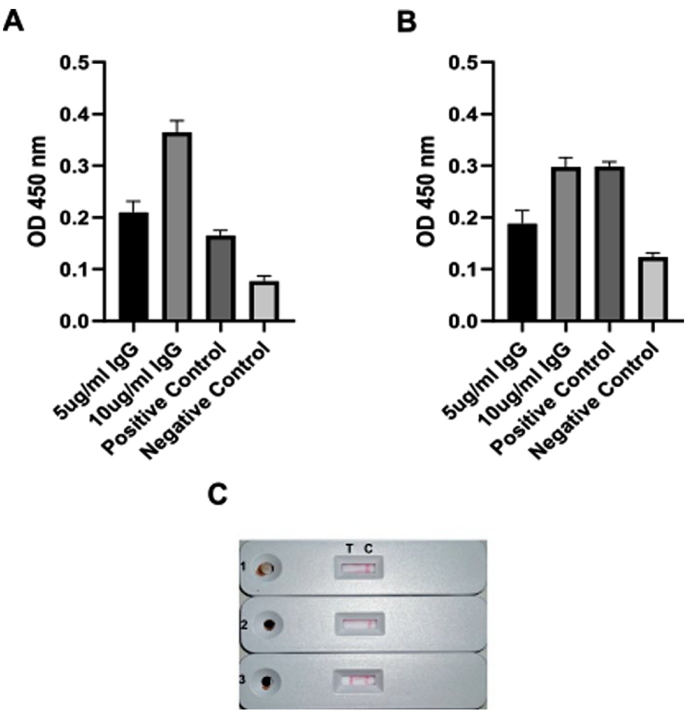

Fig. 2. ELISA test results – commercial assay (A) and in-house BcMSA (B) after antibody purification following expression in CHO-K1 cells

Explanations: Representative competitive lateral flow assay tests (C): 1-2 – negative, 3 – positive, C = control line, T = test line

Since the issue of antibody aggregation persisted despite adjustments to buffer parameters (pH and composition) as well as modifications to the purification process (including flow rate and pressure optimization), a decision was made to switch the production system to expression in CHO-K1 cells, which are widely employed in therapeutic and diagnostic antibody production (19, 22). Clone 1 cl. 5 was identified as the most promising candidate and was therefore selected for further development. The hypervariable regions (VH and VL) were amplified and cloned into expression vectors encoding the murine IgG constant regions. Following successful expression and purification of the recombinant antibodies, their antigen specificity was confirmed via ELISA (Fig. 2 A-B). Subsequently, the antibodies were evaluated in a lateral flow assay format. Initial testing using purified recombinant BcMSA1 antigen yielded an accuracy of 90%, while subsequent testing using blood samples from infected dogs resulted in an accuracy of 70% (compared with PCR) (Fig. 2 C). None of the blood samples from healthy dogs induced a positive reaction in the test, indicating that they do not produce false positive results.

The development of rapid tests for canine babesiosis is a breakthrough in the diagnosis of this disease. In clinical veterinary practice, the most commonly used method to confirm the diagnosis of canine piroplasmosis is the microscopic detection of *Babesia* in blood smear, as this technique is easy to perform and faster than laboratory sample processing. The effectiveness of the blood smear technique depends on the microscopist's experience, and it requires adequate levels of parasites in the blood, which is often sufficient in acute disease but less so in chronic babesiosis, where a high probability of false-negative results exists. *Babesia* parasites can be easily confused with artifacts, leading to false positives. Therefore, laboratory analysis using PCR is more convenient for detecting *B. canis* (3).

Molecular methods are used to detect the nucleic acids of parasites. A great advantage of using these methods is their high sensitivity and specificity (17). The polymerase chain reaction (PCR) has proven to be the most effective molecular method for *B. canis* diagnostics, and it is considered the most accurate in diagnosing babesiosis caused by *B. canis*. The PCR technique was first used to diagnose *B. bovis*, *B. bigemina*, and *B. microti* in 1992 (17), and it was later developed for the diagnosis of canine babesiosis in the early 2000s. It is characterized by high sensitivity in detecting specific parasitic DNA (0.000002% in a 2.5 µL blood sample). This technique can detect parasitemia much earlier than IFAT or microscopic examination of the blood smear, even in cases in which the IFAT examination was negative (8, 12, 15).

Immunological techniques (IFAT, ELISA) detect antibodies to *B. canis*. They are considered effective and reliable, with high sensitivity but moderate specificity due to cross-reactivity with *Babesia* spp. antigens and intact erythrocytes (1, 23). However, the disadvantage is that they rely on the presence of antibodies, which may develop over several days to weeks or may disappear completely after several months. Based on these facts, the effect of immunoassays in the case of acute infection or treated animals is quite limited and questionable. They are used especially in cases where the level of parasitemia is very low or not high enough to be detected by molecular methods (12, 17).

To date, no rapid immunochromatographic tests have been developed for *B. canis*. Such kits are available on the veterinary market for *B. gibsoni*. However, unlike the prototype test described in this article, the commercially available diagnostic kits for small *Babesia* detect antibodies to specific antigens in a small amount of serum (21). In recent years, several such immunochromatographic tests for *B. gibsoni* have been developed, using recombinant P50, BgSA1, or thrombospondin-related adhesive protein (9, 11). However, all of these methods rely on detecting antibodies to piroplasms rather than their antigens, which limits their usefulness in diagnosing the acute form of the disease.

The development of rapid diagnostic tests for detecting *B. canis* antigen in the blood of infected dogs represents a breakthrough in the clinical diagnosis of this disease. These tests are quite convenient diagnostic tools because they are rapid, easy to perform and can be conducted in any veterinary clinic. Additionally, the cost of the test is lower than that of other techniques, and results are available within 15 minutes. The tests utilize SPA, BcMSA, and Bc28.1 antigens with confirmed immunogenicity that were used in prototype vaccines against *Babesia canis* for dogs (2, 6, 18) to generate antibodies against the protozoa, which are then impregnated onto the test strips. Currently, the authors are striving to increase the sensitivity of the tests, which is already high at 70% for clinical samples.

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