NOTE *Parasitology*

Detection of *Babesia gibsoni* by Reaction of Phage Display Single Chain Antibodies with P50 Proteins

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ABSTRACT. *Babesia gibsoni* (*B. gibsoni*) is a tick-borne hemoprotozoan parasite, which causes piroplasmosis in dogs. Diagnosis of canine babesiosis is commonly carried out using Giemsa-stained thin blood smears. However, at low levels of infection, it is difficult to detect *Babesia* organisms by observation of Giemsa-stained thin blood smears. We constructed a monoclonal phage display single chain antibody (scFv) against a *B. gibsoni* merozoite antigen, P50 protein. Intraerythrocytic *B. gibsoni* organisms are clearly stained using this antibody. The monoclonal scFv facilitated the detection of *B. gibsoni* organisms in canine blood samples.
KEY WORDS: antibodies, *Babesia*, phage display.

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Babesia gibsoni (*B. gibsoni*) is a hemoprotozoan parasite carried by ticks. The parasite causes piroplasmosis in dogs. This disease is characterized by remittent fever, progressive anemia, hemoglobinuria, splenomegaly and hepatomegaly, and sometimes causes death. These clinical signs are similar to those caused by autoimmune hemolytic anemia (AIHA). Therefore, the ability to differentiate between babesiosis and AIHA is very important to ensure adequate initial treatment. However, because of similarities among the symptoms of these diseases, acute infection with *B. gibsoni* may be misdiagnosed as AIHA [1]. For example, dogs infected with *B. gibsoni* frequently test positive in the Coombs test, an indicator of AIHA [3]. Given this, clinicians must confirm the presence of *Babesia* organisms within the erythrocytes to correctly diagnose babesiosis. Traditionally, babesia is detected using a Giemsa-stained thin blood smear film. However, at low levels of infection, intraerythrocytic babesia organisms are difficult to detect using this method. To address this issue, immunostaining may be used to provide high contrast images of babesia within the erythrocytes.

We constructed a phage display single chain Fv antibody (scFv) that reacts with the P50 protein of *B. gibsoni*. The P50 protein, a major surface protein of *B. gibsoni* merozoite, is an immunodominant antigen with a molecular mass of 50 kDa [4]. The scFv antibody stains *B. gibsoni* merozoites present in canine erythrocytes. All sera samples, collected from dogs in China and the Japanese islands of Okinawa and Honshu that were infected with *B. gibsoni*, reacted with the same recombinant P50 protein [8]. Thus, the immunogenic diversity of P50 protein is likely to be low. Therefore, antibodies that are specific for P50 have a great possibility as a tool to detect *B. gibsoni* organisms in broad geographic areas. Selection of monoclonal scFvs specific for the P50 protein was conducted by biopanning, using the Human Single Fold scFv library I [2] (kindly provided by Dr Winter of the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering, Cambridge, UK) and a truncated P50 protein (P50t), expressed by *E. coli* [8]. Immunotubes (Nunc, Denmark) were coated with P50t, washed with PBS, and blocked with 2% skim milk (MPBS). We also blocked ~2.5×10^{11} to 2.5×10^{12} CFU of phages from the Human Single Fold scFv library I with MPBS and added these to the mixture. The immunotube was incubated for 2 hr then washed 20 times with PBS containing 0.1% Tween 20. The bound phages were eluted with 500 μl of elution buffer (1 mg/m*l* trypsin in PBS). *E. coli* TG1 was infected with the eluted phage during the log phase and plated onto a TYE agar plate (15 mg Bacto-Agar, 8 mg NaCl, 10 mg Tryptone, 5 mg Yeast Extract and in 1 m*l*) containing 100 μg/m*l* ampicillin and 1% glucose. The plate was then incubated overnight at 37°C. Following this, the *E. coli* were collected and stored in 15% glycerol stock at –80°C.

The phages for the second round of biopanning were obtained from the *E. coli* stock solution. The stock *E. coli* mixture was first cultured in 2x TY (16 mg Tryptone, 10 mg Yeast Extract and 5 mg NaCl in 1 m*l*) medium until the mixture reached an OD600 of 0.4. We then added 5×10^5 PFU KM13 helper phages to a 10 m*l* aliquot of the *E. coli* culture. The culture was incubated for an additional 30 min. The infected *E. coli* cells were then collected and cultured in 2x TY medium containing 100 μg/m*l* ampicillin, 50 μg/m*l* kanamycin, and 0.1% glucose at 30°C for 16 hr. Following incubation, 40 m*l* of the supernatant was mixed with 10 m*l*

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of PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) and incubated on ice for 60 min. After the incubation, the mixture was centrifuged at 3,300 g for 30 min and the precipitated phages were resuspended in 2 m*l* of PBS. These phages were used for the subsequent biopanning procedures. The biopanning was repeated four times, in total.

We used an ELISA to screen the phage binding capacity following each biopanning procedure. Briefly, a 96 well EIA/RIA plate (Corning, U.S.A.) was coated with 9 μg/well of P50t, washed with PBS, and blocked with 2% MPBS. The control wells were coated with 9 μg/well of bovine serum albumin (BSA). The control wells were washed and blocked as described above. The phages obtained during each biopanning procedure were diluted 5-fold with MPBS. One hundred μ*l* of the dilute phage preparation was added to the P50t-coated and control wells. The plate was then incubated for 40 min at 37°C and washed 3 times with PBS containing 0.1% Tween 20. The bound phages were detected using a secondary antibody, HRP/anti-M13 monoclonal conjugate (Amersham bioscience, UK) and colorimetric substrate, ortho-phenylenediamine. The scFvs antibody first reacted with P50t following the third round of biopanning (Fig. 1A). Reactivity increased following the fourth round of biopanning, suggesting that scFvs was successfully enriched by this procedure.

To confirm enrichment, we compared the scFv gene sequences present in the phages following biopanning. *E. coli* TG-1 infected with phages obtained after the first or the fourth biopannings were spread on LB agar and 10 clones each were picked from each repetition. We determined the VH and VL gene sequences in the 20 clones using the following primers: 5'-CGA CCC GCC ACC GCC GCT G-3', 5'- CTA TGC GGC CCC ATT CA -3'. All 10 clones isolated during the first biopanning procedure had nonidentical scFv gene sequences (data not shown). However, 5 of 10 clones isolated after the fourth biopanning procedure had identical DNA sequences. One of the five identical clones (clone 11) was used during the following experiments. The putative amino acid sequence of scFv clone 11 is shown in Fig. 1B. Given that the Human Single Fold scFv library I is 1.47×10^8 clones in size [2], the low level of diversity among the scFv genes following the fourth biopanning procedure suggests that enrichment of P50t specific scFvs was successful.

Clone 11 scFv was produced in a soluble form by infecting *E. coli* HB2151 with the clone 11 phage. The infected

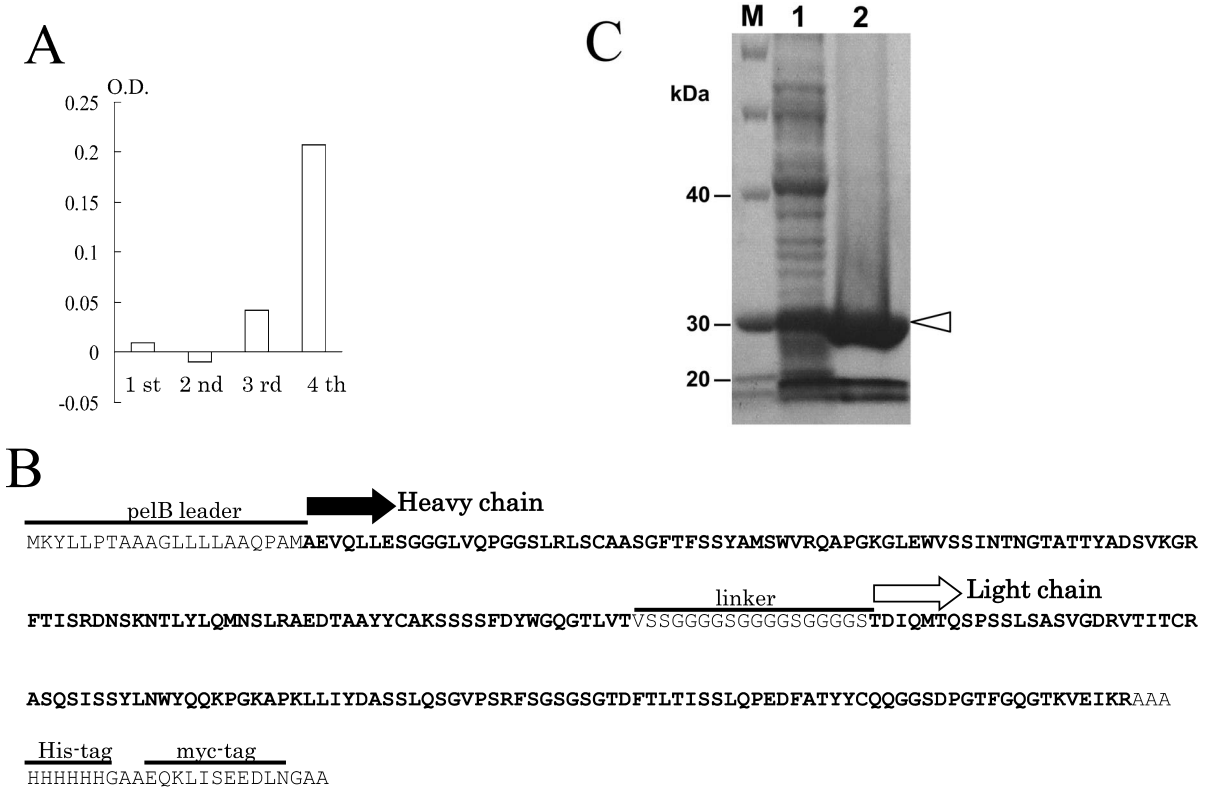

Fig. 1. Properties of the scFv clone 11. (A) The reactivity of the selected phages obtained following the first, second, third, and fourth rounds of biopanning was determined by ELISA. The differences in the OD450 value of the P50t coated and BSA coated control wells are shown. (B) Putative amino acid sequence of scFv clone 11. Bold type indicates the heavy and light chains of the scFv clone. Small type indicates the pIT2 phagemid vector derived region. (C) Purification of the soluble form scFv clone 11. Extraction of *E. coli* HB2151 infected with the clone 11 phage (left lane) and purified soluble form scFv clone 11 using His-Bind Quick Column (right lane) was analyzed by SDS-PAGE.

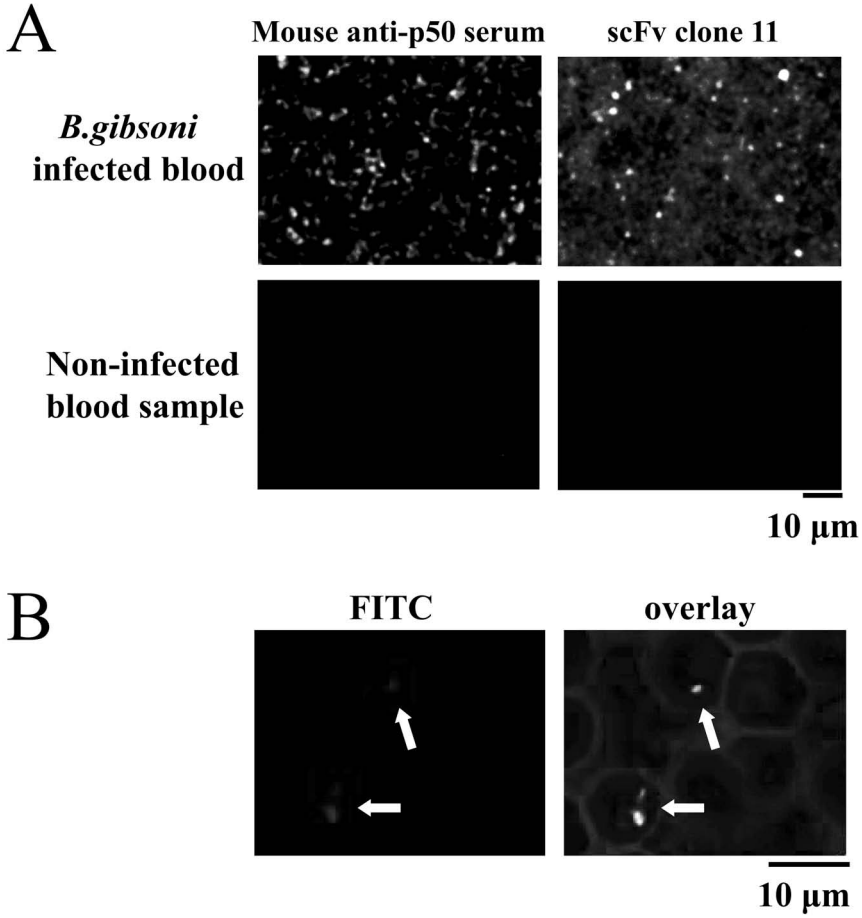

Fig. 2. Reactivity of scFv clone 11 to *B. gibsoni* merozoites. (A) Thick blood films, collected from a *B. gibsoni* infected dog (upper panels) and an un-infected dog (lower panels), were stained with anti-p50 mouse serum (left panels) or the scFv clone 11(right panels). (B) Thin blood film collected from a *B. gibsoni* infected dog and stained with the scFv clone 11. The fluorescent image (left panel) and an overlay of the fluorescent image and transmitted light image (right panel) are shown.

E. coli was cultured in 2x TY medium containing 100 μg/m*l* ampicillin and 0.1% glucose at 37°C until the OD600 reached 0.9. We then added isopropyl-β-D-thiogalactoside to the culture (final concentration of 1 mM) and incubated the culture at 30°C for 16 hr. Following this, the *E. coli* were collected by centrifugation and the bacterial protein was eluted using B-PER Reagent (Takara Bio., Japan), according to the manufacturers instructions. We observed an ~30 kDa band with the putative molecular weight of scFv (Fig. 1C, left lane). The identity of the 30 kDa band was confirmed as scFv by detection of the His tag, present on the C-terminal of the scFv antibody (Fig. 1B), in the Western blot assay (data not shown). Following extraction, scFv was purified using a His-Bind Quick Column (Novagen, Germany), according to manufacturers instructions (Fig. 1C, right lane). Using an ELISA, we confirmed that the purified soluble form scFv clone 11 reacted with P50t (data not shown).

We evaluated whether the soluble form scFv clone 11 reacted with *B. gibsoni* merozoites. In order to observe as much erythrocytes as possible in one view field, we prepared thick blood films of uninfected dogs and dogs infected with *B. gibsoni* (parasitemia was approximately 10%) and the thick blood films were stained with scFv clone 11 and anti-P50 mouse serum [4], as a positive control. To prepare the thick blood films, 10 μ*l* blood was dropped on each well of 12 well glass slide (Matsunami Glass Ind., Ltd., Osaka, Japan) and dried up. When the thick blood films were observed using microscope (400 ×), huge number of erythrocytes lying over one another could be observed in one view field (data not shown). The blood film was then hemolyzed using distilled water. The purified soluble form scFv and anti-P50 mouse serum were diluted 2- and 50-fold, respectively, with PBS containing 3% BSA. The dilute scFv and antiserum mixtures were added to the hemolyzed blood film and incubated at 37°C for 40 min. The bound

scFv was detected using secondary, anti His antibody (GE Healthcare, UK), and tertiary, FITC-Goat anti mouse IgG (ZYMED, U.S.A.), antibodies. The mouse IgG bound in the antiserum was detected using FITC-Goat anti mouse IgG (secondary antibody). The scFv clone 11 reacted with the infected blood sample but not the uninfected blood sample (Fig. 2A), as did the anti-P50 mouse serum control. Each parasite in these erythrocytes stained as like a small dot (Fig. 2 A, upper panels). The fact that the scFv clone 11 did not react with uninfected erythrocytes (Fig. 2A lower panels) indicates that the scFv clone 11 reacts with *B. gibsoni* merozoites but does not react with canine erythrocytes. Next, to observe parasites stained with the scFv clone 11 in a thin blood film (blood smear), which is used in clinical scene to detect babesia organisms, a blood sample was obtained from a dog infected with *B. gibsoni* (parasitemia was approximately 2%). The thin blood film was fixed and permealized in methanol for 10 min and reacted with the scFv clone 11, anti His antibody, and FITC-Goat anti mouse IgG, as described above. In the case of the observation of a thin blood film, comma-shaped structures, typical form of *B. gibsoni* merozoites, were clearly stained (Fig. 2B).

A recent study demonstrated that scFv was able to fuse with antiprotozoal peptides, forming a therapeutic immunotoxin, to inhibit development of the malaria parasite [7]. Thus, the scFv clone 11 may also used to develop therapeutic immunotoxins that eliminate the babesia parasite. In addition, the scFv clone 11 can be used to detect *B. gibsoni* in canine blood samples. Although methods to detect *B. gibsoni* in canine blood samples using PCR was recently developed [5] and commercially provided as a sensitive and specific tool for diagnosis of the disease, it is difficult to quantify the number of parasites using PCR. To evaluate the effectiveness of ongoing treatment, the longitudinal transition of parasitemia is an important indicator. Thus, continuous observation of parasitemia in blood samples is of clinical value, despite the development of PCR based diagnoses. Detection and quantification of intraerythrocytic *B. gibsoni* organisms in clinical samples by staining with *B. gibsoni* specific antibodies might be informative.

Relative to the traditional methods used to produce monoclonal antibodies (e.g. hybridoma), phage display antibody production is considerably faster and less expensive [6]. Thus, there is considerable scope for using the scFv clone 11 to detect *B. gibsoni* organisms in the clinical setting.

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