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Tracking *Neospora caninum* Parasites Using Chimera Monoclonal Antibodies against its Surface Antigen-related Sequences (rNcSRS2)

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Short running title: Tracking Parasites Using Chimera Monoclonal Antibodies

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Neosporosis, an infectious disease of cattle and dogs, causes an abortion in cattle, which has a major damage on the dairy industry worldwide. Tracking of *Neospora caninum* parasite that is responsible for neosporosis is required for the prevention of this infectious disease. We developed a chimera monoclonal antibodies consist of variable regions of murine antibody and constant regions of human antibody against *N. caninum*. Recombinant surface antigen-related sequence 2 (rNcSRS2) of *N. caninum* was expressed in silkworm larvae, and immunized in mice to obtain phage displaying antibody library. Through three rounds of selection, three antibodies, A6, E1 and H3, were isolated and bound to rNcSRS2 with nanomolar to micromolar affinity. In immunofluorescent staining assays, A6 and E1 bound to *N. caninum* strain Nc-Liv, demonstrating a successful tracking of the parasite. H3 clone bound to rNcSRS2 but not to a truncated protein without glycosylphosphatidylinositol (GPI) anchor domain in the carboxyl terminal. Amino acid sequences of A6 and E1 were similar, but that of H3 differed in the CDR-H1 region, which might be the reason of their difference of affinity. These antibodies are thought to be useful for prevention of cattle from neosporosis.

**Keywords:** neosporosis, parasite, antibody, phage display, immunofluorescent staining
Neosporosis is a coccidian disease caused by *Neospora caninum* in a variety of animals such as cattle, horses, deer, dogs, and so on (1), induces an abortion in cattle, which is the reason for the huge economic damage on the dairy industry in many countries (2, 3). The major mode of transfection of *N. caninum* is transplacental parasite transmission that infects in the herd over successive generations (4, 5), but horizontal transmission between cattle was not observed. There are three infectious stages in the life cycle of *N. caninum*: tachyzoite, tissue cysts, and oocysts. Tachyzoites and tissue cysts are the stages found in the intermediate hosts and they occur intracellularly (6); the unsporulated oocysts are found in domestic dogs, which are the only known definitive host for *N. caninum* (7).

Like other coccidian parasites, tachyzoites of *N. caninum* also contain the characteristic organelles such as dense granules, rhoptries, and micronemes. Those organelles produce important proteins that play important roles in infection of host cells (8). On the other hand, proteins located on the surfaces are considered to play very important roles in the infection process. They may induce the interaction with the host cell and subsequently help the parasites adhere to and invade the host cell. Surface antigen 1 (NeSAG1) of *N. caninum* is the immunodominant surface antigen of tachyzoites,
which is involved in the attachment of the parasite to host cells (9, 10). There are also
many surface proteins structurally related to NcSAG1, which are designated
NcSAG1-related sequences (NcSRSes). NcSRS2, with a molecular weight of 37 kDa,
elicit strong antibody response in infected animals and is an attractive candidate for
diagnosis and vaccine antigen (10). In vitro studies have shown that blocking these
proteins can limit the parasite’s ability to attach to and invade host cells (11-13). In
addition, in vivo studies also showed that the recombinant NcSRS2 (rNcSRS2) has
protective effects against encephalitis and transplacental transmission (14).

So far, we have successfully expressed rNcSRS2 in silkworms (15). Because
NcSRS2 was reported to have high antigenicity and plays critical roles in N. caninum
transmission, we immunized mice in this study with purified proteins and developed three
chimera monoclonal antibodies consist of variable regions of murine antibody and
constant regions of human antibody against the parasite by employing phage display
technology. The binding capacity of antibodies to parasites and the potential in practical
applications were subsequently investigated.

MATERIALS AND METHODS

Materials  The Escherichia coli strains XL10-Gold for cloning and amplification
of phagemid, and TG-1 for displaying antibody on M13 phage were purchased from Agilent Technologies (La Jolla, CA, USA). Phagemid pDong1/Fab (16), helper phage KM13 and non-suppressor *E. coli* strain HB2151 were provided kindly by Dr. Hiroshi Ueda of Chemical Resources Laboratory of Tokyo Institute of Technology. The *N. caninum* Nc-Liv strain (No. 50845) and Vero cell (No. CCL-81) were purchased from ATCC (Rockville, MD, USA). Restriction and modification enzymes were purchased from Takara-Bio (Shiga, Japan), Toyobo (Osaka, Japan), Roche Diagnostics (Tokyo, Japan), or New England Biolabs (Tokyo, Japan). Oligonucleotides were synthesized either by Operon (Tokyo, Japan) or Invitrogen (Tokyo, Japan). Other chemicals, reagents, and antibodies, unless otherwise indicated, were obtained from Sigma-Aldrich (St Louis, MO, USA) or Wako Pure Chemical (Osaka, Japan).

**Immunization of mice with rNcSRS2** The strategy for developing monoclonal antibodies is shown in Fig. 1A. Firstly, mice were immunized with rNcSRS2. After the quantitation of peptide-specific antibodies in sera, the variable region genes of the antibody heavy (*V*$_H$) and light (*V*$_L$) chains were prepared and cloned to a phagemid vector to perform phage display selection. The rNcSRS2 was expressed in silkworm according to previous report (15) and purified. Two inbred BALB/c mice (Japan SLC, Inc. Hamamatsu, Shizuoka, Japan) were immunized with purified rNcSRS2 four times at
2-week intervals with a dose of 100 μg through the subcutaneous route. The protein solutions were emulsified with a Freund’s complete adjuvant (Rockland Immunochem, Gilbertsville, PA, USA) to increase the efficiency of immunization. After the last immunization, blood samples were taken by tale bleeding and the rNcSRS2-specific antibodies in sera were confirmed by an Enzyme-Linked ImmunoSorbent Assay (ELISA) with immobilized rNcSRS2 on a microplate as described by Dong et al. (17). The experiments with animals were carried out in the Animal House of Shizuoka University in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Shizuoka University and were approved by the Committee on the Ethics of Animal Experiments of Shizuoka University (Permit Number: 24-11).

**Construction of phage display antibody library**  The total RNA was extracted from spleen cells of immunized mice with TRIzol (Invitrogen, Tokyo, Japan). The genes of V<sub>H</sub> and V<sub>L</sub> of antibodies were amplified using PrimeScript One step RT-PCR kit ver.2.0 (Takara, Shiga, Japan) according to the manufacturer’s protocol. The mouse IgG-specific primers are synthesized based on the common antibody primer sequences (17). The PCR products were then purified using Illustra<sup>TM</sup> GFX<sup>TM</sup> PCR DNA and Gel Band Purification kit (GE Healthcare). The purified V<sub>L</sub> fragments were digested with restriction enzymes SalI and NotI and were purified and ligated into a phagemid.
pDong1/Fab digested with the same enzymes using T4 DNA ligase at 16°C for 1 h. After confirmation of the inserted \( V_L \) sequence of several clones out of the obtained ones, the \( V_H \) genes were inserted into the \( V_L \)-inserted phagemid library using restriction enzymes SfiI and XhoI. Electroporation-competent \( E. coli \) TG-1 cells were transformed with the ligation product and plated on 2×YTAG agar (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH 7.2, supplemented with 100 μg/ml ampicillin, 1% glucose, and 1.5% agar) plates overnight at 37°C. The size of library was estimated from the number of colonies on the plate. \( E. coli \) TG-1 cells, transformed with the phagemid, were cultivated in 4 ml of 2×YTAG overnight at 37°C. Ten milliliters of 2×YTAG were inoculated with 100 μl of the overnight culture at 37°C at 200 rpm until \( \text{OD}_{600} \) reached \( \sim 0.5 \), when helper phage KM13 (18) was added with a multiplicity of infection (MOI) of 20. After incubation at 37°C for 30 min without shaking, the culture was centrifuged at 3700 g for 15 min. The \( E. coli \) pellet was resuspended in 50 ml of 2×YTAK (2×YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin) and incubated overnight with shaking at 30°C. The overnight culture was centrifuged at 10,800 g for 30 min. Ten milliliters of PEG/NaCl solution (20% polyethylene glycol 6000, 2.5 M NaCl) was added to 40 ml of supernatant, and the mixture was incubated on ice for 1 h. After incubation, the mixture was centrifuged at 6,000 g for 30 min. The pellet was resuspended in 2 ml of PBS and
centrifuged at 15,000 g for 10 min to pellet cell debris, and the supernatant was collected as a Fab-displaying phage solution.

**Enrichment and selection of monoclonal antibodies**  For antibody selection, 100 μl of rNcSRS2 (1 μg/ml in PBS) was immobilized on a microplate at 4°C overnight. It was washed three times with 200 μl of PBST, and then blocked with MPBS (PBS containing 2% skim milk) for 2 h, followed by adding $10^{12}$ colony forming unit (cfu) phage in 100 μl PBS and incubated for 1 h at room temperature. Phages bound to the microplate were eluted with 100 μl of 1.0 mg/ml TPCK-treated trypsin (Sigma-Aldrich) in PBS after washing with PBST for six times. *E. coli* TG-1 cells (OD$_{600}$ = 0.5 in 700 μl culture) were infected with 100 μl of eluted phage solution and cultured in 10 ml of 2×YTAG medium at 37°C with shaking at 200 rpm. When OD$_{600}$ reached 0.5, the KM13 helper phage was added at an MOI of 20, and incubated for 30 min at 37°C without shaking. After being centrifuged at 3,700 g for 10 min, the pellet was resuspended in 50 ml of 2×YTAK medium and incubated with vigorous shaking at 30°C overnight. The culture supernatant was prepared by centrifugation at 10,800 g for 30 min, and phages were precipitated with 0.2 volume of PEG/NaCl on ice for 1 h. After centrifugation at 6,000 g for 30 min, the pellet was resuspended in PBS and used as a source of Round 1 (R1) phage. Round 2 (R2) antibody selection from R1 phage was performed as described...
above and R2 phage was obtained. From the R2 phage library, Round 3 (R3) selection was also carried out to generate R3 phage. The enrichment of rNcSRS2-specific phage-antibody among the original phage library (R0), R1, R2, and R3 phages was confirmed with a polyclonal phage ELISA.

After the increase of binding capability of phage was confirmed, 96 infected *E. coli* clones at the 3rd biopanning were picked up and cultivated for making monoclonal phage. A phage ELISA was performed for 96 individual clones to select rNcSRS2-specific phage-antibodies. Nucleotide sequence of positive clones was read by Greiner Bio One (Tokyo, Japan) with primer M13rv (5’-GGAAACAGCTATGACCATG-3’) for VH and primer VLseq (5’-CACTGGCTGGTTTCGCTAC-3’) for VL, and was analyzed using a GENETYX software (Genetyx Corporation, Tokyo, Japan).

**Expression of Fab fragments of positive clones**  
pDong1/Fab was designed for the convenient expression of the Fab fragment after selection of positive clone by placing a TAG amber codon (19) between VH-CH1 and gene III of phage. With this design, Fab is expressed as a fusion protein with protein gIII of phage in suppressor strain like *E. coli* TG1, resulting in the display of Fab on the surface of phage. However, with a non-suppressor strain, such as *E. coli* HB2151, the Fab fragment will be expressed as a soluble fragment (16). Because the genes for CH1 and CL gene in pDong1/Fab system
were from human IgG (CH1 and Cκ), Fabs expressed in this study were murine-human chimera fragments. In brief, 200 µl of exponentially growing *E. coli* HB2151 was infected with 10^9 cfu of phage for 30 min at 37°C. Infected *E. coli* cells were pelleted by centrifuge at 5,000 g for 10 min, resuspended in 4 ml 2×YT medium containing 100 μg/ml of ampicillin (2×YTA), and cultivated for 3 h at 37°C. Four hundred milliliters of 2×YTA medium was inoculated with the 4-ml culture and cultivated at 37°C with shaking. Once the OD₆₀₀ reached 0.5, isopropyl β-D-thiogalactoside (IPTG), with a final concentration of 1 mM, was added and cultivated further overnight at 30°C. The *E. coli* cells were harvested by centrifugation at 4,000 g for 20 min at 4°C. The periplasmic fraction was extracted according to a general protocol. His-tagged Fabs were purified from the periplasmic fraction and concentrated supernatant with TALON Co2+-immobilized resin (Takara-Bio) according to the instructions provided by the manufacturer. Because one-step purification was not enough to achieve purity, Fabs were furthermore purified with an anti-FLAG M2 affinity gel (Sigma-Aldrich) according to the instructions provided by the manufacturer. The purified Fabs were analyzed using SDS-polyacrylamide gel electrophoresis as described by Laemmli (20).

**ELISA analysis** The antigen-binding capacity of phage-displayed Fab fragments was tested with ELISA. The microplates (NUNC, Langenselbold, Germany) were coated
overnight with 100 μl of rNcSRS2 (0.5 μg/ml) per well or 10 μg/ml of BSA in PBS at
4°C. The plate was blocked at 25°C for 2 h with 2% MPBS, washed three times with
PBST, and incubated with 100 μl/well of MPBS containing 10⁹–10¹⁰ cfu of
Fab-displaying phage at 25°C for 1 h. The plate was washed three times with PBST and
incubated with 100 μl/well of 5000-fold diluted HRP/anti-M13 monoclonal conjugate
(from Sheep; GE Healthcare UK limited, Little Chalfont, Buckinghamshire HP7 9NA,
UK) in MPBS at 25°C for 1 h. The plate was then washed three times with PBST and
developed with 100 μl/well of 3,3′,5,5′-tetramethylbenzidine (TMBZ) substrate solution.
After incubation for an appropriate time, the reaction was stopped by adding 50 μl/well of
10% sulfuric acid, and the absorbance was read using a Model 680 microplate reader
(Bio-Rad, Hercules, CA, Japan) at 450 nm with 655 nm as a control.

For evaluation of IC₅₀ of phage Fabs, competitive ELISAs were performed in which
free rNcSRS2 in a series of concentration levels competed with immobilized rNcSRS2 to
bind Fab-phages.

For confirmation of antigen-binding capacity of free Fab fragments, a Rabbit
anti-Human Kappa chain antibody (1 μg/ml; MBL, Tokyo, Japan) and an ECL™
Anti-mouse IgG, Horseradish Peroxidase linked whole antibody (from Sheep; GE
Healthcare UK Limited) were used.
Surface plasmon resonance (SPR) analysis  Binding analysis of three chimera monoclonal antibodies to rNcSRS2 was performed using BIAcore X-100 or 2000 (GE Healthcare Japan, Tokyo, Japan). In brief, purified recombinant NcSRS2 from silkworm larvae (1800-4000 RU) was immobilized on the CM5 sensor chip (GE Healthcare Japan) by amine-coupling method under pH 4.0. Serially diluted chimera monoclonal antibodies, A1, E2 at concentrations of 78, 312, 625 and 1250 nM, H3 at 179, 358, 715, 1430 and 2860 nM, was injected to the sensor chip with HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P-20 (GE Healthcare Japan), pH 7.4) at 30 μl/min, respectively. Ten mM Glycine buffer (pH2.5) was used as a regeneration buffer.

Immunofluorescent staining of parasite with Fab fragments  N. caninum strain

Nc-Liv was used to infect Vero cells. The infected cells were maintained in a CO₂ incubator at 35°C. For immunofluorescent staining, freshly purified parasites were fixed on glass slides coated with amino silane (APS), permeabilized, and blocked with 4% BSA in PBS. Immunolabeling was carried out using 10 μg/ml of purified Fabs. A commercial anti-N. caninum antibody (1 μg/ml; VMRD, Pullman, WA, USA) for positive control was used. A sample without any primary antibody was used as a negative control. After 1-h incubation, a rabbit anti-human kappa chain antibody (1 μg/ml; MBL, Tokyo, Japan) was added to samples on the glass slide, except positive control.
Rhodamine-conjugated goat IgG fraction anti-rabbit IgG (MP Biomedicals, LLC-Cappel Products, Santa Ana, CA, USA) in 200 times dilution and 4',6-diamidino-2-phenylindole (DAPI; 1000 times dilution; DOJINDO, Kumamoto, Japan) were added to all samples, except for positive control after incubation, and the samples were washed with PBS. For positive control, Alexa Fluor 594-conjugated AffiniPure Rabbit Anti-Mouse IgG (H+L) (1 µg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and DAPI were added. All samples were viewed on a confocal microscope (LSM 700, Carl-Zeiss, Oberkochen, Germany), and their images were processed by employing the software ZEN lite 2010 (Carl-Zeiss).

RESULTS

**Immunization of mice** Two inbred BALB/c mice were used for immunization. After the last immunization, blood samples were taken to check the *N. caninum* protein-specific antibodies. Specific binding capacity of sera was observed against rNcSRS2, but not to BSA which was used as negative control, suggesting that the immunization was successful (data not shown).

**Monoclonal antibody selection from phage display library** The display of the
Fab fragment on the surface of phage was achieved by using pDong1/Fab with the help of the KM13 helper phage. For construction of antibody library, the V_H and V_L genes of antibodies were amplified and detected at 350–400 bp in an agarose electrophoresis (data not shown). A phage display antibody library with a diversity of $5 \times 10^6$ was obtained.

After three rounds of selection, the enrichment of rNcSRS2-binding phage was confirmed using an ELISA with original phage library R0 and sublibraries R1, R2, and R3, which were amplified in each step of biopanning. Absorbance at 450 nm in phage ELISA for R0, R1, R2, and R3 phage against rNcSRS2 rose with the increase of the biopanning step (Fig. 1B), suggesting that three rounds of biopanning enriched the rNcSRS2-specific Fab-phages. These phage pools did not bind to BSA as a negative control.

R3 phages were used to infect *E. coli* TG-1 for forming colonies. Ninety-six colonies were picked up and cultivated for forming phage. Four clones A6, E1, H3, and H6 showed a strong signal against immobilized rNcSRS2 (Fig. 2A). The binding capacity of those Fab-phages was also reconfirmed against rNcSRS2 and BSA. A6, E1, and H3 clones bound to rNcSRS2 but not to BSA (Fig. 2B), suggesting their specificity to our target. Although H6 clone bound to rNcSRS2 in the screening, it did not bind to rNcSRS2 upon further confirmation (data not shown). The KM13 helper phage in which no antibody was displayed was used as a negative control and did not bind to immobilized
IC$_{50}$ of A6, E1, and H3 clones  To evaluate the half maximal inhibitory concentration (IC$_{50}$) of those clones, a competitive ELISA with serially diluted rNcSRS2 solutions inhibiting the binding of Fab-phage to immobilized rNcSRS2 was performed. Competition was observed between free and immobilized proteins, and the IC$_{50}$ values of A6, E1, and H3 were evaluated to be 0.54, 0.22, and 0.51 $\mu$g/ml of rNcSRS2, respectively (data not shown).

Purification of Fab antibodies and their binding to rNcSRS2  Fab antibodies were expressed in E. coli HB2151 and purified with TALON Co$^{2+}$-immobilized resin and an anti-FLAG M2 affinity gel. The purified Fab fragments showed two bands on SDS-PAGE with molecular weight of 24 and 26 kDa (Fig. 3A), which were identified as light chain and V$_{H}$-C$_{H}1$ of heavy chain, respectively. The binding of these Fabs to rNcSRS2 and a truncate rNcSRS2 without GPI anchor domain of native protein rNcSRS2 (NSNT) (unpublished data) was confirmed with an ELISA. As shown in Fig. 3B, all the three Fabs bound to the rNcSRS2 and A6, E1 also bound to rNcSRS2 (NSNT). However, the H3 clone did not bind to the truncated protein, suggesting it might bind to the anchor domain of the native protein. All Fabs did not bind to BSA as a negative control.
Antigen binding affinity of monoclonal antibodies  Rate constants of association ($k_a$) and dissociation ($k_d$), and dissociation constant ($K_D$) for each antibody against the immobilized rNcSRS2 were investigated by SPR. Using the binding sensorgrams (Fig. 4), $k_a$, $k_d$, and $K_D$ are summarized in Table 1. The $K_D$ for clone H3 was 70 nM, much lower than A6 and E1 due to its low $k_d$. $K_D$ values of A6 and E1 clones were 420 nM and 5 μM, respectively. The difference in absolute rate constants reflect their different structure, especially that of complementarity determining regions which determine the antibody's avidity and specificity for specific antigens.

Immunofluorescence assay of *N. caninum* with monoclonal antibodies  To confirm whether those clones bind to *N. caninum* parasites, immunofluorescence assays were performed using a commercial anti-*Neospora* antibody as a positive control. As shown in Fig. 5, the parasites’ nuclear was identified with DAPI-staining, showing blue fluorescence under confocal laser microscopy. Staining with the A6 and E1 Fabs, a Rabbit anti-Human Kappa chain IgG and Rhodamine-conjugated Goat IgG fractions anti-Rabbit IgG revealed red fluorescence, suggesting both A6 and E1 Fabs bound to parasites as well as the commercial anti-*N. caninum* antibody did. For the negative control without primary antibody, no fluorescence was observed. In this experiment, H3 clone did not bind to parasites.
Amino acid sequence of positive clones

The amino acid sequences of positive clones were compared and analyzed, and a small difference region was observed between A6 and E1 (Fig. 6). The difference in the N-terminal region may be caused by the primers for amplification of gene, which are thought not to be important for the affinity. However, glycine (G) at position 143 in A6 was replaced with glutamic acid (E) in E1 clone. This position is inside of the first complementarity-determining region (CDR) of heavy chain. Because most critical sequence variation associated with immunoglobulins and T cell receptors are found in the CDRs, these regions are sometimes referred to as hypervariable regions, and play a direct role in antigen binding. Therefore, this difference might be the reason for the difference of affinity. A remarkable difference was observed between the above two clones and H3, which makes them bind to different epitopes of rNcSRS2.

DISCUSSION

Neosporosis is a serious infectious disease of animals; however, so far, no efficient vaccine has been developed. Developments of detection methods or imaging technologies to monitor these kinds of infectious disease are current issues. In this study, we developed three chimera antibodies A6, E1, and H3 against rNcSRS2 with a pDong1/Fab system by phage display technology. The variable regions genes for heavy chain and light chain
were also analyzed. The sequences of these antibodies were identified to be novel. Amino acid sequences of A6 and E1 were similar; however, a change of one amino acid in the CDR-H1 region caused different affinity between A6 and E1. H3 clone has a quite different sequence with the A6 and E1 especially in CDR regions, which might bind to a different epitope of rNcSRS2.

In immunofluorescent staining assays, both A6 and E1 bound to the tachyzoites of the parasite. SRS protein of *Toxoplasma gondii*, sharing similar characteristics with *N. caninum*, has been reported to be involved in the infection of parasite to host cells (21). Therefore, there is a high possibility that these monoclonal antibodies can be used as a blocker of infection. Monoclonal antibodies are now established as a key therapeutic modality to a range of disease. Owing to the ability of these agents to selectively target tumor cells, cancer has been a major focus of development programs for monoclonal antibodies (22). Nowadays, many antibody drugs for humans have been developed and have obtained approval from governments. However, there are few for animal therapy. The antibodies developed in this study also provide a possibility for the development of antibody drugs against neosporosis. Recently, antibody fragments such as Fab and single-chain variable fragment (scFv) are more enthusiastically studied as drugs because they have small molecular weights and bear the advantages of easy detection and tracking.
in vivo, as well as the easy delivery to certain cells through antibody specificity. By adding the Fc region gene and transforming insect cell or mammal cells, the whole length antibodies for some certain applications can also be achieved.

Even though H3 clone bound to rNeSRS2, it did not bind to the truncated protein without an anchor domain. rNeSRS2 was reported to be expressed in insect cell using baculovirus expression system with all the necessary post-translational modifications (21), however, the process of GPI anchor has not been revealed yet. Therefore, this clone may be useful for studying the mechanism of the GPI anchor process.

Nowadays, most detection methods for neosporosis focus on the detection of antibodies in the serum sample of cattle. However, approaches for monitoring the infection of *N. caninum* in dairy farm could be achieved because antibodies developed in this study can directly detect *N. caninum* parasites. This would further protect livestock from infection of *N. caninum* parasite, leading to a development of antibody drugs for *N. caninum* therapy.

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Figure Legends
FIG. 1. Scheme for the development of murine anti-\textit{N. caninum} monocloal antibodies
(A) and the enrichment of rNcSRS2-specific clones (B). ELISA was performed to confirm the enrichment of rNcSRS2-specific clones. In ELISA, rNcSRS2 (0.5 μg/ml) and BSA (10 μg/ml) were immobilized on a 96-well microplate, respectively. HRP/anti-M13 monoclonal antibody conjugate was used as the secondary antibody. R0 stands for the original phage library, whereas R1, R2, and R3 stand for the amplified Fab-phage in Rounds 1, 2, and 3 of biopanning, respectively.

FIG. 2. Screening of monoclonal antibodies (A) and their bindings to rNcSRS2 and BSA. Protein rNcSRS2 (0.5 μg/ml) were immobilized on a 96-well microplate, respectively. HRP/anti-M13 monoclonal antibody conjugate was used as the secondary antibody after the addition of a 96-phage Fab solution (A). rNcSRS2 (0.5 μg/ml) and BSA (10 μg/ml) were immobilized on a 96-well microplate, respectively. HRP/anti-M13 monoclonal antibody conjugate was used as the secondary antibody after addition of phage Fab solution (B). Experimental data were presented as average values with standard error (n=3).

FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of purified Fab antibodies (A) and their bindings to rNcSRS2, rNcSRS2 (NSNT), and BSA (B). For ELISA, rNcSRS2, rNcSRS2 (NSNT) (0.5 μg/ml each), and BSA (10 μg/ml) were immobilized on
a 96-well microplate, respectively. After incubation with Fab antibodies, a Rabbit anti-Human Kappa chain antibody (1 μg/ml) and an ECL™ Anti-mouse IgG, Horseradish Peroxidase linked whole antibody (from sheep) was used as secondary and tertiary antibodies, respectively. M: Precision Plus Protein™ Dual Colors Standards.

FIG. 4. SPR sensorgrams of the purified monoclonal antibodies, A6 (A), E1 (B) and H3 (C), bound to recombinant NcSRS2. Recombinant NcSRS2 was immobilized on the CM5 sensor chip and serially diluted antibodies were injected to the sensor chip.

FIG. 5. Immunofluorescence assay of *N. caninum* with monoclonal antibodies. *N. caninum* strain Nc-Liv was immobilized and blocked followed by the addition of a commercial anti-*N. caninum* antibody (P), Fabs A6, E1, and H3, respectively. A sample without any primary antibody was a negative control (N). For Fab samples and negative control, a Rabbit anti-Human Kappa chain antibody (1 μg/ml) was used as secondary antibody and Rhodamine-conjugated Goat IgG fraction Anti-Rabbit IgG was added finally. For positive control, Alexa fluor 594-labelled anti Mouse IgG was used as a secondary antibody. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclear of parasites. The samples were observed under a confocal laser-scanning microscope. Scale bar stands for 10 μm.
FIG. 6. Amino acid sequences of anti-NcSRS2 antibody variable regions. The amino acid numbers are according to the Kabat numbering scheme. The amino acids of complementarity determining regions (CDRs) are bolded and the different amino acids between A6 and E1 are highlighted.
<table>
<thead>
<tr>
<th>Clone name</th>
<th>$K_a$ (10^4/Ms)</th>
<th>$K_d$ (10^-3/s)</th>
<th>$K_D$ (nM)</th>
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<tr>
<td>A6</td>
<td>5.80</td>
<td>24.0</td>
<td>420</td>
</tr>
<tr>
<td>E1</td>
<td>0.57</td>
<td>29.0</td>
<td>5100</td>
</tr>
<tr>
<td>H3</td>
<td>1.20</td>
<td>0.87</td>
<td>70</td>
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</table>
Dong et al. Figure 1

A

Mouse immunization

\[ \downarrow \]

Spleen cells

\[ \downarrow \]

RT-PCR

\[ \downarrow \]

Antibody gene $V_H$

\[ \downarrow \]

Antibody gene $V_L$

\[ \downarrow \]

Phage Fab Library

\[ \downarrow \]

Biopanning

Monoclonal antibodies

B

Abs 450 - Abs 655

\[ \begin{align*}
& \text{BSA} \\
& \text{rNcSRS2}
\end{align*} \]

Immobilized protein

Legend:

- R0
- R1
- R2
- R3
Dong et al. Figure 4

(A) A6

(B) E1

(C) H3

RU vs. Time (s)

Concentrations:
- 1250 nM
- 625 nM
- 312 nM
- 78 nM
- 2860 nM
- 1430 nM
- 715 nM
- 358 nM
- 179 nM
### Table: Optical Microscopy and Fluorescence Images

<table>
<thead>
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<th>Optical microscopy</th>
<th>DAPI</th>
<th>Alexa fluor 594/Rhodamine</th>
<th>Merge</th>
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