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

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(ABSTRACT)

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

(INTRODUCTION)

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(NcSAG1) 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_H and V_L 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^{TM} GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare). The purified V_L fragments were digested with restriction enzymes *Sal*I and *Not*I 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 *Sfi*I and *Xho*I. 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 OD_{600} reached ~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 V_H and primer VLseq (5'-CACTGGCTGGTTTCGCTAC-3') for $V_{L,}$ 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 V_H-C_H1 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 C_H1 and C_L gene in pDong1/Fab system

were from human IgG (C_H1 and C_κ), 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 OD_{600} 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 10^9–10^{10} 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 IC_{50} 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.

193 **Surface plasmon resonance (SPR) analysis** Binding analysis of three chimera
194 monoclonal antibodies to rNcSRS2 was performed using BIAcore X-100 or 2000 (GE
195 Healthcare Japan, Tokyo, Japan). In brief, purified recombinant NcSRS2 from silkworm
196 larvae (1800-4000 RU) was immobilized on the CM5 sensor chip (GE Healthcare Japan)
197 by amine-coupling method under pH 4.0. Serially diluted chimera monoclonal antibodies,
198 A1, E2 at concentrations of 78, 312, 625 and 1250 nM, H3 at 179, 358, 715, 1430 and
199 2860 nM, was injected to the sensor chip with HBS-EP buffer (10 mM HEPES, 150 mM
200 NaCl, 3 mM EDTA, 0.005% surfactant P-20 (GE Healthcare Japan), pH 7.4) at 30 μl/min,
201 respectively. Ten mM Glycine buffer (pH2.5) was used as a regeneration buffer.

202 **Immunofluorescent staining of parasite with Fab fragments** *N. caninum* strain
203 Nc-Liv was used to infect Vero cells. The infected cells were maintained in a CO_2
204 incubator at 35°C. For immunofluorescent staining, freshly purified parasites were fixed
205 on glass slides coated with amino silane (APS), permeabilized, and blocked with 4%
206 BSA in PBS. Immunolabeling was carried out using 10 μg/ml of purified Fabs. A
207 commercial anti-*N. caninum* antibody (1 μg/ml; VMRD, Pullman, WA, USA) for positive
208 control was used. A sample without any primary antibody was used as a negative control.
209 After 1-h incubation, a rabbit anti-human kappa chain antibody (1 μg/ml; MBL, Tokyo,
210 Japan) was added to samples on the glass slide, except positive control.

211 Rhodamine-conjugated goat IgG fraction anti-rabbit IgG (MP Biomedicals, LLC-Cappel
212 Products, Santa Ana, CA, USA) in 200 times dilution and 4',6-diamidino-2-phenylindole
213 (DAPI; 1000 times dilution; DOJINDO, Kumamoto, Japan) were added to all samples,
214 except for positive control after incubation, and the samples were washed with PBS. For
215 positive control, Alexa Fluor 594-conjugated AffiniPure Rabbit Anti-Mouse IgG (H+L)
216 (1 μg/ml; Jackson ImmunoReasearch Laboratories, West Grove, PA, USA) and DAPI
217 were added. All samples were viewed on a confocal microscope (LSM 700, Carl-Zeiss,
218 Oberkochen, Germany), and their images were processed by employing the software
219 ZEN lite 2010 (Carl-Zeiss).
220

221 ## RESULTS

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

227 **Monoclonal antibody selection from phage display library** The display of the

228 Fab fragment on the surface of phage was achieved by using pDong1/Fab with the help of
229 the KM13 helper phage. For construction of antibody library, the V_H and V_L genes of
230 antibodies were amplified and detected at 350–400 bp in an agarose electrophoresis (data
231 not shown). A phage display antibody library with a diversity of 5×10^6 was obtained.
232 After three rounds of selection, the enrichment of rNcSRS2-binding phage was confirmed
233 using an ELISA with original phage library R0 and sublibraries R1, R2, and R3, which
234 were amplified in each step of biopanning. Absorbance at 450 nm in phage ELISA for R0,
235 R1, R2, and R3 phage against rNcSRS2 rose with the increase of the biopanning step (Fig.
236 1B), suggesting that three rounds of biopanning enriched the rNcSRS2-specific
237 Fab-phages. These phage pools did not bind to BSA as a negative control.
238 R3 phages were used to infect *E. coli* TG-1 for forming colonies. Ninety-six colonies
239 were picked up and cultivated for forming phage. Four clones A6, E1, H3, and H6
240 showed a strong signal against immobilized rNcSRS2 (Fig. 2A). The binding capacity of
241 those Fab-phages was also reconfirmed against rNcSRS2 and BSA. A6, E1, and H3
242 clones bound to rNcSRS2 but not to BSA (Fig. 2B), suggesting their specificity to our
243 target. Although H6 clone bound to rNcSRS2 in the screening, it did not bind to rNcSRS2
244 upon further confirmation (data not shown). The KM13 helper phage in which no
245 antibody was displayed was used as a negative control and did not bind to immobilized

246 rNcSRS2.

247 **IC_{50} of A6, E1, and H3 clones** To evaluate the half maximal inhibitory
248 concentration (IC_{50}) of those clones, a competitive ELISA with serially diluted rNcSRS2
249 solutions inhibiting the binding of Fab-phage to immobilized rNcSRS2 was performed.
250 Competition was observed between free and immobilized proteins, and the IC_{50} values of
251 A6, E1, and H3 were evaluated to be 0.54, 0.22, and 0.51 μg/ml of rNcSRS2,
252 respectively (data not shown).

253 **Purification of Fab antibodies and their binding to rNcSRS2** Fab antibodies
254 were expressed in *E. coli* HB2151 and purified with TALON Co^{2+}-immobilized resin and
255 an anti-FLAG M2 affinity gel. The purified Fab fragments showed two bands on
256 SDS-PAGE with molecular weight of 24 and 26 kDa (Fig. 3A), which were identified as
257 light chain and V_H-C_H1 of heavy chain, respectively. The binding of these Fabs to
258 rNcSRS2 and a truncate rNcSRS2 without GPI anchor domain of native protein rNcSRS2
259 (NSNT) (unpublished data) was confirmed with an ELISA. As shown in Fig. 3B, all the
260 three Fabs bound to the rNcSRS2 and A6, El also bound to rNcSRS2 (NSNT). However,
261 the H3 clone did not bind to the truncated protein, suggesting it might bind to the anchor
262 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

316 in vivo, as well as the easy delivery to certain cells through antibody specificity. By
317 adding the Fc region gene and transforming insect cell or mammal cells, the whole length
318 antibodies for some certain applications can also be achieved.

319 Even though H3 clone bound to rNcSRS2, it did not bind to the truncated protein
320 without an anchor domain. rNcSRS2 was reported to be expressed in insect cell using
321 baculovirus expression system with all the necessary post-translational modifications (21),
322 however, the process of GPI anchor has not been revealed yet. Therefore, this clone may
323 be useful for studying the mechanism of the GPI anchor process.

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

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Figure Legends

FIG. 1. Scheme for the development of murine anti-*N. caninum* monocloal antibodies

406 (A) and the enrichment of rNcSRS2-specific clones (B). ELISA was performed to
407 confirm the enrichment of rNcSRS2-specific clones. In ELISA, rNcSRS2 (0.5 μg/ml) and
408 BSA (10 μg/ml) were immobilized on a 96-well microplate, respectively. HRP/anti-M13
409 monoclonal antibody conjugate was used as the secondary antibody. R0 stands for the
410 original phage library, whereas R1, R2, and R3 stand for the amplified Fab-phage in
411 Rounds 1, 2, and 3 of biopanning, respectively.

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

420 FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of purified Fab antibodies
421 (A) and their bindings to rNcSRS2, rNcSRS2 (NSNT), and BSA (B). For ELISA,
422 rNcSRS2, rNcSRS2 (NSNT) (0.5 μg/ml each), and BSA (10 μg/ml) were immobilized on

423 a 96-well microplate, respectively. After incubation with Fab antibodies, a Rabbit
424 anti-Human Kappa chain antibody (1 μg/ml) and an ECL™ Anti-mouse IgG, Horseradish
425 Peroxidase linked whole antibody (from sheep) was used as secondary and tertiary
426 antibodies, respectively. M: Precision Plus Protein™ Dual Colors Standards.

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

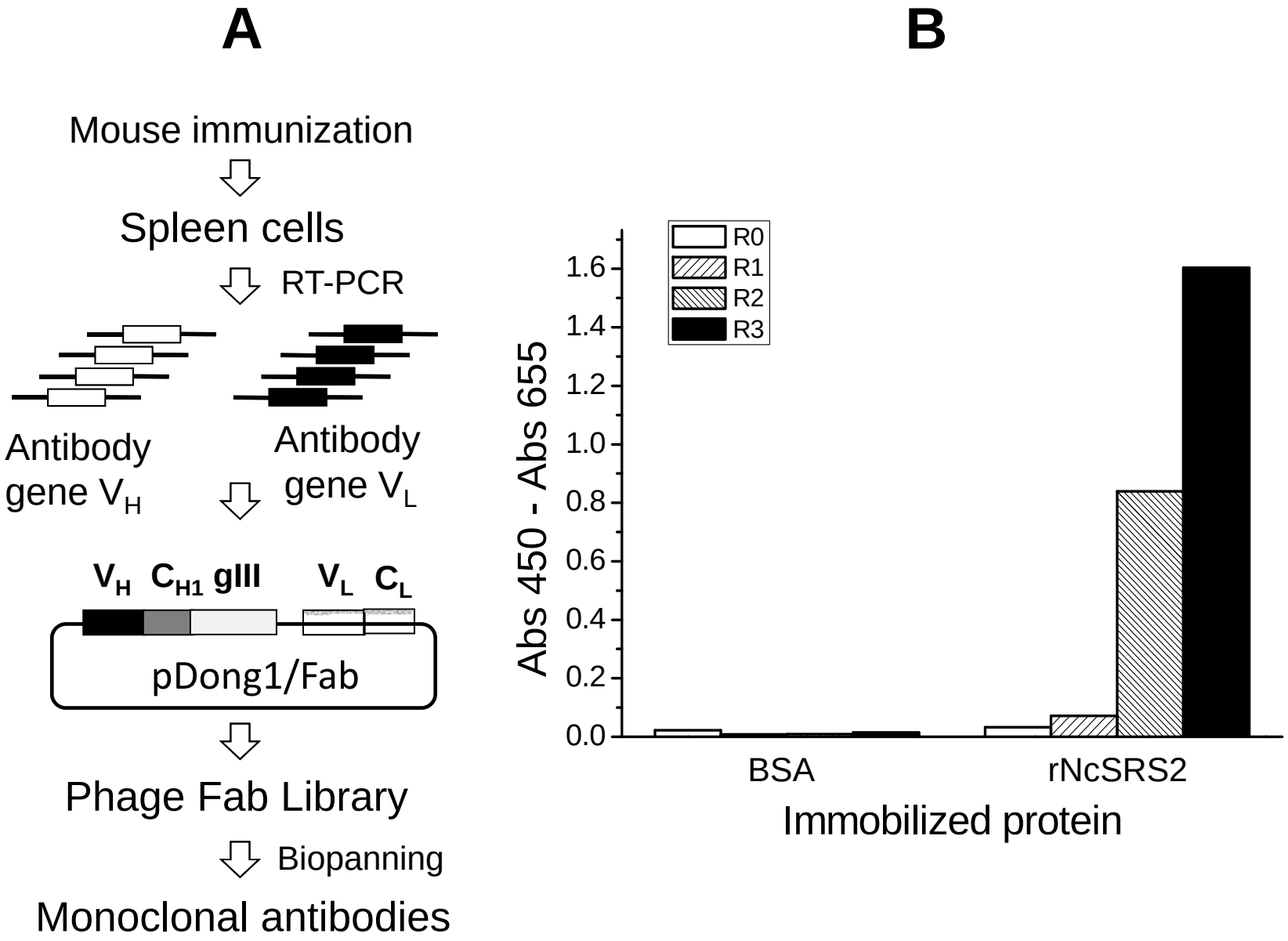
430 FIG. 5. Immunofluorescence assay of *N. caninum* with monoclonal antibodies. *N.*
431 *caninum* strain Nc-Liv was immobilized and blocked followed by the addition of a
432 commercial anti-*N. caninum* antibody (P), Fabs A6, E1, and H3, respectively. A sample
433 without any primary antibody was a negative control (N). For Fab samples and negative
434 control, a Rabbit anti-Human Kappa chain antibody (1 μg/ml) was used as secondary
435 antibody and Rhodamine-conjugated Goat IgG fraction Anti-Rabbit IgG was added
436 finally. For positive control, Alexa fluor 594-labelled anti Mouse IgG was used as a
437 secondary antibody. 4',6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclear
438 of parasites. The samples were observed under a confocal laser-scanning microscope.
439 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.

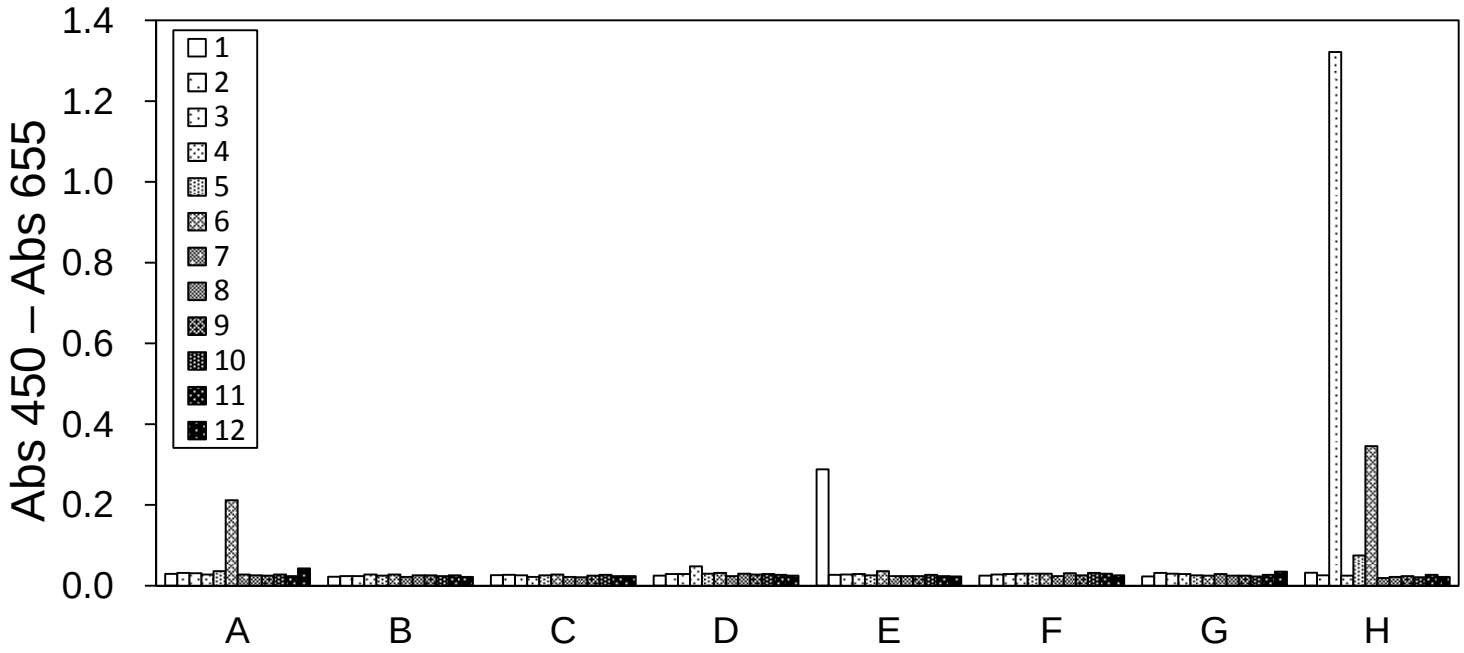
445

TABLE 1. Kinetic parameters of the monoclonal antibodies

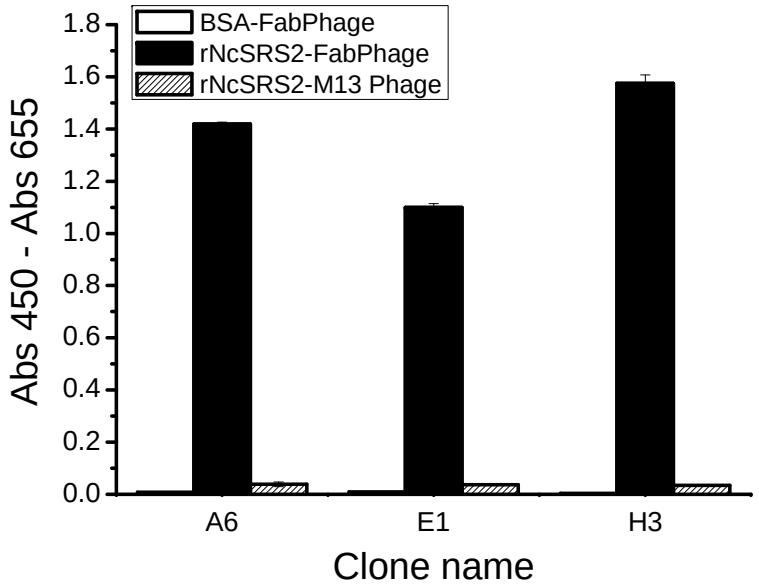
Clone name	K_a (10^4/Ms)	K_d (10^{-3}/s)	K_D (nM)
A6	5.80	24.0	420
E1	0.57	29.0	5100
H3	1.20	0.87	70

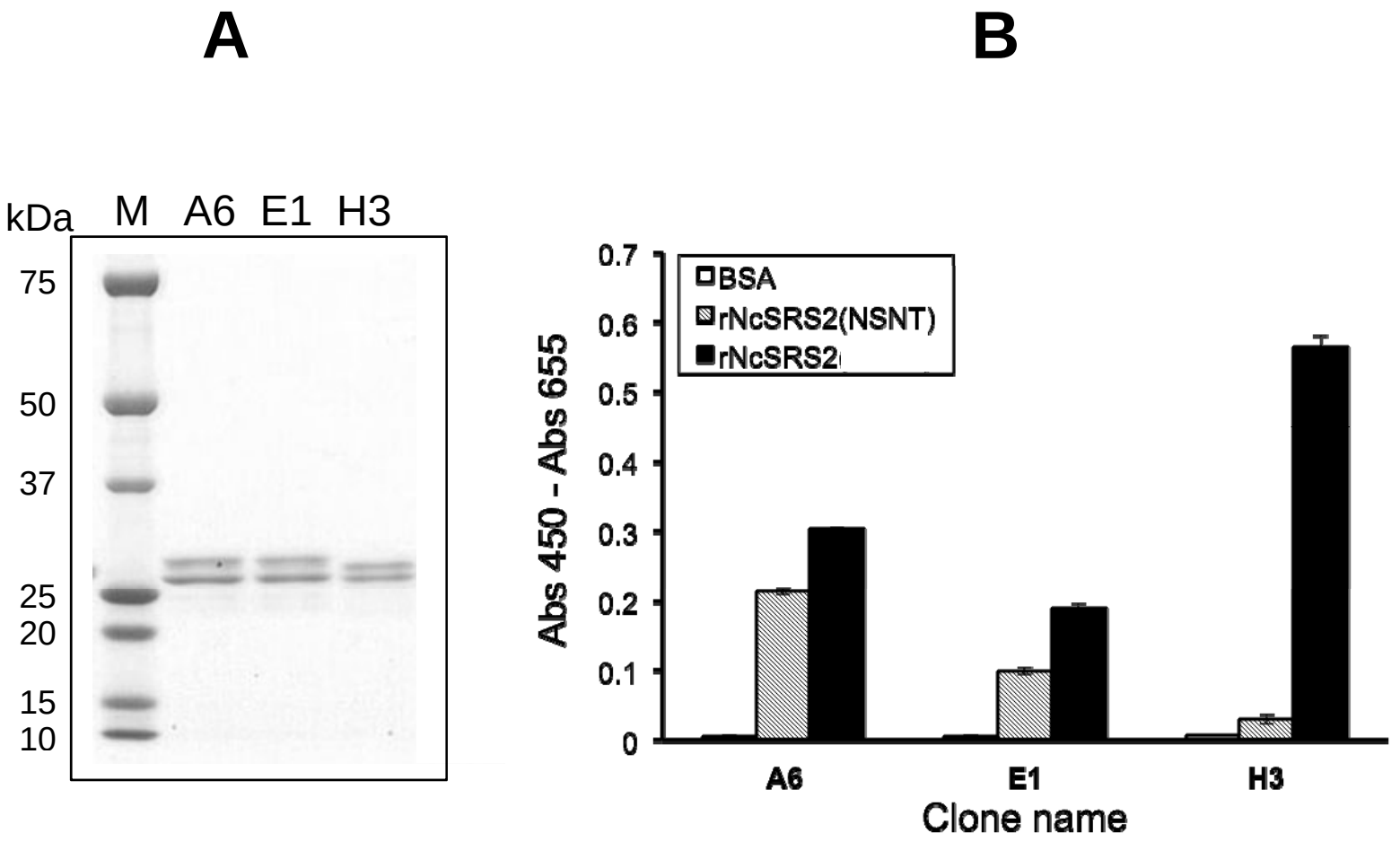

A
Mouse immunization
Spleen cells
RT-PCR
Antibody gene V_H
Antibody gene V_L
V_H C_{H1} gIII V_L C_L
pDong1/Fab
Phage Fab Library
Biopanning
Monoclonal antibodies
B
R0
R1
R2
R3
Abs 450 - Abs 655
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
BSA
rNcSRS2
Immobilized protein

A

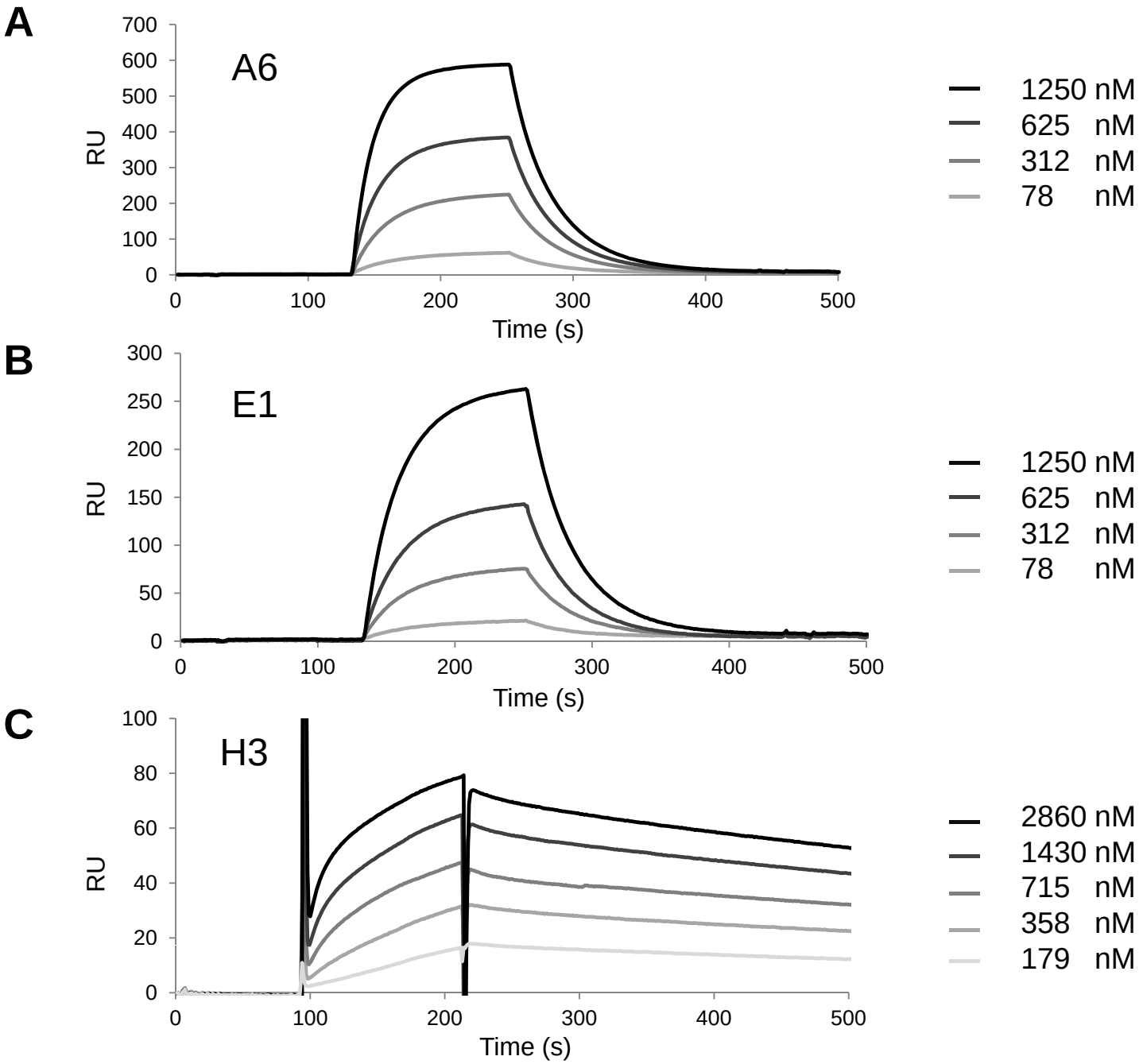

Abs 450 – Abs 655
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H

B

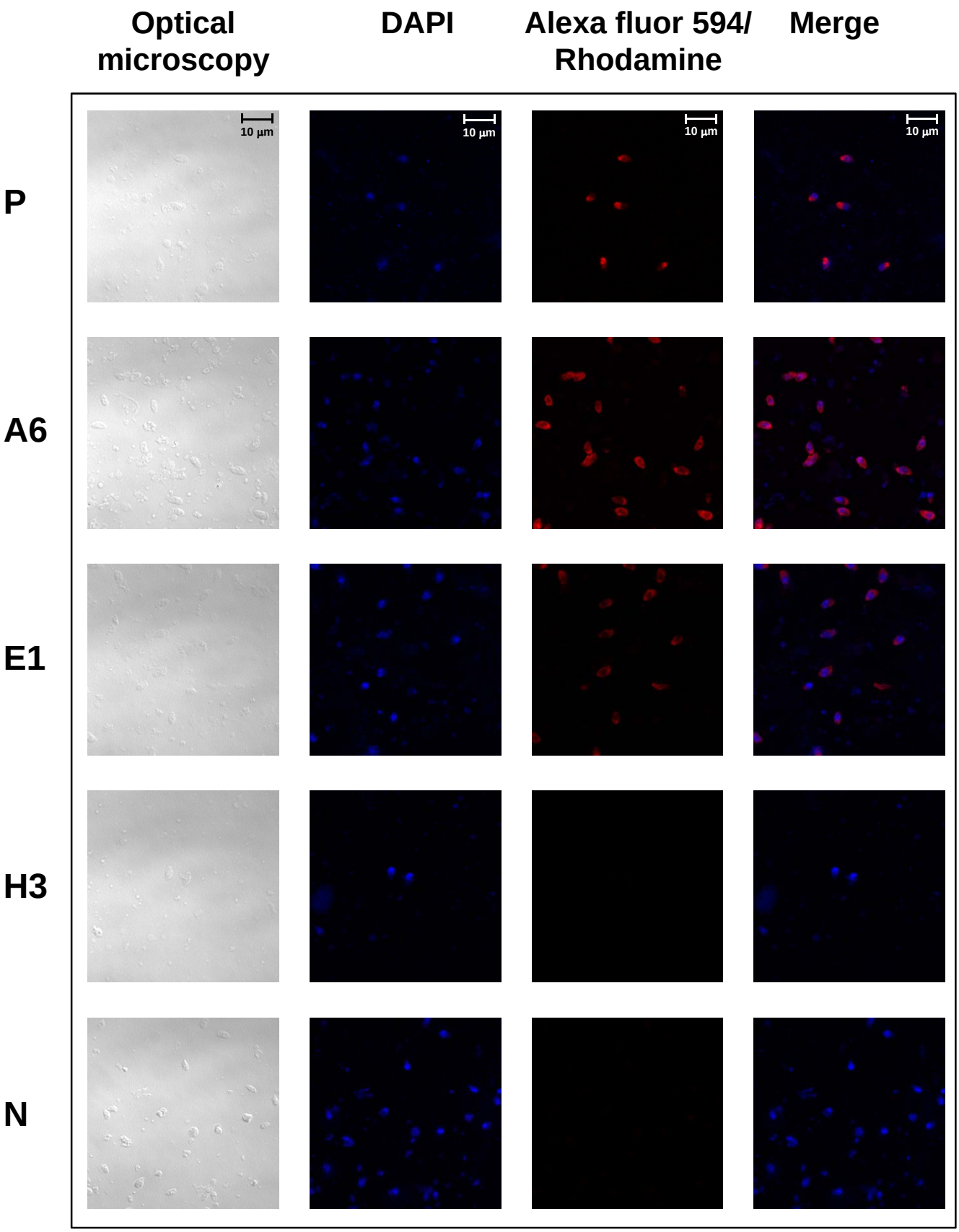

BSA-FabPhage
rNcSRS2-FabPhage
rNcSRS2-M13 Phage
Abs 450 - Abs 655
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A6
E1
H3
Clone name

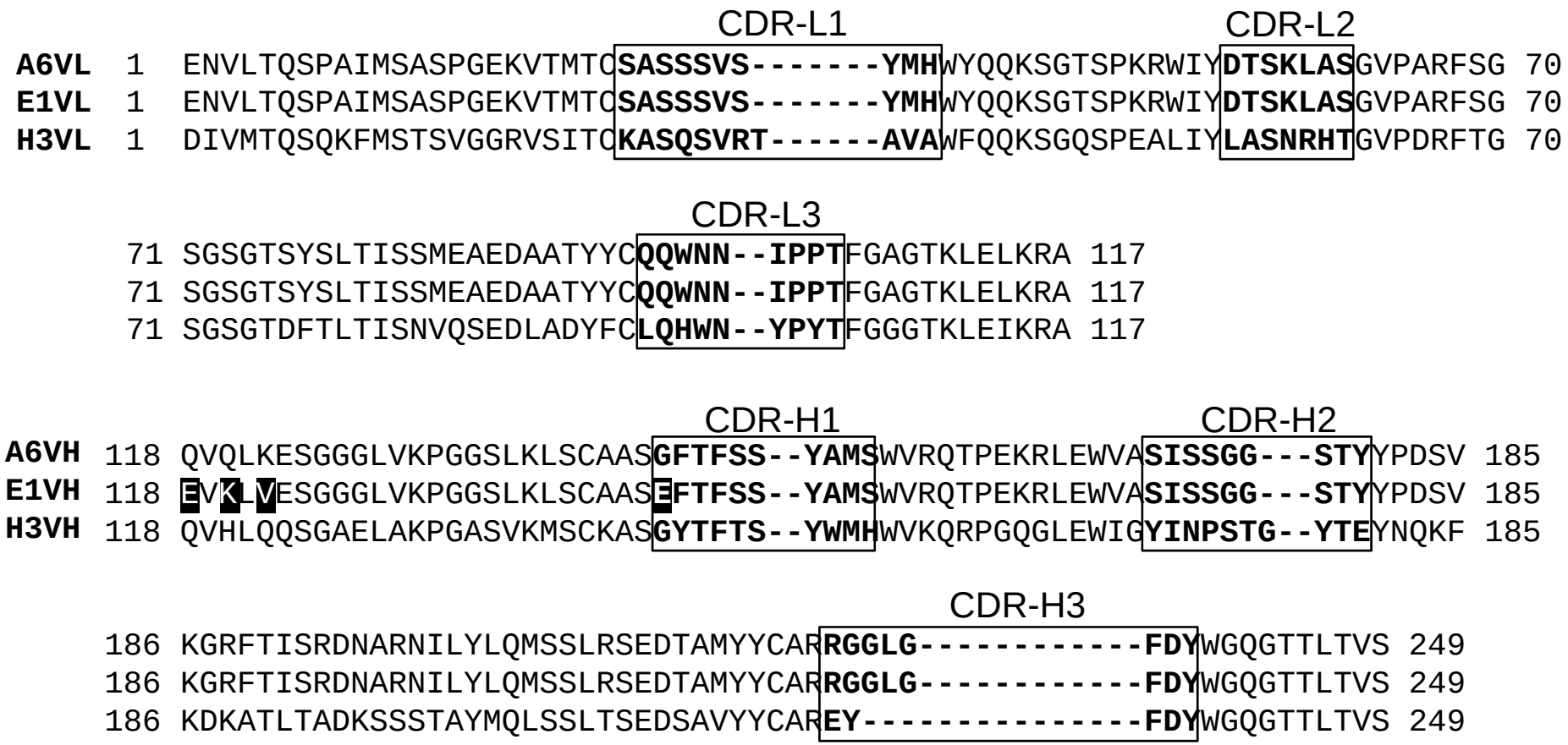

A
kDa
M
A6
E1
H3
75
50
37
25
20
15
10
B
BSA
rNcSRS2(NSNT)
rNcSRS2
Abs 450 - Abs 655
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A6
E1
H3
Clone name

Dong et al. Figure 4



Dong et al., Figure 5




CDR-L1
CDR-L2
A6VL 1 ENVLTQSPAIMSASPGEKVTMTCSASSSVS-------YMHWYQQKSGTSPKRWIYDTSKLASGVPARFSG 70
E1VL 1 ENVLTQSPAIMSASPGEKVTMTCSASSSVS-------YMHWYQQKSGTSPKRWIYDTSKLASGVPARFSG 70
H3VL 1 DIVMTQSQKFMSTSVGGRVSITCKASQSVRT------AVAWFQQKSGQSPEALIYLASNRHTGVPDRFTG 70
CDR-L3
71 SGSGTSYSLTISSMEAEDAATYYCQQWNN--IPPTFGAGTKLELKRA 117
71 SGSGTSYSLTISSMEAEDAATYYCQQWNN--IPPTFGAGTKLELKRA 117
71 SGSGTDFTLTISNVQSEDLADYFCLQHWN--YPYTFGGGTKLEIKRA 117
CDR-H1
CDR-H2
A6VH 118 QVQLKESGGGLVKPGGSLKLSCAASGFTFSS--YAMSWVRQTPEKRLEWVASISSGG---STYYPDSV 185
E1VH 118 EVKLVESGGGLVKPGGSLKLSCAASEFTFSS--YAMSWVRQTPEKRLEWVASISSGG---STYYPDSV 185
H3VH 118 QVHLQQSGAELAKPGASVKMSCKASGYTFTS--YWMHWVKQRPGQGLEWIGYINPSTG--YTEYNQKF 185
CDR-H3
186 KGRFTISRDNARNILYLQMSSLRSEDTAMYYCARRGGLG----------FDYWGQGTTLTVS 249
186 KGRFTISRDNARNILYLQMSSLRSEDTAMYYCARRGGLG----------FDYWGQGTTLTVS 249
186 KDKATLTADKSSSTAYMQLSSLTSEDSAVYYCAREY--------------FDYWGQGTTLTVS 249