

## Identification of single-chain antibody fragments specific against SARS-associated coronavirus from phage-displayed antibody library

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### Abstract

To develop early diagnostic reagents, effective vaccines, and even drugs against SARS-associated coronavirus (SARS-CoV), the human single fold single-chain antibody fragments, (scFv) libraries I + J (Tomlinson I + J) were used to identify novel scFvs, which can specifically bind to SARS-CoV. Interestingly, two scFvs (B5 and B9) exhibited higher binding specificity to SARS-CoV with the OD<sub>450</sub> value 0.608 and 0.545, respectively, and their coding sequences shared the identical sequence composed of V<sub>H</sub> gene (351 bp) and V<sub>L</sub> gene (327 bp), so the two scFvs were uniformly named as SA59B and chosen for further analysis. SA59B scFv was expressed in soluble form in *Escherichia coli* HB2151 and purified by immobilized metal affinity chromatography. The soluble 30 kDa SA59B scFv-antibody was verified in SDS-PAGE and Western-blot. The purified SA59B scFv-antibody was labeled with HRP by the glutaraldehyde method, and the concentration of HRP and SA59B scFv-antibody in the SA59B-HRP solution reached 2.4 and 2.28 mg/ml, respectively. Then, the binding ability of SA59B-HRP to SARS-CoV was evaluated by ELISA with S/N of 11.6, indicating higher binding specificity between them. Finally, both the SA59B sequence specificity and its application for diagnosis, prophylaxis or therapy of SARS were discussed.

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Severe acute respiratory syndrome (SARS) with a significant morbidity and mortality, caused by SARS-CoV, is responsible for the first pandemic of the 21st century, which had caused over 8000 probable cases worldwide and more than 800 deaths [1,2]. However, there are currently no effective vaccines and antiviral compounds to prevent or cure the disease [3]. Furthermore, currently there are no reliable diagnostic tests for diagnosing patients and monitoring its spread. Hence, single-chain antibody fragments specific against SARS-CoV from phage-displayed antibody library have potential for

exploitation as diagnostic or even antiviral therapeutic reagents.

Phage display is an *in vivo* selection technique in which billions of peptides or proteins are fused to one of the capsid proteins, expressed at the N-terminus of the capsid proteins (pVIII or pIII) of filamentous bacteriophage such as Fd or M13, and then displayed on the surface of a phage particle [4,5]. There are two most significant points in this technique one provides the natural linkage between the phenotype and genotype [6]; the other lies in the fact that it allows specific screening based on binding affinity to a given target molecular by an *in vivo* selection process called panning [7–9]. With the rapid development and maturation of this technique, it is often an effective way to screen proteins or peptides for

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important applications. For example, this technique can be used in antibody engineering, peptide and protein drugs' finding, vaccine manufacture, and so on [10].

Antibody engineering has made it possible to operate genes encoding antibodies and to construct antibody derivatives, which retain entire antigen binding function [11]. These derivatives include single-chain antibody fragments (scFvs) which consist of variable regions of heavy and light chains with a flexible linker for connecting [12]. Functional scFv-antibody is expressed from a single cDNA sequence, enabling gene manipulation. Thus, in this manner, this technique has great potential in both basic and applied research work.

Up to now, there has not been any research group utilizing this technique to find new reagents directed against SARS-CoV. In this report, we describe the use of the synthetic phagemid library for the generation of high-affinity scFv antibodies directed against this new coronavirus. Their affinity was measured by plasmon surface resonance, and the specificity was confirmed by ELISA. Two stronger positive scFvs designated as B5 and B9 were sequenced, uniformly designated as SA59B because of their 100% homology, and further chosen for expression, purification, labeling, and detecting research. Furthermore, we discussed the possible applications of SA59B scFv-antibody as diagnostic or even antiviral therapeutic reagents.

## Materials and methods

**Library, bacteria, and reagents.** The Human Single Fold scFv libraries I + J (Tomlinson I + J), *Escherichia coli* TG1 and HB2151 were kindly provided by MRC Geneservice. The ELISA plates coated with purified SARS-CoV lysate, positive and negative sera were provided by Military Medical Science Academic and HuaDa Gene Company (Beijing, China). NBT/BCIP (Sigma), polyhistidine mouse antibody (Sigma), AP-labeled anti-mouse antibody (SABC), and anti-M13 (Pharmacia) were all purchased from distributors. Other commonly used reagents are all of analytic purification grade and made in China.

**Panning assay.** The panning procedure was performed on a 96-well flexible assay plate coated with purified SARS-CoV lysate, which was blocked directly with MPBS (PBS containing 4% skimmed milk powder) at room temperature for 2 h. After removal of the blocking solution, the plate was washed three times with PBS. Then 200  $\mu$ l of the primary library solution ( $10^{13}$  phages in 4% MPBS) was added into each well of the plate. After standing at room temperature for 2 h, the unspecific binding phages were washed away with PBS containing 0.1% Tween for 10 times (20 times in the second panning and 30 times in the third panning). The remaining phages were eluted with 1 ml of 0.2 M glycine-HCl buffer (pH 2.2), and the elution fraction was subsequently neutralized with 0.5 ml of 1 M Tris-HCl (pH 9.5).

In parallel, 10 ml of *E. coli* TG1 cultured in 2 $\times$  TY ( $OD_{600} = 0.4$ ) was infected with the eluted scFv-phages at 37  $^{\circ}$ C for 30 min without shaking. Then a small proportion of infected *E. coli* TG1 was titrated in 4-fold dilutions, starting with 1:10, to determine the scFv-phage titer. Each dilution was spotted 10  $\mu$ l separately on TYE plates (including 100  $\mu$ g/ml ampicillin and 1% glucose), which were cultured at 37  $^{\circ}$ C overnight to titer the eluted scFv-phage by calculating clones on TYE plates. Meanwhile, the rest of infected *E. coli* TG1 was all spread on TYE plates. The next day, bacteria on the plates were

scrapped and added into 100 ml of 2 $\times$  TY with 100  $\mu$ g/ml ampicillin and 1% glucose for amplification. After this procedure, the phages were rescued by helper phage M13K07 for the next round of selection. Three rounds of selections were performed.

**Preparation of scFv-phage.** In the last selection round, individual clones were randomly picked out from the TYE plate to different wells (100  $\mu$ l of 2 $\times$  TY with 100  $\mu$ g/ml ampicillin and 1% glucose was added in advance) of a flexible ELISA plate. After culturing, a small inoculum (2  $\mu$ l) from each well was transferred to a second ELISA plate containing 200  $\mu$ l of 2 $\times$  TY with 100  $\mu$ g/ml ampicillin and 1% glucose per well. The original ELISA plate was stored at 4  $^{\circ}$ C temporarily. The transferred plate was shaken at 37  $^{\circ}$ C for 2 h, and phages were rescued in *E. coli* TG1 by adding  $10^9$  helper phages to each well, the plate was shaken at 37  $^{\circ}$ C for 1 h before spinning the plate at 1800g for 10 min. The supernatant was thrown away and the pellet was suspended in 200  $\mu$ l of 2 $\times$  TY containing 100  $\mu$ g/ml ampicillin and 50  $\mu$ g/ml kanamycin, and cultured at 30  $^{\circ}$ C, 250 rpm overnight. The overnight culture was spun at 1800g for 10 min and 50  $\mu$ l of the supernatant containing scFv-phage was used in monoclonal phage ELISA.

**Monoclonal scFv-phage ELISA.** A 72-well flexible ELISA plate coated with SARS-CoV lysate was blocked and washed as described above. Then 50  $\mu$ l scFv-phage supernatant was added into each well of the plate. After incubation for 1 h at room temperature, the plate was washed three times with PBS containing 0.1% Tween. Subsequently, 100  $\mu$ l HRP-anti-M13 (1:5000 dilution in 4% MPBS) was added to each well. After incubation for an additional 1 h at 37  $^{\circ}$ C, the wells were washed again, and 100  $\mu$ l fresh substrate solution (100  $\mu$ g/ml in 100 mM sodium acetate, pH 6.0, 30% hydrogen peroxide) was added to each well. Enzyme reaction was terminated by adding 50  $\mu$ l of 2 N  $H_2SO_4$ . Absorbance was measured at 450 nm and evaluated by S/N (sample/negative).

**DNA sequencing.** Two stronger positive clones (B5 and B9) in monoclonal phage ELISA were sequenced by Shanghai Casarray and Gene (Shanghai, China). Two sequencing primers were used: forward primer 5'-CGA CCC GCC ACC GCC GCT G-3' and reverse primer 5'-CTA TGC GGC CCC ATT CA-3'.

**Expression and Western-blot assay.** The selected scFv-phage was inoculated into *E. coli* HB2151 to express a soluble scFv antibody. In briefly, *E. coli* HB2151 was grown in 2 $\times$  TY media at 37  $^{\circ}$ C until  $OD_{600} = 0.4$ . Fifty microliters of the culture was infected with 1  $\mu$ l scFv-phage from a single colony, incubated for 30 min at 37  $^{\circ}$ C without shaking, and plated on TYE agar with 100  $\mu$ g/ml ampicillin, a single colony was picked out from this plate, inoculated into 5 ml of 2 $\times$  TY with 1% glucose and 100  $\mu$ g/ml ampicillin, and grown overnight at 37  $^{\circ}$ C with shaking (250 rpm). The next day, 50  $\mu$ l of the overnight culture was transferred to another fresh 2 $\times$  TY (0.1% glucose and 100  $\mu$ g/ml ampicillin) and grown with shaking (250 rpm) at 37  $^{\circ}$ C until  $OD_{600} = 0.9$ . IPTG was added into the culture to 1 mM final concentration, and shaking was continued (200 rpm) at 30  $^{\circ}$ C. One milliliter of the culture was aspirated after 0, 2, 4, and 6 h of adding IPTG. The obtained samples were disposed off by the traditional methods and then subjected to electrophoresis in 12% SDS-PAGE. The whole bacteria protein was electrophoretically transferred to nitrocellulose membrane from the gel in 25 mM Tris base, 192 mM glycine, and 2% methanol at 65 V for 4 h. The membrane was blocked with 5% MPBS for 2 h before washing with PBS. Then, 5 ml of anti-His-tag mouse antibody (1:1000 dilution in 5% MPBS) was incubated with the membrane at room temperature for 2 h. Bound polyhistidine mouse antibody was detected with anti-mouse IgG alkaline phosphatase conjugate (1:1000). Bands were detected with the addition of BCIP/NBT.

**Production and purification of SA59B scFv-antibody.** In order to acquire large quantities of purified SA59B scFv-antibody, overnight activated *E. coli* HB2151 (infected by positive scFv-phage) culture was transferred to 300 ml of 2 $\times$  TY (0.1% glucose and 100  $\mu$ g/ml ampicillin) in a 1:100 mass ratio, and grown at 37  $^{\circ}$ C, 250 rpm to an  $A_{600}$  of 0.6. Expression of the scFv-antibody was induced by addition of IPTG

at a 1 mM final concentration. After induction at 30 °C for 6 h, the cultured cells were harvested by centrifugation at 4 °C and 4000g for 10 min, and were washed with a suspension in 40 ml PBS, pH 7.4, and centrifuged at 4 °C and 10,000g for 10 min. The cells pelleted were stored at –20 °C for further use.

The cells were suspended in 40 ml PBS, pH 7.4, containing 5 mM EDTA and 0.1% Triton X-100, sonicated, and centrifuged at 25,000g for 20 min. The supernatant fluid was applied to a column (1.0 by 10.0 cm) with His-bond Ni Affinity Resin in the presence of 10 mM sodium phosphate, pH 8.0, 0.3 mM sodium chloride, and 250 mM imidazole. After extensive washing with about 100 ml of 50 mM sodium phosphate, pH 8.0, 0.3 mM sodium chloride, and 20 mM imidazole, bound protein was eluted with approximately 100 ml of 50 mM sodium phosphate, pH 8.0, 0.3 mM sodium chloride, and 250 mM imidazole followed by extensive dialysis against PBS, pH 7.4. Purity of all proteins was evaluated by 12% SDS-PAGE. Protein bands were visualized with Coomassie blue stain.

**Labeling of SA59B scFv-antibody with HRP.** The purified SA59B scFv-antibody was labeled with HRP by the glutaraldehyde method. Briefly, 10 mg of HRP (Cat. No. BE1842; Wuhan, China) was dissolved in 0.1 ml of 0.2 M PBS, pH 6.8, and 0.1 ml of a freshly prepared 2.5% glutaraldehyde (Cat. No. 830119, Shanghai, China; dissolved in Millipore-quality water) was added and kept at room temperature for 18 h; the reaction mixture was dialyzed twice against 1 L of 0.8% NaCl in MilliQ water, pH 7.0, at 4 °C for 24 h. The dialysate (0.3 ml) was collected and adjusted to 1.0 ml with MilliQ water. Subsequently, 1.0 ml of SA59B scFv-antibody (5 mg/ml) was added and mixed; 0.1 ml of 1.0 M CBS, pH 9.5, was added, mixed, and reacted at 4 °C for 24 h; 0.1 ml of 0.2 M lysine was added, mixed, dialyzed against 1 L of 0.15 M PBS, pH 7.2, for 24 h, and centrifuged at 4 °C and 4000g for 20 min; the supernatant fluid (2.0 ml) was brought to 2.0 ml of 100% saturation of  $(\text{NH}_4)_2\text{SO}_4$ , pH 7.2, stirred for 20 min, and centrifuged at 4000g for 20 min; the precipitate was washed twice with 4.0 ml of 50% saturation of  $(\text{NH}_4)_2\text{SO}_4$ , pH 7.2, and dissolved in 4.0 ml of 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA followed by dialysis against 1 L of 0.01 M PBS, pH 7.2, at 4 °C for 24 h. Finally, the dialysate (1.0 ml) was collected. After addition of BSA at a final concentration of 1%, it was filtered with a 0.22- $\mu\text{m}$  membrane. The aliquots were made and stored at –20 °C.

**SA59B-HRP ELISA.** ELISA was used to further measure HRP-labeled SA59B scFv-antibody (SA59B-HRP) response to SARS-CoV lysate. SA59B-HRP solution was titrated in 4-fold dilutions, starting with 1:10, to determine the antibody titer. A flexible ELISA plate coated with SARS-CoV lysate was prepared. As negative control, three wells were coated with control lysate. After three washes with PBS, the plates were blocked with 4% MPBS. The wells were emptied and incubated for 1 h at 37 °C with SA59B-HRP conjugates at various concentrations. A commercially available SARS convalescent serum-HRP conjugate was used as a control. The wells were washed three times with PBS and incubated for 20 min at room temperature with 100  $\mu\text{l}$  substrate solution (see Monoclonal scFv-phage ELISA). Fifty microliters of 2 M sulfuric acid was added to stop the reaction. Absorbance was measured at 450 nm.

**Statistical assay.** The Student *t* test was used for Statistical analysis. All data are shown as means  $\pm$  SD. Significance was defined as  $P < 0.05$ .

## Results

### Selection of SARS-CoV-specific binding clones from phage display library

The human synthetic scFv Griffin.1 library was used for selection of SARS-CoV-specific scFvs. Three

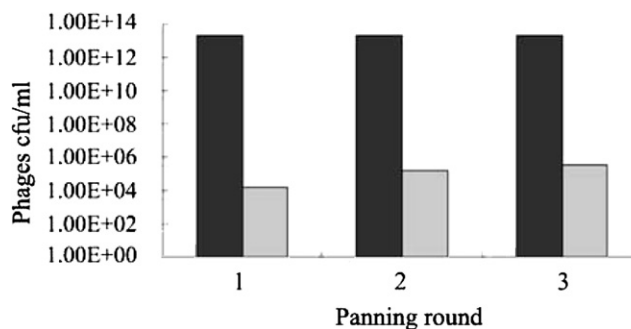


Fig. 1. Numbers of eluted and amplified scFv phages and their specificity after each of three rounds of selection. The left columns indicate the number of amplified scFv-phages after each panning round, which was then used in another panning round. The right columns show the number of eluted scFv-phages from each panning round. The titer was determined as cfu/ml.

rounds of panning were done on a flexible ELISA plate coated with purified SARS-CoV lysate. After each round of panning, the titer of the eluted scFv-phage was measured to monitor the efficiency of the selection process. Compared with the second round, the number of scFv-phages eluted after the third round did not increase, which indicated that the library was already enriched in SARS-CoV-specific phages (Fig. 1). From the third round of panning, 68 clones were randomly picked out and assessed for binding to SARS-CoV in monoclonal scFv-phage ELISA. The results (Fig. 2) suggested that A3, A5, A7, A8, B5, B9, E6, and F9 were positive clones ( $S/N > 2.1$ ). Also, B5 and B9 showed higher SARS-CoV-binding specificity with the  $\text{OD}_{450}$  value 0.608 ( $S/N$ , 10.4) and 0.306 ( $S/N$ , 6.0), respectively, and so were chosen for further analysis.

### Sequences analysis

The complete nucleotide sequences of the heavy and light chain variable region from the two stronger positive clones B5 and B9 were determined. The results indicated that the coding sequences of B5 and B9 share identical sequence (Fig. 3), which is composed of  $V_H$  gene (351 bp) and  $V_L$  gene (327 bp), and the two scFvs were uniformly designated as SA59B. The closest germline sequences for SA59B scFv-antibody gene could be identified by comparison with the database [13–15]. The  $V_H$  gene use, the  $V_H3$  family-derived germline V3-23, D3-9, D7-27, and  $J_H4b$  genes, while the  $V_L$  gene uses the  $V_L1$  ( $V$ -kappa subgroup I,  $V_k$ -1) family-derived germline 02 (012) and  $J_k1$  genes (Fig. 3). Also, a high degree of mutation appeared in the  $V_H\text{CDR2}$  (CDR, complementarity-determining region) with 70.6% (bases) and 58.8% (amino acids) sequence homology to the closest germline match (Table 1). In addition, there was a replacement mutation

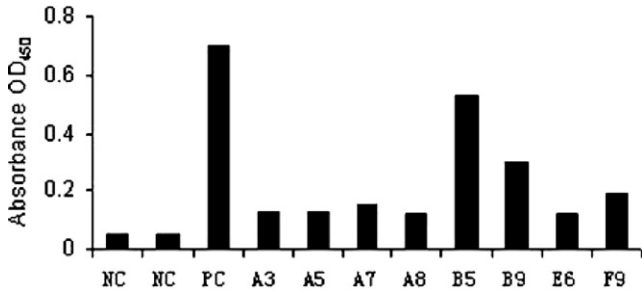


Fig. 2. Reactivity of eight SARS-CoV-specific clones selected from the third round of panning given as absorbance in monoclonal phage ELISA. NC, negative control. PC, positive control. A3, A5, A7, A8, B5, B9, E6, and F9 are monoclones which give positive signal. Optical density was measured as OD<sub>450</sub>.

(position 282, T → G) that led to generation of a stop codon TGA in the V<sub>H</sub>FWR3 (FWR, functional framework region), which could surprisingly be suppressed when the scFv-phages were expressed in *E. coli* TG1 or HB2151. Any errors introduced during the amplification of DNA can be excluded, because we sequenced B5 and B9 scFvs repeatedly in two different companies (see Materials and methods) and obtained the same results. The deduced amino acid sequence of heavy and light chains of SA59B is shown in Fig. 3, and the predicted molecular weight of the whole soluble scFv was about 30 kDa.

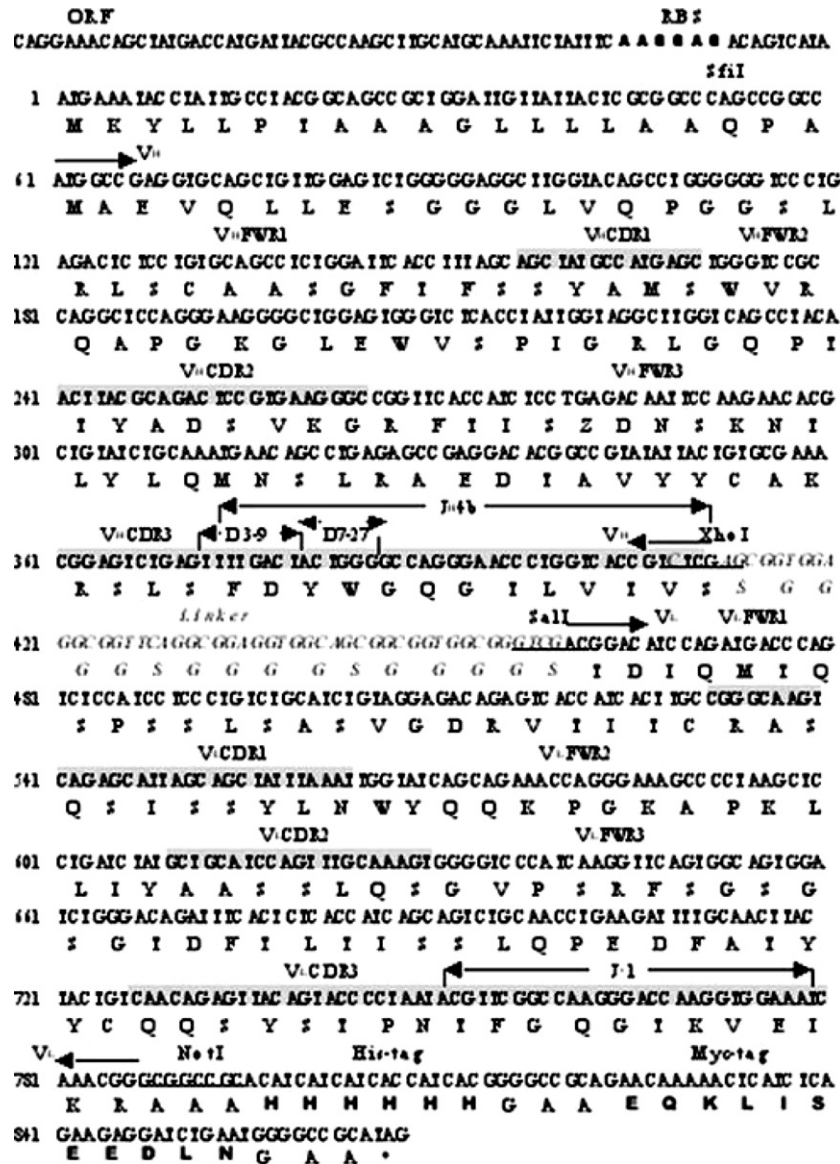


Fig. 3. Complete nucleotide sequence of SA59B scFv-antibody gene and derived amino acid sequence of the protein. The deduced amino acid sequence is listed starting with the initiation methionine and shown below the nucleotide sequence. All the annotations are displayed above the nucleotide and amino acid sequence. The presumed ribosome binding site is labeled as RBS. Arrows above the DNA sequence represent V<sub>H</sub> and V<sub>L</sub> regions. Lighter shaded areas depict complementary determinant regions (CDR) of V<sub>H</sub> and V<sub>L</sub>. The blank regions demarcate the region (FWR) of V<sub>H</sub> and V<sub>L</sub>. The linker sequence is in italic. His-tag and Myc-tag are in Arial black. The locations of various restriction sites are underlined.

Table 1  
Analysis of nucleotide and amino acid sequences of V<sub>H</sub>CDR2 region of SA59B single-stranded antibody gene

Gene	IV <sub>H</sub> CDR2 region (5' → 3' or -NH <sub>2</sub> → -COOH)	Homology (%)
V <sub>H</sub> 3-23	B GCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGC	
SA59B	C*****G**A*GCT****CAGCCT***ACT*****	70.6
V <sub>H</sub> 3-23	AA P I G R L G Q P T T Y A D S V K G	
SA59B	C * S G S * G S * Y * * * * * * *	58.8

“B” and “AA” are the abbreviations of “base” and “amino acid,” respectively. The sign “\*” expresses homology.

### The expression of soluble SA59B scFv-antibody and its identification

SA59B was chosen for expression analysis. The samples aspirated according to induced time were subjected to 12% SDS-PAGE gel. Western blot analysis (Fig. 4) was performed according to the former described method. We concluded that the scFv-antibody was expressed with MW of 30 kDa since its polyhistidine tail was confirmed in Western-blot.

### Purification of soluble SA59B scFv-antibody

The purification results of SA59B scFv-antibody are shown in Fig. 5. The purified SA59B scFv-antibody migrated as a single protein band on SDS-PAGE (Fig. 5A). It had an estimated molecular mass of 30 kDa

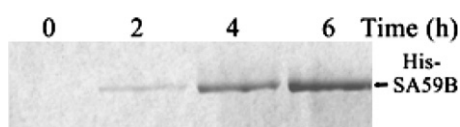


Fig. 4. SA59B scFv-antibody expression by Western-blot analysis. Analysis of reactivity of the total cell lysates with polyhistidine antibody. Total cell lysates from *E. coli* HB2151 cells after 0, 2, 4, and 6 h induced by IPTG, respectively. Induction after 2, 4, and 6 h gives a positive signal.

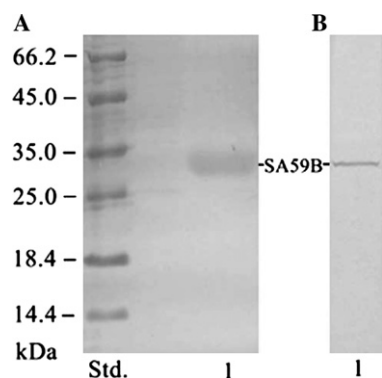


Fig. 5. Purification results of His<sub>6</sub>-tagged SA59B scFv from total cell lysates. Commassie brilliant blue-stained 12.0% SDS-PAGE gel (A) and Western blot (B). Std., molecular mass standards with masses of (from top to bottom) 66.2, 45.0, 35.0, 25.0, 18.4, and 14.4 kDa, lane 1, SA59B scFv.

and possessed a high degree of purity. As shown in Fig. 5B, Western-blot analysis of the purified SA59B scFv-antibody also verified the result of its SDS-PAGE. The production yield of purified SA59B scFv-antibody was around 1–1.5 mg/L of production culture valued by absorbance at 280 nm of the purity solution.

### Labeling of SA59B scFv-antibody with HRP

The purified SA59B scFv-antibody was labeled with HRP by the glutaraldehyde method. After that, the concentration of HRP and SA59B scFv-antibody in the SA59B-HRP solution was 2.4 and 2.28 g/L, respectively. SDS/PAGE result of SA59B-HRP solution is shown in Fig. 6. The SA59B-HRP migrated as a single protein band on SDS-PAGE, and it had an estimated molecular mass of about 66.2 kDa with evidence for the binding of HRP to SA59B scFv (HRP, 44 kDa; SA59B scFv-antibody, 30 kDa).

### The binding activity of SA59B-HRP to SARS-CoV

To primarily examine the effects of SA59B-HRP on binding to SARS-CoV, we still used the ELISA plate coated with purified SARS-CoV lysate. SA59B-HRP solution was titrated in 4-fold dilutions, starting with 1:10, to determine the antibody titer (data not shown), the optimal dilution degree of SA59B-HRP solution was in a ratio of 1:100 and chosen for further ELISA.

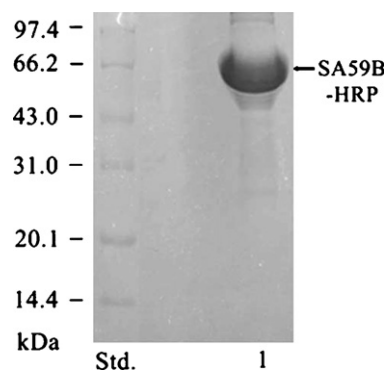


Fig. 6. SDS-PAGE of SA59B scFv-antibody labeled with HRP. Std., molecular protein mass standards, lane 1, SA59B-HRP. The numbers on the left refer to the molecular masses of the standards.

Table 2  
Results of SA59B-HRP ELISA ( $X \pm SD$ )

List of tests	<i>n</i>	Absorbance OD <sub>450</sub>			$X \pm SD$	S/N
SA59B-HRP (1:100)	3	0.791	0.776	0.783	$0.783 \pm 0.006^{**}$	11.6
Positive control	3	0.538	0.535	0.541	$0.535 \pm 0.004^{**}$	7.9
Negative control	3	0.066	0.069	0.070	$0.068 \pm 0.002$	
Blank control	3	0.052	0.052	0.050	$0.051 \pm 0.001$	

“*n*” expresses test number. “S/N” is an absorbance OD<sub>450</sub> ratio of sample to negative control. The sign“\*\*” indicates the deviation from the absorbance OD<sub>450</sub> between experimental groups and negative controls was dramatically significant ( $P < 0.01$ ). The ELISA positive was defined as  $S/N > 2.1$ .

As seen in Table 2, comparison with the positive controls (SARS convalescent serum-HRP conjugate), the absorbance OD<sub>450</sub> ( $A_{450}$ ,  $X \pm SD$ ) of SA59B-HRP (1:100) tests reached  $0.783 \pm 0.006$  increasing by ~46.4%. However, there was a remarkable significant difference in  $A_{450}$  levels observed between either SA59B-HRP (1:100) tests or positive controls and the negative controls with S/N of 11.6 and 7.9, respectively ( $P < 0.01$ ). These results indicated that SA59B-HRP also bore high specificity against SARS-CoV.

## Discussion

After three rounds of affinity selection, we obtained eight positive scFvs specific against SARS-CoV. Interestingly, two scFvs of them designated as B5 and B9 exhibited higher binding specificity to SARS-CoV with the OD<sub>450</sub> value 0.608 and 0.545, respectively. Their coding sequences shared the identical sequence composed of V<sub>H</sub> gene (351 bp) and V<sub>L</sub> gene (327 bp). Moreover, comparison with the closest Ig germline V genes showed that the V<sub>H</sub> gene was derived from V<sub>H</sub>3 family, and a high degree of mutation appeared in the V<sub>H</sub>CDR2 with 70.6% (bases) and 58.8% (amino acids) sequence homology to the closest germline match, which indicated that the V<sub>H</sub>CDR2 of SA59B scFv-antibody gene may be a critical region of specific binding of SA59B scFv to SARS-CoV.

Although the precise mechanism of this action was unknown, our study suggested that SA59B scFv-antibody or SA59B-HRP had a high binding specificity to SARS-CoV in ELISA. Since it reacted well with SARS-CoV, it is also conceivable that it might restore its binding properties in the form of fusion proteins after expression as soluble scFvs in *E. coli* HB2151 or labeling as scFv-horseradish peroxidase, which was described by others [16–18]. Notably, the SDS-PAGE results of SA59B-HRP solution may also provide evidence for this point. The SA59B-HRP migrated as a single protein band on SDS-PAGE, and it had an estimated molecular mass of 66.2 kDa, which was obviously less than 74 kDa, the total molecular weight of SA59B scFv-antibody (30 kDa) and HRP (44 kDa). Furthermore, the

creation of scFvs fused to other protein is believed to lead to the formation of dimers [16], which should have greater avidity and stability than original monovalent scFvs, and can be used, for example, for detection purposes. At present, the laboratory tests that can be applied for routine diagnosis of SARS mainly include detecting viral genome by PCR involving in RT-PCR [19–22] and RT-LAMP [23], and detecting serum antibodies using SARS-CoV from Vero cell culture by ELISA [24–26], IFA [27–29], and NT [2]. However, there is little progress in detection of SARS-CoV using scFv-antibodies. So, we anticipate that SA59B scFv-antibody or SA59B-HRP described here will be useful in detection of SARS-CoV.

In addition, the direction of SARS research has now moved from identifying the virus and sequencing its genome to analyzing the viral proteins and their roles in virus replication and pathogenesis with the aim to develop new drugs and vaccines against SARS [30]. Fortunately, progresses are being made in the development of new drugs and vaccines against SARS although no antiviral treatments are currently available against SARS-CoV. For example, complete inhibition of cytopathic effects of SARS-CoV in culture was observed for interferon subtypes,  $\beta$ -1b,  $\alpha$ -n1,  $\alpha$ -n3, and human leukocyte interferon  $\alpha$  [31]; the in vitro efficacy of a recombinant human type I interferon IFN- $\beta$  1a (Serono International, Geneva, Switzerland) against three different isolates of SARS-CoV (Tor2, Tor7, and Urbani) using yield reduction assays was evaluated [32]; a three-dimensional model of the SARS-CoV-encoded 3 CL proteinase has been constructed to direct the design of protease inhibitors that may block coronavirus replication [32], and so on. Our scFv-antibodies obtained by us directed against purified SARS-CoV lysate may also be ideal vaccine candidates or effective molecule guiders for prophylaxis or therapy.

In summary, our study demonstrated that SA59B scFv-antibody or SA59B-HRP had a high binding specificity to SARS-CoV in ELISA and the potential applications for detection reagents, vaccines, and drugs against SARS-CoV were further discussed. Currently, efforts are underway to improve the sensitivity of SA59B-HRP ELISA assay and to further confirm its acting target(s) to develop their clinical applications.

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## References

- [1] A. Berger, C. Drosten, H.W. Doerr, M. Sturmer, W. Preiser, Severe acute respiratory syndrome (SARS)–paradigm of an emerging viral infection, *J. Clin. Virol.* 29 (2004) 13–22.
- [2] H.S. Wu, S.C. Chiu, T.C. Tseng, S.F. Lin, J.H. Lin, Y.F. Hsu, M.C. Wang, T.L. Lin, W.Z. Yang, T.L. Ferng, K.H. Huang, L.C. Hsu, L.L. Lee, J.Y. Yang, H.Y. Chen, S.P. Su, S.Y. Yang, T.H. Lin, I.J. Su, Serologic and molecular biologic methods for SARS-associated coronavirus infection, Taiwan, *Emerg. Infect. Dis.* 10 (2004) 304–310.
- [3] R.F. Breiman, M.R. Evans, W. Preiser, J. Maguire, A. Schnur, A. Li, H. Bekedam, J.S. MacKenzie, Role of China quest to define and control severe acute respiratory syndrome, *Emerg. Infect. Dis.* 9 (2003) 1037–1041.
- [4] G.P. Smith, Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface, *Science* 228 (1985) 1315–1317.
- [5] J. McCafferty, A.D. Griffiths, G. Winter, D.J. Chiswell, Phage antibodies: filamentous phage displaying antibody variable domains, *Nature* 348 (1990) 552–554.
- [6] G. Winter, Synthetic human antibodies and a strategy for protein engineering, *FEBS Lett.* 430 (1998) 92–94.
- [7] H.R. Hoogenboom, Designing and optimising library selection strategies for generating high-affinity antibodies, *TIBTECH* 15 (1997) 62–70.
- [8] H.R. Hoogenboom, A.P. de Bruine, S.E. Hufton, R.M. Hoet, J.W. Arends, R.C. Roovers, Antibody phage display and its applications, *Immunotechnology* 4 (1998) 1–20.
- [9] A.B. Sparks, N.B. Adey, L.A. Quilliam, J.M. Thorn, B.K. Kay, Screening phage-displayed random peptide libraries for SH3 ligands, *Methods Enzymol.* 255 (1995) 498–509.
- [10] M. Hassan, E. Azzazy, W.E. Highsmith, Phage display technology: clinical application and recent innovations, *Clin. Biochem.* 55 (2002) 425–445.
- [11] B.H. Muller, F. Lafay, C. Demangel, P. Perrin, N. Tordo, A. Flamand, P. Lafaye, J.L. Guesdon, Phage-displayed and soluble mouse scFv fragments neutralize rabies virus, *J. Virol. Methods* 67 (1997) 221–233.
- [12] R.E. Bird, K.D. Hardman, J.W. Jacobson, S. Johnson, B.M. Kaufman, S.M. Lee, T. Lee, S.H. Pope, G.S. Riordan, M. Whitlow, Single-chain antigen-binding proteins, *Science* 242 (1988) 423–426.
- [13] I.M. Tomlinson, G.P. Cook, G. Walter, N.P. Carter, H. Riethman, L. Buluwela, T.H. Rabbitts, G. Winter, A complete map of the human immunoglobulin VH locus, *Ann. N.Y. Acad. Sci.* 764 (1995) 43–46.
- [14] F. Matsuda, E.K. Shin, Y. Hirabayashi, H. Nagaoka, M.C. Yoshida, S.Q. Zong, T. Honjo, Organization of variable region segments of the human immunoglobulin heavy chain: duplication of the D5 cluster within the locus and interchromosomal translocation of variable region segments, *EMBO J.* 9 (1990) 2501–2506.
- [15] G.P. Cook, I.M. Tomlinson, G. Walter, H. Riethman, N.P. Carter, L. Buluwela, G. Winter, T.H. Rabbitts, A map of the human immunoglobulin V (H) locus completed by analysis of the telomeric region of chromosome 14q, *Nat. Genet.* 7 (1994) 162.
- [16] D. Blazek, V. Celer, I. Navratilova, P. Skladal, Generation and characterization of single-chain antibody fragments specific against transmembrane envelope glycoprotein gp46 of measles virus, *J. Virol. Methods* 115 (2000) 83–92.
- [17] K. Harper, R.L. Toth, M.A. Mayo, L. Torrance, Properties of a panel of single chain variable fragments against Potato leafroll virus obtained from two phage display libraries, *J. Virol. Methods* 81 (1999) 159–168.
- [18] R.A. Griep, C. Van Twisk, R.J. Kerschbaumer, K. Harper, L. Torrance, G. Himmler, J.M. Van der Wolf, A. Schots, pSKAP/S: an expression vector for the production of single-chain Fv alkaline phosphatase fusion proteins, *Protein Expr.* 16 (1999) 63–69.
- [19] K.W.C. Yam, H. Chan, L.L.M. Poon, Y. Guan, K.Y. Yuen, W.H. Seto, J.S.M. Peiris, Evaluation of reverse transcription-PCR assays for rapid diagnosis of severe acute respiratory syndrome associated with a novel coronavirus, *J. Clin. Microbiol.* 41 (2003) 4521–4524.
- [20] P.M. Hawkey, S. Bhagani, S.H. Gillespie, Severe acute respiratory syndrome (SARS): breathtaking progress, *J. Med. Microbiol.* 52 (2003) 609–613.
- [21] D.S.C. Hui, M.C.H. Chan, A.K. Wu, P.C. Ng, Severe acute respiratory syndrome (SARS): epidemiology and clinical features, *Postgraduate Med. J.* 80 (2004) 373–381.
- [22] P. Tang, M. Louie, E.S. Richardson, M. Smieja, A.E. Simor, F. Jamieson, M. Fearon, S.M. Poutanen, T. Mazzulli, R. Tellier, J. Mahony, M. Loeb, A. Petrich, M. Chernesky, A. McGeer, D.E. Low, E. Phillips, S. Jones, N. Bastien, Y. Li, D. Dick, A. Grolla, L. Fernando, T.F. Booth, B. Henry, A.R. Rachlis, L.M. Matukas, D.B. Rose, R. Lovinsky, S. Walmsley, W.L. Gold, S. Krajden, Interpretation of diagnostic laboratory tests for severe acute respiratory syndrome: the Toronto experience, *Can. Med. Assoc. J.* 170 (2004) 47–54.
- [23] H.T.C. Thai, M.Q. Le, C.D. Vuong, M. Parida, H. Minekawa, T. Notomi, F. Hasebe, K. Morita, Amplification method for rapid detection of severe acute respiratory syndrome coronavirus, *J. Clin. Microbiol.* 42 (2004) 1956–1961.
- [24] M. Guan, H.Y. Chen, S.Y. Foo, Y.J. Tan, P.Y. Goh, S.H. Wee, Recombinant protein-based enzyme-linked immunosorbent assay and immunochromatographic tests for detection of immunoglobulin G antibodies to severe acute respiratory syndrome (SARS) coronavirus in SARS patients, *Clin. Diagn. Lab. Immunol.* 11 (2004) 287–291.
- [25] Y. Shi, Y. Yanping, P. Li, T. Kuang, L. Li, M. Dong, Q. Ma, C. Cao, Diagnosis of severe acute respiratory syndrome (SARS) by detection of SARS coronavirus nucleocapsid antibodies in an antigen-capturing enzyme-linked immunosorbent assay, *J. Clin. Microbiol.* 41 (2003) 5781–5782.
- [26] Z. Chen, D. Pei, L. Jiang, Y. Song, J. Wang, H. Wang, D. Zhou, J. Zhai, Z. Du, B. Li, M. Qiu, Y. Han, Z. Guo, R. Yang, Antigenicity analysis of different regions of the severe acute respiratory syndrome coronavirus nucleocapsid protein, *Clin. Chem.* 50 (2004) 988–995.
- [27] W.T. Chang, C.L. Kao, M.Y. Chung, S.C. Chen, S.J. Lin, W.C. Chiang, S.Y. Chen, C.P. Su, P.R. Hsueh, W.J. Chen, P.J. Chen, P.C. Yang, SARS exposure and emergency department workers, *Emerg. Infect. Dis.* 10 (2004) 1117–1119.
- [28] P.R. Hsueh, C.L. Kao, C.N. Lee, L.K. Chen, M.S. Ho, C. Sia, X.D. Fang, S. Lynn, T.Y. Chang, S.K. Liu, A.M. Walfield, C.Y. Wang, SARS antibody test for serosurveillance, *Emerg. Infect. Dis.* 10 (2004) 1558–1562.
- [29] G.M. Leung, P.H. Chung, T. Tsang, W. Lim, S.K.K. Chan, P. Chau, C.A. Donnelly, A.C. Ghani, C. Fraser, S. Riley, N.M.

- Ferguson, R.M. Anderson, Y.I. Law, T. Mok, T. Ng, A. Fu, P.Y. Leung, J.S.M. Peiris, T.H. Lam, A.J. Hedley, SARS-CoV antibody prevalence in all Hong Kong patient contacts, *Emerg. Infect. Dis.* 10 (2004) 1653–1656.
- [30] K.V. Holmes, L. Enjuanes, *VIROLOGY: The SARS coronavirus: A postgenomic era*, *Science* 300 (2003) 1377–1378.
- [31] E.L.C. Tan, E.E. Ooi, C.Y. Lin, H.C. Tan, A.E. Ling, B. Lim, L.W. Stanton, Infection in vitro with clinically approved antiviral drugs, *Emerg. Infect. Dis.* 10 (2004) 581–586.
- [32] L.E. Hensley, E.A. Fritz, P.B. Jahrling, C.L. Karp, J.W. Huggins, T.W. Geisbert, Interferon- $\beta$  1a and SARS coronavirus replication, *Emerg. Infect. Dis.* 10 (2004) 317–319.