Construction of immune human library and screening of neutralizing antibodies specific to glycoprotein of severe fever with thrombocytopenia syndrome virus

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Abstract

Background

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease circulated in eastern Asian countries including China, Japan and South Korea. Currently, there are no effective prophylactic or therapeutic measurements available in the clinical settings. Antibody-mediated prevention and treatment can be an effective complement to the clinical supporting strategies. Glycoprotein N (Gn) and Glycoprotein C (Gc) are two of the highly antigenic envelope proteins on the SFTS virus (SFTSV) surface and contain neutralizing epitopes that can induce neutralizing antibodies.

Methods

To obtain the neutralizing antibodies specific to glycoprotein of SFTSV, we used phage display technology to generate a human phage antibody library with ScFv format from the peripheral lymphocytes of 8 patients who had recovered from SFTS. The library was bio-panned against recombinant Gn and Gc proteins for four rounds to find their specific antibody clones. Finally, the selected clones were characterized by binding activity test, virus neutralization and Competitive ELISA.

Results

An immune human ScFv antibody library against SFTSV with high capacity and diversity was constructed. After 4 rounds of panning, 6 distinct clones were found. Of them, 5 were specific to Gn, whereas only 1 was specific to Gc. The immunofluorescence assay showed only three clones with Gn specificity called MAb 4A6, MAb 2B6 and MAb 1F2, respectively, could bind nature virion. All these clones showed broad neutralization activity against the SFTSV, and had different antigenic epitopes from MAb 4-5, a previous identified antibody clone.

Conclusions

Three new monoclonal antibodies described in our study could be used as potential agents in immunotherapy against SFTSV infection.

Background

Severe fever with thrombocytopenia syndrome (SFTS) is a tick-borne emerging infectious disease with fatality of up to 30% and its etiological agent proven to be a phlebovirus in Bunyaviridae called SFTS virus (SFTSV) [1]. SFTS were first reported in China, followed by Japan, South Korea with more evidences indicating its world-wide distribution[2]. The incidence has been annually increased in these countries since then [3,4,5]. Clinically it is characterized by the sudden onset of a fever, headache and gastrointestinal symptoms, and a decrease of whole white blood cell and platelet counts that gradually progressed into hemorrhage and multi-organ failure at the end stage [6]. Humans' primary infection have been considered to be the tick bites; however, cases of person-to-person transmission of SFTSV have also been reported [7,8].

There are no effective prophylactic or therapeutic agents available in nowdays, and posing threats to public health [9].

SFTSV is a member of the Phlebovirus genus in the Bunyaviridae. Like other members in this family, the L segment within its genome encodes the RNA-dependent RNA polymerase; the S segment which uses ambi-sense encoding strategy for two proteins: nucleocapsid protein (NP) and the nonstructural protein (NS). The M segment has an open reading frame (ORF) coding for a glycoprotein precursor in the order Gn and Gc [10], which are responsible for receptor binding and membrane fusion, respectively, mediating the whole process of virus infection. Gn and Gc are now the important targets for vaccine research [11], and can stimulate host to produce neutralizing antibodies.

Methods

Cell culture

293T cells and Vero cells (Invitrogen, USA) were retrieved from storage in liquid nitrogen and maintained in 12 mL DMEM medium containing with 100 U/mL of penicillin and 100 U/mL streptomycin, 0.2% sodium bicarbonate and 10% heat- inactivated fetal bovine serum at 37 °C under an atmosphere of 5% CO_2.

Virus strains

Table.1 lists the 6 SFTSV strains from 4 different provinces, Jiangsu, Anhui, Henan, and Zhejiang, in China. The pathogens were propagated at 37 °C in Vero cells at a multiplicity of infection (MOI) of 1.0 and cultivated for 7 days. Supernatants containing viral particles were harvested, aliquoted, and stored at –70 °C until use. 50% tissue culture infective doses ($TCID_{50}$) of working stocks of each strain were titrated on Vero cells. The operation of live virus was performed under bio-safety containment conditions.

Recombinant Gn and Gc protein expression and purification

The RNA was extracted from SFTSV-infected cell culture supernatant by TRIzol reagent (Invitrogen, USA). Gn and Gc encoding genes were amplified by one-step RT-PCR by using specific primers. After sequencing, they were cloned into expression vector *pcDNA3.1(*+), respectively. The recombinant vector *pcDNA3.1*-Gn and *pcDNA3.1*-Gc were transinfected into 293T cells for transient expression, respectively. The cultural supernatants containing recombinant glycoproteins were used to load nickel ion affinity column (GE Healthcare, USA) for histidine tag fusion protein purification. All operations were followed the instructions of manufacturer.

Construction of the human single chain fragment (ScFv) antibody library

Human lymphocytes were isolated from 8 convalescent SFTS patients in Jiangsu Province. The total RNA was extracted from lymphocytes by using an RNA purification kit (Qiagen, USA) and first-strand cDNA was synthesized (Invitrogen, USA) with oligo(dT). A protocol describing the ScFv antibody construction was strictly followed [12], and two parameters to define the library quality called diversity and size, were also characterized [13].

Bio-panning of library against recombinant SFTSV glycoprotein

For phage bio-panning, immunotube (Thermo, USA) was coated with Gn, and Gc proteins, respectively, at a concentration of 10 μg/mL. Amplified phage mixtures were then incubated in the coated tube for 1.5 h at room temperature, and the tube was washed ten times with phosphate-buffer saline with 0.05% Tween–20 (PBST). Bound phages were eluted with glycine–HCl (pH 2.2). After adjusted to pH 7.0 with Tris base, the eluted phages were used to infect fresh prepared *E. coli* XL1-Blue (OD_{600} = 0.8) for a new round of panning. After four rounds of panning, the single clone of eluted phages was picked up to produce phage antibody, and tested for their binding activity to Gn or Gc by phage-ELISA[14]. For the positive clones, the genes of V_H and V_L chains were sequenced, and their corresponding amino acid sequences were aligned.

Expression of human IgG1 whole molecule of positive clones

The vector for Gn-specific IgG1 antibody expression was established by cloning the V_H and V_L region of the positive clone, respectively, into pCAGGS, an eukaryotic vector, and then transfected into 293T cells for transient expression at 37 °C for 72 h in atmosphere of 5 % CO_2 [15]. The supernatant containing recombinant human IgG1 was purified by Protein G column (GE Healthcare, USA) according to the manufacturer's instructions. The purified antibodies were kept at –20 °C until use.

Immunofluorescence assay

The SFTSV-infected and non-infected Vero cells were grown on an 24-well Millicell EZ slide (Millipore, Billerica, MA) for 48 h at 37 °C, and cells were fixed by treatment with paraformaldehyde for 30 min at room temperature. Anti-Gn IgG1 MAbs were incubated with the fixed cells for 1 h at 37 °C. Bound antibodies were stained using goat anti-human antibodies-fluorescein isothiocyanate (FITC) conjugate at 1:10 dilution at 4 °C for 30 min, and observed under an immune-fluorescence microscope. mAb 4–5[16] and an irrelevant human IgG1 were used as positive and negative control, respectively.

Virus neutralization test

For the binding-positive clones, 50 μl of anti-Gn IgG1 MAb (100 μg/ml) was mixed with an equal volume of SFTSV strain (JS–2010–014) suspension containing 100$TCID_{50}$, and incubated at 37 °C for 1 h. The virus-

antibody mixture was then transferred onto Vero cell monolayers in 96-well plates and incubated at 37 °C for 2 h. After being washed with minimal essential medium (DMEM), a maintenance medium, the cells were incubated for 48 h before fixation with paraformaldehyde for 30 min at room temperature. For detection of virus proliferation, the fixed cells were consecutively incubated with mouse anti-NP antibody (prepared in our laboratory) and goat anti-mouse antibodies-fluorescein isothiocyanate (FITC) conjugate as described elsewhere [17]. The plates were then observed under a microscope for fluorescence. To further determine their functionality coverage, each Gn-specific neutralizing MAb was evaluated against the six different SFTSV strains (Table. 1) respectively, by the method described above.

Competitive ELISA

Costar 96-well EIA/RIA Strip well immune-plate (Corning, USA) was coated with Gn protein at a concentration of 1 μg/mL by carbonate-bicarbonate buffer, pH 9.6. After washing and blocking, each Gn-specific neutralizing MAb with a concentration of 10 μg/mL was mixed with equal volume of MAb 4–5-HRP conjugate (prepared in our laboratory), and 100 μl of the mixture was added to the well. The plate was incubated for 30 min at 37°C and washed 5 times, and 100 μL per well of tetramethylbenzidine (TMB) substrate (Thermo, USA) was used for detection. The plate was incubated at room temperature for 5 min, and 50 μL of 1M H_2SO_4 was added to stop the reaction. Absorbance was read at 450 nm. MAb4–5, anti-SFTSV-NP MAb (prepared in our laboratory), and PBS was used as positive, negative, and blank control, respectively.

Results

Recombinant Gn and Gc protein expression and purification

The recombinant Gn and Gc proteins were expressed in 293T cell mammalian host, respectively, and purified to homogeneity by Ni-based affinity chromatography (Fig.1A-B).

Construction of ScFv antibody library

V_H and V_L (including V_κ and V_λ) chain genes with a size of ~400 bp and ~350 bp, respectively, were amplified by PCR from the mixed cDNA. The ScFv genes containing V_H, V_L gene, and the linker to connect them, with a size of ~750 bp were achieved by a second round of overlap PCR (Fig.2). The ScFv gene repertoires were cloned into phagemid pComb3XSS vector in *Sfi* I site. After 5 rounds of electro-poration, a human phage antibody library containing 2.8×10^7 clones was constructed successfully. 45 clones were randomly selected and ScFv gene inserts were sequenced. The DNAMAN software analysis showed that 41 of their sequences were unique (Fig.3), with a theoretical diversity of 91% (41/45), indicating a good diversity of this library for panning.

Phage library bio-panning

For each target, Gn and Gc, 200 clones were randomly picked up from the fourth round eluted phages and tested for their binding activity by phage-ELISA. 17 of them showed high positivity against SFTSV-Gn protein (Fig.4), while 2 Gc-specific phage clones were ever found (Fig. 5). After fully sequenced, 5 distinct ScFv antibody clones with Gn-specific binding activity called MAb 4A6, MAb 1F5, MAb 2E9, MAb 2B6, MAb 1F2, and 1 ScFv antibody clone with Gc-specific binding activity called MAb 1A7,were obtained (Fig.6).

Immunofluorescence assay

The six clones in their full molecule format of IgG1 were tested for their binding activity in immunofluorescence assay. The SFTSV-infected Vero cells fixed to slides were moderately stained when treated by MAb 4–5 IgG1,MAb 4A6 IgG1, MAb 2B6 IgG1 and MAb 1F2 IgG1, respectively, while no fluorescence could be detected when treated by MAb 1A7 IgG1, MAb 1F5 IgG1 or MAb 2E9 IgG1. The irrelevant human IgG1 did not stain any infected cells (Fig. 7A-H), indicating only MAb 4A6 IgG1, MAb 2B6 IgG1 and MAb 1F2 IgG1 had binding specificity against SFTSV.

Microneutralization assay

The three clones including MAb 4A6 IgG1, MAb 2B6 IgG1 and MAb 1F2 IgG1, which showed specific binding in immunofluorescence assay, were further tested for functionality to neutralize virus *in vitro.* As shown in Fig. 8A-E, all the three clones demonstrated high neutralizing activity by exhibiting decreased fluorescence intensity, comparing to the negative control. Besides JS–2010–014, a Jiangsu isolate, these three clones can also neutralize SFTSV strains derived from Anhui, Henan and Zhejiang between 2010 and 2012, indicating their broad neutralization spectrum against this new emerging pathogen (Fig. 9).

Competitive ELISA

Compared to the positive control, these three antibodies didn't show any competition with MAb4–5, a well characterized human antibody, in reacting to Gn protein, demonstrating their binding epitope sites are unique to MAb4–5's (Fig. 10).

Discussion

In this study, a couple of human neutralizing antibodies against SFTSV have been developed and characterized. As a new emerging infectious disease, SFTS poses a high public health threat to its endemic areas including China, Japan and Korea. Additionally, since SFTSV, the etiological agent for this disease, is transmitted by a biological vector, tick, this pathogen possesses also a significant potential to spread to as yet non-endemic regions, just like Crimean-Congo Hemorrhagic Fever Virus, a similar virus in Bunyaviridae, which presents a world-wide transmission profile. Currently, vaccine is not yet available for prophylactic purposes. For clinical human cases, there is no any effective anti-viral agent for this disease besides the supporting treatment strategies. Immunotherapy, including antibody-based applications, has been an effective way for a broad range of conditions. Historically, convalescent sera from patients who recovered

from viral infection have been used to treat acute patients, and now several antiviral monoclonal antibodies are commercially available. Recent technical improvements in the discovery and manufacture of antibody have also allowed the development of therapeutic antibody product more rapidly and successfully to combat emerging infectious diseases [2,18,19].

Similar to the other virus, influenza virus, for instance, the glycoproteins anchored on the virion surface mediate the interaction between pathogens and susceptible receptor cells, and deliver the viral genome to host cell's cytoplasm to establish a steady infection. In this context, it is believed that Gn of SFTSV mainly mediates receptor attachment, while Gc facilitates the fusion of viral and cellular membranes by its conformational changes in a low-pH micro-environment[20]. Accordingly, antibodies pursue their anti-viral capabilities by blocking either viral Gn attachment to target cells, or Gc conformational changes required for fusion. However, in this study no any Gc-specific neutralizing antibody clones were ever found. To our best estimation, the recombinant Gc protein itself can't keep its conserved functional epitopes to maintain the Gn/Gc hetero-dimer structure without the Gn presence. Additionally, some functional sites with a hydrophobic property can be exposed only under the low-pH induced condition to fulfill membrane fusion event[21]. In this study, however, the bio-panning has been operated in a physiological similar condition, and the potential antibody clones for these antigenic sites conceivably have no chance to be selected out and enriched.

SFTSV is a single-stranded negative-sense tripartite RNA virus that is prone to mutation[22]. All the three antibody clones identified in this study showed broad neutralizing activities by covering different SFTSV strains isolated in 4 provinces in China, demonstrating that their binding epitopes should be conserved through the whole viral evolution process. The competitive experiment showed that the epitopes for these three antibody clones are unique to MAb 4–5, a clone screened out by our team previously. Additionally, the sequences of these three antibody clones are different, demonstrating their binding epitopes on Gn protein are disparate. The mixture of these noncompeting antibodies, when used in combination, can exert a synergistic effect and cover escape mutants.

For their efficacy and safety when used as prophylactic and therapeutic agent, these antibody clones have not been evaluated *in vivo,* due to the lack of appropriate animal models in China now.

Conclusions

Three human neutralizing monoclonal antibodies had broad neutralization activity against the SFTSV, were finally obtained, our data provide a rationale to develop antibody cocktails in future for human clinical SFTSV infection treatment.

Abbreviations

SFTS: Severe fever with thrombocytopenia syndrome; SFTSV: Severe fever with thrombocytopenia syndrome virus; ScFv: single-chain variable fragment; Gn: Glycoprotein N; G_C: Glycoprotein C; PCR: Polymerase chain reaction. MAb: monoclonal antibody.

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of Jiangsu Provincial Center for Disease Control.(approval number:JSCDCLL2014015). The written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that there are no conflicts of interest exist.

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Authors’ contributions

WSZ and YJJ conceived and designed the study. WSZ collected the data. WSZ, YJJ, XLG, YC implemented the study. WSZ prepared the manuscript. YJJ revised and finalized the manuscript. All of the authors read and approved the final version of the manuscript.

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Tables

Table 1. SFTS virus strains used in this study

Virus strain	Area	Year
JS-2010-014	Jiangsu	2010
JS-2012-035	Jiangsu	2012
JS-2010-006	Anhui	2010
JS-2011-034	Anhui	2011
HN01	Henan	2010
ZJZYT	Zhejiang	2010

Figures

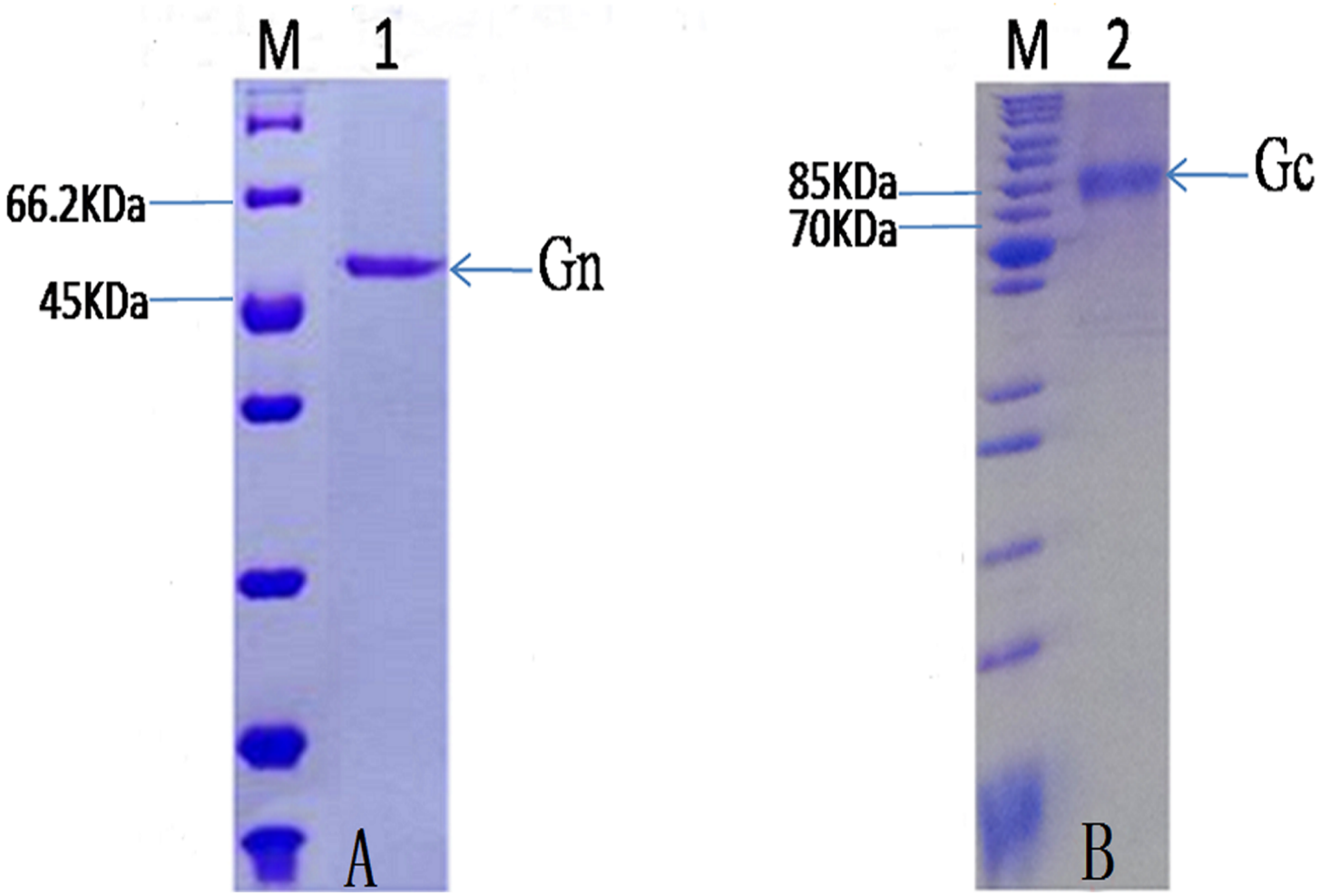

Figure 1

SDS–PAGE analysis of the purified recombinant Gn and Gc protein.A: Lane M, protein marker; lane 1, recombinant Gn protein.B: Lane M, protein marker;lane 2, recombinant Gc protein.

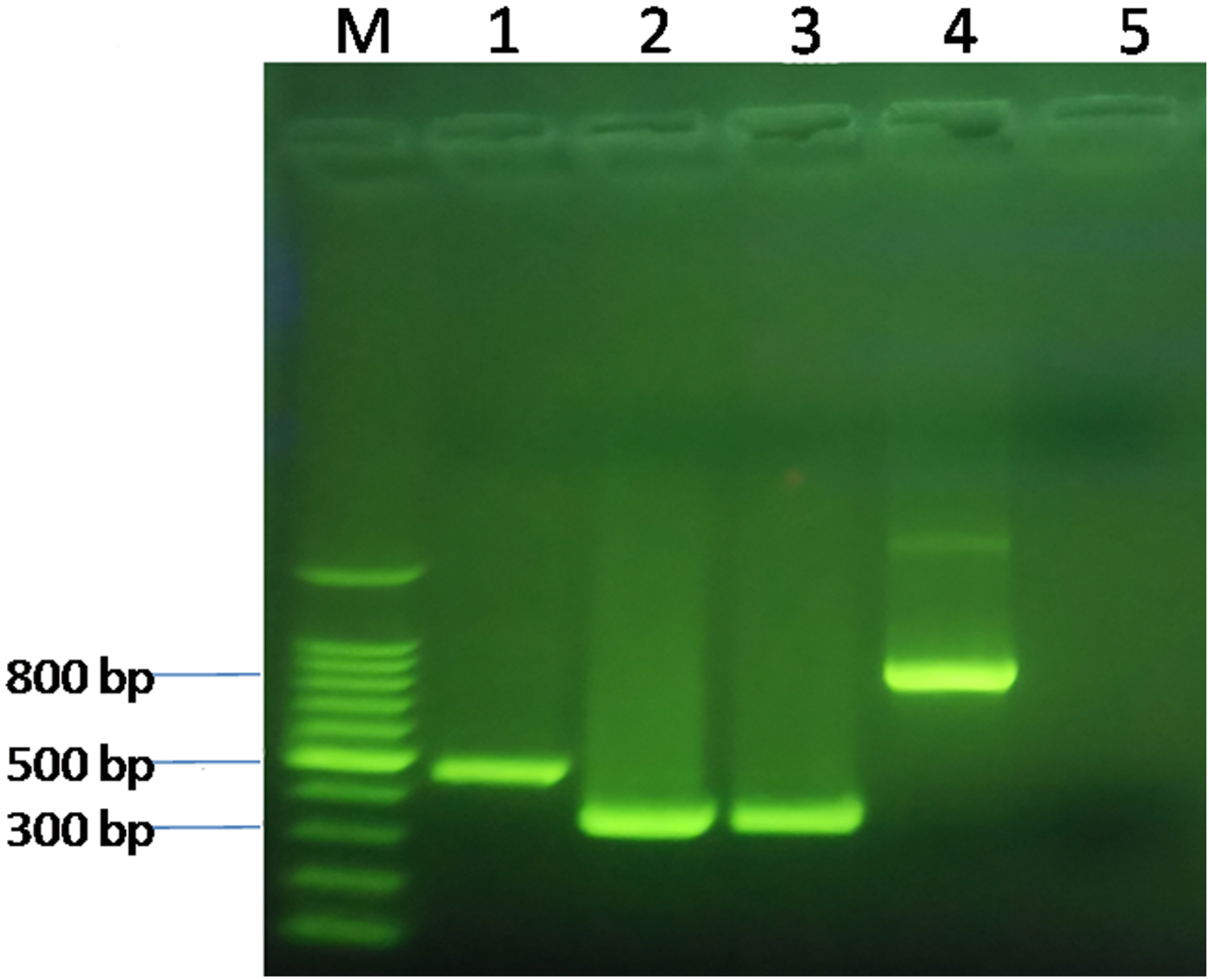

Figure 2

Agarose gels showing gene amplicons. Lane M, 100 bp DNA ladder; lane 1, VH gene amplicon (≈400 bp) ; Lane 2, Vκ gene amplicon (≈350 bp) ; Lane 3, Vλ gene amplicon (≈350 bp) ; Lane 4, ScFv gene amplicon (≈ 750 bp) ; Lane 5, negative control (template was water).

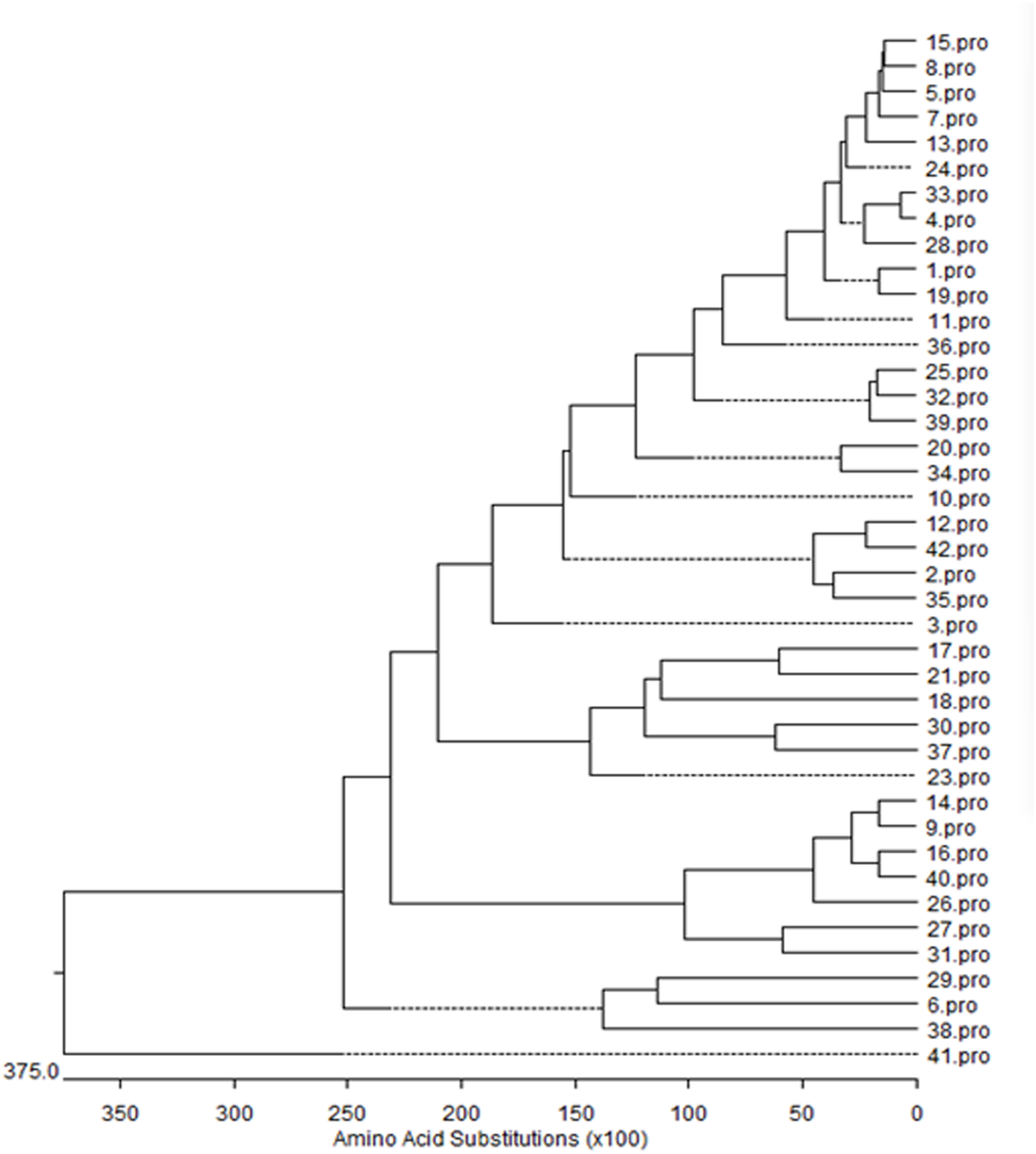

Figure 3

Evolutionary Tree Analysis of Amino Acid Sequences in Antibody Library.

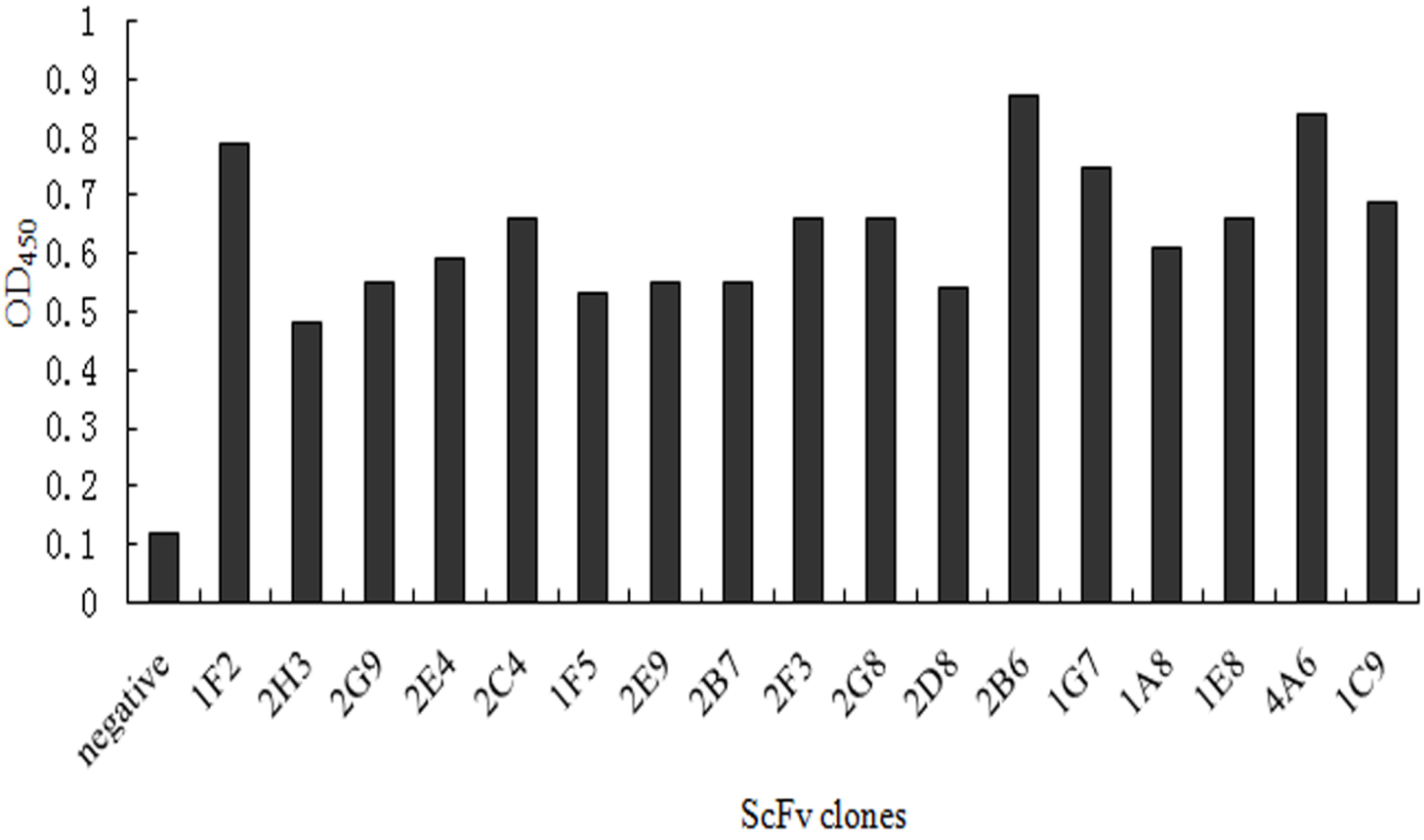

Figure 4

Phage-ELISA binding of ScFv antibodies to SFTSV-Gn protein.

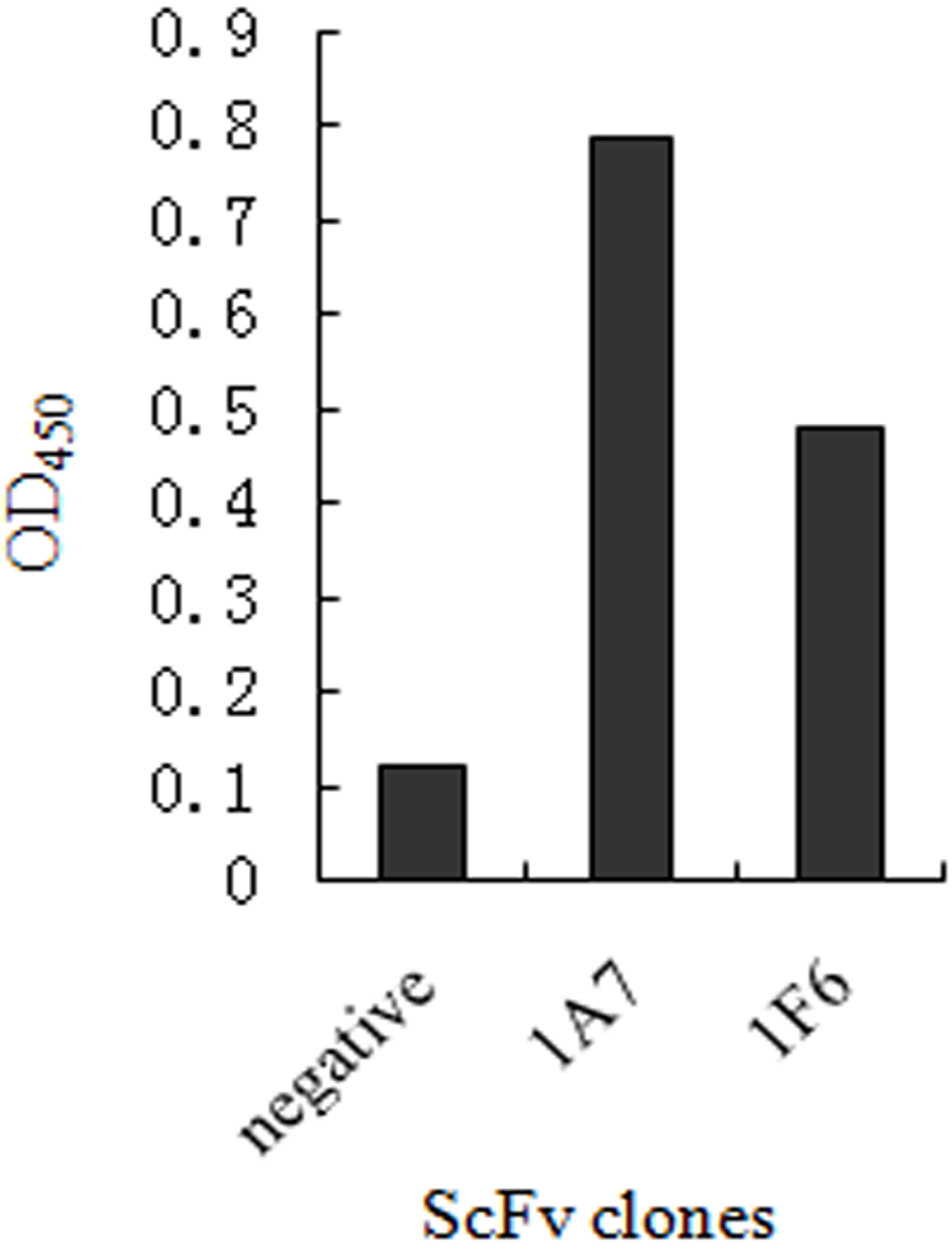

Figure 5

Phage-ELISA binding of ScFv antibodies to SFTSV-Gc protein.

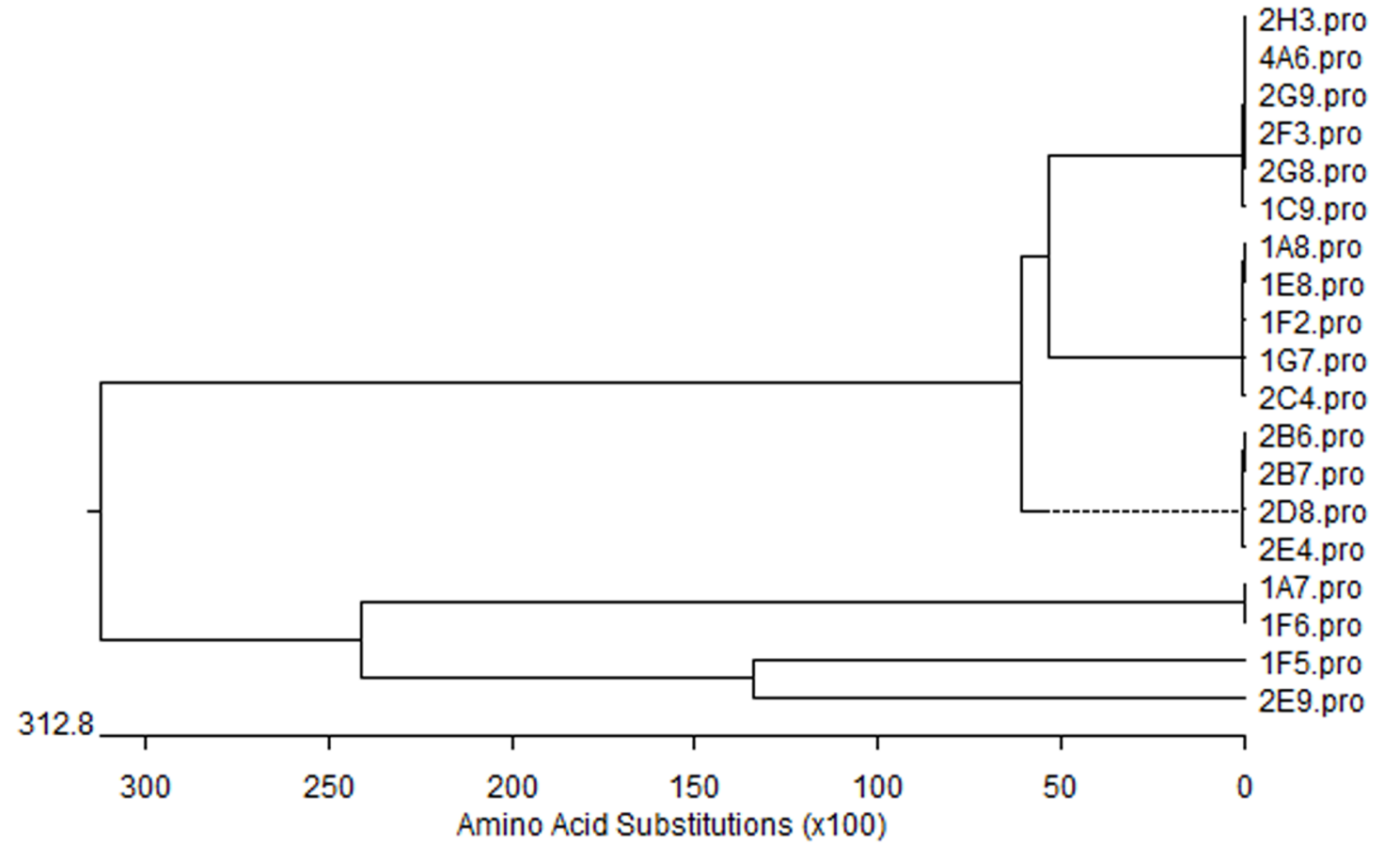

Figure 6

Amino acid Sequences analysis of the positive clones.

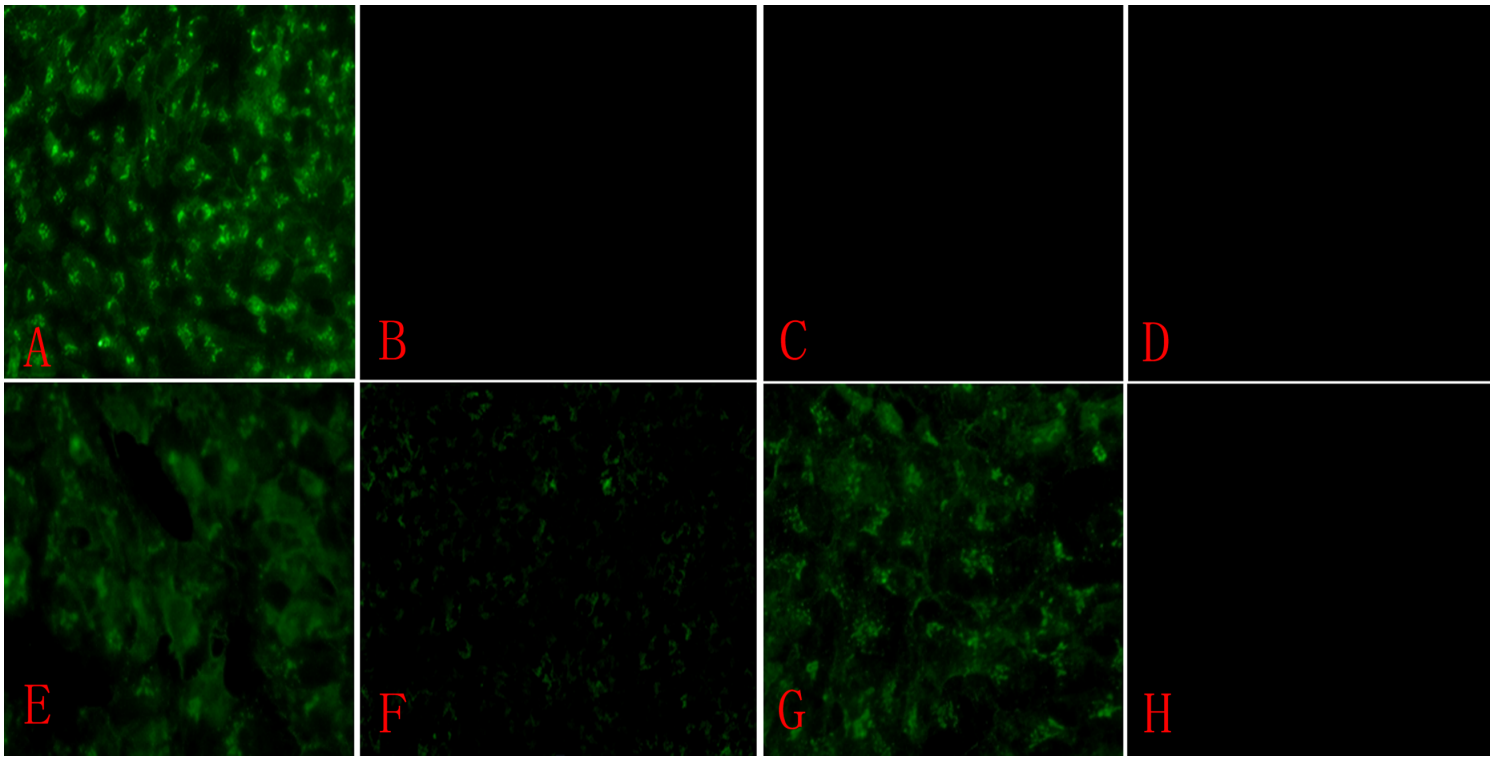

Figure 7

Characterization of IgG1 MAbs in IFA.A, Infected Vero cells were fixed on slides and incubated with MAb 4A6 IgG1; B, Infected Vero cells were fixed on slides and incubated with MAb 1A7 IgG1;C, Infected Vero cells were fixed on slides and incubated with MAb 1F5 IgG1;D, Infected Vero cells were fixed on slides and incubated with MAb 2E9 IgG1;E, Infected Vero cells were fixed on slides and incubated with MAb 2B6 IgG1; F, Infected Vero cells were fixed on slides and incubated with MAb 1F2 IgG1; G, Infected Vero cells were fixed on slides and incubated with MAb 4-5 IgG1;H, Infected Vero cells were fixed on slides and incubated with an irrelevant human IgG1.

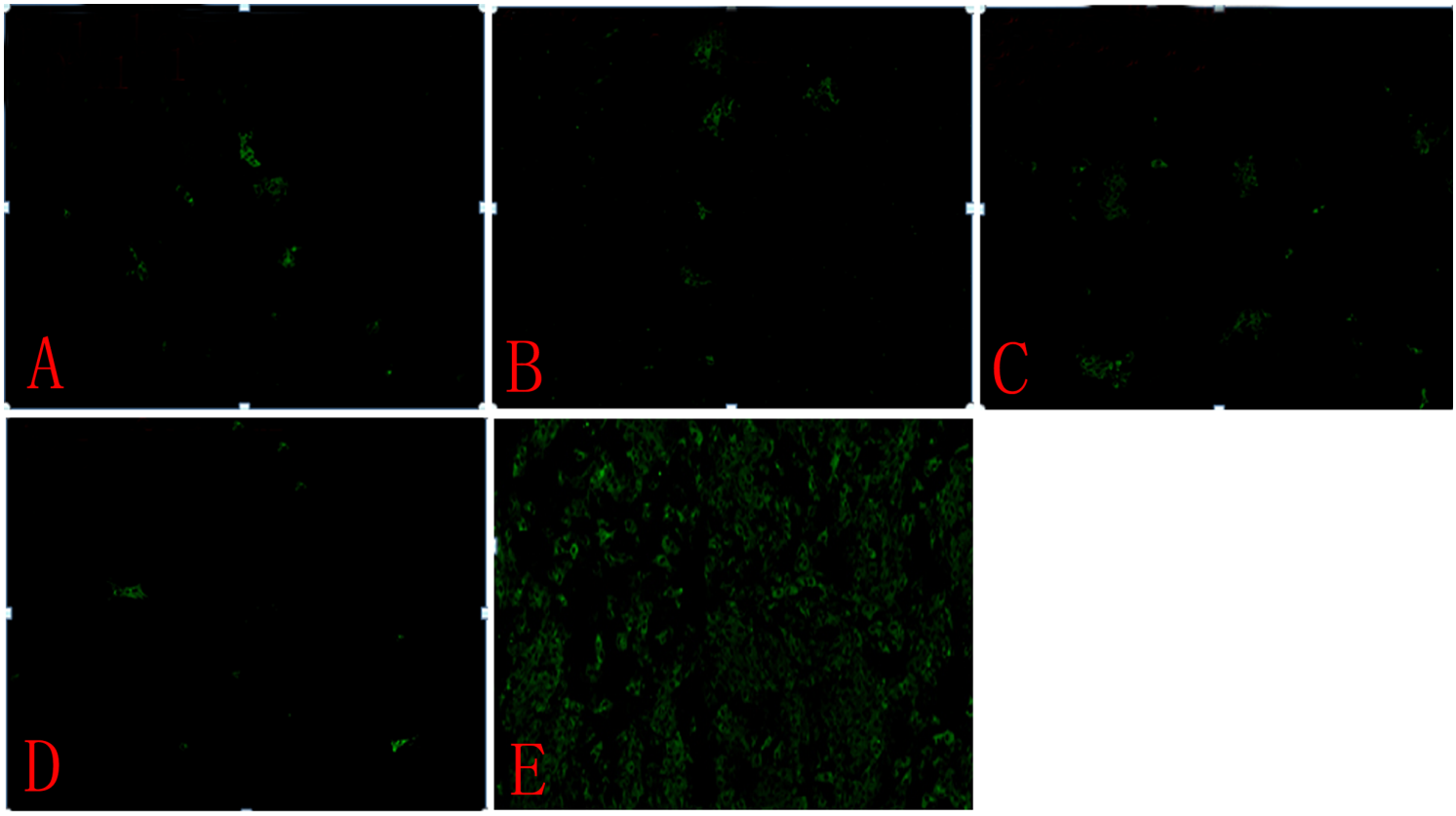

Figure 8

Analyses of the neutralizing activity of MAb 4A6 IgG1, MAb 2B6 IgG1 and MAb 1F2 IgG1 against SFTSV strain JS-2010-014.A, MAb 4A6 IgG1 was mixed with SFTSV strain JS-2010-014; B, MAb 2B6 IgG1 was mixed with SFTSV strain JS-2010-014; C, MAb 1F2 IgG1 was mixed with SFTSV strain JS-2010-014; D, MAb 4-5 IgG1 was mixed with SFTSV strain JS-2010-014; E, an irrelevant human IgG1 was mixed with SFTSV strain JS-2010-014.

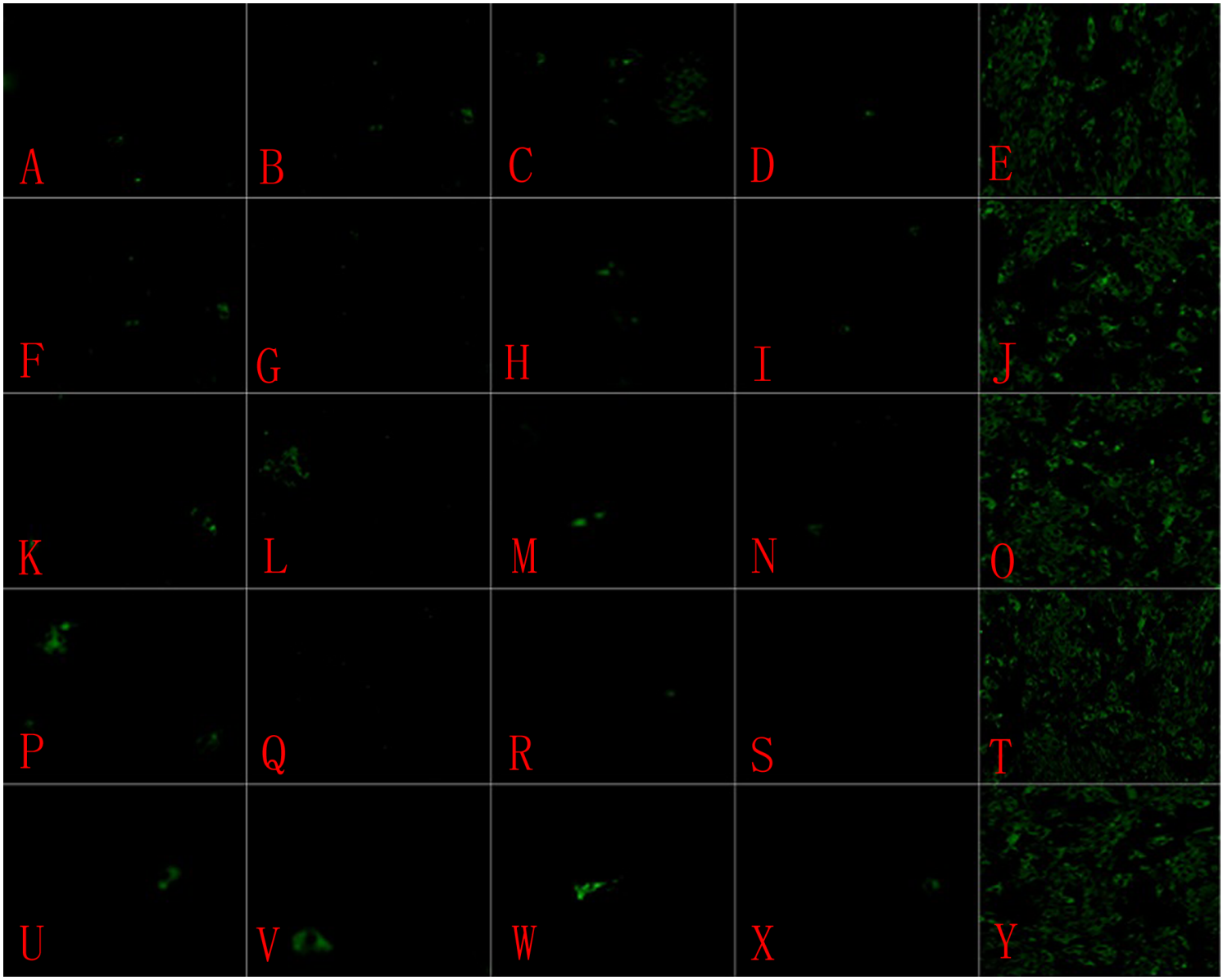

Figure 9

Analyses of the neutralizing activity of MAb 4A6 IgG1, MAb 2B6 IgG1 and MAb 1F2 IgG1 against SFTSV.A, MAb 4A6 IgG1 was mixed with SFTSV strain JS-2012-035; B, MAb 2B6 IgG1 was mixed with SFTSV strain JS-2012-035; C, MAb 1F2 IgG1 was mixed with SFTSV strain JS-2012-035; D, MAb 4-5 IgG1 was mixed with SFTSV strain JS-2012-035; E, an irrelevant human IgG1 was mixed with SFTSV strain JS-2012-035; F, MAb 4A6 IgG1 was mixed with SFTSV strain JS-2010-006; G, MAb 2B6 IgG1 was mixed with SFTSV strain JS-2010-006; H, MAb 1F2 IgG1 was mixed with SFTSV strain JS-2010-006; I, MAb 4-5 IgG1 was mixed with SFTSV strain JS-2010-006; J, an irrelevant human IgG1 was mixed with SFTSV strain JS-2010-006; K, MAb 4A6 IgG1 was mixed with SFTSV strain JS-2011-034; L, MAb 2B6 IgG1 was mixed with SFTSV strain JS-2011-034; M, MAb 1F2 IgG1 was mixed with SFTSV strain JS-2011-034; N, MAb 4-5 IgG1 was mixed with SFTSV strain JS-2011-034; O, an irrelevant human IgG1 was mixed with SFTSV strain JS-2011-034; P, MAb 4A6 IgG1 was mixed with SFTSV strain HN01; Q, MAb 2B6 IgG1 was mixed with SFTSV strain HN01; R, MAb 1F2 IgG1 was mixed with SFTSV strain HN01; S, MAb 4-5 IgG1 was mixed with SFTSV strain HN01; T, an irrelevant human IgG1 was mixed with SFTSV strain HN01; U, MAb 4A6 IgG1 was mixed with SFTSV strain

ZJZYT; V, MAb 2B6 IgG1 was mixed with SFTSV strain ZJZYT; W, MAb 1F2 IgG1 was mixed with SFTSV strain ZJZYT; X, MAb 4-5 IgG1 was mixed with SFTSV strain ZJZYT; Y, an irrelevant human IgG1 was mixed with SFTSV strain ZJZYT.

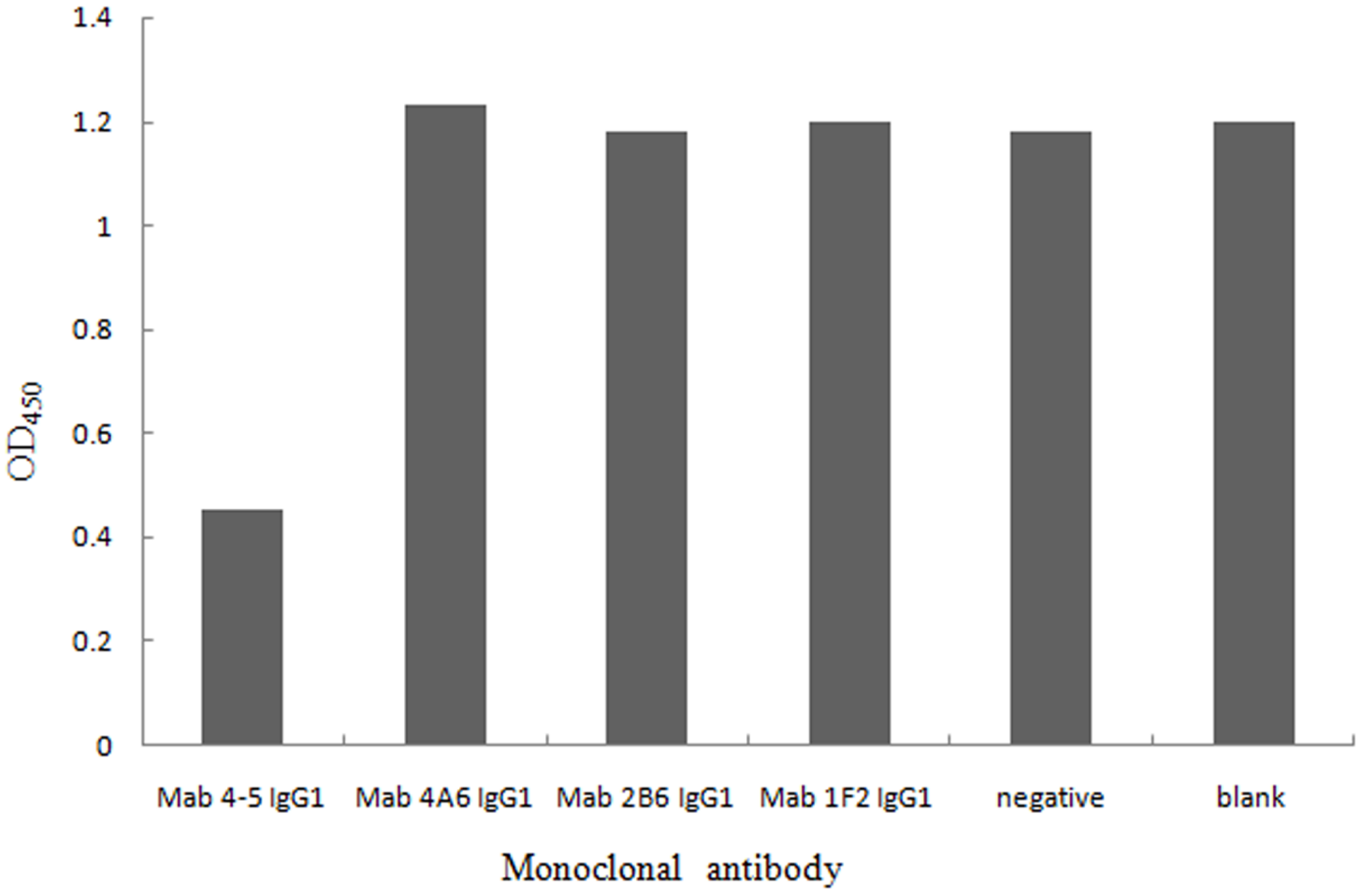

Figure 10

Analyses of binding epitope sites for these antibody clones.