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Original Article

# Generation and characterization of the human neutralizing antibody fragment Fab091 against rabies virus

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**Aim:** To transform the human anti-rabies virus glycoprotein (anti-RABVG) single-chain variable fragment (scFv) into a Fab fragment and to analyze its immunological activity.
**Methods:** The Fab gene was amplified using overlap PCR and inserted into the vector pComb3XSS. The recombinant vector was then transformed into *E coli* Top10F′ for expression and purification. The purified Fab was characterized using SDS-PAGE, Western blotting, indirect ELISA, competitive ELISA, and the fluorescent antibody virus neutralization test (FAVN), respectively, and examined in a Kunming mouse challenge model *in vivo*.
**Results:** A recombinant vector was constructed. The Fab was expressed in soluble form in *E coli* Top10F′. Specific binding of the Fab to rabies virus was confirmed by indirect ELISA and immunoprecipitation (IP). The neutralizing antibody titer of Fab was 10.26 IU/mL. The mouse group treated with both vaccine and human rabies immunoglobulin (HRIG)/Fab091 (32 IU/kg) showed protection against rabies, compared with the control group ($P<0.05$, Logrank test).
**Conclusion:** The antibody fragment Fab was shown to be a neutralizing antibody against RABVG. It can be used together with other monoclonal antibodies for post-exposure prophylaxis of rabies virus in future studies.

**Keywords:** rabies; Fab engineered antibody; neutralizing antibodies

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## Introduction

Rabies is one of the most fatal central nervous system diseases and is a threat to humans and other mammals. The rabies virus (RABV) belongs to the *Rhabdoviridae* family and constitutes the prototype of the lyssa viruses[1]. Rabies kills more than 50 000 people and millions of animals worldwide every year[2]. The progress of infection is rapid, and the mortality rate is nearly 100%. The glycoprotein of the rabies virus (RABVG) has been studied extensively for many years. It is a crucial protein for determining the neurovirulent nature of the rabies virus and is an important antigen for inducing protective immunity[3]. Among the different antibodies elicited after immunization, neutralizing antibodies specific to the RABVG are thought to provide protection[4]. We screened out a human anti-RABVG single-chain variable fragment (scFv) from an immune phage antibody library[5]. Based on the discrepancies between the native conformations of scFv and IgG, if the scFv was shown to be a neutralizing antibody, we would not consider the IgG, for scFv having the same neutralizing activity. In addition, the small molecular weight, short half-life, and expression type of the inclusion body also restrained the therapeutic application of the scFv[6].

In the present study, the scFv was transformed into a Fab fragment with a larger molecular weight and longer half-life. Fab has the same native conformation as IgG. Accordingly, exploration of the immunological activity of Fab will be helpful in preparing the human IgG. In this study, we transformed the human anti-RABVG scFv into a Fab fragment and to analyze its immunological activity.

## Materials and methods

The rabies virus strain CTN was provided by the Wuhan

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Institute of Biologic Products, Wuhan, China. The rabies virus strains (CVS-11 and CVS-24) and BHK-21 cells were obtained from the Veterinary Institute of the Academy of Military Medical Sciences, Changchun, China. The XL1-Blue and Top10F′ *Escherichia coli* strains were obtained from the Medical Research Council, Lab of Molecular Biology, University of Cambridge, Cambridge, UK. The plasmids pComb3XSS and pComb3Xλ were obtained from the Barbas Laboratory, TSRI, La Jolla, CA, USA. The horseradish peroxidase-conjugated goat anti-human IgG (Fab specific) was obtained from Sigma, St Louis, MO, USA. The competitive ELISA kit (20080526) was purchased from the Veterinary Institute of the Academy of Military Medical Sciences, Changchun, China. The Kunming mice were provided by the Medical College of Jilin University, Changchun, China. In addition, all the *in vivo* experiments were approved by the Ethics Committee on Laboratory Animals of Nanjing Medical University.

### Construction of human anti-RABV antibody Fab fragment

The human $V_H$ and $V_L$ genes were amplified from the anti-RABVG scFv plasmid by PCR. The forward primer of $V_H$ was $V_H$F: 5′-*GCTGCCCAACCAGCCATGGCC*CAGGTGCAGCTGGTGCAGTCTGG-3′, which contained 21 complementary bases to the reverse primer of human IgG1 $C_L$ (italicized). The reverse primer of $V_H$ was $V_H$R: 5′-*CGATGGGCCCTTGGTGGAGGC*TGAGGAGACGGTGACCAGGGTTCC-3′, which contained 21 complementary bases to the forward primer of human IgG1 $C_H1$ (italicized) for overlap PCR. The $V_L$ gene was amplified using the forward primer $V_L$F: 5′-GGGCCCAGGCGGCCCAGTCTGCCCTGACTCAGCCTCGCTCAGTGTCCGGG-3′, which contained the restriction endonuclease *Sfi* I site (underlined), and the reverse primer $V_L$R: 5′-*CGAGGGGGCAGCCTTGGGCTG*ACCTAGGACGGTCAGCTTGGTCCCTCCGCCGAAAACCAC-3′, which contained 21 complementary bases to the forward primer of human IgG1 $C_L$ (italicized) for overlap PCR. The human IgG constant domains $C_H1$ and $C_L$ were amplified from a recombinant vector pComb3Xλ. The PCR conditions were repeated for 25 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final extension time of 10 min at 72 °C. The genes of $C_H1$ (human IgG1) and $V_H$ were used as templates for generation of the Fd fragment by overlap PCR with a pair of primers — FdF: 5′-GCTGCCCAACCAGCCATGGCCCTCGAGGTGAAGCTGGTGGAGTC-3′ and FdR: 5′-AGAAGCGTAGTCCGGAACGTC-3′. The assembly of $V_H$ and $C_H1$ was performed by PCR for 15 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 2 min, followed by a final extension time of 10 min at 72 °C. Similarly, human $C_L$ and $V_L$ fragments were joined to generate the light chain with the pair of primers LF: 5′-GGGCCCAGGCGGCCGAGCTCGACATTGTGATGCACAGTC-3′ and LR: 5′-GGCCATGGCTGGTTGGGCAGC-3′. In the third round of PCR, the Fd and L chains were mixed in equal parts to generate the overlap full-length Fab products. The conditions for PCR was 6 cycles without primers at 94 °C for 50 s, 56 °C for 30 s, 72 °C for 3 min, and then 20 cycles with a pair of human primers — FabF: 5′-GGGCCCAGGCGGCCGAGCTCGACATTGTGATGACACAGTC-3′ and FabR: 5′-AGAAGCGTAGTCCGGAACGTC-3′ [7].

### Construction of recombinant plasmid

A phagemid pComb3XSS was used for expression of the Fab fragment[8]. The vector pComb3XSS and the Fab fragments were digested by the restriction endonuclease *Sfi* I (New England Biolabs, Ipswich, MA, USA)[9, 10] and ligated to create recombinants. The recombinants were transformed into competent *E coli* XL1-Blue cells by standard chemical methods ($CaCl_2$/heat shock)[11]. After overnight incubation, the clones were checked for the presence of the insert by colony PCR and DNA sequencing.

### Expression and purification of Fab fragment

The recombinant phagemid, which was confirmed to contain the correct sequence by DNA sequencing, was transformed into *E coli* Top10F′ for expression by way of soluble protein expression[12]. The cells were harvested by centrifugation, and the cell pellet was suspended in PBS. The periplasmic extract was obtained by sonication and centrifugation of the suspended products. Twenty microliters of the samples were used for denaturing polyacrylamide gel analysis. The gels were analyzed by staining with Coomassie blue and Western blotting. The Fab fragment was purified from the supernatant (150 mL) by affinity chromatography using a HisTrap HP column (1 mL, GE Healthcare, Piscataway, NJ, USA) with a flow rate of 1 mL/min. The binding buffer was 20 mmol/L of phosphate buffer (pH 7.4) with 20 mmol/L imidazole and 500 mmol/L NaCl. The Fab was eluted using 20 mmol/L phosphate buffer (pH 7.4) with imidazole at different concentrations (50, 100, 200, 300, 400, and 500 mmol/L). The eluted Fab fractions were concentrated using an Amicon Ultra centrifugal filter device (10 kDa cut-off, Millipore, Bedford, MA, USA) and dissolved in PBS. The purified Fab fragment was named Fab091.

### SDS-PAGE and Western blot analysis

The purified Fab091 fragment was resolved by 12% SDS-PAGE under reducing conditions with β-mercaptoethanol (β-ME). For Western blot analysis, Fab091 was detected by HRP-conjugated goat anti-human IgG (1:2000), and the blot was developed using the DAB/$H_2O_2$ system.

### Antigen binding assays for Fab091

#### *Indirect ELISA*

A 96-well microplate (Costar, Washington, DC, USA) was coated with 2 μg/mL of rabies virus strain CTN. The Fab091 fragment (0.2 mg/mL) and *E coli*-negative supernatant was added to antigen-coated wells in serial two-fold dilutions (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640), and bound antibodies were detected by HRP-conjugated goat anti-human IgG (1:5000). Each sample had two duplicate wells. PBS was used as a blank control, and its $OD_{450}$ value was assigned as 0.

### *Competitive ELISA (C-ELISA) of Fab091 and scFv*

Fab091 (0.5 mg/mL) was added into a 96-well microplate plate coated with RABV. Then the original anti-RABVG scFv was added at dilution ratios of 1:1, 1:5, 1:25, and 1:125 and incubated in these wells for 1 h at room temperature. After washing five times with PBST (20 mmol/L PBS, 0.05% Tween 20), HRP-conjugated mouse anti-human IgG (Fab specific) was added to the wells and incubated for 1 h at room temperature. An anti-Met scFv antibody[13] was used as a negative control in lieu of anti-RABVG scFv, and the blank control lacked scFv. The final results were expressed as percentages of inhibition (PI). The PI values for the Fab091 were calculated with the following formula:

$$PI = 1-(OD_b-OD_s)/OD_b\times 100\%$$

(s: sample, b: blank control)

A competitive ELISA kit was used to detect the neutralizing titer of Fab091. For the ELISA, the standard serum was obtained from volunteers who had been inoculated with rabies vaccine. The titers of neutralizing antibodies in the serum were detected by the FAVN method, and the serum was diluted to 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 IU/mL. The neutralizing antibodies in the ELISA were murine monoclonal antibodies against RABVG conjugated with horseradish peroxidase. One hundred microliters of HRP-conjugated antibodies against RABVG were mixed with 100 μL of different titers of standard serum (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 IU/mL) and added to a RABVG-coated ELISA microplate. Meanwhile, 100 μL of HRP-conjugated antibodies and 100 μL of Fab091 were mixed in the coated microplate. Every sample had two duplicate wells. Following incubation for 45 min at 37 °C, the plates were washed, and a tetramethylbenzidine (TMB) substrate solution was added. After incubation for 15 min at 37 °C, the reaction was stopped by adding 2.0 mol/L sulfuric acid, and the $OD_{450}$ was measured by a Multiskan Spectrum Microplate Photometer. The neutralizing antibody titer of Fab091 was calculated according to the $OD_{450}$ values and the titers of the standard samples[14, 15].

### *Immunoprecipitation (IP) and mass spectrometry (MS)*

A mixture of 20 μL of Fab091 and 40 μL of CTN was resuspended in 1 mL of PBS and incubated for 1 h at 4 °C. Protein G magnetic beads (30 μL) were added to the mixture for immunoprecipitation (IP). The mixture was whirled gently and incubated with agitation at 4 °C for 12 h. The beads were collected by centrifugation at 4000×*g* for 5 min and then washed three times with PBS. The supernatant was removed. Finally, the beads were resuspended with loading buffer and boiled, and the proteins were resolved by 12% SDS-PAGE. The resolved proteins were transferred onto a nitrocellulose membrane and detected by Rab-50 (sc-57994, Santa Cruz, CA, USA), which is a mouse monoclonal anti-RABVG antibody. After blocking in 5% milk in PBST for 30 min and washing three times with PBST, the membrane was incubated with the HRP-conjugated goat anti-mouse IgG (Sigma, St Louis, MO, USA). Finally, the blot was developed using the DAB/$H_2O_2$ system. The band that corresponded to the blot (67 kDa) on a separate polyacrylamide gel stained with Coomassie blue was analyzed by mass spectrometry (MS). Detected spots were excised from the gels, which were stained with Coomassie Brilliant blue. The gel chips were excised and destained with a solution containing 100 mmol/L $NH_4HCO_3$ and 50% ethane nitrile (pH 8.0). After hydrating with ethane nitrile and drying, the gel chips were hydrated in a minimal volume of trypsin (Promega Corporation, Madison, WI, USA) solution and incubated at 37 °C overnight. The gel pieces were extracted with 50% acetonitrile/2.5% trifluoroacetic acid at 37 °C for one hour with sonication, and the supernatant was removed. The extraction was repeated twice. A gel slice was dissolved in 0.1% trifluoroacetic acid (Sigma, St Louis, MO, USA), desalted, and concentrated using ZipTips (Millipore, Bedford, MA, USA). The peptide solution (0.5 μL) was mixed with 0.5 μL of matrix (5 mg/mL α-cyano-4-hydroxycinnamic acid in 30% acetonitrile/0.1% TFA), spotted on a target disk, and allowed to air-dry. Samples were analyzed by MS (Bruker Daltonics, Leipzig, Germany). Protein database searching was performed with the MASCOT search engine (http://www.matrix science.com; Matrix Science, UK)[16].

### Affinity analysis by surface plasmon resonance (SPR)

SPR was performed on a BIAcore T100 (GE, Piscataway, NJ, USA) analytical system. RABV was diluted to 5 μg/mL with acetate buffer (10 mmol/L sodium acetate, pH 5.5, GE, Piscataway, NJ, USA) and immobilized on the surface of a CM5 sensor chip (GE, Piscataway, NJ, USA) to capture purified Fab091. Fab091 was diluted by HBS-EP buffer (GE, USA) at concentrations ranging from 31.25 to 1000 nmol/L and performed at a constant flow rate of 30 μL/min for 3 min at 25 °C. The association time was 180 s, and the dissociation time was 600 s. The constants for association ($k_a$) and dissociation rate ($k_d$) and the equilibrium constant for dissociation ($K_D$, $K_D=k_d/k_a$) were obtained using global fittings from the Langmuir binding model (1:1 binding model). The sensor grams were evaluated using the BIAcore T100 evaluation software (GE, Piscataway, NJ, USA).

### Detection of Fab091 neutralizing activity

To perform the FAVN test, BHK-21 cells and the RABV strain CVS-11 were used. The positive control serum against RABV was obtained from adult dogs vaccinated with rabies vaccine. The neutralizing antibody titers were determined by the Weybridge Laboratory (UK), a rabies reference laboratory of OIE, the World Organization for Animal Health. The serum was then diluted to 0.5 IU/mL and used as a standard serum. Serial three-fold dilutions of the positive and negative control sera and the Fab091 were made in 100-μL volumes in microplate wells. Each sample was added to four adjacent wells and serially diluted five times using a multichannel pipette. Fifty-microliter challenge virus suspensions containing 100 $TCID_{50}$ were added to each well. The microplates were incubated for one hour at 37 °C in a humidified incubator with 5% $CO_2$. Following incubation, $2\times10^4$ BHK-21 cells in 50 μL of culture medium were added to each well, and the plates

were incubated for another 48 h. Thereafter, the medium was discarded, and the plates were rinsed in PBS (pH 7.2) and in acetone (80% in distilled water). The plates were then fixed in 80% acetone for 30 min at room temperature and air-dried. The staining was carried out by adding 50 μL of fluorescein-isothiocyanate-conjugated anti-rabies serum. After incubation for 30 min at 37 °C, the plates were washed twice with PBS. The FAVN result was assessed with a fluorescence microscope (image A.l; Zeiss, Germany). The total area of each well was examined. The well was considered positive if one or more fluorescent cells were observed; otherwise, it was considered negative. The 50% endpoint of the antibody ($D_{50}$) content of the test sample and virus titers ($TCID_{50}$) were calculated according to the Spearman-Karber method[17]. The neutralizing titer of Fab091 was calculated based on the number of negative wells and their dilutions, compared with those of the standard serum included in the test[18].

### Animals and inoculation

A lethal animal model mimicking rabies exposure was used as described elsewhere[19–21]. The mice were treated as described in Table 1. Four-week-old pathogen-free Kunming mice (10–12 g, 8 mice/group, 9 groups) were infected with 100 $LD_{50}$/0.05 mL CVS-24 on d 0 and d 7. The mice were inoculated in the hind limb with rabies vaccine (Sanofi-PASTEUR SA, France). In addition, human rabies immunoglobulin (HRIG, Taibang Health, Taian, China) at 20 IU/kg and Fab091 (treated with 32, 20, 8, 2, and 0.5 IU/kg) were administered on d 0. The mice were evaluated for 28 d for clinical signs of neurology and death. In the control group, the mice were injected with 100 $LD_{50}$/0.05 mL of the CVS-24 strain. The experiments using the CVS-24 strain were performed in a Biosafety Level 3 Laboratory (BSL-3)[22].

**Table 1.** Rabies virus postexposure prophylactic trial.

| Treatment | Vacc | Fab091+vacc | | | | | Fab091 | HRIG+ vacc | PBS |
|---|---|---|---|---|---|---|---|---|---|
| dose | A | B | C | D | E | F | G | H | I |
| (IU/kg) | 0 | 0.5 | 2 | 8 | 20 | 32 | 20 | 20 | 0 |
| Virus | + | + | + | + | + | + | + | + | + |
| Vaccine | + | + | + | + | + | + | – | + | – |
| Fab091 | – | + | + | + | + | + | + | – | – |
| HRIG | – | – | – | – | – | – | – | + | – |
| PBS | – | – | – | – | – | – | – | – | + |

+, disposed; –, undisposed; HRIG, human rabies immunoglobulin; PBS, phosphate-buffered saline.

### Statistical analysis

Kaplan-meier method was used for survival analysis of Kunming mice after rabies virus challenge. Statistical analyses were performed with SPSS 11.5 statistical software package (SPSS Inc, Chicago, IL, USA). A $P$ value <0.05 was accepted as statistically significant.

## Results

### Amplification of the human anti-RABV antibody Fab gene fragment and construction of the expression vector

The human anti-RABV antibody Fab gene fragment was amplified in three rounds of PCR, as described in Materials and methods. The cloning strategy is shown in Figure 1.

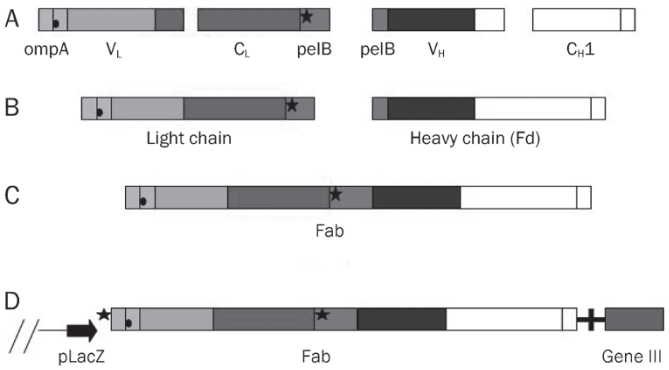


**Figure 1.** Construction of plasmid pComb3XSS-Fab for the production of Fab antibody fragments in *E coli*. Genes of light-chain and heavy-chain Fd fragments were fused by overlap-extension PCR, and cloned directionally by using two asymmetric sites of the rare cutter *Sfi* I. Fab was transcribed as a single transcript under the control of one LacZ promoter. The amber stop codon (cross) between the antibody genes and bacteriophage gene III enables the production of soluble Fab fragments in a non suppressor strain of *E coli*. (A) The genes for the variable and constant regions were amplified separately. (B) Heavy-chain Fd and light chain DNA were assembled by variable regions and their constant counterpart respectively by using overlap PCR. (C) Fd and light chain were fused to form Fab-encoding sequences by overlap PCR. Fab genes were directionally cloned into pComb3XSS phagemid by using the *Sfi* I site. (D) Both L chain fragment and Fd fragment were transported to the periplasm of *E coli*.

### Purification and detection of soluble Fab fragment

Purified Fab091 was resolved by 12% SDS-PAGE under reducing conditions. Both the Fd (34 kDa) and L chains (26 kDa) were detected in the monomeric form (Figure 2A). The expression of Fab091 was confirmed using Western blotting (Figure 2B).

### Specific binding of Fab091 fragment to RABV

Binding of the Fab091 fragment to RABV was detected by indirect ELISA as shown in Figure 3. The $OD_{450}$ values showed a gradient change that was accompanied by a decreasing concentration of Fab091.

In the competitive ELISA, as shown in Figure 4A, the PI of Fab091 ranged from 73.65% to 11.83% concomitant with the decreasing concentration of scFv. Less than 10% inhibition was observed for the anti-Met scFv negative control.

A standard curve for the competitive ELISA is shown in Figure 4B. The range of the standard curve was 0 to 8 IU/mL. If the $OD_{450}$ value was between 0 to 8 IU/mL, the neutralizing antibody titers could be read from the curve. In Figure 4B, the titers of standard serum decreased with increasing $OD_{450}$ val-

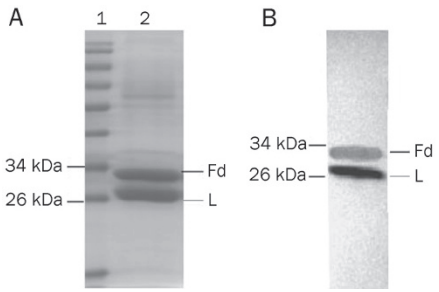


**Figure 2.** SDS-PAGE and Western blot of the purified Fab091 fragment after purification. The purified Fab091 was resolved in 12% SDS-PAGE under reducing conditions and stained with Coomassie blue. The heterodimer was dissociated into light chain (26 kDa) and Fd (34 kDa). (A) Lane 1: protein marker (#0671, Fermantas, Burlington, Ontario, Canada); Lane 2: the purified Fab091 fragment. (B) Electrobloted Fab091 fragments were detected by goat anti-human IgG-HRP conjugate (1:2000 dilution).

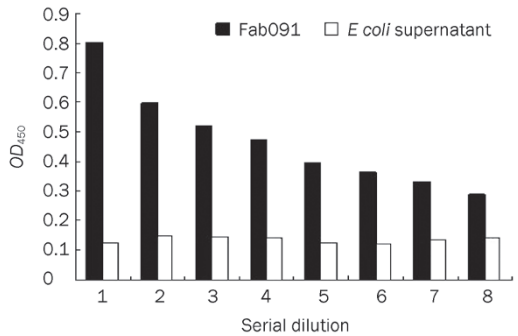


**Figure 3.** Indirect ELISA of the different dilutions of Fab091 fragment. The ELISA plate was coated with rabies virus strain CTN at 2 μg/mL. (1–8): The virus reacted with Fab091 which was in serial two-fold dilution (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640) and incubated for 2 h at room temperature. *E coli* Top10F′ supernatant was used as control. The plate was washed five times with PBST, followed by incubation with goat anti-human IgG HRP-conjugated (1:5000).

ues. The $OD_{450}$ of the test sample was 1.022, so the result was negative.

IP was also used for the detection of the specific binding of Fab to the rabies virus strain CTN (Figure 5A). Four peptide sequences (Table 2) matched with RABVG by MS analysis (Figure 5B) were found when the identified peptides were compared with the known sequences of RABVG in the SWISS PROT database. Mass tolerance was allowed within 0.05%.

**Table 2.** Amino-acid residue sequences of matched peptides.

| | Relative intensity | Amino-acid residue |
|---|---|---|
| 1 | 983.431 | TCGFVDER |
| 2 | 1070.575 | STQHGLGGTGR |
| 3 | 2118.972 | YEESLHNPYPDYHWLR |
| 4 | 2708.251 | YVLMSAGVLIALMLTIFLMTCCR |

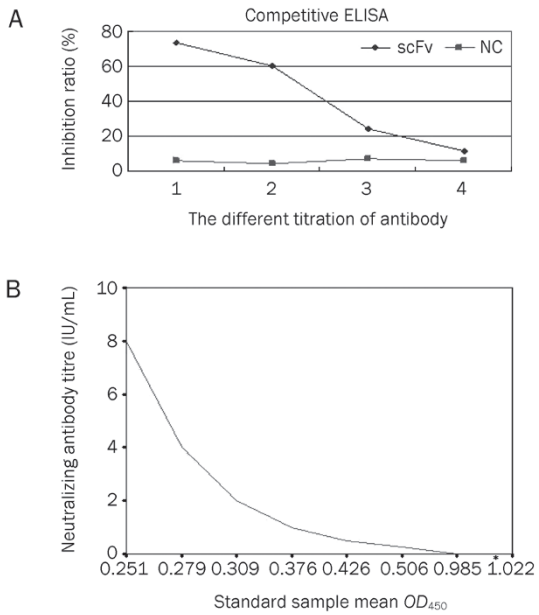


**Figure 4.** Competitive ELISA detection. (A) The rabies virus strain CTN was used as coated antigen for Competitive ELISA of scFv and Fab091. (1–4): The scFv was diluted to 1:1, 1:5, 1:25, 1:125, and incubated with Fab091. PI of Fab091 ranged from 73.65% to 11.83% (73.65%, 60%, 24.95%, and 11.83%) with decreasing concentration of scFv. Less than 10% inhibition was observed for negative anti-Met scFv. (B) Standard curve for Competitive ELISA: The 96-well microplate was coated with RABVG. HRP-conjugated antibodies against RABVG were mixed with different titers of standard serum (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 IU/mL). Meanwhile, HRP-conjugated antibodies and Fab091 were mixed in the coated microplate and incubated. The mean $OD_{450}$ value of Fab091 well was 1.022, according to the standard curve ranging from 0 to 8 IU/mL, so the corresponding titre was 0.

Protein matching with a MASCOT score of >69 was considered statistically significant ($P$<0.05).

### Affinity assay of Fab091

The binding affinities between rabies protein and purified Fab091 were analyzed by Biacore T100 (GE, Piscataway, NJ, USA). Fab091 had a high affinity with a $K_D$ of 6.268E-10 M. ($\chi^2$=1.09, U=5). The interaction between Fab091 and rabies virus is shown in Figure 6.

### Neutralizing activity of Fab091

In the FAVN, no fluorescence was seen in the wells until the standard serum was diluted nine times. There were still two negative wells when the serum concentration was at 1:27 dilution. For the test sample Fab091, no fluorescence was seen until the wells were diluted to 1:81 (12 μg/mL). There was only one negative well at 1:243 dilution. According to the dilution rates and the total number of negative wells, compared with the standard serum (0.5 IU/mL), the neutralizing antibody titer of Fab091 was calculated to be 10.26 IU/mL.

### Activity identification *in vivo*

The number of deaths observed in Kunming mice after infec-

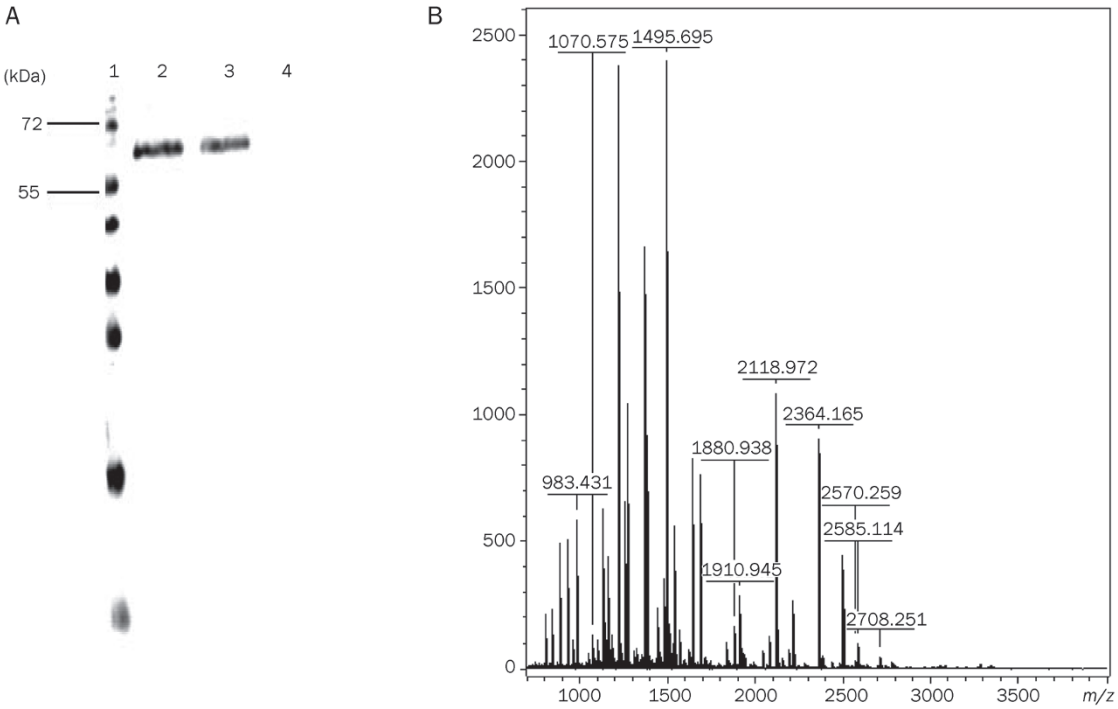


**Figure 5.** IP and MS analysis of RABVG. (A) Proteins immunoprecipitated by Fab091 were separated by SDS-PAGE and probed with RAB-50 by Western blot. (1): Protein marker; (2–3): One protein was recognized by RAB-50. The molecular weight of the protein was about 67 kDa. (4): BHK-21 lysate was used as the negative control to replace Fab091 in the IP. (B) The corresponding 67 kDa band on the polyacrylamide gel was analyzed by mass spectrometry, which was identified as RABVG. MS spectrums of fragment ions were from the 67 kDa protein. Four major (*m/z*=983.431, 1070.575, 2118.972, 2708.251) ions were detected.

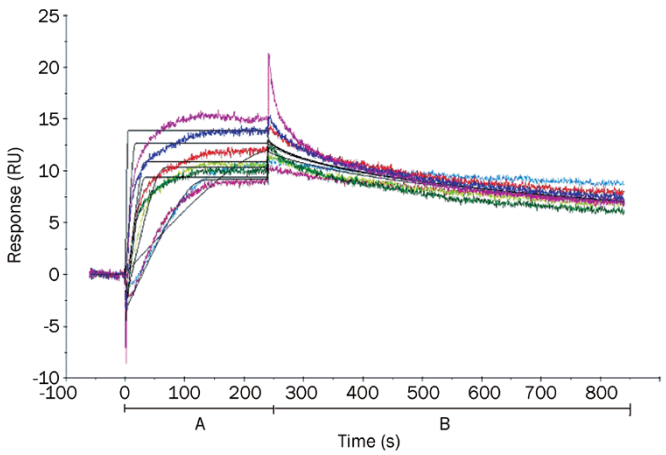


**Figure 6.** SPR analysis of Fab091 and rabies virus interaction. Fab091 (1 mg/mL) was subjected to SPR analysis. CM5 sensor chip was activated by the injection of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS). Rabies virus was coated on the CM5 chip, and the surface was washed with 10 mmol/L HEPES (pH 7.4) for 200 s and then Fab091 was injected. Dilution rates of Fab091 were 31.25, 62.5, 125, 250, 500, 1000 nmol/L ($Chi^2$=1.09, U=5, $R_{max}$=13.05 RU). (A) Association stage; (B) Dissociation stage.

tion with CVS-24 at 28 d is shown in Table 3, and a survival curve is shown in Figure 7 for the Kunming mice, according to the Kaplan-Meier method. A survival rate of 12.5% (1/8) was observed in the group with CVS-24 infection. A survival rate of 25% (2/8) was observed in the control group, which

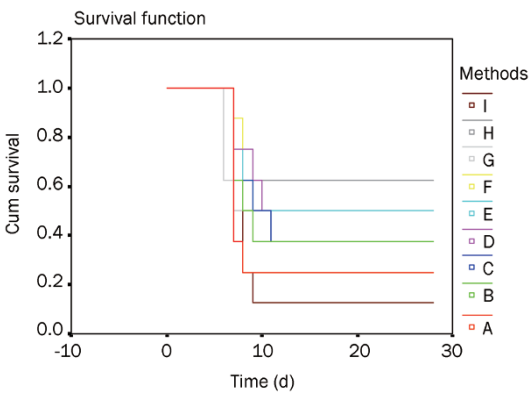


**Figure 7.** Kaplan-Meier survival curve for Kunming mice after rabies virus challenge. Mice (*n*=8 per group) were challenged with CVS-24 strain on d 0. Three hours later (d 0), the mice in treatment groups were inoculated with rabies vaccine and treated either with 32, 20, 8, 2, 0.5 IU/kg Fab091 or 20 IU/kg HRIG. The treatment groups also included 20 IU/kg Fab91 group. The mice in control groups received only PBS. The mice were evaluated twice daily and were sacrificed when neurological signs appeared. Kaplan-Meier survival curves were shown for d 0 to d 28. (A) Vaccine; (B) Vaccine+0.5 IU/kg Fab091; (C) Vaccine+2 IU/kg Fab091; (D) Vaccine+8 IU/kg Fab091; (E) Vaccine+20 IU/kg Fab091; (F) Vaccine+32 IU/kg Fab091 ($P$<0.05, Logrank test); G: 20 IU/kg Fab091; H: Vaccine+20 IU/kg HRIG ($P$<0.05, Logrank test); I: PBS.

**Table 3.** Deaths of Kunming mice after infected with CVS-24 in 28 d.

| | Vaccine | | | Fab091+vaccine | | | Fab091 | HRIG+vaccine | PBS |
|---|---|---|---|---|---|---|---|---|---|
| Days | A | B | C | D | E | F | G | H | I |
| | 0 | 0.5 | 2 | 8 | 20 | 32 | 20 | 20 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 4 |
| 7 | 5 | 3 | 3 | 2 | 2 | 1 | 1 | 1 | 2 |
| 8 | 1 | 2 | 0 | 0 | 0 | 1 | 0 | 2 | 1 |
| 9 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| 10 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| 11 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 12–28 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

A: vaccine; B–F: vaccine+Fab091 (different dilution rates from 0.5 to 32 IU/kg); G: Fab091; H: vaccine+HRIG (20 IU/kg); I: PBS

received vaccine only. The group treated with vaccine and HRIG had a survival rate of 62.5% (5/8). The survival rates were 50% (4/8), 50% (4/8), 37.5% (3/8), 37.5% (3/8), and 25% (2/8) when the mice were treated with 32, 20, 8, 2, and 0.5 IU/kg Fab091, respectively, and vaccine. The groups that received vaccine and 32 IU/kg Fab091 (Figure 7) or vaccine and 20 IU/kg HRIG (Figure 7) were provided with a level of protection against the rabies virus compared to the PBS group ($P$=0.0485 and $P$=0.038, respectively; Logrank test).

## Discussion

Equine anti-RABV immunoglobulin (ERIG) and human anti-RABV immunoglobulin (HRIG) have been used for passive rabies immunotherapy. However, ERIG can lead to allergic reactions and blood diseases, and HRIG is expensive and often in short supply[23–25]. Recombinant DNA technology and bacterial expression systems used to obtain active antibodies were very attractive compared to hybridoma technology, and antibody molecules can be further engineered to increase their binding affinity. Smaller antibody fragments (such as Fab and scFv) can be effectively used for diagnostic and therapeutic application due to their low immunogenicity and better tissue penetration[26]. ScFv fragments are composed of $V_H$ and $V_L$ with a connecting peptide link. Fab fragments are more stable due to an additional domain-domain interface resulting from the $C_H1$-$C_L$ association. This structure resulted in higher affinity of purified antibodies[27–30].

Expression of heterologous proteins in bacterial systems can be influenced by many factors such as codon usage, DNA-protein interactions, regulatory factors for transcription and translation, and culture conditions. In the present study, the amber stop codon (cross) enabled the production of soluble Fab fragments in a non-suppressor strain of *E coli*. We optimized the expression conditions and found that Fab could be expressed in a soluble form at room temperature (22 to 25 °C). If the inducing temperature was 30 or 37 °C, Fab was most often expressed in the form of inclusion bodies. In the process of purification, it was found that the amount of purified Fd fragments was less than that of the light chain. This result was possibly due to the pH value of the binding or washing buffer used for purification being unsuitable for purification of the Fd fragment. Furthermore, we found that the Fab091 fragment was easily eluted by phosphate buffer with a concentration of 100 mmol/L imidazole and that the eluted protein had a higher purity. In Figure 3, $OD_{450}$ values showed a gradient change that was accompanied by a decreasing concentration of Fab091, which illustrated the high specificity of Fab091 against the RABV. Furthermore, IP and MS analysis results also demonstrated that Fab091 binds specifically to RABVG. The competitive ELISA indicated that Fab091 and the original scFv shared the same epitope. The neutralizing antibodies in the ELISA kit had different epitopes against RABVG from Fab091.

The most critical property of Fab091 was its neutralizing potency to inhibit the entry of CVS-11 into BHK-21 cells. A sufficient level of antibody to inhibit entry is considered to be 0.5 IU/mL, according to the recommendations of the World Health Organization and the Centers for Disease Control and Prevention[31]. Compared with the reported neutralizing concentrations of FabRV01 and FabRV02, which neutralized the CVS-11 at 146 μg/mL[32], Fab091 was used to neutralize the virus at a concentration of only 12 μg/mL. FabRV01, FabRV02, and FabRV03 were isolated from a recombinant immune antibody library, differing from the altered Fab091, which was from scFv. These results may provide insight into the neutralization activity of recombinant antibodies.

Vaccination alone in the *in vivo* study was not sufficient to protect mice from rabies, whereas the treatment of mice with both vaccine and HRIG (20 IU/kg)/Fab091 (32 IU/kg) provided better protection against rabies than vaccine alone ($P$<0.05, Logrank test). However, the survival rates of the

groups treated with vaccine and Fab091 (32 IU/kg) were lower than that of the vaccine and HRIG (20 IU/kg)-treated group. This result could be interpreted as follows. First, the *in vivo* clearance of RABV is a complicated process. Compared with small-molecular-weight antibodies, the IgG antibody (HRIG), with antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity, has a greater scavenging capacity *in vivo*[33, 34]. The neutralizing activity of Fab is usually less than that of full-length IgG because of the monovalence of the Fab fragments and the lack of Fc fragment on an IgG molecule. Human Fab may exhibit even greater functional activity when converted into IgG[35]. Second, HRIG consists of polyclonal antibodies from human plasma with a number of epitopes of RABVG, and the function of each epitope is independent of the others. Antibodies can effectively neutralize free virus by macrophage phagocytosis or complement-mediated cytotoxicity. However, Fab091, a monoclonal antibody, can only bind to a single epitope, limiting its ability to remove the virus.

In future studies, the Fab091 and human IgG Fc fragments could be fused and expressed in eukaryotic cells. The reconstitution of Fab091 to IgG is a strategy that could be used for future passive immunotherapy against RABV infection[36–39]. Although Fab091 is different from scFv and IgG in biological function, tissue penetration, and half-life, the three together will not only retain the same binding specificity but also play complementary functional roles. They, together with other monoclonal antibodies, in accordance with the WHO-recommended "cocktail" therapy, can be used for post-exposure prophylaxis of rabies virus.

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## Author contribution

Chen LI carried out the molecular biology experiments and drafted the manuscript. Jin ZHU designed the study. Zhen-qing FENG and Xiao-hong GUAN were involved in the design of the study, and Feng ZHANG, Hong LIN, Jin ZHU, Zhong-can WANG, Xin-jian LIU, Zhen-qing FENG, and Xiao-hong GUAN helped to draft the manuscript.

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