



Construction and characterization of porcine single-chain fragment variable antibodies that neutralize transmissible gastroenteritis virus *in vitro*

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Abstract

Transmissible gastroenteritis virus (TGEV) infection causes severe diarrhea in piglets and imposes a significant economic burden on pig farms. Single-chain fragment variable (scFv) antibodies effectively inhibit virus infection and could be a potential therapeutic reagent for preventing disease. In this study, a recombinant scFv antibody phage display library was constructed from peripheral blood lymphocytes of piglets infected with TGEV. The library was screened with four rounds of biopanning using purified TGEV antigen, and scFv antibodies that bound to TGEV were obtained. The scFv gene was subcloned into the pET-28a(+), and the constituted plasmid was introduced into *Escherichia coli* BL21 (DE3) for protein expression. All three scFv clones identified had neutralizing activity against TGEV. An immunofluorescence assay and western blot analysis demonstrated that two scFv antibodies reacted with the spike protein of TGEV. These results indicate that scFv antibodies provide protection against viral infection *in vitro* and may be a therapeutic candidate for both prevention and treatment of TGEV infection in swine.

Introduction

Transmissible gastroenteritis virus (TGEV) belongs to the genus *Alphacoronavirus* in the family *Coronaviridae* of the order *Nidovirales* [1]. It is an etiological agent of transmissible gastroenteritis (TGE), which causes watery diarrhea, dehydration and vomiting in pigs. Notably, neonatal piglets have a mortality rate of up to 100% following infection by TGEV [2, 3]. TGEV infection often results in significant economic losses for pig farms.

The TGEV genome is composed of positive-stranded RNA that is ~28.5 kb in length and encodes four major structural proteins: the spike (S) protein, the membrane (M) protein, the minor envelope (E) protein, and the nucleocapsid (N) protein [4, 5]. Among these, the surface protein S, which is also an integral membrane protein, is the major inducer of neutralizing antibodies in a host. It mediates the attachment of viral particles to host cells via binding to the cellular receptor porcine aminopeptidase N (pAPN) before cell invasion [6, 7]. Consequently, the S protein could be an excellent target for therapeutics that can block viral entry or vaccines that induce protective immunity against TGEV [8]. The TGEV S protein is artificially divided into the S1 domain (residues 1-790) and S2 domain (residues 790-1,383) [8, 9]. The S1 domain contains several neutralization epitopes, and four major antigenic sites (in the order C, B, D, A) are located in the S1 protein [10, 11].

Passive immunization with antibodies is a particularly effective method for protecting newborn piglets against TGEV infection, which can be achieved by immunizing pregnant sows with inactivated or attenuated vaccines [12]. Consequently, antibodies are produced in the colostrum or milk and newborn piglets are passively protected from TGEV when they suckle immune dams (via lactogenic immunity) [13, 14]. Previous studies have demonstrated

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that neonatal piglets that receive high titers of TGEV-specific antibodies in colostrum and milk are protected from TGEV-induced diarrhea [15, 16]. Therefore, oral antibody administration may represent an alternative strategy for the prevention and treatment of TGEV infection.

Genetically engineered recombinant antibody fragments are increasingly used in medical therapy of many diseases, such as enteric colibacillosis, influenza, and Glässer's disease [17–21]. Single-chain fragment variable (scFv) antibodies constitute one of the most commonly used types of genetically engineered antibodies for treatment of disease [22, 23]. The scFv antibody is a small engineered antibody (26–28 kDa) that includes a variable heavy (VH) chain and variable light (VL) chain linked by a flexible peptide linker. Compared with intact IgG molecules, scFv antibodies retain their antigen-binding capability despite removal of the constant regions and are genetically modified to increase affinity and specificity [24]. Moreover, scFv antibodies are easily expressed in a functional form in *Escherichia coli* [25]. scFv antibodies can neutralize various viruses, inhibit viral infection and protect hosts from infectious disease [26–28]. These studies indicate that scFv antibodies could act as a protective therapeutic treatment for virus infection.

In this study, a porcine antibody phage library was constructed from peripheral blood lymphocytes of TGEV-infected piglets. scFv antibodies that bound to TGEV were screened using the phage display method, and their TGEV neutralization capacity and specificity were determined. We identified and characterize porcine scFv antibodies that neutralize TGEV *in vitro*, and these might be promising therapeutic reagents for use in the prevention and treatment of porcine viral gastroenteritis.

Materials and methods

Cells, viruses, plasmids and antibodies

Swine testicle (ST) cells were purchased from Wuhan Boster Biological Technology Co. Ltd. The cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C. 293T cells (CRL-3216) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in DMEM supplemented with 10% FBS and 2 mM L-glutamine in a humidified atmosphere containing 5% CO₂ at 37 °C.

The swine transmissible gastroenteritis virus (TGEV, China strain, SHXB) (GenBank accession no. KP202848.1) stock was purchased from the Jiangsu Academy of Agricultural Sciences (JAAS) and propagated in ST cells.

The plasmid p3 × FLAG-TGEV-S was constructed in our laboratory using common cloning techniques. The nucleotide sequence of the S gene from the TGEV SHXB strain was amplified using a pair of primers: (i) SF (GGGGGCTAGCCCATGAAAAA ACTATTTG; *Nhe* I site underlined) and (ii) SR (CCCCGAATTCGTTTGTCTAATAA; *Xho* I site underlined). PCR products were inserted into the p3 × FLAG-CMV-14 plasmid (Sigma-Aldrich, St. Louis, MO, USA) and p3 × FLAG-TGEV-S was generated.

Horseradish peroxidase (HRP)-conjugated anti-M13 antibody (Abcam, Cambridge, MA, USA), HRP-conjugated anti-His Tag antibody (Abcam), fluorescein isothiocyanate (FITC)-conjugated anti-His-tag antibody (Abcam), HRP-conjugated goat anti-mouse IgG (Beyotime Biotech, Shanghai, China), FITC-conjugated goat anti-mouse IgG (Beyotime Biotech), and HRP-conjugated goat anti-pig IgG (Jackson ImmunoResearch, West Grove, PA, USA) were used in this study. Mouse anti-TGEV S monoclonal antibody 3D11 was kindly provided by associate Prof. Zhibiao Yang and stored at our laboratory. TGEV-negative serum was collected from a 1-month-old piglet uninfected with TGEV.

Preparation of TGEV antigen

Confluent monolayers of ST cells were infected with TGEV strain SHXB at a multiplicity of infection (MOI) of 0.01. After incubation for 1 h at 37 °C in DMEM without FBS, complete medium was added. When the cytopathic effect reached 80%, the culture fluids were harvested by centrifugation at 5,000 × *g* for 10 min. Viruses were purified using sucrose density gradient centrifugation as described previously and stored at -80 °C until use [29].

Phage library construction

A total of 10 TGEV serum-negative piglets (large white) were purchased from a local breeding farm after birth. Each piglet was fed with commercial sterile milk and water. Piglets grown to fourteen days old were infected orally with 1 × 10⁵ PFU of TGEV. All piglets developed diarrhea, lethargy, dehydration and weight loss. No mortality was observed, and these animals recovered beginning at 10 days postinfection. At four weeks postinfection, piglets were inoculated orally with 1 × 10⁷ PFU of TGEV, and none of the piglets developed diarrhea after challenge. Four mL of blood was collected from each piglet at two weeks after the second infection and used to isolate peripheral blood lymphocytes (PBL) using lymphocyte separation reagents (Beyotime). Total RNA was extracted from the PBLs using TRIzol Reagent (Invitrogen) and reverse transcribed to cDNA using a reverse transcription kit (Takara, Qingdao, China).

Heavy-chain variable region (VH) primers were designed to imitate the porcine VH gene sequence from the IMGT

database (<http://www.imgt.org>). The light-chain variable region kappa (VL κ) primer and light-chain variable region lambda (VL λ) primer were designed using data from the GenBank database (accession no. AF334738-AF334742 and accession no. NM_001243319, respectively). The VH and VL sequences were amplified using the cDNA as template and combined with a DNA sequence encoding a (Gly₄Ser)₃ peptide linker to form scFv fragments in an overlap PCR that was described by Wang *et al.* [30]. The primer sequences are listed in Table 1.

The scFv fragments were ligated into phagemid pCANTAB5e (Biovector Inc., Beijing, China), and the resultant recombinant DNA was used to transform *Escherichia coli* strain TG1 (Biovector) by electroporation. A portion of the transformed cells was plated onto an agar medium containing ampicillin (100 μ g/mL) and 2% glucose, and library sizes were estimated by counting the colonies on the plates. The remaining culture was infected with M13KO7 helper phage (Biovector). After overnight cultivation, the rescued phages were precipitated by addition of PEG/NaCl (20 % PEG 8000 and 15% NaCl). The precipitates were collected by centrifugation at 8000 $\times g$ for 15 min, suspended in phosphate-buffered saline (PBS), and used for biopanning.

Biopanning of the antibody phage library

The scFv antibody phage library was subjected to four rounds of biopanning against TGEV, as described in the standard protocol [31]. In the first round, a 96-well ELISA plate (Santa Cruz Biotech, Dallas, TX, USA) was coated with TGEV (20 μ g/mL) at 4 °C overnight and blocked with 5 % skimmed milk in PBS. Recombinant phages were added and incubated at 37 °C for 2 h and the plate was then washed with PBST (PBS containing 0.05 % Tween-20) to remove unbound phages. Bound phages were eluted from the plates with 100 μ L of 0.1 M glycine-HCl (pH 2.2), followed by

neutralization with 50 μ L of 1 M Tris-HCl (pH 8.8). Eluted phages were then used to infect *E. coli* TG1 and rescued by M13KO7 helper phage, and the generated phages were used for the next round of panning.

To select scFv antibodies with high affinity and specificity, the antigen concentration used was reduced to 10 μ g/mL, 5 μ g/mL and 2 μ g/mL in the following rounds of biopanning, and the number of washing steps was increased to 10, 15, and 20 times for stringent selection. Input and output phages for each round were quantified by counting the colonies on the plate, and the ratio of the output titer to the input titer was calculated.

Phage ELISA

Indirect ELISA was used to examine the phages that remained after several rounds of biopanning. TGEV antigens (2 μ g/mL) as determined by checkerboard titration or ovalbumin (10 μ g/mL) as a negative control in 0.1 M NaHCO₃ (pH 8.6) was coated onto an ELISA plate and incubated at 4 °C overnight. Subsequently, wells were blocked with 5% skimmed milk in PBST for 2 h, washed with PBS and then incubated with 50 μ L of phage solution in PBS at 37 °C for 2 h. Polyclonal serum against TGEV obtained from experimentally infected piglets and an unrelated scFv, ZW 88, were used as positive and negative controls, respectively [32]. After washing the plate five times with PBST, a horseradish peroxidase (HRP)-conjugated anti-M13 antibody, HRP-conjugated anti-His monoclonal antibody, or HRP-conjugated anti-swine IgG (1:3000 dilution) was added to each well, and the mixture was incubated for 2 h. The assays were detected by adding 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate to each well (Tiangen, Beijing, China) and stopped by adding 2 M H₂SO₄. The absorbance at 450 nm wavelength was measured using a plate reader (Biotek, Winooski, VT, USA).

Table 1 Primers used for the construction of the porcine antibody library

Primer	Sequence (5'-3')
VH-Back-linker	ATGGCCGAGGWGAAGCTGGTGGAGTCYGG
VH-For-linker	GGATCCACCACCGCCGAGCCACCGCCACCACGACTTCAACGCCTGG
VL κ 1-Back-linker	GGCGGTGGTGGATCCGGTGGCGGCGGGTCTGCCATYGTGCTGACCCAGASTCC
VL κ 2-Back-linker	GGCGGTGGTGGATCCGGTGGCGGCGGGTCTGAGACTCGTSATGACCCAGTCTCC
VL κ 3-Back-linker	GGCGGTGGTGGATCCGGTGGCGGCGGGTCTGAGCTGCGTGATACACAGTCTCC
VL κ -For-linker	CGTTTGAKYTCCAGCTTGGTCCC
VL λ -Back-linker	GGCGGTGGTGGATCCGGTGGCGGCGGGTCTCAGRCTGTGGTGACVCAGGAGCC
VL λ -For-linker	ACCGAGGACGGTCAGCTGGGTGC
VH-Backward(<i>Sfi</i> I)	<u>GCGGCCAGCCGGCC</u> ATGGCCGAGGWGAAGCTGGTGGAGTCYGG
VL κ -Forward(<i>Not</i> I)	<u>TTGCGGCCGC</u> CACGTTTGAKYTCCAGCTTGGTCCC
VL λ -Forward(<i>Not</i> I)	<u>TTGCGGCCGC</u> ACCGAGGACGGTCAGCTGGGTGC

The recognition sites for the restriction enzymes *Sfi*I and *Not*I are underlined. The linker sequence is indicated in bold

scFv expression and purification

The bound phage clones were selected for further analysis. The scFv fragments were ligated into the *Bam*HI and *Xho*I sites of the pET-28a(+). The ligation mixture was transformed into BL21 (DE3) competent cells (Tiangen). For protein expression, transformed *E. coli* were grown in 200 mL of 2× YT medium containing 50 µg of kanamycin per mL at 37 °C. When the optical density reached 0.6 at 600 nm, 1.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression. The soluble scFv proteins were extracted from the bacteria and further purified using Ni-NTA agarose resin (Merck, Madison, WI, USA). The eluted products were quantified using a BCA protein assay kit (Thermo Scientific-Pierce, Rockford, IL, USA) and analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Neutralization test

To determine whether scFv antibodies showed neutralization activity against TGEV, a plaque-reduction neutralization (PRN) assay was performed as described by Hofmann and Wyler [33]. Briefly, 100 µL of scFv antibody (2 mg/mL) was serially diluted twofold (1:20–1:2560) and pretreated with an equal volume of TGEV (1000 TCID₅₀/mL) at 37 °C. Serum from an uninfected piglet and an unrelated scFv, ZW88, were used as negative controls. One hour later, the mixture was transferred to an ST cell monolayer in a 24-well plate and then incubated for another 1 h. After washing with PBS, the cells were overlaid with 1% agar medium containing 0.5% TPCK-treated trypsin. When viral plaques became visible, the cells were fixed with 4% formaldehyde and stained with 0.5% crystal violet. The inhibition rate (in percent) for each scFv was calculated as follows: [1-(average number of plaques in the treated well/average number of plaques in the negative control scFv ZW 88 well)] × 100. Each scFv was tested in triplicate.

Immunofluorescence staining

Selected scFv antibodies were screened for the binding of TGEV-infected cells. For this, ST cells were grown to 60 to 70% confluence on coverslips and infected with TGEV at an MOI of 0.01 for 24 h. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 10 min. The cells were then incubated with 100 µL of purified scFv antibody (20 µg/mL), and an unrelated scFv, ZW 88, was used as a negative-control antibody. The bound scFv antibodies were visualized using FITC-conjugated anti-His-tag antibody (Abcam) and a Nikon Eclipse 80i fluorescence microscope. The 293T cells transfected with p3 × FLAG-TGEV-S were also analyzed by IFA using scFv antibody as the primary antibody at 48 h post-transfection.

Western blot analysis

ST cell lysates were harvested and lysed in RIPA lysis buffer (Beyotime). Equal amounts of protein were separated on SDS-PAGE gels and transferred to nitrocellulose filter membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 2 h at room temperature and incubated with primary antibodies (purified scFv antibody, unrelated scFv ZW 88, or TGEV-specific monoclonal antibody) and appropriate secondary antibodies (HRP-conjugated anti-His Tag antibody or HRP-conjugated goat anti-mouse IgG). Signals were detected using a SuperSignal West Pico Kit (Thermo Scientific, Inc., Waltham, MA, USA).

Evaluation of the specificity of purified scFv by ELISA

The specificity of the anti-TGEV scFv antibodies was determined using other porcine pathogens (see Table 2). Each well of a 96-well ELISA plate was coated with 100 µL of a 2 µg/mL suspension of virus or bacteria (n = 3), and purified soluble scFv antibodies were added to each well to evaluate their cross-reactivity with other pathogens. Wells coated with TGEV antigen were used as a positive control.

Table 2 Bacteria and viruses used in this study

Species	Strain	Source ^a
Transmissible gastroenteritis virus	SHXB	Jiangsu Academy of Agricultural Science
Porcine epidemic diarrhea virus	HLJBY	Northeast Agricultural University
Porcine respiratory syndrome virus	JXA1-R	Pulike Biological Engineering, INC.
Porcine circovirus type 2	WG09	Isolated strain
Enterotoxigenic <i>Escherichia coli</i> K88	C83902	CVCC
Enterotoxigenic <i>Escherichia coli</i> K99	C83912	CVCC

^aCVCC = China Veterinary Culture Collection Center, Beijing, China

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD). Statistical analysis was performed using Student's *t*-test, whereby $p < 0.01$ is considered to be statistically significant.

Results

Construction of a phage library and selection of anti-TGEV scFv antibodies

The lengths of VH-linker, VL-linker and scFv were approximately 390, 360 and 740 bp (Fig. 1A and B). Two porcine scFv phage libraries were constructed. The library size was

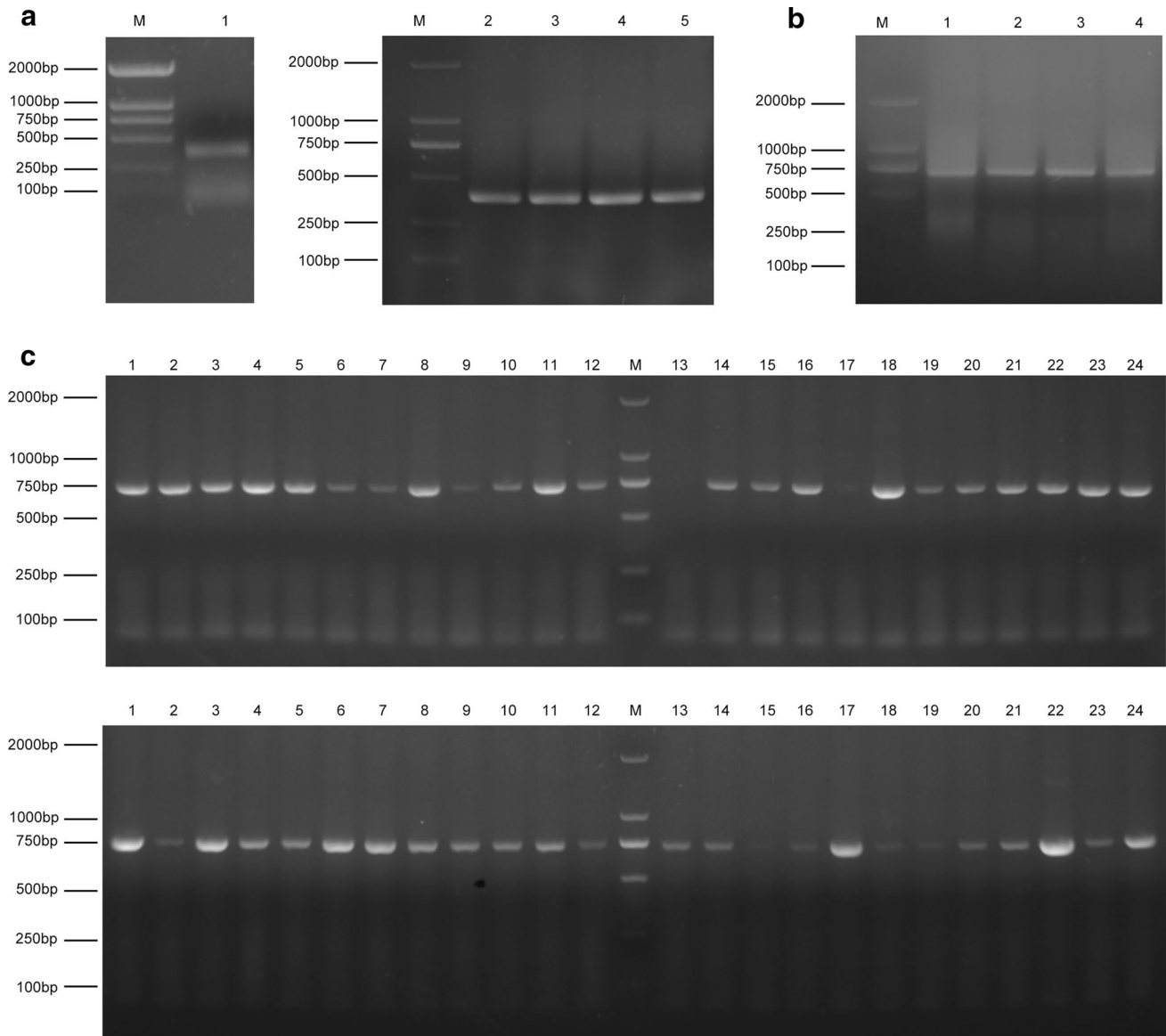


Fig. 1 Construction of the scFv antibody phage display library. (A) PCR amplification of VH-linker and VL-linker from cDNA of porcine peripheral blood lymphocytes. Lane M, 2000-bp DNA ladder marker (Takara); lane 1, VH-linker fragments; lane 2, VLκ1-linker fragments; lane 3, VLκ2-linker fragments; lane 4, VLκ3-linker fragments; lane 5, VLλ-linker fragments. (B) Lane M, 2000-bp DNA ladder marker (Takara); lane 1, assembled scFv genes containing VLκ1 fragments; lane 2, assembled scFv genes containing VLκ2

fragments; lane 3, assembled scFv genes containing VLκ3 fragments; lane 4, assembled scFv genes containing VLλ fragments. (C) PCR amplification of scFv genes from the phage display library for assessing insertion frequency. Lane M, 2000-bp DNA ladder marker (Takara); lanes 1-24, scFv genes containing VLκ fragments amplified by PCR; lanes 25-48, scFv genes containing VLλ fragments amplified by PCR

5.5×10^6 cfu/mL for scFv containing VL κ and 7.2×10^6 cfu/mL for scFv containing VL λ . Twenty-four clones were picked randomly from each library and subjected to PCR amplification in order to evaluate the efficiency of the library construction. The results showed that 23 out of 24 individual clones in the VL κ library contained an insert of the expected size of scFv. All 24 of the clones in the VL λ library contained an insert of the expected size of scFv (Fig. 1C). These results indicate that the porcine scFv libraries were successfully constructed.

The two porcine scFv antibody phage libraries (VL kappa library and VL lambda library) were mixed together, and the mixed library was used to screen scFv antibodies that bound to TGEV. In the first round of biopanning, the titer of the eluted phage was 5.75×10^4 cfu/mL (Table 3). Significant enrichment of the specific scFv phage antibody was observed after each round of biopanning, as indicated by the higher output/input ratio. In the fourth round of panning, the titer of output phage reached 10^7 cfu/mL, suggesting that specific bound phages were successfully enriched throughout the panning procedure.

In order to confirm the enrichment of TGEV-specific scFv, the pooled amplified phage from the fourth round of panning was tested by phage ELISA. Forty-eight clones were randomly selected from the library and screened. Three clones with relatively high binding affinity were selected for further analysis (OD_{450} value >2.5 times the negative control scFv ZW 88), and those were designated as TZZ 14, TZZ 19 and TZZ 43 (Fig. 2A). No TGEV binding was detected using the unrelated scFv ZW88.

Sequence analysis, expression and purification of selected scFv antibodies

The amino acid sequences of the scFv antibodies are listed in Fig. 2B. Sequence analysis of the three clones confirmed that the scFv VH gene fragments were connected with the VL gene fragments via a linker. The VH and VL domains each consisted of four framework regions (FRs) and three complementarity-determining regions (CDRs). The TZZ 14 contained a VL λ chain, while TZZ 19 and TZZ 43 contained VL κ chains. The amino acid sequences

of the three scFv antibodies showed that the FRs were highly conserved and that amino acid sequences in the CDR3 regions of both heavy and light variable chains were diverse.

SDS-PAGE analysis showed that the scFv antibodies had a molecular weight of ~30 kDa, were purified in a soluble form, and produced a single band (Fig. 2C). In western blot analysis, three scFv antibodies were recognized in the bacterial lysates by an anti-his monoclonal antibody and were absent in lysates of bacteria transformed with empty pET-28a (+) (Fig. 2D).

Neutralization test

To determine whether scFv antibodies neutralized TGEV infection, a PRN assay was performed. An unrelated scFv, ZW 88, and TGEV-negative serum were used as negative controls. Two serial dilutions of the scFv working stocks (1: 20-1: 2560) were tested in triplicate. The results showed that TZZ 14, TZZ 19 and TZZ 43 significantly inhibit TGEV infection in a dose-dependent manner ($P < 0.01$) (Fig. 3A). Notably, TZZ 14 and TZZ 19 completely neutralized virus replication at a dilution of 1:40 and 1:80, respectively (Fig. 3B). No neutralizing activity was observed in the negative controls. Taken together, these results demonstrate the neutralizing activity of TZZ 14, TZZ 19 and TZZ 43 against TGEV *in vitro*.

Identification of a viral protein recognized by scFv

IFA results showed that the scFv antibody binds to TGEV-infected cells, but not uninfected cells, indicating that it specifically recognizes a TGEV protein (Fig. 4A). Since the S protein of coronaviruses is the major inducer of neutralizing antibodies, it was investigated whether scFv antibody binds to S protein.

293T cells transfected with plasmid p3 \times Flag-TGEV-S were stained with TZZ 14 and TZZ 19, and specific fluorescence signals were observed in the cytoplasm. No distinct green signals were observed in cells transfected with S followed by incubation with TZZ 43 or ZW 88 (Fig. 4B). The reactivity of the selected scFv antibodies

Table 3 Enrichment of phages during each round of the biopanning process [31]

Round of screening	Coating antigen ^a (μ g/mL)	Input (cfu/mL)	Output (cfu/mL)	Output/Input (%) ^b
1 st	20	1.32×10^{12}	5.75×10^4	4.36×10^{-6}
2 nd	15	1.87×10^{12}	3.12×10^5	1.67×10^{-5}
3 rd	10	1.43×10^{12}	2.25×10^6	1.57×10^{-4}
4 th	5	1.12×10^{12}	2.78×10^7	2.48×10^{-3}

^aIn each round of biopanning, the amount of coating antigen was reduced to select ScFvs with high affinity

^bOutput/Input (%) = (Output number \times 100)/(Input number)

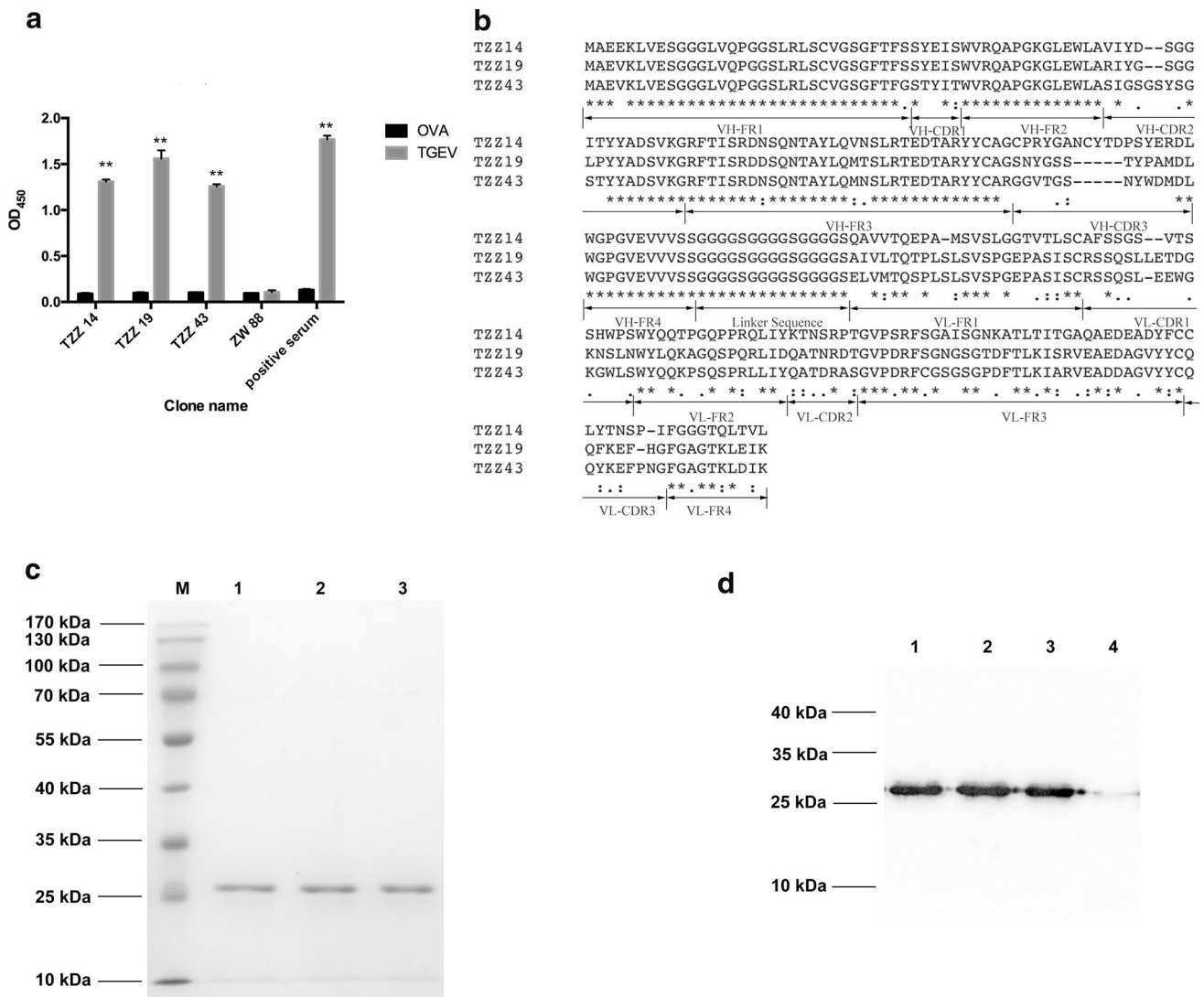


Fig. 2 Screening of scFv antibodies bound to TGEV. (A) Binding analysis of scFv antibodies to TGEV by phage ELISA. Forty-eight clones were randomly selected from the library and screened, and three clones (TZZ14, TZZ 19 and TZZ43) with relatively high binding affinity were identified. The data represent the means of three independent experiments; “***” represents a statistically significant difference ($p < 0.01$). Ovalbumin was served as a control antigen. Polyclonal serum against TGEV obtained from experimentally infected piglets and an unrelated scFv, ZW 88, were used as positive and negative control, respectively. (B) Amino acid sequences of

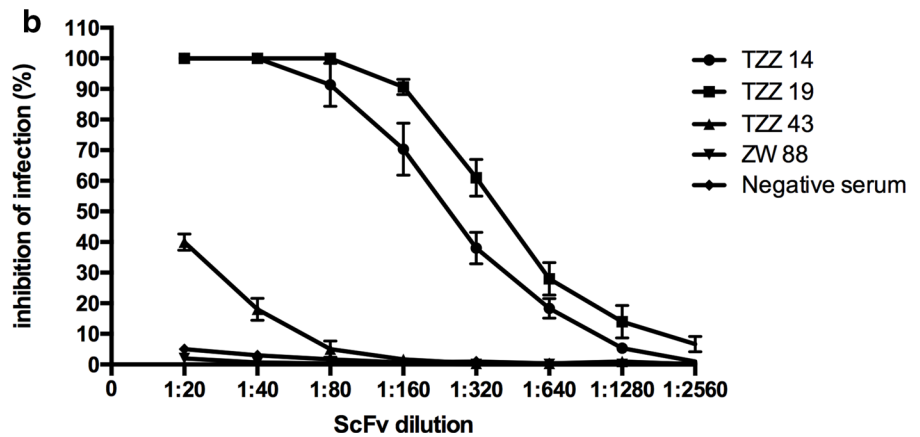
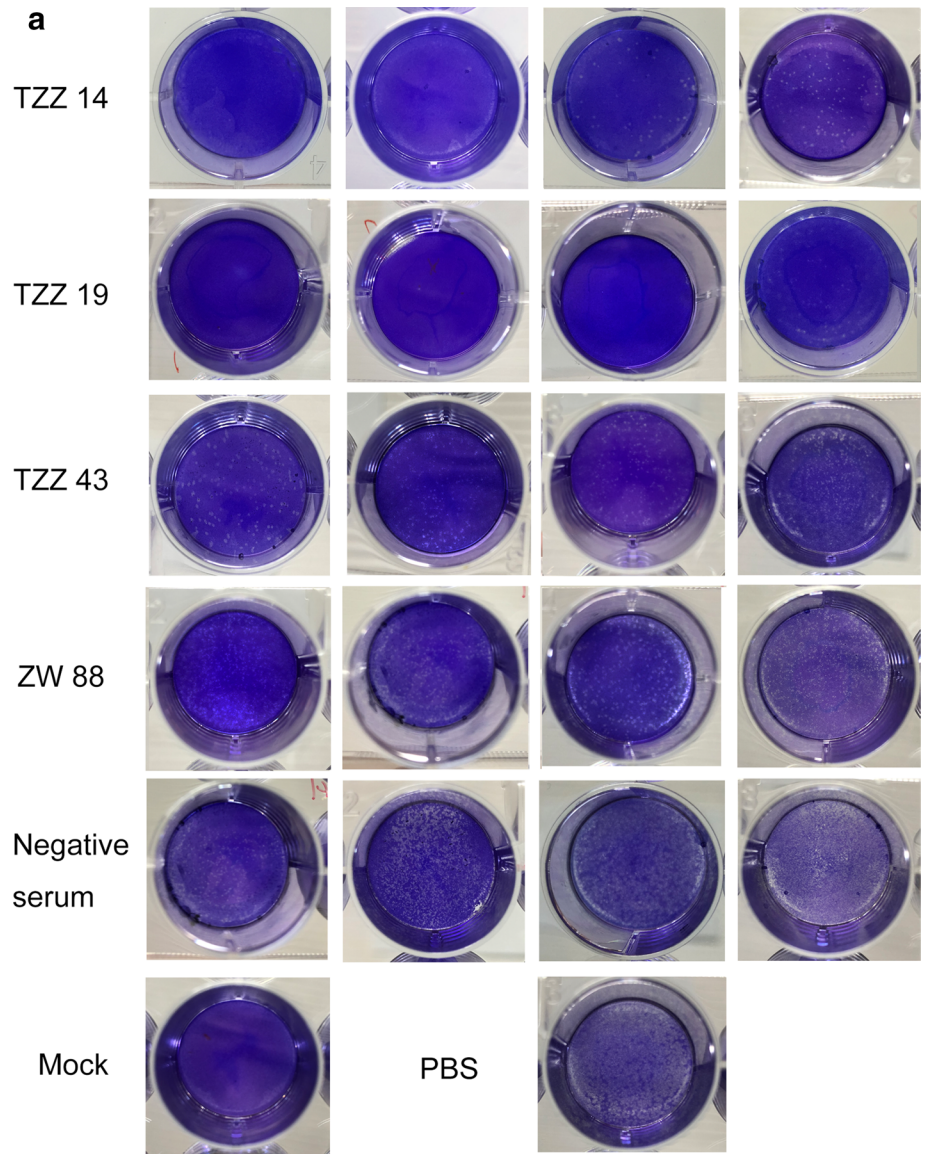
three scFv antibodies. A glycine-rich linker (G_4S)₃ that joins the VH fragment and VL is indicated. Sequences were aligned using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Dashes (-) indicate missing amino acids. (C) SDS-PAGE analysis of purified scFv antibodies against TGEV. Lane M, prestained protein ladder marker (Thermo Scientific); lane 1, purified scFv TZZ 14; lane 2, purified scFv TZZ 19; purified scFv TZZ 43. (D) Western blot analysis of purified scFv antibodies against TGEV. Lane 1, purified scFv TZZ 14; lane 2, purified scFv TZZ 19; lane 3, purified scFv TZZ 43; lane 4, *E. coli* BL21(DE3) transformed with pET-28a (+)

with the S protein was also evaluated using western blot (Fig. 4C). S protein was detected by TZZ 14, TZZ 19, and mouse anti-S monoclonal antibody, yielding a visible band at the predicted molecular weight (~160 kDa). S protein was not detected with TZZ 43 or ZW 88, and no band was observed on the NC membrane. These results indicate that TZZ 14 and TZZ 19 interact with the TGEV S protein.

Specificity of the scFv antibody

To confirm the specificity of the purified scFv antibodies, an indirect ELISA was performed. The results showed that PZZ 14, PZZ 19 and PZZ 43 specifically bind to TGEV with no cross-reactivity with other available porcine pathogens (including PEDV, PRRSV, PCV2), suggesting that scFv antibodies could be used in the diagnosis of TGEV infection (Fig. 5).

Fig. 3 Neutralizing activity of purified scFv antibodies. (A) The neutralization activity was measured by plaque reduction neutralization (PRN) assay on ST cells. Diluted scFv antibody was incubated with TGEV; this was followed by infection of ST cells. The scFv antibodies showed no affinity for TGEV and were used as a negative control. (B) The reduction in virus titer corresponded to changing scFv antibody concentration. scFv TZZ 14, TZZ 19 and TZZ 43 inhibited TGEV infection. An unrelated scFv ZW 88 and TGEV-negative porcine serum were used as a negative control. Inhibition rates (in percent) were calculated as follows: $[1 - (\text{plaques in treated wells}/\text{plaques in control scFv ZW 88 wells})] \times 100$. The means and standard deviations of the three experiments are shown



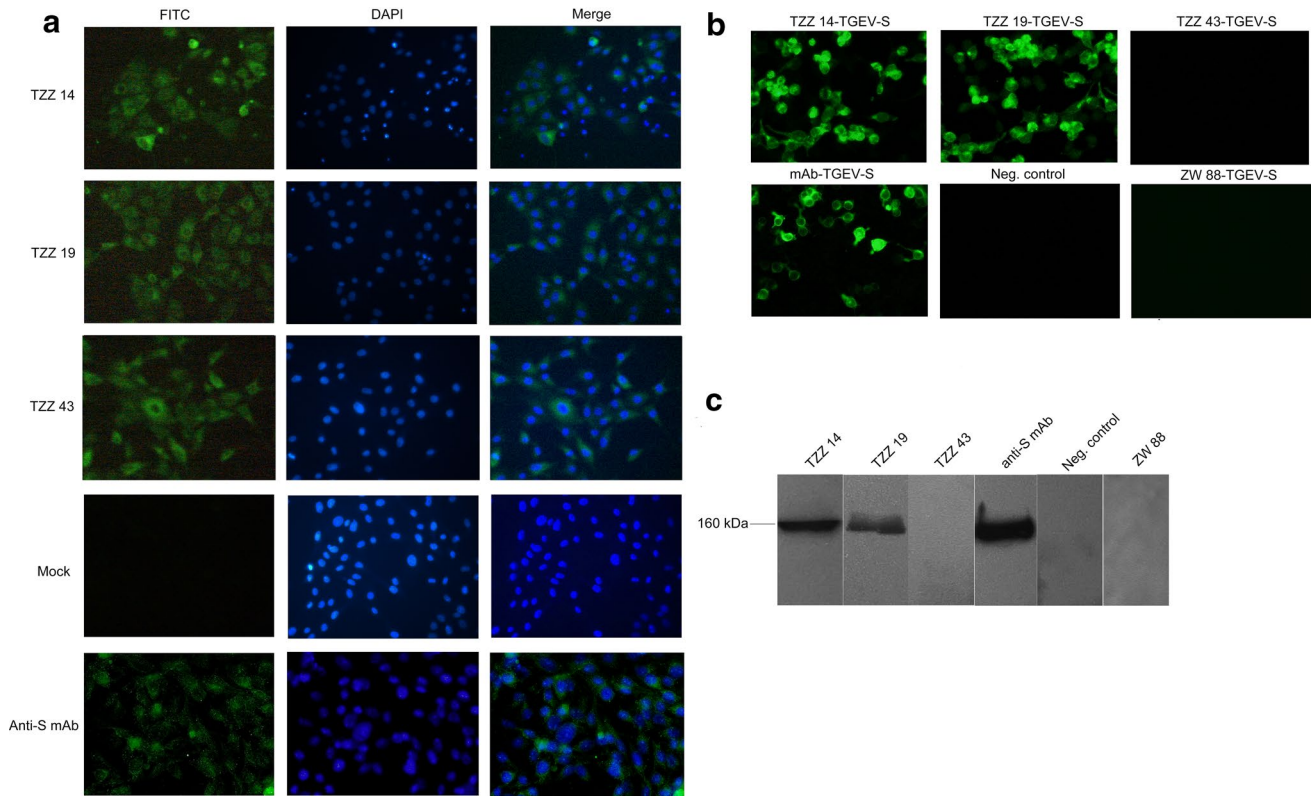


Fig. 4 Purified scFv antibodies bound to the TGEV S protein. (A) All three scFv antibodies bound specifically to TGEV-infected cells. ST cells were infected with TGEV SHXB strains and incubated with purified scFv or anti-TGEV spike (S) protein monoclonal antibody followed by FITC-conjugated secondary antibody. The nuclei were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI). Cells that were not infected with TGEV were used as a negative control and examined under a fluorescence microscope. (B) Identification of the viral protein recognized by the scFv antibody. 293T cells were transfected with recombinant plasmid pCDNA3.1(+)-S and incubated with scFv antibody, an unrelated scFv, ZW 88, or anti-TGEV spike

(S) protein monoclonal antibody, followed by staining with FITC-conjugated secondary antibody. The cells were examined under a fluorescence microscope. Cells transfected with $p3 \times \text{FLAG-CMV-14}$ followed by staining with mouse anti-TGEV S monoclonal antibody were used as an additional negative control. (C) The binding of scFv antibody to viral proteins was measured by Western blot analysis. The membrane containing the proteins was incubated with purified scFv, an unrelated scFv, ZW 88, or anti-TGEV spike (S) protein monoclonal antibody. Cells transfected with $p3 \times \text{FLAG-CMV-14}$ followed by staining with mouse anti-TGEV S monoclonal antibody were used as an additional negative control

Discussion

TGEV infection is characterized by severe diarrhea, vomiting and dehydration, with high morbidity and mortality in suckling piglets (especially in piglets younger than two weeks of age). This can cause significant economic losses on affected farms [3]. In neonatal piglets, the main mechanism of protection is mediated by lactogenic immunity. Sows are vaccinated with inactivated or attenuated vaccine in order to induce the production of secretory IgA in colostrum and milk. The neonatal piglets are passively protected from TGEV when they ingest colostrum and/or milk with high titers of anti-TGEV secretory IgA antibodies [15]. Sows inoculated with inactivated vaccine do not generate the required local immune response in the small intestine and the replication of the attenuated viruses is limited, resulting in an inadequate immune response. Piglets cannot overcome

infection unless adequate maternal antibodies are acquired. Passive immunization by oral administration of specific antibodies or antibody derivatives represents an attractive approach against TGEV infection. Egg yolk antibodies (IgY) against TGEV that are administered orally to suckling piglets show a prophylactic effect on the piglets. Lee *et al.* demonstrated that oral administration of antibodies can protect piglets from diarrhea, which indicates that antibody administration is an alternative way of controlling TGEV infection [34]. Besides traditional polyclonal serum and monoclonal antibodies, oral administration of recombinant antibodies can also protect the host from diarrhea. Garai-coechea *et al.* demonstrated that mice that were inoculated orally with single-chain antibody fragments (VHH) against the inner capsid protein VP6 of rotavirus (RV) protected mice from rotavirus diarrhea [35]. This study offers promising alternative strategies to prevent RV-induced diarrhea and

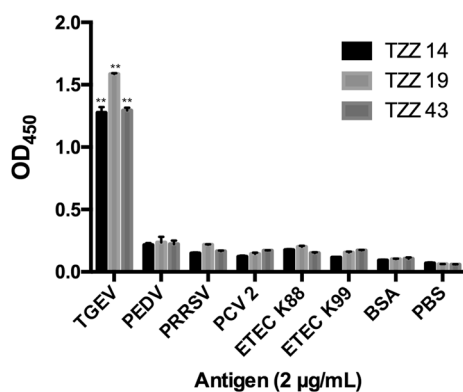


Fig. 5 Binding specificity of purified scFv antibodies. Three scFv antibodies were evaluated for cross-reactivity against the following swine pathogens: transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), porcine respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), ETEC enterotoxigenic *Escherichia coli* (ETEC) K88, and ETEC K99. BSA was used as a negative control. The data represent the means from three independent experiments. “***” indicates a statistically significant difference ($p < 0.01$)

suggests that our selected scFv antibodies may be used as a novel therapeutic reagent to control porcine transmissible gastroenteritis in suckling piglets.

Phage display technology dates back 30 years to the discovery that foreign DNA fragments can be fused to the gene encoding the pIII coat protein of a nonlytic filamentous phage and expressed as a fusion protein on the virion surface [36]. A few years later, McCafferty *et al.* first reported that scFv antibodies can be displayed on the surface of the phage through phage display technology and that scFv antibodies can be obtained after biopanning [37]. This technique for the production of scFv antibodies has several advantages: rapid culture of phage clones, easy handling and detection of secreted antibodies, genetic stability, and lower production costs compared with monoclonal antibodies [38, 39]. Lika *et al.* first attempted to construct a porcine phage library by analyzing sequence data in the IMGT database [40]. These authors reported that information on the kappa and lambda chain repertoire is scant and that some of the kappa/lambda chains might not be recovered from the sites of the transcripts of porcine spleen [40]. The porcine scFv antibody library is incomplete, and further research is required. In this study, we constructed a porcine scFv antibody library against TGEV. The library size was large enough to select specific scFv antibodies despite being smaller than the recommended size (6×10^8 pfu/mL). To our knowledge, this is the first study using a porcine antibody phage library to select scFv antibodies against TGEV.

The scFv is a small, genetically engineered recombinant antibody in which the VH and VL chain of the antibody are connected by a flexible peptide linker. The scFv has

considerable potential for use in receptor blockades, pathogen neutralization and therapeutic antigen targeting *in vivo* [41, 42]. In this study, phages expressing scFv antibodies were selected after four rounds of biopanning. TZZ 14, TZZ 19 and TZZ 43 were chosen because of their high binding affinity to TGEV antigen. The DNA sequences of the scFv antibodies were analyzed and their amino acid sequences were deduced. Our results showed that sequences of these scFv antibodies share similarities in the FRs and that their CDRs are highly variable, especially the CDR3 of VH and VL. Since CDRs contribute to the specificity for binding to a specific epitope, a difference in the diversity of CDR3 in the VL and VH domains reflects differences in antigen binding. We did not determine the affinity of this interaction, but we infer from the phage ELISA and sequence analysis that these two antibodies were variable. Previous studies indicate that scFv antibodies are able to neutralize various viral infections, including influenza A virus (H5N1 subtype), human immunodeficiency virus 1, respiratory syncytial virus, rabies virus, and infectious bronchitis virus [43–47]. Notably, most scFv antibodies can target viral surface glycoproteins, which in turn play a role in blocking virus adherence to receptors of host cells. These scFv antibodies with high affinity to TGEV antigen were further analyzed using a neutralization assay. Our results indicate that three scFv antibodies have a neutralization effect on TGEV infection.

The S protein of the porcine coronavirus TGEV is a large surface glycoprotein. It is responsible for inducing neutralizing antibodies, and it also affects viral binding to host cells before invasion, and thus pathogenicity [6, 48]. The neutralization assay indicated that three scFv antibodies exert neutralization activity. IFA results showed that ST cells transfected with p3XFLAG-TGEV-S was stained by TZZ 14 and TZZ 19. The exact epitope recognized by the scFv antibodies is still unknown, and current work is ongoing to map the epitopes recognized by each scFv. IFA and western blot results showed that scFv TZZ 43 do not bind to S protein-transfected cells. Some studies have shown that the nucleocapsid (N) protein of TGEV is also an inducer of neutralizing antibodies, and swine vaccinated with the N protein produce a mucosal immune responses against TGEV [49, 50]. Thus, TZZ 43 might neutralize virus infection through interacting with another TGEV viral protein (e.g. the N protein).

In conclusion, a porcine scFv antibody phage display library was successfully constructed. scFv antibodies were selected from the antibody library by several rounds of biopanning using purified TGEV as the antigen. The scFv antibody was expressed in *E. coli* in a soluble form. PRN assay results showed that TZZ 14, TZZ 19 and TZZ 43 could neutralize virus replication. IFA results showed that TZZ 14, TZZ 19 and TZZ 43 bind to TGEV-infected cells and TZZ 14 and TZZ 19 bind to TGEV S protein-transfected

cells. Our research lays the foundation for further scFv development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The animal experiment was performed in accordance with the recommendations in the Guidelines for the Use of Laboratory Animals provided by the Science and Technology Commission of Shanghai Municipality (STCSM). The protocol was approved by the ethics committee of the Shanghai JiaoTong University, School of Agriculture and Biology.

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