

Acquisition and bioactivity analysis of single-chain fragment variable antibody against CSFV

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Abstract

Background Classical swine fever (CSF) is a highly contagious disease that threatens the pig industry. Passive immunization with neutralizing antibodies against classical swine fever virus (CSFV) is an effective prevention and therapeutic measure.

Results In this study, to prepare a single-chain fragment variable (scFv) antibody against CSFV, two weanling piglets were injected twice with a CSFV attenuated vaccine. After the second immunization, peripheral blood lymphocytes were separated from the blood of the piglets by lymphocyte separation medium. After FACS, B lymphocytes with FITC labelled-E2 and FITC goat anti-swine IgG were sorted, extracted and cultivated on 96-well plates for 72 h. Then, the culture supernatants of B lymphocytes were screened by indirect ELISA with the purified CSFV-E2 antigen, and the target scFv antibody was bound to CSFV and obtained. Then, the scFv gene was inserted into the eukaryotic expression plasmid pCAGGS, and the constituted plasmid pCAGGS-scFv was transfected into ST cells. Western blot analysis showed that an approximately 31 kDa fusion protein was detected in the cell supernatant. Addition, the neutralizing capacity was measured in vitro. Indirect immunofluorescence assay (IFA) results showed that scFv-16 was able to neutralize CSFV.

Conclusion Our data demonstrated that scFv-16 would be an effective diagnostic tool and potential therapeutic reagent for the CSFV infection in swine.

Background

Classical swine fever, historically called hog cholera, is caused by the classical swine fever virus, and can lead to high morbidity and mortality in swine[1, 2]. CSFV is a small, enveloped virus with a nonsegmented, single-stranded positive RNA genome and belongs to the Pestivirus genus in the Flaviviridae family. This virus is prevalent around the world, including Central America, Africa and Asia[3]. In China, there are four subgenotypes of CSFV leading to CSF outbreaks[4]. Since nationwide vaccinations were implemented in China, large-scale CSF outbreaks are rare[5]. However, none of the regions in China have been declared free of CSF. There is still a long way to go to control and ultimately eradicate CSF in China[6].

The World Organization for Animal Health (OIE) reported that CSFV can severely diminish the export of pork. Therefore, the control and therapy of this disease is very important. Vaccination is a very effective way to prevent viruses and bacteria. In the process of antibody recognizing and neutralizing antigen, the "lock-key" relationship is formed by the binding of the antibody to the epitope on the surface of the Antigen[7, 8]. So the structure of antigen and antibody recognition is very important. The Fv fragment is the smallest unit of immunoglobulin molecule with antigen-binding activities[9]. An antibody in a scFv (single chain fragment variable) format consists of variable regions of heavy(VH) and light (VL) chains, which are joined by a flexible peptide linker and can be easily expressed by E.coli in their by functional form. Protein engineering has improved scFv properties, such as increased of affinities and alterations in the specificity[7, 10].

The CSFV vaccine cannot arise from an ineffective neutralizing antibody as it cannot block the transmission of CSFV, thus leading to immune failure. Therefore, the purpose of this study is to develop a method that can rapidly generate neutralizing antibodies rapidly and improve the effective immune level to provide a new means for the prevention of CSFV. This study provides a new view on the prevention and treatment of animal diseases and provides technical information for the development of animal vaccinology.

Methods

Virus and cell culture

The CSFV strain was maintained in our laboratory. Swine testicle (ST) cells were routinely cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and propagated in a humidified atmosphere containing 5% CO₂ at 37°C.

Animal immunization

All animal experiments were performed in accordance with the guide for the Institutional Animal Care and Use Committee at Centers for Disease Control and Prevention (CDC) and in a facility accredited by the Laboratory Animal Care International. Two weanling piglets (thirty-days old) were purchased from CSFV seronegative pregnant sows at a local breeding farm and housed in separate sterile isolators and fed with commercial fodder and water. injected twice with CSFV attenuated vaccine. After the second immunization, peripheral blood lymphocytes (PBLs) were separated from the blood of the pigs by a lymphocyte separation medium. After this experiment completed, all animals were euthanized humanely using sodium pentoarbiital anesthesia to reduce suffering.

B lymphocyte sorting

An equal amount of anticoagulation and lymphocyte separation fluid were gently mixed and horizontally centrifuged at 4000 rpm for 20 min. Then, lymphocytes in the middle layer were gently pipetted into a new microtube and washed with PBS. Finally, the cell pellet was dissolved in 0.5 ml PBS. After FACS B lymphocytes positive for FITC-E2 and FITC-IgG (sheep anti-swine) were sorted, extracted, and cultivated on 96-well plate for 72 h.

Generation of the VH and VL chains

According to the sequences of the pig antibody coding for the variable regions of the heavy chain(AF064688.1; AF064687.1; AF064690.1; and AF064689.1) and light chain (GQ867595.1; KF561242.1; and GQ867594.1) that were entered into GenBank, primers were designed to amplify the light and heavy chains of the CSFV antibody(listed in Table 1).Primers were synthesized by Shanghai Biotechnology Service Co., Ltd.

Table 1

Primers for Amplifying Antibody Variable Region and Their Amplified Fragment Sizes

Primer	Primer sequence	Size(bp)
VH backward	GCGGCCAGCCGGCCATGGCCGAGGWGAAGCTGGTGGAGTCYGG	
VH forward 1	GGATCCACCACCGCCCGAGCCACCGCCACCACGACGACTTCAACGCCTGG	380
VH forward 2	GGATCCACCACCGCCCGAGCCACCGCCACCACGACGACTTCAACG	380
Vk backward 1	GGCGGTGGTGGATCCGGTGGCGGGGGTCTGCCATYGTGCTGACCCAGASTCC	350
Vk forward	TTGCGGCCGCACGTTTGAKYTCCAGCTTGGTCCC	350

Molecular cloning of scFv

Total RNA was extracted from the Lymphocyte fluid by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and the cDNA was synthesized according to the instructions for the PrimeScript RT Master Mix (Takara, Japan). Using cDNA as the template, Vk-Linker and VH-Linker genes were amplified. The experimental conditions were as follows: predenaturation at 95°C for 5 minutes; 30 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute (annealing of the VH-Linker at 66°C for 1 minute), extension at 72°C for 1 minute; and final extension at 72°C. Agarose gel electrophoresis(1.5%) was used to identify the products(120V. 20 min). The results were displayed by a UV gel imaging system. Furthermore the target scFv gene was acquired by SOE-PCR using the VH-Linker and Vk-Linker genes as templates.

Construction of recombinant eukaryotic expression pCAGGS-scFv plasmid

After being digested with NheI and EcoRI restriction endonucleases, the target scFv gene was cloned into the pCAGGS vector by the T4 DNA ligase. Furthermore, the recombinant plasmids were identified by visualizing with digestion, PCR and sequencing.

Cytotoxicity Assay

The cytotoxic activity of the pCAGGS-scFv-16 plasmid on ST cells was determined using an MTT assay (Beyotime). Briefly, 2 µg pCAGGS-scFv-16 plasmid was transfected into ST cells by Lipofectamine 2000. After 48 h, approximately 2×10^4 cells/well were seeded in a 96-well cell culture plate and cultured overnight. The next morning, the MTT solution (0.2 mg/ml) was added to each well, and the plate was further incubated at 37 °C for 4 h. The supernatant was then removed, and the colored formazan crystal in each well was dissolved by adding 100 µl of the SDS-HCl solution (Sigma-Aldrich). The OD value at 570 nm was measured using a microplate reader (BioTek). The cell survival rate was calculated as $\frac{\text{OD}_{570} \text{ of scFv treatment}}{\text{OD}_{570} \text{ of PBS treatment}} \times 100\%$.

Obtaining purified pCAGGS-scFv-16

After transfecting the pCAGGS-scFv-16 plasmid into ST cells, the cellular supernatants were collected after 48 h. Then the expression of the pCAGGS-scFv-16 protein was verified by Western blotting. Equal amounts of protein were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. These membranes were blocked with 5% skimmed milk for 1.5 h at 37 °C and incubated with primary antibody and appropriate secondary antibodies. Signals were detected using a Supersignal West Pico Kit. Then the verified proteins were purified by concentrated column for further use.

Neutralization Test

The purified pCAGGS-scFv-16 protein was mixed with 100 TCID₅₀ CSFV at a 1:1 ratio at 37°C for 1 h, and the mixtures were added to ST cells. After 1 h of incubation, the ST cells were washed twice with PBS and then cultured for 48 h. Then, the neutralization effect of the purified pCAGGS-scFv protein was detected by indirect immunofluorescence. That is, the anti-CSFV-E2 was the primary antibody that was incubated with ST cell for 1.5 h, and after washing, FITC-sheep anti-swine was used as the secondary antibody and incubated for 1 h. Then, fluorescence images were acquired using a Nikon fluorescence microscope. Fluorescence intensity was detected and compared between different groups in different concentrations of the pCAGGS-scFv-16 protein. Positive fluorescence indicates an infection with CSFV. ST cells without CSFV (with negative fluorescence) were used as negative controls; anti-CSFV serum was used as positive controls.

Results

Screening of IgG + B Lymphocytes

After separated from the pig peripheral blood, 2 µl anti-pig FITC-IgG and 2 µl FITC-E2 antibodies were added and incubated with 2×10^6 lymphocytes for 21 min at room temperature. Then the mixture was centrifuged at 1000 rpm and washed 3 times with PBS. Finally, the lymphocytes were resuspended in 200 µl PBS and sorted into the IgG positive lymphocytes by flow cytometry. The results indicated that the positive rate of IgG positive B lymphocytes against porcine IgG-FITC was 30% (Figs. 1A and 1B).

Screening for Positive Clones of CSFV by ELISA

The sorted B lymphocytes were transferred to a 96-well cell plate, at 1 lymphocyte/well, and 0.35 µg/ml PMW was added, and cultured for 72 hours, the cell supernatant was collected and tested by ELISA. The supernatants with an

OD value surpassing the negative control (NC) were sorted as a positive antibody. The results showed that eight positive antibodies containing 2-16, 1-17, 1-9, 2-20, 3-15, 3-18, 2-19 and 4-14 were screened and removed. Among them, 2-16 had the highest OD value(Figs. 2A).

Construction of Single Chain Antibody

Total RNA from single-chain antibody-positive cytosol was extracted by an RNA extraction kit, and reverse transcription was used to prepare the cDNA. The VH and VL genes were amplified using the RNA extraction kit. VH-Linker-VL fragments were constructed by SOE-PCR. As shown in Fig. 4A, PCR amplified an approximately 730 bp band, which is correct and expect size of the fragment(Figs. 2B and 2C).

Sequencing analysis of single chain antibody positive clones

The positive clones were connected to the T vector and then sequenced and analyzed. The results were as follows: compared with the single chain Fv antibody published in the NCBI database, the 5 McAbs obtained from pigs were also single chain Fv antibodies. These include the largest variation in the CDR region, less variation in the FR region, and the largest change of amino acids in the CDR3 region of heavy and light chains(Fig. 3).

Construction of a eukaryotic expression vector

As shown in Fig. 4B, the pCAGGS vector and scFv genes were simultaneously digested by NheI and EcoRI restriction endonucleases. The corresponding fragments were recovered and ligated. The correct recombinant cloning plasmid pCAGGS-scFv was screened by plasmid extraction.

Evaluation of the in vitro cytotoxicity of scFv-16

The MTT assay was performed to investigate the possible cytotoxicity of pCAGGS-scFv-16 toward ST cell growth. The MTT assay showed no cytotoxic effects toward ST cells, suggesting that pCAGGS-scFv-16 was also safe for ST cells.

The expression of scFv-16 in ST cells

The expression of pCAGGS-scFv in ST cells was analyzed by Western blotting. Cells transfected with an empty vector were used as a control. 24 h, 36 h, 48 h, 60 h, the cell lysates were collected, and these samples were subsequently identified by means of protein gel electrophoresis. The CSFV serum was incubated with primary antibody, followed by incubation with secondary antibody (HRP sheep anti-swine). As shown in Fig. 2D, successful transfection and effective expression of pCAGGS-scFv were confirmed by Western blotting.

Viral Neutralization assay

The purified scFv was mixed with CSFV, and the neutralization index was detected in ST cells. As the scFv concentration decreased, the amount of green fluorescence in ST cells increased gradually. No green fluorescence appeared in cells, indicating that scFv (1.142 $\mu\text{g}/\mu\text{l}$) could fully neutralize CSFV. As shown in Figs. 4.1-7.7, the concentrations of the scFv-16 protein were double and decreased from 1.142 $\mu\text{g}/\mu\text{l}$ to 0.0178 $\mu\text{g}/\mu\text{l}$. When the concentration of scFv-16 reached to 1.142 $\mu\text{g}/\mu\text{l}$ (as shown in Fig. 7.1), the fluorescence could not be detected. This results indicates the complete neutralization of CSFV by the scFv-16 protein. The number of FITC-positive

cells showed a negative correlation with the concentration of the scFv-16 protein. Therefore, the scFv-16 protein can determinately neutralize CSFV.

Discussion

Classical swine fever (CSF) is a highly contagious disease caused by the classical swine fever virus (CSFV)[11]. The high incidence and mortality of CSF threatens the pig industry, and CSF is an acute and highly infectious disease among farmed animal industries worldwide[12, 13]. Vaccination against CSFV has controlled the epidemic of classical swine fever to a certain extent. However, because of the immune suppression caused by CSFV, the level of effective neutralized antibodies caused by the vaccine is low, so immunization cannot adequately prevent a CSF epidemic.

As an important tool in modern life science research, the monoclonal antibody technique covers the hybridoma technique, phage display technology, single B cell antibody preparation technique and so on[14]. Antibodies prepared by the hybridoma technique have high immunogenicity and a short half-life period, which leads to an unsatisfactory clinical efficacy[15]. Because of its simplicity and convenience, phage display technology is the most widely used method in antibody preparation. However, due to the random assortment of heavy and light chains, the native mating of heavy and light chains in antibodies cannot be maintained[16]. Currently, as an emerging technology, single B cell antibody preparation is a technique that clones and expresses single antigen-specific B cell antibody genes in vitro. This technique reserves the native mating of heavy and light chains, and has the advantages of good genetic diversity, high efficiency and low cell requirement[17]. Therefore, in this study, we used a B cell antibody as a focus to acquire a single-chain antibody to CSFV.

A neutralizing antibody is an important factor in antiviral immunity and is also a key index to analyze the immune effect of vaccines[18]. Neutralizing antibodies kill viruses by neutralizing infectious virus particles, thus preventing viral infection and eliminating pathogens[19]. Currently, the vaccine is mainly developed by selecting protective antigens and inducing immune neutralizing antibodies to neutralize a viral infection. However, the protective antigens of viruses that can induce immunosuppression often fail to induce effective neutralizing antibodies, resulting in an unsatisfactory immune response. Therefore, designing the design of vaccines that can induce highly effective neutralizing antibodies has attracted more attention. Research on neutralizing antibodies in humans has developed rapidly, especially in the fields of AIDS, hepatitis A and hepatitis B. The current method is to establish an antibody gene library through a phage vector expression system, especially the pCom3 expression system, to screen and obtain anti-viral neutralizing and monoclonal antibodies, which can directly prepare antibodies or antibody vaccines in vitro for emergency immunoprophylaxis and treatment. Currently, human monoclonal antibodies against influenza virus, anti-HbsAg, anti-respiratory syncytial virus (RSV) F protein, anti-herpes simplex virus, anti-HIV gp120 and anti-hepatitis C virus have been successfully screened by this technology[20–24].

Currently, the idea of animal vaccine research is to express protective antigens of pathogens and to stimulate the body to produce antibodies. However, only some of these antibodies have a neutralizing viral activity, and can be identified as neutralizing antibodies, addition, the remaining antibodies have no neutralizing activity. Therefore, although the vaccine can induce a high level of antibodies, the immune effect is not ideal. In addition, pathogens with an immunosuppressive function can inhibit immunity. In that case, the immune system cannot arouse an effective immune response to eliminate pathogens, so the protective antigen cannot be used as an ideal vaccine to obtain an effective immune reaction.

In summary, the development of neutralizing antibodies and human vaccines has shown good prospects for their use. However, further research is needed for antibody vaccines. There are few studies on neutralizing antibodies in animal diseases, and no antibody vaccines have been reported. In this study, we renovated the design idea of vaccines using protective antigens as an immunogen and constructed a neutralizing antibody vaccine against CSF. The vaccine has the characteristics of producing a rapid neutralizing antibody and strong pertinence of antibodies. This method improves the effective immune levels and provides a new means for the immune prevention of CSF. On the one hand, neutralizing antibody production is effective and targeted; on the other hand, neutralizing antibodies can directly neutralize the virus without influencing the body's immune system.

Conclusion

In this article, the scFv-16 protein against CSFV was screened by ELISA and neutralized with CSFV in ST cells. The research and design of the swine fever antibody vaccine developed in this project are novel. Next, we will conduct further research on swine, such as regularly collecting pig blood, measuring the titer of the CSFV antibody and neutralization virus, monitoring the antibody content and duration, and evaluating the protective effect of the purified scFv-16 protein from the recombinant eukaryotic expression plasmid.

Abbreviations

scFv: single-chain fragment variable antibody; CSFV: classical swine fever virus; ST: Swine testicle; FBS: fetal bovine serum

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and use committee of Shanghai Academy of Agricultural Science. No specific permissions were required for the collection of samples. All sampling and publication of the data were approved by the farm owners.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HL conceived and designed the experiments. BL and JT performed the experiments. JC and YS contributed data analysis. All authors have read and approved the final version of manuscript.

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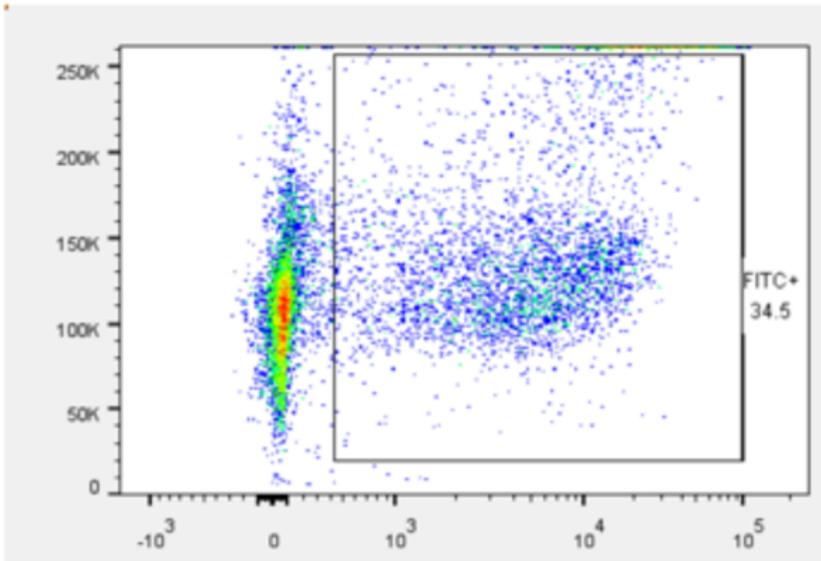
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Figures

A



B

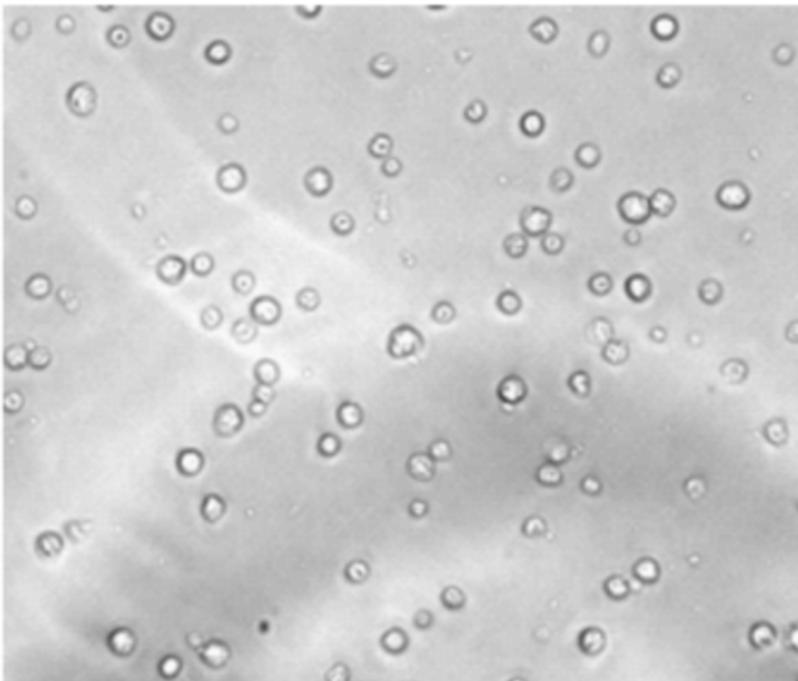
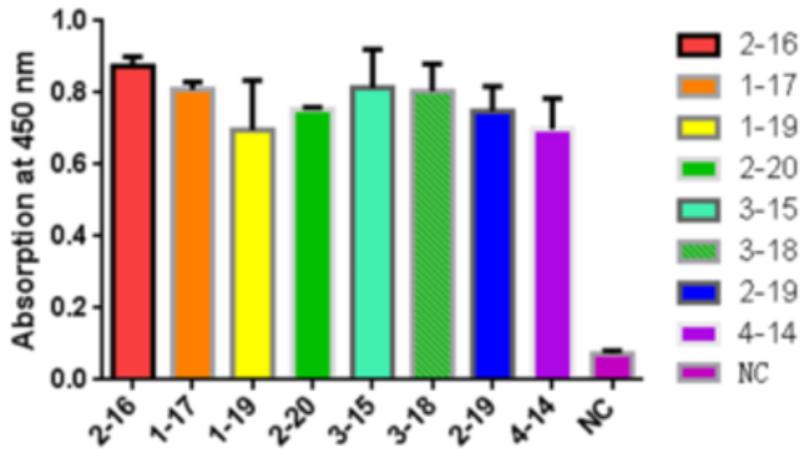


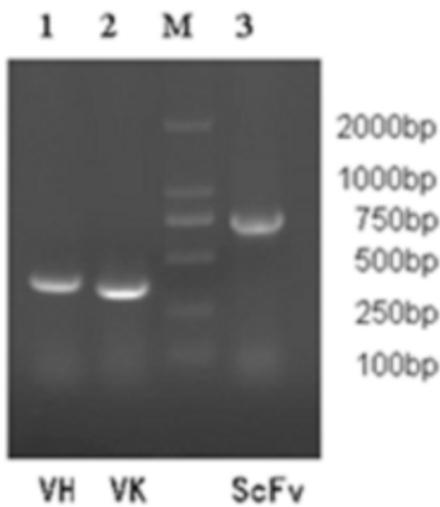
Figure 1

A. Separation of IgG positive B Lymphocytes by Flow Cytometry B. Lymphocyte sorted by flow cytometry

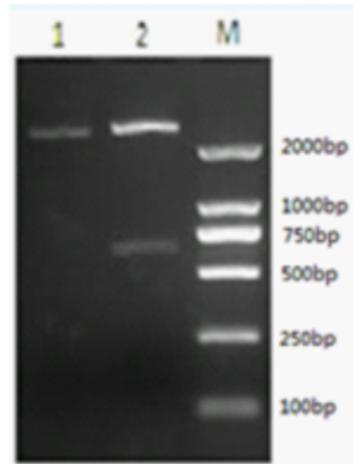
A



B



C



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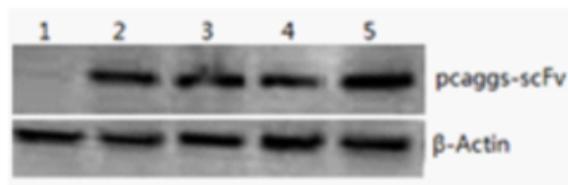


Figure 2

A. Functional of anti-CSFV scFv by indirect ELISA method B. M; Marker DL2000. Lane 1. The PCR product of VH gene; Lane 2. The PCR product of VK gene; Lane 3. The PCR product of scFv gene C. The PCR product of pCAGGS-scFv gene D. Line 1. Negative control; Line 2-5. 24h, 36h, 48h, 60h,. The expression of pCAGGS-scFv in ST cells were analyzed by western blotting



Figure 3

Amino acid sequence coding for the six positive anti-CSFV scFvs derived from an antibody library based on the immunoglobulin genes of the swine. Variable heavy chains and variable light chains(VH and VL) are indicated, as are framework regions(FR)

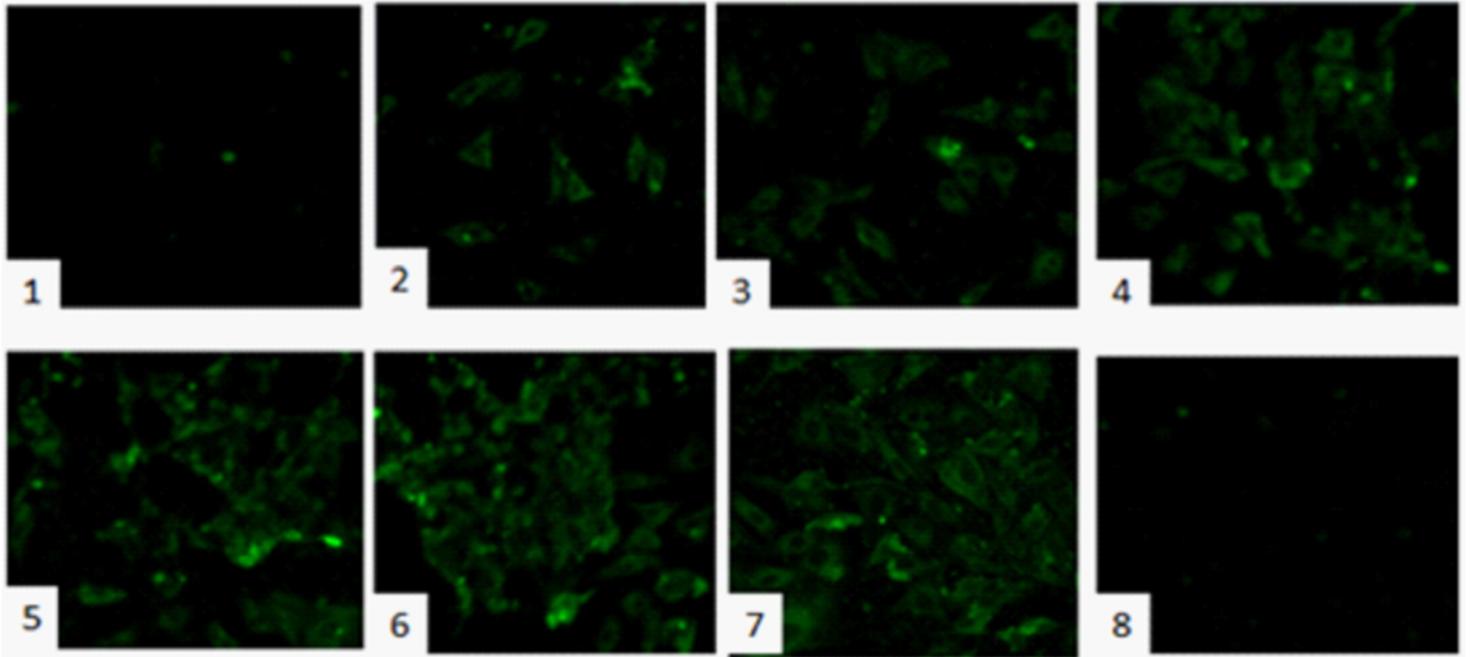


Figure 4

Neutralization ability of scFv-16 by indirect immunofluorescence. 1-7. the concentrations of scFv-16 protein were serially diluted from 1.142µg/µl to 0.0178µg/µl. 8. negative control

Supplementary Files

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