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Selection and identification of single-domain antibody against Peste des Petits Ruminants virus

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ABSTRACT

Background: Peste des petits ruminants (PPR) is an infectious disease caused by the peste des petits ruminants virus (PPRV) that mainly produces respiratory symptoms in affected animals, resulting in great losses in the world's agriculture industry every year. Single-domain variable heavy chain (VHH) antibody fragments, also referred to as nanobodies, have high expression yields and other advantages including ease of purification and high solubility.

Objectives: The purpose of this study is to obtain a single-domain antibody with good reactivity and high specificity against PPRV.

Methods: A VHH cDNA library was established by immunizing camels with PPRV vaccine, and the capacity and diversity of the library were examined. Four PPRV VHHs were selected, and the biological activity and antigen-binding capacity of the four VHHs were identified by western blot, indirect immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) analyses. ELISA was used to identify whether the four VHHs were specific for PPRV, and VHH neutralization tests were carried out. ELISA and western blot analyses were used to identify which PPRV protein was targeted by VHH2.

Results: The PPRV cDNA library was constructed successfully. The library capacity was greater than 2.0×10^6 cfu/mL, and the inserted fragment size was approximately 400 bp to 2000 bp. The average length of the cDNA library fragment was about 1000 bp, and the recombination rate was approximately 100%. Four single-domain antibody sequences were selected, and proteins expressed in the supernatant were obtained. The four VHHs were shown to have biological activity, close affinity to PPRV, and no cross-reaction with common sheep diseases. All four VHHs had neutralization activity, and VHH2 was specific to the PPRV M protein.

Conclusions: The results of this preliminary research of PPRV VHHs showed that four screened VHH antibodies could be useful in future applications. This study provided new materials for inclusion in PPRV research.

Keywords: Peste des petits ruminants virus; cDNA library; single-domain antibody

INTRODUCTION

Peste des petites ruminants (PPR), an acute and highly contagious infectious disease of small ruminants, is caused by the peste des petites ruminants virus (PPRV) that mainly infects

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

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sheep, cattle, and some wild animals, such as white-tailed deer, African grey duiker, and Dorcas gazelle [1]. Morbidity and mortality rates can be 90%–100% in naïve populations, dropping to about 20% in PPRV endemic areas. PPR is one of the most important challenges facing sustainable small-scale agriculture, especially in sheep and goat breeding, in developing countries and has caused considerable losses in the animal husbandry-related global economy. The Office International Des Epizooties or World Organisation for Animal Health (OIE) and the Food and Agriculture Organization (FAO) have sought to eradicate PPR globally by 2030.

PPRV exists serologically as a single serotype but can be genetically divided into four distinct lineages (I, II, III, and IV) [2]. PPRV is an enveloped RNA virus with a non-segmented single-stranded negative-sense RNA genome that contains six contiguous non-overlapping transcription units that encode six structural proteins: the nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin protein (H), and the large (L) polymerase protein as well as two non-structural proteins (V and C proteins) that are encoded from the P-gene transcription unit by using overlapping open reading frames (ORFs) and co-transcriptional editing to access the third ORF to generate protein C [3,4].

Conventional antibodies are composed of two identical light (L) chains and two identical heavy (H) chains in many animals and humans. However, a special antibody, which is a dimer without a light chain and only containing the variable region of a heavy chain, has been observed in the sera of Camelidae and cartilaginous fish [5,6]. Compared with conventional antibodies, the molecular weight of a special dimer antibody (sdAb) is notably smaller (approximately 12–15 kDa), and sdAbs are also known as heavy chain antibodies (HcAb) or variable domain of heavy chain (VHH) antibodies [7,8]. The long CDR3 region in the variable region of a heavy chain can form a stable and exposed convex ring structure, and the stable disulfide bond in that structure can penetrate the interior of an antigen. Compared with the concave topological structure formed between a common antibody and its antigen, the affinity between a VHH antibody and its antigen is higher. The VHH antibody has the advantage of high affinity, and its recombinant expression form has been easily prepared in *Escherichia coli*, yeasts, mammalian cell lines, and plants [5]. Due to its structure, VHH antibodies can be used to reveal hidden epitopes of traditional immunoglobulins, which has allowed significant progress in the fields of immune research, diagnostic testing, medical and biological imaging, and therapeutic antibody development [9,10].

Since the first cDNA library was constructed in 1976, the construction method has improved continuously. A cDNA library provides particular advantages in the study of gene expression state and gene function identification of genes expressed in specific cells; thus, it is widely used in research into life phenomena such as individual development, cell differentiation, cell cycle regulation, cell aging, and cell death regulation. Moreover, a cDNA library can be used when screening specific VHHs.

MATERIALS AND METHODS

Preparation of concentrated PPRV antigens

A PPRV vaccine was inoculated into monolayer Vero cells at a multiplicity of infection (MOI) of 2. Viruses were harvested when 80% lesions appeared in 4–6 days, and the MOI was 200

MOI when the viruses were harvested. According to the above rules, PPRV was subcultured to the 10th generation, and the harvested virus in each generation needed to be frozen and thawed three times at -80°C . After freezing and thawing, collection, centrifugation, concentration, and purification, the viruses were stored at -80°C until used.

Immunization of camel and serum antibody titer identification

Blood (100 mL) was collected from a 3-month-old female Bactrian camel to act as a negative control. Camels were vaccinated three times, once every two months. Each immunization dose was 2 mL, but the first immunization dose was three times the usual immune level, which was 6 mL. In addition, three Bactrian camels were subcutaneously injected with PPRV vaccine three times at two-month intervals in the right neck area. Serum samples were obtained two weeks after each immunization and analyzed to monitor humoral immune responses [6]. Before immunization, a dose of 2.5×10^5 cfu Bacillus Calmette Guerin vaccine was injected intradermally into the deltoid muscle of the left foreleg of camel. The serum antibody titer identifications were determined by performing ID Screen PPR Competition ELISA. A 96-well microplate was loaded with positive and negative control and serum samples and the plate incubated at 37°C for 45 min. The microplate was washed three times after incubation, after which TMB and stop solutions were added. The OD_{450} value was then determined.

Isolation of peripheral blood lymphocyte

Camel lymphocyte separation liquid (10 mL) was added to 30 mL centrifuge tube before adding diluted blood gently. The mixture was then centrifuged at 1,800 r/min and 18°C – 22°C for 20 min. The lymphocyte layer was carefully collected in a new collecting tube and washed three times with cell cleaning solution to remove red blood cells. The precipitate containing peripheral blood lymphocytes (PBLs) was suspended in an RNA sample preservation solution and stored at -80°C until RNA extraction.

Extraction of total mRNA

Total RNA was isolated by adding TRIzol reagent (Invitrogen) to 400 μL of peripheral blood mononuclear cells. Guanidine isothiocyanate, β -mercaptoethanol, and sodium N-dodecyl sarcosine were added to the PBLs to prevent RNA degradation by RNase. The concentration and purity of the obtained total RNA were measured by using a NanoDrop-2000 ultraviolet-visible spectrophotometer (Thermo Fisher, USA).

Separation and purification of total mRNA

To lysate RNA and fully expose the poly A tail, improve the recovery of poly A, and dissociate the binding of mRNA to rRNA, total mRNA was heated at 65°C for 15 min, and then cooled rapidly to 0°C for RNA denaturation. Oligomerization (DT)-cellulose column chromatography was used for RNA separation and purification. During that process, the total denatured RNA was mixed with 0.02M Tris-HCl and 1M KCl (pH 7.5) of equal volumes, transferred to a 2 mL glass wool sealed syringe, and shaken in a rotating shaker for 10 min. The column was washed twice with 4 mL of high salt buffer and discarded eluent. The column was then washed with 3 mL of low salt buffer and centrifugated at $500 \times g$ for 1 min. The precipitate was collected after centrifugation. After denaturation, 0.01M Tris-HCl was added to the column mentioned above. 1M KCl (pH 7.5) was added to the second oligomeric (DT)-cellulose column. The mixture in the first column was then transferred to the second column. Through the first column elution procedure, mRNA was obtained by 0.01M Tris-HCl (pH 7.5), which was increased to 2.5 times the original volume by adding cold ethanol and 0.1 times the volume of 3 M sodium acetate (pH 5.2). The mixture was preserved at -20°C for 2

h, then centrifugated at $10000 \times g$ at 4°C for 20 min to precipitate the mRNA. After washing with 75% ethanol and drying, the purified mRNA was obtained by dissolving the precipitate in 2 μL of DNase-free water. The purified product was used for cDNA synthesis.

Synthesis and purification of cDNA

cDNA was synthesized according to instructions contained in the SMART™ cDNA Library Construction kit (Clontech, USA). SMART primer and CDS4M adapter primer (**Table 1**) 3 μL mRNA, 1 μL SMART primer with restriction site 5'GGCCATTACGGCC3', and 1 μL CDS primer with restriction site 5'GGCCCCTCGGCC3' were added successively to a 200 μL centrifuge tube. The mixture was then cooled on ice for 2 min, before adding reverse transcriptase, 5 \times first strand buffer, and DTT and dNTP mix. The above liquid was incubated at 42°C for 1 h, cultured at room temperature, and then the RNA enzyme inhibitor was added. Full-length cDNA was obtained and stored at -20°C . The Advantage 2 PCR kit (Clontech, USA) was used for double-chain amplification using the 95°C for 1 min, 95°C for 30 sec, 68°C for 3 min, 25–30 cycles (each cycle extension time increased by 5 sec), and 68°C for 3min amplification procedure. The double-stranded cDNA (8 μL) was identified by performing 1% agarose gel electrophoresis. The VHH-F and VHH-R primers contained the restriction enzyme cutting sites, which were used to synthesize double-strand cDNA using MiniBEST DNA (**Table 1**). A fragment purification kit (TaKaRa, China) was used to purify and recycle the amplified cDNA according to the manufacturer's instructions.

Construction of cDNA library

The double-stranded cDNA fragments and the pGADT7-SfiI vector were digested with SfiI A and B restriction enzymes (SfiIA site: 5'-GGCCATTACGGCC-3', SfiIB site: 5'-GGCCGCCTCGGCC-3'), and the obtained cDNA was treated using CHROMA SPIN-1000-TE Columns (Clontech, USA) to remove fragments less than 400bp. A 1 μL cDNA sample was used to carry out 1.5% agarose gel electrophoresis. The pGADT7-SfiI vector was connected to a certain amount of the cDNA after column treatment using a DNA ligation kit (TaKaRa, Dalian, China) at 12°C . The obtained product was purified and refined to construct the primary cDNA library. A small amount of primary library connection fluid was transformed into HST08 *E. coli* competent cells (TaKaRa, Dalian, China) under reaction conditions of 1.8 kV, 200 Ω , and 25 μF during electric rotation. Subsequently, the primary cDNA library was obtained by adding the transformed product to 1 mL LB ampicillin and shaking the mixture for 1 h at 225 r/min and 37°C . The final product was coated on LB ampicillin (100 g/ mL ampicillin) agar plates overnight at 37°C . The primary library was diluted from 10^{-1} times to 10^{-5} times, coated on an LB ampicillin plate, and cultured at 30°C for 3-5 days until clones appeared. Finally, sixteen single clones were randomly selected, and single-strand DNA was amplified using pGADT7-F and pGADT7-R primers (**Table 1**), which allowed analysis of the storage capacity of and diversity in the library. Finally, 8 μL of the PCR product were analyzed by performing 1.5% agarose gel electrophoresis.

Table 1. Primers used in this study

Primers	Primer sequences (5'-3')
CDS4M adapter primer	AAGCAGTGGTATCAACGCAGAGTGGCCGAGGCGGCC(T)4G(T)6C(T)13VN (N=A, C, G or T; V=A, G or C.)
VHH-F	TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG
VHH-R	GTATCGATGCCACCCTCTAGAGGCCGAGGCGGCCGACA
pGADT7-F	GGAGTACCCATACGACGTACC
pGADT7-R	TATCTACGATTCATCTGCAGC

VHH, variable domain of heavy chain.

Megaclone amplification and plasmid extraction from the primary cDNA library

Based on the results of the storage capacity test, 1 million clones were obtained. The required connecting liquid volume was then calculated, and the systems were transformed into the HST08 competent cells (TaKaRa, Dalian, China). The cells were coated on 10 LB ampicillin (100 µg/mL) 24.5 cm agar plates and cultured overnight at 37°C. The number of amplified clones obtained from the actual transformation was calculated. LB liquid medium (5 mL) was added to each plate and all clones were washed with a glass rod and collected into a triangular flask. The transferred clones were shaken at $225 \times g$ and 37°C for 3 h, and the plasmids of the amplified library were extracted by using a Nucleobond Xtra MIDI EF kit. The concentration and purity of the extracted plasmid were determined by using a NanoDrop-2000 ultraviolet-visible spectrophotometer (Thermo Fisher, USA). The plasmids in 100 ng of the amplified library were detected by performing 1.5% agarose gel electrophoresis.

Screening and expression of VHH antibodies

The constructed PPRV single-domain antibody cDNA library was amplified to 900 bp using primers with an upstream sequence of 5-GTCCTGGCTGCTCTTCTACAAGG-3, and a downstream sequence of 5-GGTACGTGCTGTTGAACTGTTCC-3, which allowed annealing at the framework 1 and 4 regions of the VHH. The above 900 bp fragment was amplified to 400 bp by using the upstream primer sequence 5- TTCCACCCAAGCAGTGGTATCAACGCAGAGTGGGAGTCTGGGGGAGG-3, and the downstream primer sequence 5- GTATCGATGCCAC CCTCTAGAGGCCGAGGCGGCCGACATGGAGACGGTGACCTGGGT-3, which contained the restriction sites used for the further cloning steps. The final PCR fragments contained the NdeI/BamHI restriction sites and were ligated to the pMAC-c2x vector. The 400 bp plasmid was transformed into TOP10 competent cells. For sequencing, 100 clones were randomly selected. The most repetitious sequences were selected and transformed into *E. coli* BL21 cells, which were then induced by IPTG to express the VHH antibody.

ELISA assay

The affinity and specificity of the VHHs combined with the PPRV were detected by using an ELISA process. The PPRV was diluted 1:100 with phosphate buffered saline (PBS), and then coated onto 96-well enzyme-labeled plates. The primary antibody with continuously diluted purified VHHs was added and incubated for 30 min at 37°C. HRP-conjugated anti-his mouse monoclonal antibody as the second antibody was added after washing 96-well enzyme-labeled plates three times with PBST. The purified VHHs were then added to microtiter plates that were coated with PPRV, goat pox virus (GPV), or sheep mouth sore virus (ORFV) (diluted to 1:100 with serum dilution) and incubated for 30 min at 37°C. After washing with PBST three times, the bound VHHs were detected by adding 100 µL of HRP conjugate anti-his mouse monoclonal antibody.

Immunofluorescence assay (IFA)

Goat tracheal epithelial cells (GTC) infected with PPRV were cultured at 37°C for 48 h. The cells were fixed with 4% paraformaldehyde, washed with PBS (pH = 7.2) three times, and incubated with 3% skimmed milk powder at 37°C for 1 h. In the negative control group, PBS was used as the primary antibody and the secondary antibody was goat anti-rabbit IgM, IgG fraction-FITC conjugate diluted 1:500. Rabbit polyclonal antibody with a titer of 1:64000 obtained after immunization was used as the primary antibody at dilution of 1:100 in the positive control group, and the second antibody was the goat anti-rabbit IgM, IgG fraction-FITC conjugate. In the experiment group, purified VHHs (20 µg/mL) were added to the

cell plate as the primary antibody. The second antibody was rabbit anti-His-tag polyclonal antibody, and the third antibody was goat anti-rabbit IgM, IgG fraction-FITC conjugate.

Neutralization test

The four types of VHHs were diluted from 1:10 to 1:1,280 in serum-free MEM medium. The negative control was serum from non-PPRV vaccinated sheep, and the positive control was serum from PPRV vaccinated sheep. The positive and negative serum control were not diluted. A Vero cell suspension with a concentration of 2×10^5 cells/mL (100 μ L/well) was inoculated into a 96-well cell culture plate. The plates were cultured in a 5% CO₂ incubator at 37°C for 1–2 days until 70%–80% of the cells had formed a monolayer. A 50 μ L equi-volume mixture of diluted VHHs and PPRV suspension containing 100 TCID₅₀/50 μ L (the same as used for the serum samples) was placed in a 5% CO₂ incubator at 37°C for 1 h. In addition, 100 μ L of the above media with different dilutions were added to Vero cells, which were then cultured in a 5% CO₂ incubator at 37°C. Seven days after inoculation, the results were observed and assessed based on the following criteria: if the cells in the negative control group exhibited complete pathological changes and the cells in the positive control group did not, the test was valid, If the test was valid and the VHHs diluted with 1:8 or greater dilutions inhibited 50% or more of the CPE cells, the test result was positive.

Identification of which PPRV protein is the VHH target

For western blot analysis, the PPRV-attenuated vaccine was passaged and centrifuged at 8000 r/min for 2 min to remove cellular debris. Then, the protein obtained via SDS-PAGE was transferred to an NC membrane, and TBST containing 50 g/L skimmed milk was applied at 37°C for 2 h, and the membrane was washed three times with TBST. After blocking at 37°C for 2 h, the NC membrane was washed three times with TBST, and VHH2 diluted to 20 μ g/mL was then added and the mixture incubated overnight at 4°C. Then, TBST was used to wash the NC membrane three times, and an anti-His monoclonal antibody (conjugated HRP) was added and the membrane incubated for 1 h at 37°C.

For the ELISA process, the ELISA plate was coated with VHH2 (1:100) overnight at 4°C, then 10 μ g/mL of N, P, M, F, or H protein were added, followed by incubating and washing. The mixed PPRV-negative serum samples and PPRV-positive serum samples were then added to the plate, followed by incubation and washing. Finally, anti-sheep secondary antibody (1:10,000) was added to the plate.

RESULTS

Construction of cDNA library

The PPRV-attenuated vaccine Nigeria 75/1 was uploaded to Vero cells (**Fig. 1A**) and inoculated in Bactrian camels with a second immunization and a third was two months later. Samples were taken 21 days after a third immunization. The serum titer before immunization was much lower than that after immunization, and the polyclonal serum titer was 1:16,000 (**Fig. 1B**); thus, the immunization was considered effective and specific for PPRV. The total RNA concentration extracted from PBL was 5,162 ng/ μ L by ND2000, and 251 ng/ μ L of mRNA was obtained by oligomerization (DT)-cellulose column chromatography. Three mRNA bands were visible (28S, 18S, and 5S) (**Fig. 1C**). These results showed that the quality of the purified mRNA was suitable for library construction. Double-stranded cDNA was obtained by reverse transcription synthesis and LD amplification (**Fig. 1D**), and after the short segment was

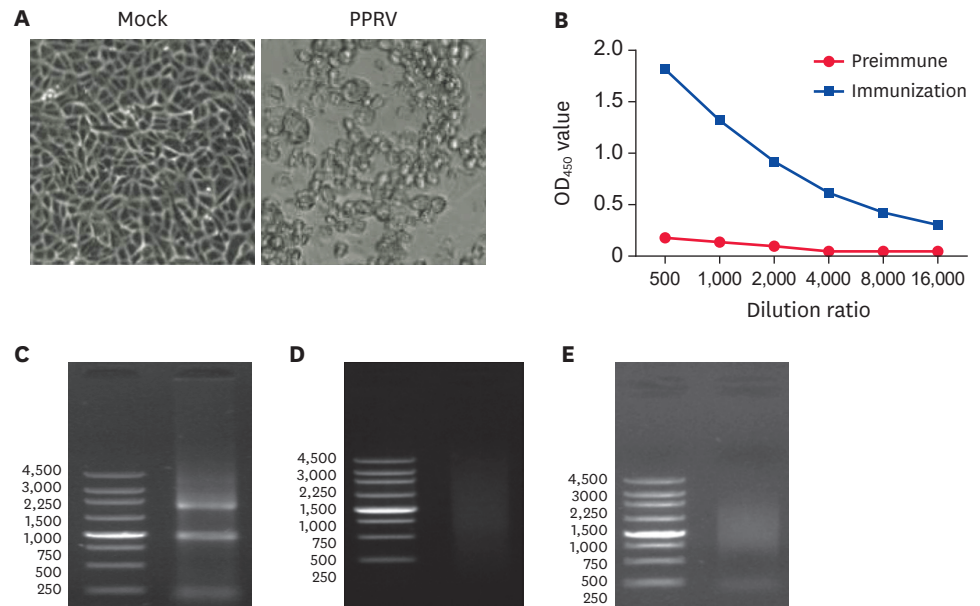


Fig. 1. Construction of cDNA library. (A) White light diagram of normal Vero cells and lesions after immunization with attenuated PPRV vaccine. (B) Enzyme-linked immunosorbent assay was used to detect the reaction of polyclonal serum to PPRV. At 21 days after the third immunization, samples were collected to determine the antibody titer. The x-axis shows dilutions from 1:500 to 1:16,000. PPRV was used as the coating antigen. Serum obtained before immunization was used as the negative control. (C) The total RNA extracted from peripheral blood lymphocytes was purified to obtain an electrophoretic map of the mRNA. Lane 1: mRNA, the three bands are 28S, 18S, and 5S from top to bottom. (D) Electrophoresis of double-stranded cDNA. Lane 1: double-stranded cDNA. (E) Electrophoresis of purified double-stranded cDNA. Lane 1: purified double-stranded cDNA. Lane M: 250 bp DNA Ladder. PPRV, peste des petits ruminants virus.

removed by the CHROMA SPIN-1000-TE column, the purified cDNA showed obvious smear bands (**Fig. 1E**).

The pGADT7 vector was connected to the above purified cDNA to form the primary cDNA library, which was then transformed into HST08 competent cells. The transformation solution was diluted from 10^{-1} to 10^{-5} , followed by coating with LB medium and overnight culture at 37°C. Twenty clones were grown on each plate at a dilution ratio of 10^{-5} , and the cDNA library capacity was calculated to be 2.0×10^6 cfu/mL. Sixteen clones were randomly selected to examine the diversity of the cDNA library, and the electrophoresis results demonstrated that the cDNA library included 400–2,000 bp with an average insert fragment of about 1,000 bp. The recombination rate of the library was 100% (**Fig. 2A**). The amplified clones were recovered and plasmids were extracted according to the Nucleobond Xtra MIDI EF kit instructions (**Fig. 2B**).

Screening and selection of single-domain antibodies

The plasmid extracted during cDNA library construction was screened twice by PCR analysis, and the plasmid size was determined to be 400 bp. Each examined clone contained different CDR sequences. The 400 bp plasmid was transformed into TOP10 competent cells, and 100 clones were randomly selected for sequencing. Four VHH sequences were obtained by screening (VHH1, VHH2, VHH3, and VHH4), and the deduced amino acid sequences are illustrated in **Fig. 3**.

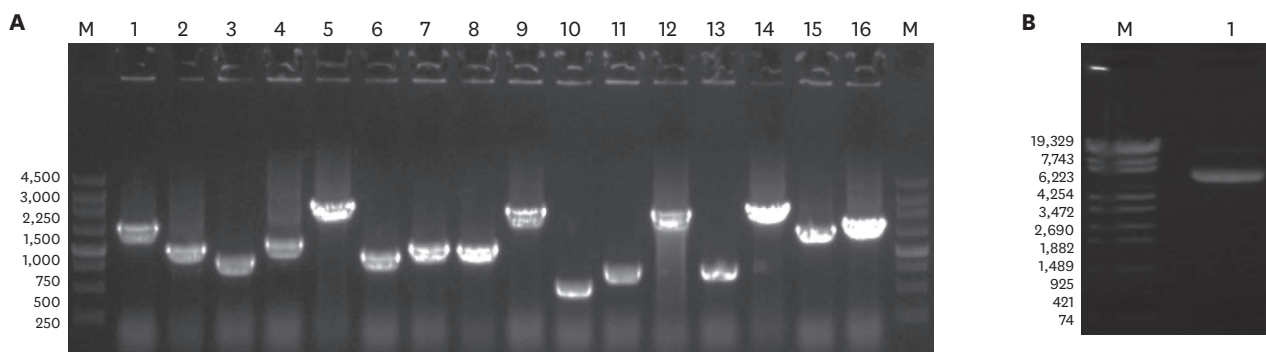


Fig. 2. Identification of cDNA library. (A) Library diversity detection. Lanes 1–16: 16 of the mono-clonal electrophoresis results were randomly selected after the library's construction. Lane M: 250 bp DNA Ladder. (B) Lane 1: plasmid electrophoresis results extracted from the library. Lane M: Lambda EcoT14 I digest.

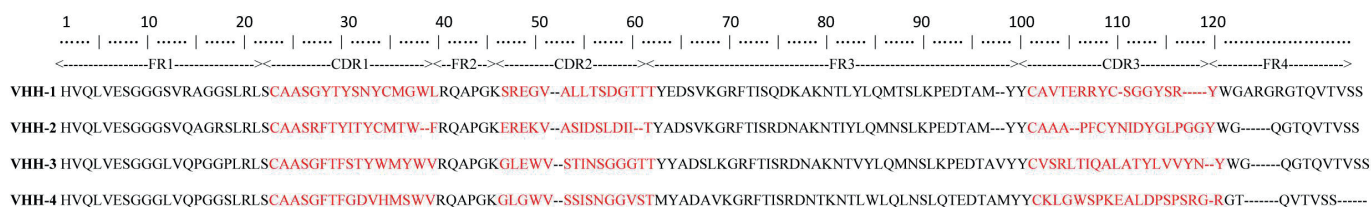


Fig. 3. Alignments of the amino acid sequences of VHHs. Thick-lined boxes indicate the specific VHH amino acids, the CDRs are shown in thin-lined boxes. FR, framework region; CDR, complementarity determining region; VHH, variable domain of heavy chain.

Cloning and expression of VHHs

Three of the VHH sequences from the isolated clones were inserted into the pCold-c2x vector and transformed into BL21-codon-Plus (DE3)-RIL strain cells. Proteins were induced with IPTG and analyzed via SDS-PAGE. The soluble analysis showed that four recombinant proteins were expressed in the supernatant, and the expressed protein size was approximately 56 kDa (Fig. 4A-C), which corresponded to the predicted molecular weight of the protein predicted from the DNA sequence. As shown in Fig. 4D, the purified VHH proteins were single banded. The VHH expression levels were approximately 30% of the total *E. coli* protein content.

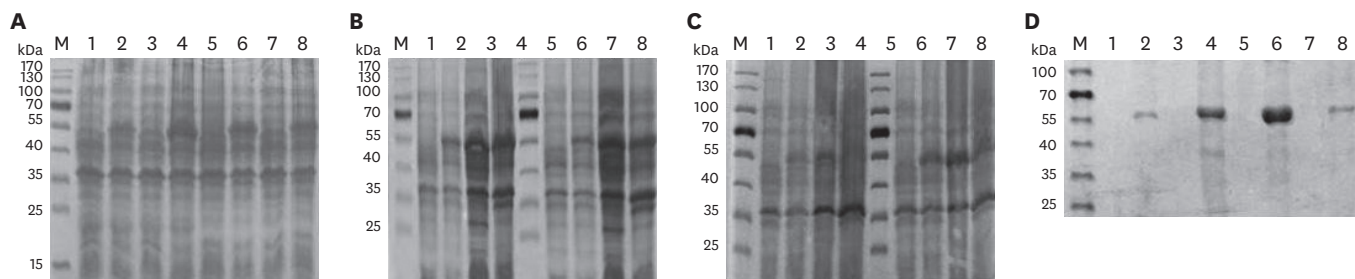


Fig. 4. Soluble analysis and expression of VHH proteins. (A) IPTG induces the expression of four proteins in *E. coli*. Lanes 1, 3, 5, 7: bacterial lysates of uninduced VHH1, VHH2, VHH3, and VHH4. Lanes 2, 4, 6, 8: bacterial lysates of VHH1, VHH2, VHH3, and VHH4 induced with IPTG. (B) Soluble expression analysis of VHH1 and VHH2. Lanes 1 and 5: bacterial lysates from groups VHH1 and VHH2 were not induced with IPTG. Lanes 2 and 5, expression of VHH1 and VHH2 in *E. coli* induced by IPTG. Lane 3, 4, 7, and 8, VHH1 and VHH2 supernatant and precipitated expression products after IPTG induction. (C) Soluble expression analysis of VHH3 and VHH4. Lanes 1 and 5: bacterial lysates from groups VHH3 and VHH4 were not induced with IPTG. Lanes 2 and 5, expression of VHH3 and VHH4 in *E. coli* induced by IPTG. Lane 3, 4, 7, and 8, VHH3 and VHH4 supernatant and precipitated expression products after IPTG induction. (D) Purification of VHH1 and VHH2 antibodies. Lanes 1 and 6, the supernatant of bacterial lysates of VHH1 and VHH2. Lanes 2 and 5, the supernatant of bacterial lysates of VHH1 and VHH2 combined with the purification column results of VHH1 and VHH2. (E) Lanes 2 and 8, the supernatant of bacterial lysates of VHH3 and VHH4. Lanes 1 and 6, the supernatant of bacterial lysates combined with the purification column results of VHH3 and VHH4. Lane M: Protein marker. VHH, variable domain of heavy chain.

Characterization and application of purified VHHs

The expression of the target proteins was also evaluated by western blot analysis using HRP conjugate anti-his mouse monoclonal antibody. The results showed that four VHH proteins had a specific reaction with the antibody (Fig. 5A). The affinities of the four recombinant proteins to PPRV were detected by ELISA. All of the selected VHHs showed high OD₄₅₀ values, demonstrating that the selected VHHs had a high affinity to PPRV (Fig. 5B). Among the four VHHs strains, VHH2 had the highest affinity to PPRV. The specificities of the four VHHs were determined via ELISA by comparison with cross-reactions with other pathogens, including ORFV, foot-and-mouth disease (FMDV), and GPV. The results showed a specific binding activity with PPRV with no other cross-activity detected in the ELISA results (Fig. 5C). In our previous study, GTCs were infected with PPRV [11] and were shown to be useful in detecting PPRV infection. The results showed that the nucleus of the negative control group was clear blue with no green fluorescence. In comparison, the positive control group and the experimental group nuclei had obvious green fluorescence. In conclusion, VHHs did not react with cell proteins but did react with PPRV (Fig. 6).

Neutralization test

The results showed that VHH1 and VHH4 could inhibit 50% of cell lesions at 1:160, while VHH2 and VHH3 could inhibit 50% of cell lesions at 1:640 and 1:320, respectively. It was concluded that all four VHHs had neutralizing activity.

Specificity identification of VHH

In this study, VHH2 was identified as PPRV M protein-specific by western blot and ELISA analyses. The western blot results showed a single band at 38 kDa, which was the closest to the length of M protein (Fig. 7B); the lengths of the PPRV proteins were: N, 58 kDa; P, 60 kDa; C, 20 kDa; V, 32 kDa; M, 38 kDa; F, 59 kDa; H, 67 kDa; and L, 247 kDa. The ELISA results showed that the antibody had good reactivity with the PPRV M protein, and the reactivity with the other proteins was negative result (Fig. 7A).

To summarize, VHH2 is considered to have PPRV M protein specificity. The other three VHHs are still being analyzed and the PPRV-associated results will be reflected in a subsequent paper.

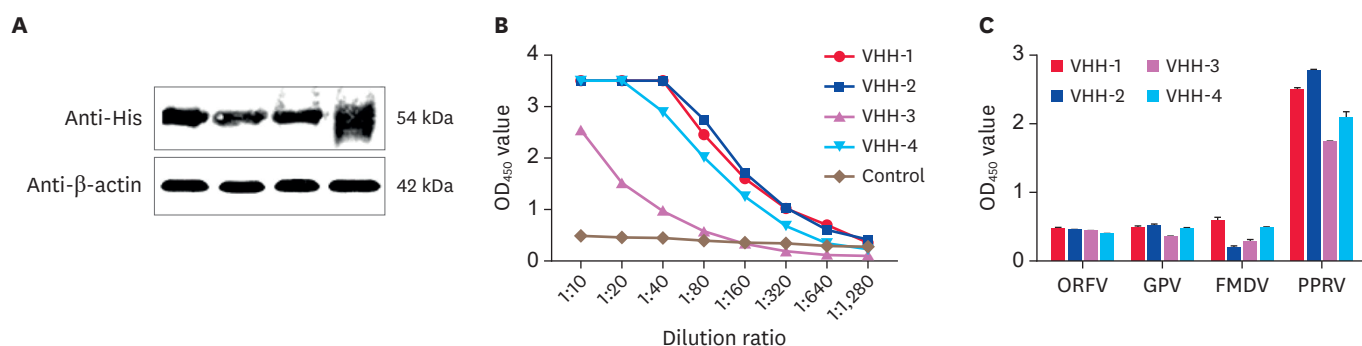


Fig. 5. Characterization and application of purified VHHs. (A) Western blot analysis was performed with HRP conjugate anti-His mouse monoclonal antibody. (B) The affinity of VHH1, VHH2, VHH3, and VHH4 with PPRV was determined by indirect ELISA. PPRV was used as an enzyme plate-coated antigen and was diluted at the ratio of 1: 80. Four single-domain antibodies were added to the plate as the primary antibody, and anti-His mouse monoclonal antibody was added as the secondary antibody. (C) Cross-reactions with other viral antigens. ELISA detected the specificity of the VHH antibodies. The antigens of sheep and GPV, ORFV, FMDV, PEDV, and PPRV were used to evaluate PPRV specificity. The OD₄₅₀ value was determined as the average of three wells. Error bars represent mean ± SD values. VHH, variable domain of heavy chain; PPRV, peste des petits ruminants virus; ELISA, enzyme-linked immunosorbent assay; GPV, goat pox virus; ORFV, sheep mouth sore virus; FMDV, foot and mouth disease virus; PEDV, porcine epizootic diarrhea virus.

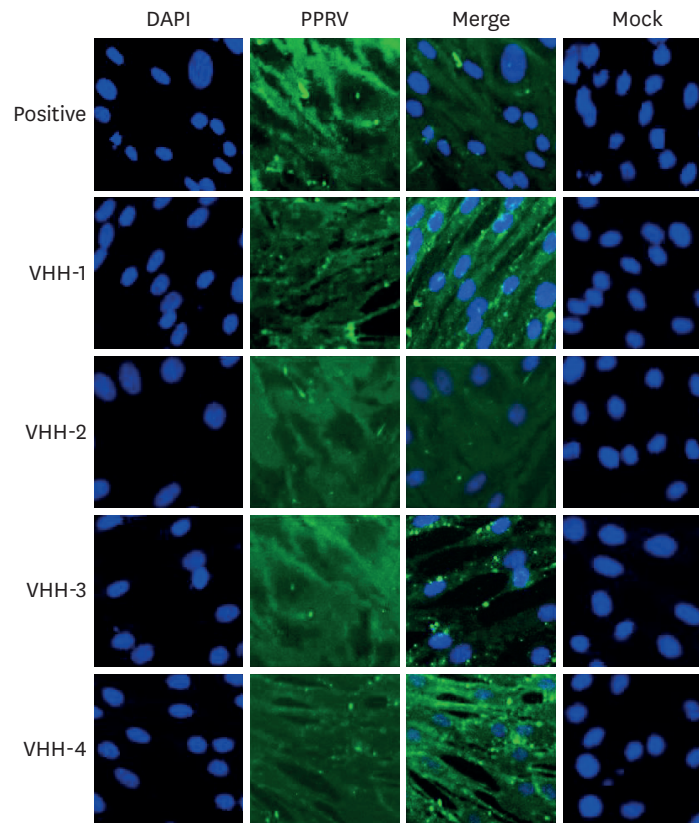


Fig. 6. The reaction of VHH antibodies with PPRV was determined by indirect immunofluorescence assay. GTC was infected with PPRV at 37°C for 48 h. Four selected VHH antibodies were used as primary antibodies and DAPI was used to stain nuclei. After 48 h, conspicuous immunofluorescent nuclei were observed. Scale bar is 100 μm. VHH, variable domain of heavy chain; PPRV, peste des petits ruminants virus; GTC, goat tracheal epithelial cells.

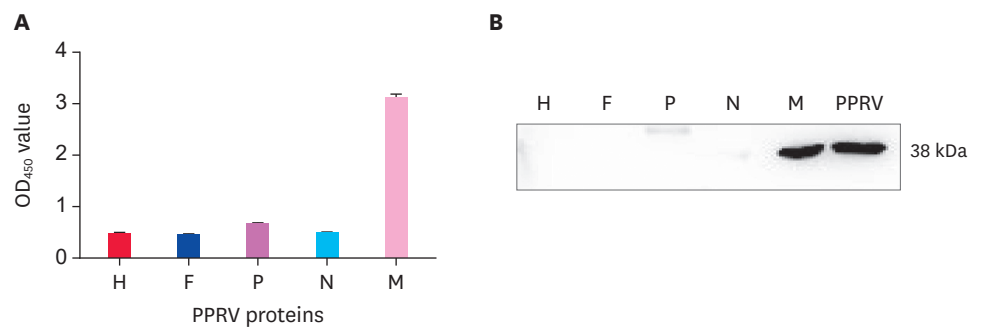


Fig. 7. (A) The results of enzyme-linked immunosorbent assay test to detect the affinity of VHH2 with five proteins (H, F, P, N, M) of PPRV. (B) The results of western blot test to detect the affinity of VHH2 with five proteins (H, F, P, N, M) of PPRV. VHH, variable domain of heavy chain; PPRV, peste des petits ruminants virus; H, hemagglutinin protein; F, the fusion protein; P, the phosphoprotein; N, the nucleocapsid protein; M, the matrix protein.

DISCUSSION

Among the infections affecting small ruminants, FMDV, small ruminant disease virus (PPRV), sheep pox virus (SPV), goat pox virus (GPV), and blue tongue disease (BTV) are likely to occur as mixed infections [12]. About 62.5% of the world's goat and sheep populations are at risk of PPR, which is considered a significant infectious disease by the global goat and

sheep industry. According to the FAO, production loss and treatment cost associated with PPR in the SAARC region may amount to \$29 billion 725 million per year in 2012–2017 and may have caused economic losses of \$25 billion 690 million per year in India alone [12]. With the successful eradication of rinderpest from the world, PPR has become a prime animal disease, as proposed by the FAO, OIE, and other international organizations, to be controlled and eradicated globally.

A cDNA library refers to a collection of clones formed by the reverse transcription of all of the mRNA transcribed during a certain development period of an organism and a vector. Each clone contains only one kind of mRNA information, and the sum of a sufficient number of clones will contain all of the cell's mRNA information. A cDNA library constructed by SMART (switching mechanism at 5' end of the RNA transcript) and CDS methods can indicate the abundance of mRNA in the cell samples. In this study, we constructed a VHH cDNA library from the PBLs of Bactrian camels. The results indicate that the diversity of the library is good, and the library capacity is limited (approximately 2.0×10^6), which is about 1×10^{13} times that of those reported previously [13,14], indicating there is a need for improvement. Compared with traditional antibodies, the molecular weight of single-domain antibodies from camels is approximately 1/5 of that of conventional antibodies. In this study, to promote the expression of single-domain antibodies, an MBP tag (42.5 kDa) was introduced into the recombinant antibody vector, resulting in producing a final VHH molecular weight of about 56 kDa.

Single-domain antibodies have a special feature: they can maintain stable three-level folding while supporting extreme sequence diversity in numerous binding-specific immune systems [15]. Based on the four amino acid sequences of the four VHHs in this study, the amino acid sequences of the CDRs are different, indicating that these four VHHs likely target different proteins of the PPRV or different binding sites of the same protein. The above observations need further study to verify these suggestions.

In this study, ELISA and IFA verified the affinity between PPRV vaccine and VHH antibodies. It was shown that VHH2, among the four VHH, had the best affinity to PPRV. At the same time, in order to access specificity, we used PPRV and three other pathogens of common sheep diseases. The results showed that the specificity of VHH2 was higher than that of the other three VHH antibodies. Thus, we speculate that VHH2 is a promising antibody for further PPRV research.

Following the discovery of its potential, sdAbs have been widely used in many fields. Yang et al. [16] showed that fusion of porcine circovirus type-2 specific sdAb (psdAb) with an alkaline phosphatase produced binding activity and specificity levels that were about five times that of the original single-domain antibody. The small size of sdAbs allows them to be used as nano-scale detection tools, after being appropriately labeled. Moreover, an sdAb can stabilize the antigen's structure after combining with the antigen; thus, it can stabilize proteins with unstable structures to allow analysis of the protein's structure [17]. Yin et al. [18] combined an sdAb with Zn/CdSe quantum dots (QDs) to form the QDs-C6 probe, which has been used to track and image the subcellular localization of FMDV in BHK-21 cells. Furthermore, sdAbs have an important role in medical diagnosis, drug delivery, and disease treatment, thereby promoting technology updates and indicating novel possibilities for disease treatment and diagnosis [19-21]. Puttemans et al. [22] showed that a radiolabeled sdAb can be used as a valuable additional therapy for patients with HER2pos metastatic cancer, which can be challenging to treat. In addition, O'Sullivan et al. [23] reported that the production of

thrombin in the plasma of hemophilia mice was successfully restored using a monodomain antibody of llama, and their results showed that the bleeding phenotype of the hemophilia mice was corrected. Due to its neutralizing activity, sdAbs have been used as reagents in the specific detection of bacteria, parasites, and viruses and in biotechnological applications related to virus infection therapy in humans [24].

In conclusion, this study confirmed that a PPRV-specific VHH fragment can be selected from a PPRV-immune Bactrian camel VHH library. In this study, four PPRV VHHs were selected and characterized. All four were easy to express and purify, and they had a high affinity to and specificity for PPRV, indicating that they have potential as diagnostic reagents for PPRV detection.

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