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Design and Selection of a Camelid Single-Chain Antibody Yeast Two-Hybrid Library Produced *De Novo* for the Cap Protein of Porcine Circovirus Type 2 (PCV2)

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Abstract

Nanobodies (or variable domain of the heavy chain of the heavy-chain antibodies, VHHs) are single-domain antigen-binding fragments derived from camelid heavy chain antibodies. Their comparatively small size, monomeric behavior, high stability, high solubility, and ability to bind epitopes inaccessible to conventional antibodies make them especially suitable for many therapeutic and biotechnological applications. In this paper, for the first time, we created the immunized *Camelus Bactrianus* VHH yeast two-hybrid (Y2H) library according to the Clontech Mate & Plate library construction system. The transformation efficiency and titer of the VHH Y2H library were 7.26×10^6 cfu/3 μg and 2×10^9 cfu/ml, which met the demand for Y2H library screening. Using as an example the porcine circovirus type 2 (PCV2) Cap protein as bait, we screened 21 positive Cap-specific VHH sequences. Among these sequences, 7 of 9 randomly selected clones were strongly positive as indicated by enzyme-linked immunosorbent assay, either using PCV2 viral lysis or purified Cap protein as coated antigen. Additionally, the immunocytochemistry results further indicated that the screened VHHs could specifically detected PCV2 in the infected cells. All this suggests the feasibility of *in vivo* VHH throughput screening based on Y2H strategy.

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Introduction

Heavy-chain antibodies (HCAbs) were discovered in camelids such as camel, llamas, alpacas, and sharks in the 1990s [1]. Unlike conventional immunoglobulin G (IgG), these antibody molecules are naturally devoid of light chains. Therefore, VHH is solely responsible for antigen recognition [2]. With a molecular weight of approximately 15 kDa (compared with the 150 kDa of IgG), VHH is currently the smallest naturally occurring intact antigen-binding units [3]. On average, VHH has longer complementarity determining region 3 [4], a feature that may facilitate binding into deeper cavities on the antigen surface that are inaccessible for conventional antibodies. Moreover, VHH is characterized by low immunogenicity in primates, high stability, and often excellent expression yield in prokaryotic and eukaryotic hosts. Thus, VHH is realistic candidates for disease diagnosis and treatment [5,6,7].

Methods of camelid VHH library construction are normally based on phage display. Following mRNA isolation and CH2 gene specific reverse transcription, usually two successive PCRs are performed [8]. The first is used to discriminate VH from VHH based on amplicon sizes which differ by about 300 bp in length (about 900 bp and 600 bp). Shorter amplicons encoding VHHs are subsequently re-amplified through a second nested PCR with primers annealing at the codons of FR1 and FR4 (about 400 bp), because all variable domains of camelid heavy chain antibodies belong to a single family (family III). Such a nested PCR approach could be mutagenic and disadvantageous for molecular diversity, but is expected to eliminate VH derived contributors which could lead to sticky proteins through exposed hydrophobic amino acids on their surface lacking VL domain. Afterwards, the conventional procedure is to clone VHH fragments into phage plasmid (such as pHEN2 or pHEN4) and transform *E.coli* competent cells to construct phage display library.

In the present study, a novel method of generating a VHH library was reported because of the fast and direct selection of VHHs in yeast without any *in vitro* pre-selection. Compared with the conventional VHH phage library, the VHH Y2H library in this study had the following distinct features. First, the antigen gene was expressed *in vivo* by fusion with GAL4 DNA-binding domains (DNA-BD/bait), which avoided time-consuming antigen expression and purification *in vitro*. The yeast VHH library had a unique advantage for difficult-to-express proteins or proteins with complex post-translational modifications, or for the determination of VHHs targets [9]. Second, the repertoire cloning of VHH into the DNA-AD/prey plasmid was based on SMARTer^{TM} and recombination technology (Clontech), which was more efficient than conventional restriction enzyme digestion and T4 ligase ligation [10]. Third, compared with the currently available golden

standard (i.e., analysis of the crystal structure of a given antigen-antibody complex), this approach was more effective and realistic for the precise domain mapping of linear epitopes, confirmation of non-linear epitopes or conformational epitope sensors, and detection of secondary binding partners [11]. The general construction procedures of a VHH phage library and a Y2H library were shown in Figure 1, the screening procedures comparisons of the two libraries were shown in Figure 2 and Table S1 (Table S1 in File SI), and the advantages and disadvantages comparisons of the two libraries were shown in Table S2 (Table S2 in File SI).

To further confirm the feasibility of selecting VHHs targeting a specific antigen based on the Y2H system, we took as an example porcine circovirus type 2 (PCV2) Cap protein, PCV2 is the primary causative agent of several syndromes collectively known as porcine circovirus-associated disease (PCVAD), currently considered the most economically important disease affecting the global swine industry [12,13]. PCV2 has two major open reading frames (ORFs). ORF1 is essential for viral DNA replication, whereas ORF2 (the Cap gene, which encodes the only structural protein of viral capsid), has become the major target for developing PCV2 vaccines and serological diagnostic reagents [14].

The present study aimed to create an immunized *Camelus Bactrianus* VHH Y2H library for the *in vivo* VHHs throughput screening using the yeast two-hybrid system. The selected VHHs specific to Cap protein were found to have great potential for the development of PCV2 therapeutic vaccine or diagnostic reagent.

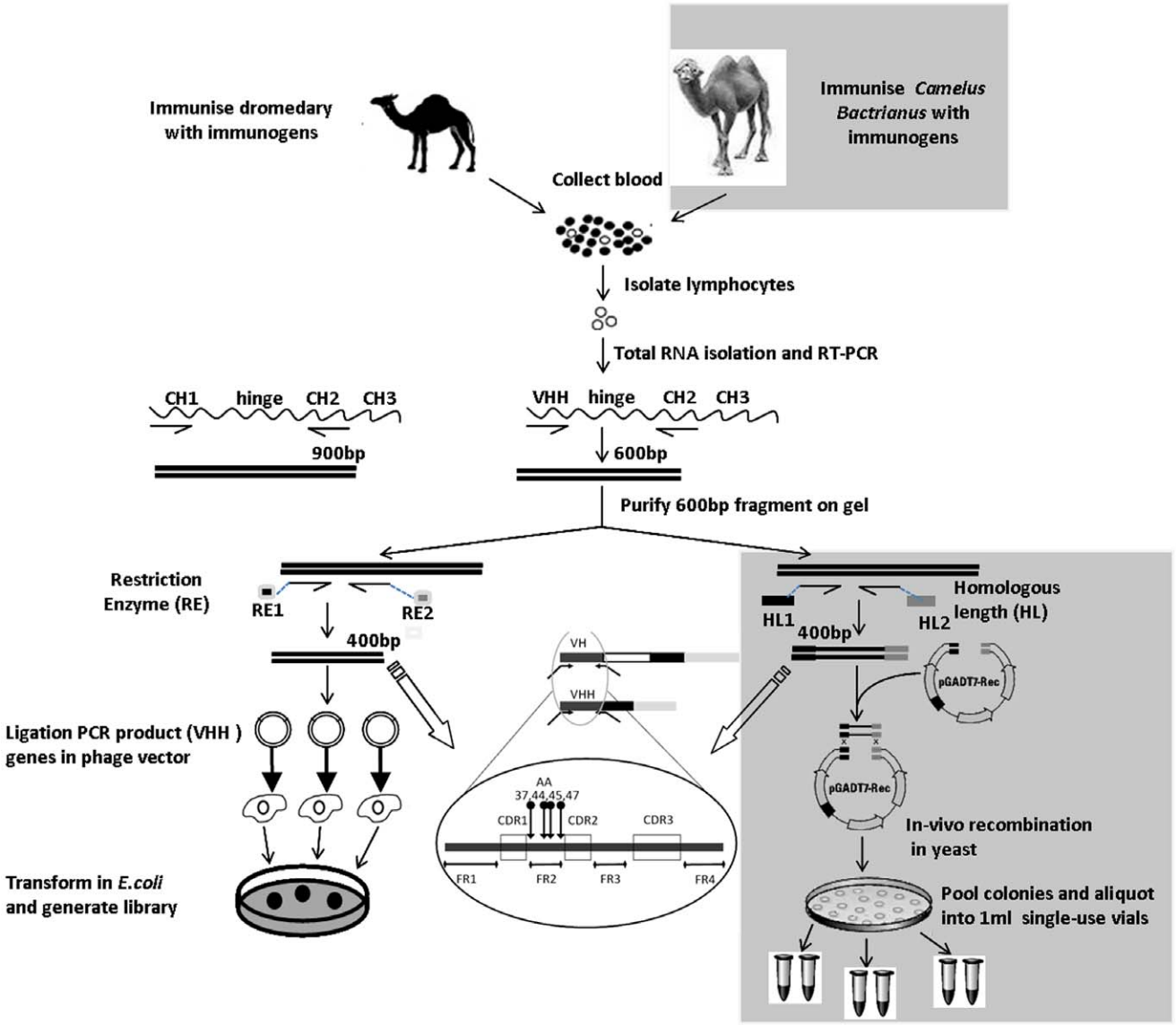

Figure 1. Schematic of strategies for constructing a phage library and a Y2H library. On the left side, the conventional method of constructing a VHH phage library is presented. On the right side, the novel method of constructing a VHH Y2H library is presented. The differences in the procedure for *Camelus Bactrianus* VHH Y2H library construction are in the shaded box. The discontinuous arrow shows the schematic of the hallmark VHH domains.
doi:10.1371/journal.pone.0056222.g001

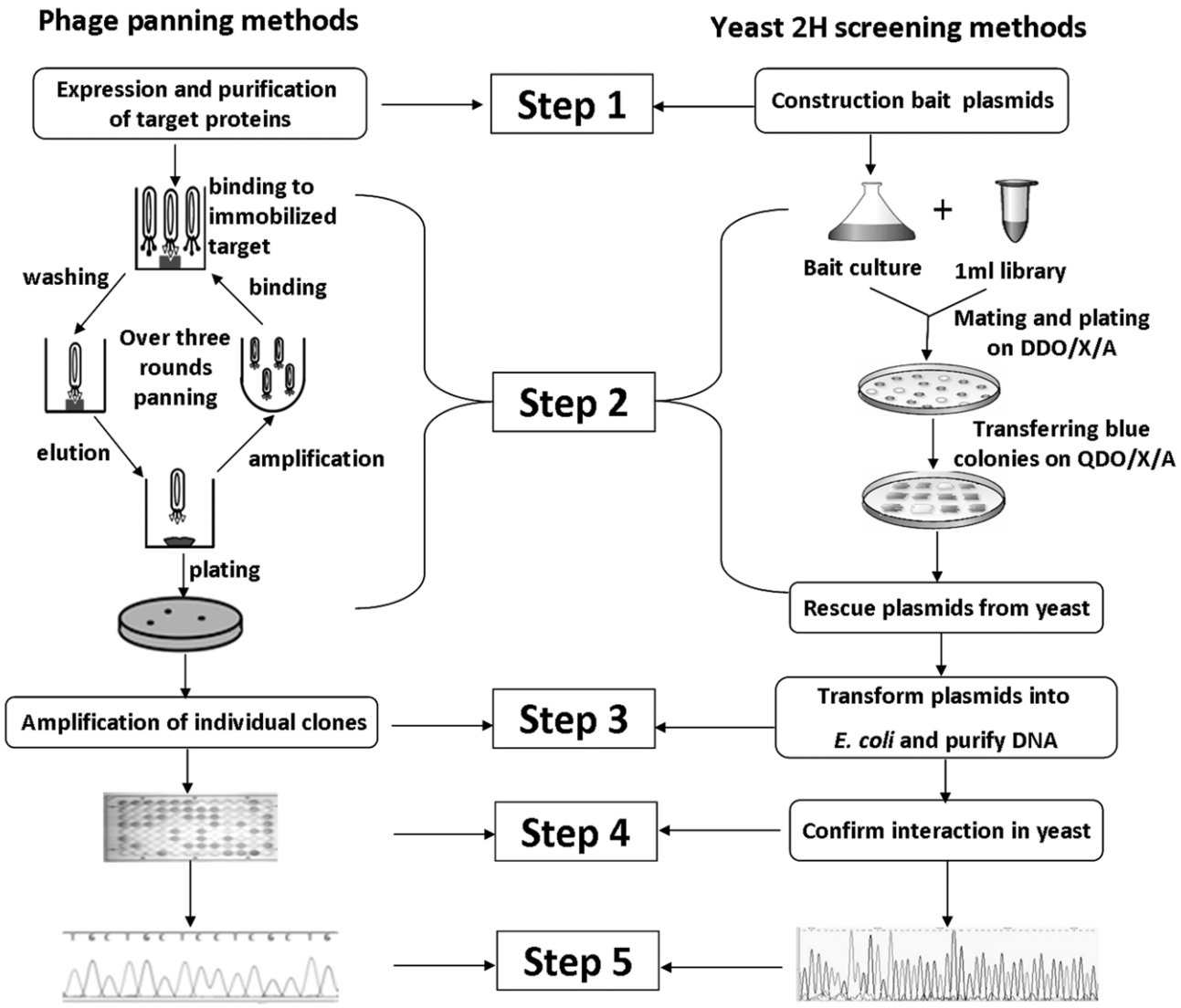

Figure 2. Schematic of two alternate approaches for the selection of antigen-specific VHHs (continuation of Figure 1). On the left side, a VHH screening approach based on phage panning is presented. On the right side, a VHH screening approach based on Clontech Matchmaker™ Gold Yeast Two-Hybrid System is presented.
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Materials and Methods

Camelus Bactrianus Immunization

A 6 month-old male *Camelus Bactrianus* was immunized with PCV2, FMDV, and CSFV vaccines (provided by the Chinese Academy of Agricultural Sciences, Lanzhou Veterinary Research Institute, China) five times with 2 weeks intervals. The immunized dose was based on the weight ratio between porcine and *Camelus Bactrianus*. The humoral immune response was monitored in serially diluted serum by enzyme-linked immunosorbent assay (ELISA) on microtiter plates coated with PCV2-lysed fetal porcine retina cells (FPRC) [15]. The animal with the stronger response received a final boost and bled 20 days later. All animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China. This study was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (No. LVRIAEC2012-006).

RNA Isolation and VHH Amplification

About 100 ml of blood was collected 20 days after the last injection. 10^8 mononuclear cells were extracted by Ficoll-Paque gradient centrifugation, pelleted, frozen in liquid nitrogen, and then kept at $-80^{\circ}C$. Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen), and the first-strand cDNA was synthesized using Reverse Transcriptase M-MLV (TaKaRa) with Oligo-dT primers. The cDNA encoding VHH and VH was specifically amplified from the first-strand reaction, using the primers CALL01 and CALL02 (Table 1) annealed from the 5′ leader sequences to the CH2 domain [6]. The resulting polymerase chain reaction (PCR) products included a 900 bp fragment for VH-CH1-CH2 exons and a 600 bp fragment for VHH-CH2 exons. The 600 bp fragment was collected as a template to re-amplify the VHH fragments with primers VHH-up and VHH-down (Table 1). The resulting 400 bp fragment of VHH pool was extracted from the gel and purified by a commercial gel extraction kit (OMEGA).

Table 1. Primers used in this work.

Primer	Primer Sequence (5′-3′)
CALL01	GTCCTGGCTGCTCTTCTACAAGG
CALL02	GGTACGTGCTGTTGAACTGTTCC
VHH-up	TTCCACCCAAGCAGTGGTATCAACGCAGAGTGGGAGTCTGGRGGAGG
VHH-down	GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACATGGAGACGGTGACCWGGGT
T7	TAATACGACTCACTATAGGG
3′AD	AGATGGTGCACGATGCACAG
VHH-Cold-F	GGATCCATGCAGGTGCAGCTGGTGGAGTCTGGRGGAGG
VHH-Cold-R	GTCGACTTATGTGGAGACGGTGACCWGGGT
Cap-F	CGCGGATCCATGACGTATCCAAG
Cap-R	AAAAGTCGACCTTAATGGTTAAGT

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VHH Y2H Library Construction, Complexity, Titering, and Diversity Evaluation

Y187 yeast competent cells were prepared according to the protocol of the Yeastmaker Yeast Transformation System 2 (Clontech). About 20 µl of purified VHH fragments (2–5 µg) and 3 µg of Sma I Linearized pGADT7-Rec were co-transformed into the yeast strain Y187 to construct the VHH Y2H library according to the Make Your Own "Mate & Plate^{TM}" Library System User Manual (Clontech). To determine the complexity of the library, 100 µl of 1/10 and 1/100 dilutions of transformed cells were spread on SD/−Leu (synthetically defined medium lacking leucine) 100 mm agar plates. After incubation at 30°C for 3–4 days, numbers of colonies on the dilution plates were counted and the transformation efficiency was calculated. To identify the titer of the constructed VHH Y2H library, 10 µl of library aliquot was taken out and diluted to 1/100, 1/1000, 1/10 000, and 1/1000 000. The last two dilutions were spread in duplicate on SD/−Leu 100 mm agar plates, and the library titer was calculated according to the colonies appearing.

Meanwhile, at least 20 random clones were picked for insertion diversity assay by PCR using the universal primers 3′AD and T7 (Table 1). The PCR products were further sequenced and analyzed using the NCBI BLASTP program to verify whether the insertions have the signature of VHH.

Yeast Two-hybrid Screening

To screen Cap proteins targeting VHH antibodies from the above Y2H library, the bait protein with complete coding sequences of Cap from PCV2 was cloned into the BamH I and Sal I sites of pGBKT7 (Clontech) and expressed as a fusion to the yeast GAL4 DNA-BD. Before screening, the bait protein was tested against the autoactivity and toxicity of Cap in the absence of a prey VHH library. A concentrated Y2HGold (pBD-Cap) culture with 1 ml of Y187 (pAD-VHHs) Y2H library was mixed for mating in accordance to the Matchmaker^{TM} Gold Yeast Two-Hybrid protocol (Clontech) (Figure 2). Sixty 150 mm DDO/X/A (double dropout medium lacking tryptophan and leucine and supplemented with X-α-Gal and Aureobasidin A) plates were used to screen the clones after mating for 3–5 days. All blue colonies were then patched out and allowed to grow on QDO/X/A (quadruple dropout medium lacking adenine, histidine, tryptophan and leucine and supplemented with X-α-Gal and Aureobasidin A) plates. The blue colonies were screened twice on QDO/X/A plates to rescue additional library plasmids and eliminate false positives. The bait plasmid (pBD-Cap) and prey plasmid (pAD-VHHs) were co-transformed into the yeast strain (Y2HGold) to validate the interactions in yeast (Figure 2). Meanwhile, the bait plasmid (pGBKT7-53 or pGBKT7-Lam) was co-transformed into Y2HGold with the prey plasmid (pGADT7-T) to serve as positive or negative control, respectively. The AD/library VHH inserts were further sequenced and analyzed using the NCBI BLASTP program.

Expression and Purification of Recombinant VHHs and Cap

The positive VHH fragments were amplified (using the primers VHH-Cold-F and VHH-Cold-R; Table 1) and cloned into BamH I and Sal I restriction enzyme sites of plasmid pCold I DNA (TaKaRa) for fusion to 6×His tag at the N-terminal, respectively. The recombinant plasmids were transformed into *E. coli* BL21 (DE3) (Novagen) and grown at 15°C by inducing 0.4 mM isopropyl-D-thiogalactopyranoside (IPTG) for 10 h. The cell pellets were harvested to check the expression of VHH antibodies by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The VHH antibodies were further purified on Talon Metal Affinity Resin (Clontech) and eluted with phosphate buffer containing 200 mM imidazole. For Cap expression, the full-length Cap gene from the bait pGBKT7-Cap plasmid was cloned into BamH I and Sal I restriction enzyme sites (using the primers Cap-F and Cap-R; Table 1) of the pMAL-c2X vector (NEB). The protocol for Cap-inducing expression and purification was in accordance to the pMAL^{TM} Protein Fusion and Purification System (NEB).

ELISAs

To verify the specific interactions between the selected VHH clones with Cap, PCV2 viral lysis (PCV2-lysed FPRC cells) or purified Cap protein (0.5 µg/ml) was coated overnight at 4°C with 100 µl/well onto Maxisorp 96-well plates (Nunc) in Na_2CO_3 (0.05 M, pH 9.6). Additionally, the lysis of uninfected FPRC (Mock), BSA, or MBP tag served as a negative control, and the PCV2 positive serum (provided by Wuhan Keqian Animal Biological Products Co., Ltd) served as a positive control. The plates were blocked with 1% BSA- phosphate-buffered saline with 0.05% Tween 20 (PBST) for 1 h at 37°C. After washing three times with PBST, the 6×His tag fused with VHH fragments were loaded into 100 µl/well for 2 h at 37°C in triplicate and serially diluted up to log2. The wells were washed three times with PBST

and treated with 100 µl of horseradish peroxidase (HRP)-Conjugated Anti His-Tag Mouse Monoclonal Antibody (CWBIO) at a 1:2000 dilution for 1 h at 37°C. At the same time, the positive control wells were treated with HRP-conjugated goat anti-swine antibody. After 3 min of washing five times with PBST, the reaction was developed by adding tetramethylbenzidine (TMB) and stopped by adding 2 N H_2SO_4 after 30 min of incubation. The absorbance was measured in a microplate reader at 450 nm (Bio-Rad). Student's t-test was used to compare the ELISA results of different samples. Calculated P values of <0.05 were considered to be statistically different.

Immunocytochemistry Assay

To verify the specific intracellular binding activity between selected VHHs and PCV2 virus, PCV2-infected FPRC cells and mock control (1×10^5 cells/well) were grown on six-well dishes for the immunoperoxidase assay. After 48h, the cells were fixed with 4% paraformaldehyde on six-well dishes and were permeabilized with PBS-0.5% Triton X-100. The fixed cells were incubated with blocking solution followed by the selected VHHs over night at 4°C. The next morning, the treated cells were then incubated with horseradish peroxidase (HRP)-Conjugated Anti His-Tag Mouse Monoclonal Antibody (CWBIO) at a 1:2000 dilution for 1 h at room temperature. The detection of the antigen was carried out using DAB Kits (BOSTER). At last, the nuclear of cells were stained by hematoxylin. The results were examined under an inverted light microscope.

Results

Camelus Bactrianus VHH Amplification and VHH Y2H Library Construction

According to the procedure in Figure 1, two PCR products including a 900 bp fragment for VH-CH1-CH2 exons and 600 bp for VHH-CH2 exons, were amplified with primers CALL01 and CALL02 (Table 1 and Figure 3A). The only VHH domain (about 400 bp) was further amplified using the template of the 600 bp fragment isolated from Figure 3A as well as the primers VHH-up and VHH-down (Table 1 and Figure 3B).

For library construction, 3.76 µg of the purified VHH PCR repertoire was co-transformed with 3 µg of Sma I Linearized pGADT7-Rec plasmid into Y187 yeast competent cells for *in vivo* recombination. The quality of Y2H library was checked through three parameters: the transformation efficiency, the library titer, and the percentage of library clones that contain an insert. In our VHH Y2H library, the transformation efficiency were 7.26×10^6/3 µg, the titer of the constructed library was 2×10^9 cfu/ml as determined by the number of independent clones in the dilution plates (Figure S1A in File SI). Meanwhile, 20 clones randomly picked from the SD/−Leu plates were used to determine the library functional diversity with universal primers T7 and 3'AD (Table 1) by PCR. All 20 clones had about 400 bp bands similar to the size of the VHH domain (Figure S1B in File SI), so the percentage of library insertion diversity was more than 95%. These clones had unique sequences by DNA sequencing, indicating that the library was diverse (not shown). From the above, the quality of our VHH Y2H library met the demand for Y2H library screening.

Selection of Cap-specific VHH Antibody Fragments

Before Y2H experiments, we first confirmed that the BD-Cap fusion protein had no autoactivator activity in yeast (Figure 4, lane A). After mating the Y2HGold (pBD-Cap) and Y187 (pAD-VHHs) yeast libraries and incubating on DDO/X/A plates for 3–5 days, hundreds of yeast blue colonies were selected. However, only 36 clones were recovered after two more rounds of screening on QDO/X/A plates. The prey plasmids from the above 36 clones were isolated, recovered, and co-transformed with the bait plasmid (pBD-Cap) or pGBKT7 (empty insertion) to verify the positive interactions between VHHs and Cap protein in yeast strain Y2HGold. The co-transformation results showed that all 36 clones rescued from QDO/X/A plates were genuine positive clones. Lanes B-M of Figure 4 show 12 randomly selected positive clones. Meanwhile, both positive (pGBKT7-53 and pGADT7-T co-transformants) and negative (pGBKT7-Lam and pGADT7-T co-transformants) control groups were found eligible (Figure 4, lane N). The results for intracellular interaction between Cap and anti-Cap VHHs in yeast are also summarized in the Table S3 (Table S3 in File SI). Sequencing results showed that the 36 positive clones were of 21 different clonal origins and the insertions were homologous to the properties of camel VHH (Figure 5).

Expression and Purification of VHH and Cap Proteins

To further validate the interactions between Cap and selected VHHs *in vitro*, 9 VHH fragments from 21 positive sequences were randomly selected for expression by pCold DNA I system (TaKaRa) and purified using Talon Metal Affinity Resin (Clontech). As 6×His tag was fused with the VHH fragments at the N-terminal, Cap gene was cloned into the pMAL-c2X vector for fusion expression with MBP tag in *E. coli* Rosetta (Novagen) and was purified by using Amylose Resin (NEB). After running on 12% SDS-PAGE, the 6×His-VHH fusion proteins (pCold-VHHs) showed 16 kDa-18 kDa bands (Figure 6, lanes A-I), and MBP or MBP-Cap fusion protein showed 42 kDa (lane K, Figure 6) or 70 kDa bands (42 kDa MBP tag plus 28 kDa Cap) (Figure 6, lane J), respectively. All proteins were expressed in soluble form as predicted sizes.

ELISAs

Indirect ELISA was used to detect the specific interaction between VHH antibodies and PCV2 viral lysis or purified Cap protein. For plate coated with PCV2 viral lysis, the measurement of virus titer was $10^{-6.4}$ $TCID_{50}$ and the best coated concentration of the virus was 1:800 (data not shown). The ELISA results indicated that most of the selected VHHs interacted with PCV2 viral lysis, among which 7 of 9 VHH clones were significantly stronger than the commercial PCV2 positive serum control ($OD_{450}>1.50$, $P<0.05$) (Figure 7A). The reaction in the wells coated with BSA (negative control 1) or the lysis of PCV2 uninfected FPRC (negative control 2) was very weak ($OD_{450}<0.10$).

For the plate coated with purified Cap protein (0.5 µg/ml), the Ag-Abs interaction results were similar to the plate coated with PCV2 viral lysis, except for a slightly decreased in response activity (Figure 7B). The interaction in the positive control wells also decreased from 1.30 (Figure 7A) to about 1.00 (Figure 7B), but VHH3, VHH1, VHH9, VHH2, and VHH11 were still considered strongly positive ($P<0.05$). The reaction in the wells coated with BSA (negative control 1) or 0.5 µg/ml purified MBP tag (negative control 2) was as weak, as seen in Figure 7A ($OD_{450}<0.10$).

Detection of PCV2-infected Cells by Immunocytochemistry

Using positive VHH3 clone as model, the intracellular specificity and binding affinity of selected VHHs were further detected by immunocytochemistry. As shown in Figure 8, in PCV2-infection group, most of infected cells had already

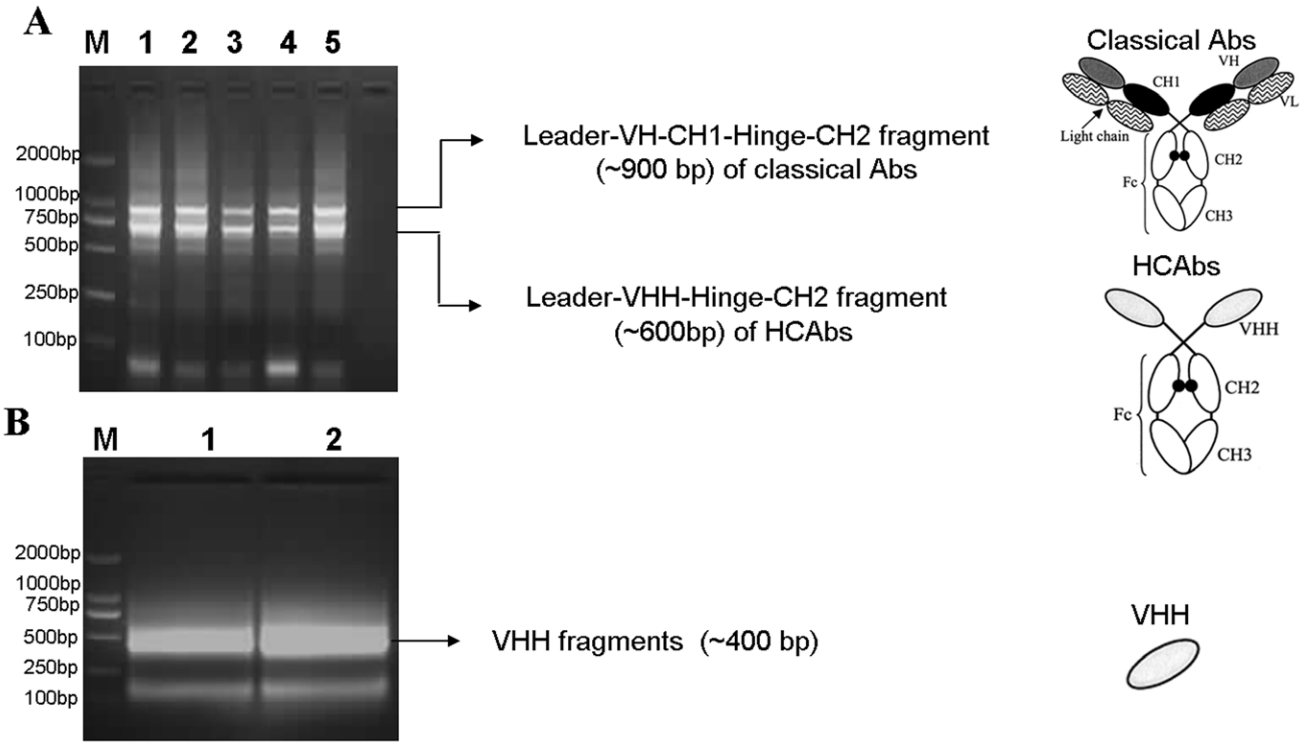

Figure 3. Agarose gel electrophoresis of *Camelus Bactrianus* VHH repertoire amplified by two successive PCRs. A, First round PCR to distinguish VH from VHH based on amplicon sizes. The upper bands in lanes 1–5 (~900 bp) represent the Leader-VH-CH1-Hinge-CH2 region of classical Abs. The lower band (~600 bp) in lanes 1–5 represents the Leader-VHH-Hinge-CH2 region of HCAbs. B, The complete VHH fragments is amplified (~400 bp in lanes 1–2) by a second nested PCR using the purified 600 bp DNA from Figure 3A as template. M in A and B indicate the DL2000 DNA marker. The primers used in two successive PCRs are from Table 1, and the schematics on the right of Figure 3A and Figure 3B represent the classical Abs (top), HCAbs (middle), and VHH (bottom).
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detached and lysed. So the cell coverage was much less than mock control. Meanwhile, the strong nuclear brownish staining was specifically appeared in PCV2-infected FPRC cells group but not in the mock control (only FPRC cells). These results indicated the feasibility of detection intracellular PCV2 antigen with selected VHHs.

Discussion

The single variable domain of camelid heavy chain antibodies (HCAbs) is generated from a V-D-JH gene rearrangement for which a distinct set of V genes, the VHH germline genes, is available in the camelid genome [16]. Different subfamilies have been distinguished for these VHH genes, but all are closely related to the human VH (3) family of clan III. In particular, the hallmark

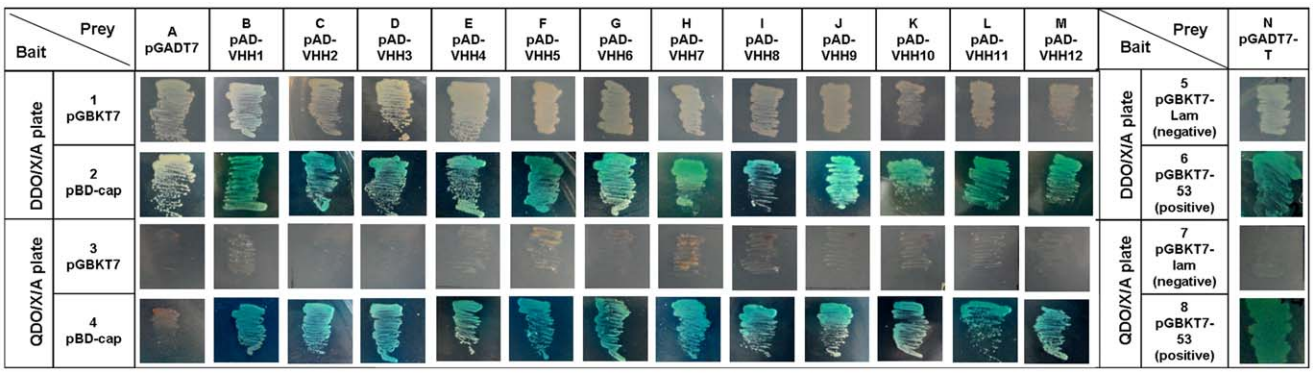

Figure 4. Intracellular interaction of the selected VHHs with PCV2 Cap protein. The white clones in lane A indicate that the bait (pBD-Cap) has no autoactivator activity in yeast. When DNA-BD/Cap was cotransformed into Y2HGold yeast strain with 12 random positive AD/VHH plasmids, all co-transformants appeared blue on both DDO/X/A and QDO/X/A plates (Figure 4, lanes B-M). In the control group (Figure 4, lane N), the pGBKT7-53 and pGADT7-T co-transformants (positive control) appeared blue on both DDO/X/A and QDO/X/A plates, whereas pGBKT7-Lam and pGADT7-T co-transformants (negative control) appeared white on the DDO/X/A plate and did not grow on the QDO/X/A plate.
doi:10.1371/journal.pone.0056222.g004

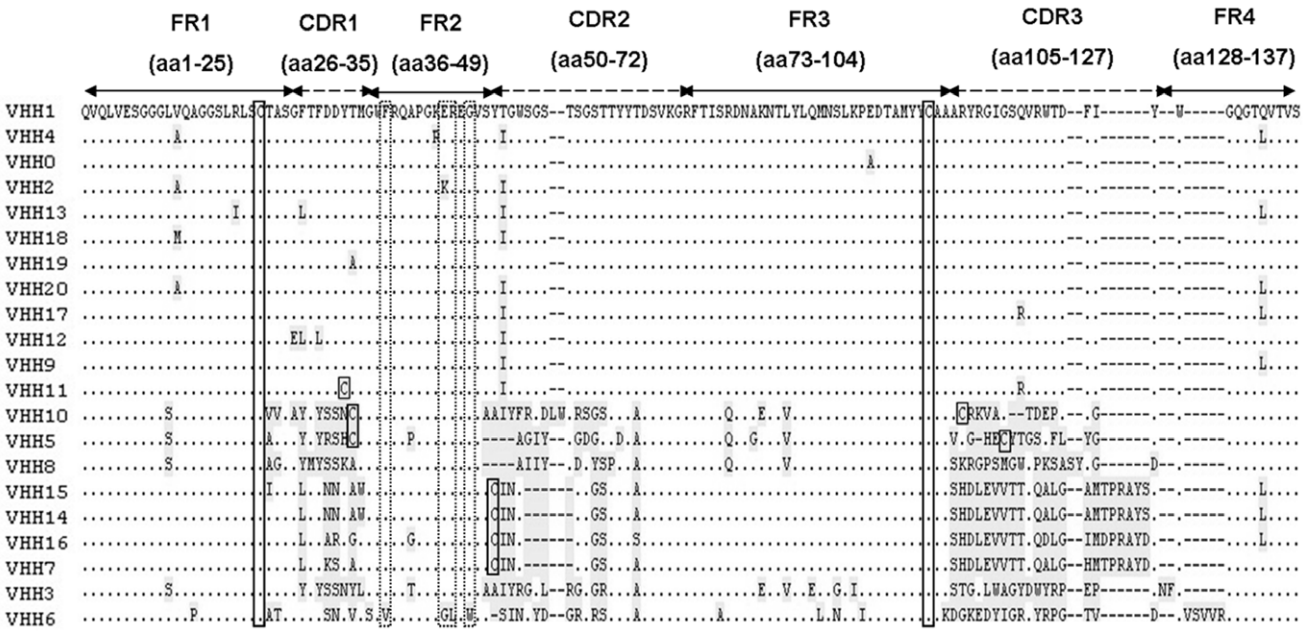

Figure 5. Aligned amino acid sequence of 21 anti-Cap specific VHH antibody fragments. Amino acid sequences were aligned according to the Kabat numbering [16]. The dots denote the same sequences compared with VHH1. Differences in the sequence are shadowed, and the dash shows the missing sequences. The hallmark Cys residues are denoted by the thick-line boxes. The four conservative hallmark residues of VHH in FR2 (Val37Phe, Gly44Glu/Lys, Arg45Leu, and Trp47Gly) are denoted by the dotted line boxes.
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residues of Val37Phe/Tyr, Gly44Glu, Leu45Arg, Trp47Gly substitutions in framework region 2 (FR2) were highly conserved [17]. In this work, we firstly screened 21 VHH antibodies from *Camelus Bactrianus*, the four conservative hallmark residues of VHH in FR2 were Val37Phe, Gly44Glu/Lys, Arg45Leu and Trp47Gly. Except for the number 44 residue in FR2 of one sequence that was mutated into Lys, all remaining sequences were in complete accordance with a previous report [18].

The longer flexible loop on camelid VHH might compensate for the loss of VL CDRs and increase the feasibility to screen VHHs with better specificity and binding affinity. However, the very flexible loop might cause a high frequency of amino acid mutation and insertion as well, especially the high frequency of Cys appearing in the camelid longer CDR3 loop (at position 106, such as VHH10). All of the selected VHHs of *Camelus Bactrianus* which contained a Cys at position 22 in FR1 also had a second Cys at position 102 in the FR3 (Kabat numbering), and the two Cys residues formed the conserved disulfide bridge to stabilize the structure of CDR3 loop [19]. Generally, some of the selected VHHs had an extended CDR3 that was often stabilized by an additional disulfide bond with a Cys in either the CDRl (at position 33 or 34, such as VHH11, VHH10, and VHH5) or the

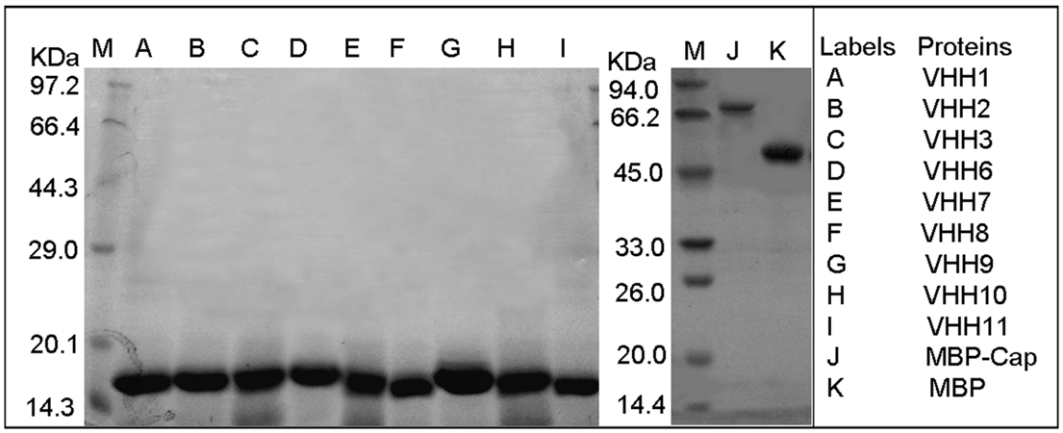

Figure 6. Purification of positive VHHs and Cap proteins. Lanes A–I, The purification of VHHs was identified by 12% SDS-PAGE. VHHs were expressed by fusion of 6×His tag at N- terminus and purified on Talon Metal Affinity Resin (Clontech).The molecular weight of the 6×His-VHH fusion proteins were 16 kDa–18 kDa. Lane J and K, Purification of 70 kDa MBP-Cap fusion protein (42 kDa MBP tag plus 28 kDa Cap) and 42 kDa MBP tag on Amylose Resin (NEB), respectively. The two M's represented the protein MW markers (sizes in kilodalton are indicated on the left side). On the right box, the letters represent the names of the corresponding proteins.
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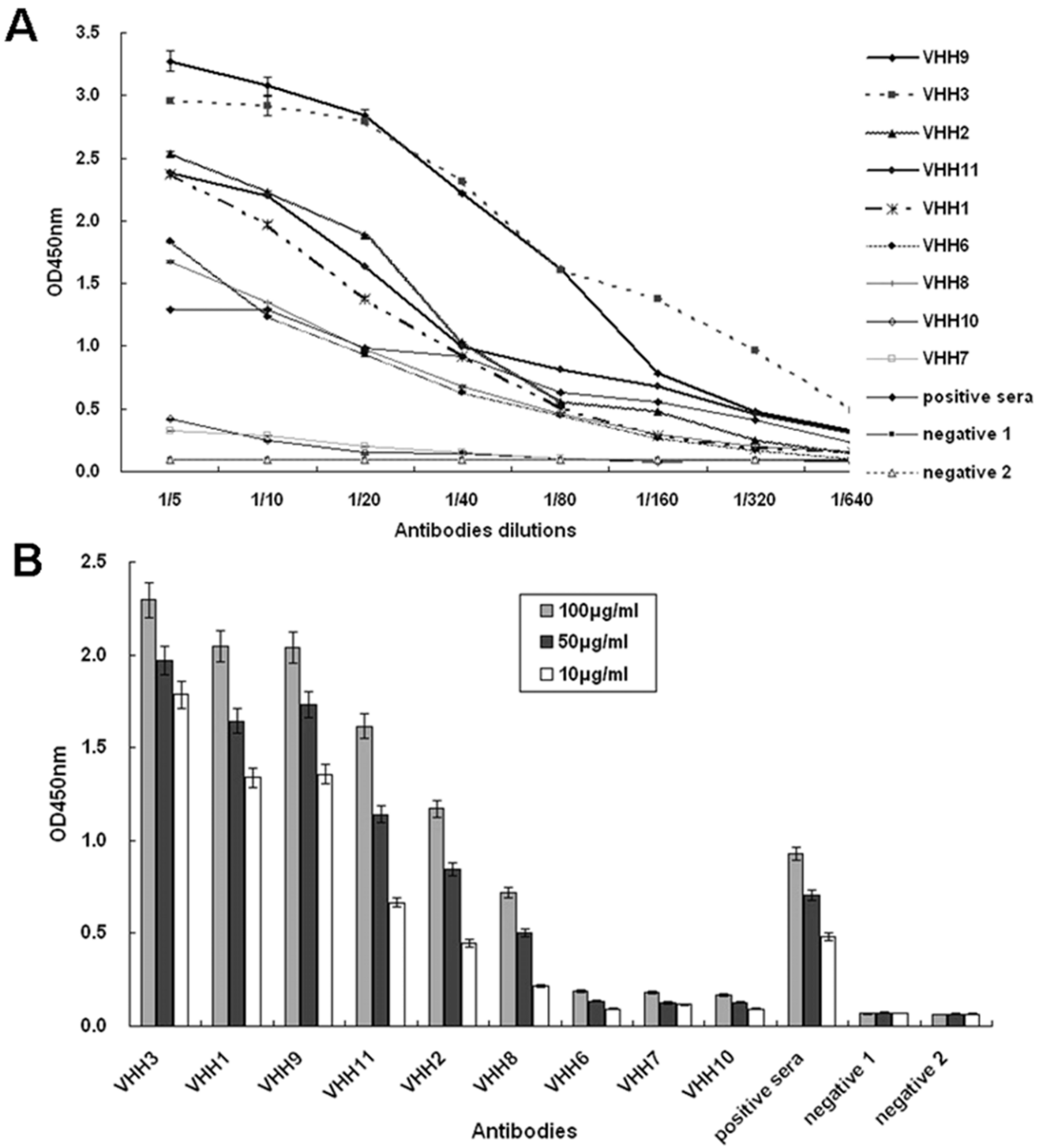

Figure 7. Determination of the specific interaction between Cap and anti-Cap VHHs by indirect ELISA. A, The plate was coated with PCV2 viral lysis ($10^{-6.4}$ $TCID_{50}$, 1:800 dilution). B, The plate was coated with purified Cap protein (0.5 μg/ml). The positive serum in A and B was the anti-Cap serum taken from a pig. Negative 1 and 2 in A represent the wells coated with BSA (negative control 1) or the lysis of PCV2 uninfected FPRC cells (negative control 2), respectively. Negative 1 and in B represent the wells coated with BSA (negative control 1) and 0.5 μg/ml purified MBP tag (negative control 2). The absorbance at 450 nm indicated the binding activity between selected VHHs and Cap. Data is plotted as the average of 3 wells, with error bars representing the standard deviation.
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CDR2 loop (at position 50, such as VHH14, VHH15, VHH16, and VHH7), which were identical with those reported in literatures [17,20,21].

For disease treatment and functional genomic research wherein proteins of unknown function are predicted from genomic sequences, a major hindrance is the paucity of antibodies that

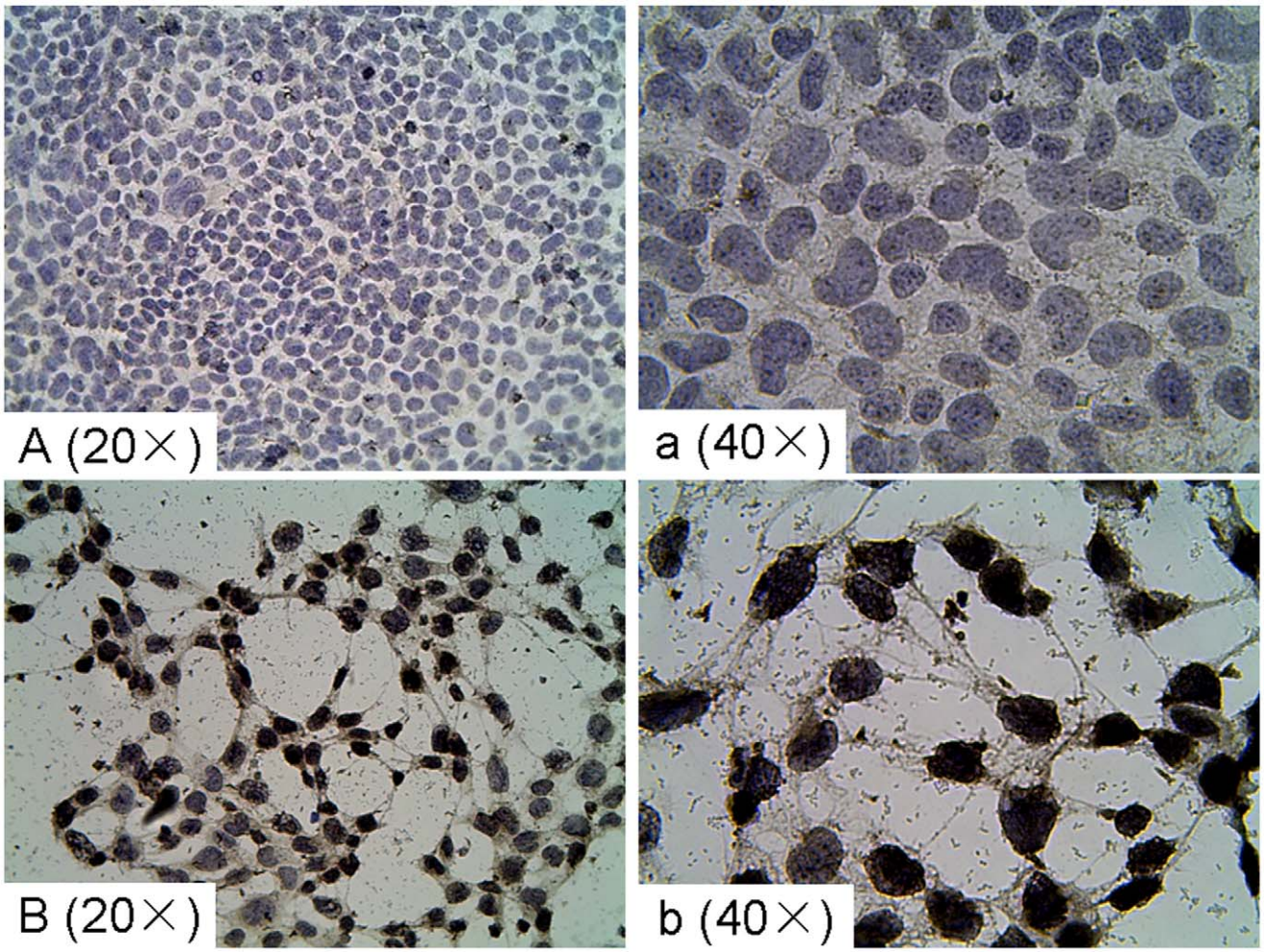

Figure 8. Immunocytochemistry analysis with VHH3 mAb. A and a, mock-infected FPRC cells; B and b, PCV2-infected FPRC cells. All cells were examined under an inverted light microscope. The expression of PCV2 Cap protein is visualized as a brown color in the nucleus, and cell nuclei stained with hematoxylin are shown in blue in the mock-infected FPRC cells. In PCV2-infection group, most of infected cells had already detached and lysed. So the cell coverage was much less than mock control.
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function in eukaryotic cells, presumably because the antibodies incorrectly fold in the cytoplasm [22]. Thus, the intracellular expression of highly specific antibody fragments (''intrabodies'') in eukaryotes has become an increasing concern. Currently, VHH antibodies are mainly used in bioscience as *in vitro* tools for recognizing target antigens and for medical applications, such as diagnosis and therapeutics. Although a few VHH antibodies have been reported to be functional in eukaryotes, the platform for intracellular VHH antibody capture is still not established [23]. To date, most intracellular antibody capture technologies (IACTs) are based on the yeast two-hybrid system, and the specific intrabodies were isolated from *in vivo* single chain fragmented variable (scFv) library (VL-VH format) [11,24]. Although the scFv is one of successful forms for intracellular expression, not all mAbs can be made as scFv and maintain their function in the cells because of the lack of disulfide bonding, which causes scFv incorrect folding of scFv, less stability, and solubility under conditions of intracellular expression [22]. Compared with the scFv format, recombinant VHHs are generally expressed in high levels with soluble and functional form within eukaryotic systems because the domain naturally folds and functions independently from VL interactions [2]. Combining the unique advantages of VHH in the intrabody format and successful IACT based on the yeast two-hybrid approach, we created a novel *in vivo* VHH library from immunized *Camelus Bactrianus* for intracellular antibody capture. According to existing literature, we believe this study is the first to design and select an intrabody (as VHH format) library produced *de novo* for PCV2 Cap protein.

The similarity between the properties of Ag-Abs interaction *in vivo* microenvironment and those *in vitro* is still unclear. However, this work, we confirmed that the two circumstances were consistent in most cases, i.e., most VHH positive clones (7/9) from the *in vivo* yeast two-hybrid were also strongly positive *in vitro* as determined by ELISA. Before construction of the VHH Y2H library, we also have entrusted Novavac Biotec Co., Ltd (Beijing, China) to screen the VHH antibodies targeting the Cap protein purified from Rosetta *E.coli*. After three rounds of panning, we screened over 10 ELISA positive clones that covered only one VHH origin (data not shown). Compared with the VHH phage library screening results, we obtained 36 positive clones on QDO/X/A plates, which covered 21 different VHH sequences, including the only one VHH sequence screened by phage library. The differences between two VHH screening methods can be attributed to two aspects. First, the N-terminal with 41 amino

acid (aa) residues of the PCV2 Cap protein possesses a nuclear localization signal (NLS) containing high incidence of arginine residues and rare codons for *E. coli* that is disadvantageous for full-length Cap expression [25]. Thus, the poor quality of antigen expression in prokaryotic system may influence the panning results. Second, at least five conformational epitopes are reportedly present on Cap protein (47–72, 80–94, 110–154 190–210, and 230–235 aa) [26]. Given that prokaryotic expression normally collapses the conformational epitopes, the VHHs targeting Cap conformational epitopes may not be captured.

In conclusion, we successfully constructed a novel VHH library based on the yeast two-hybrid system. The system did not only strengthen the VHH conventional extra-cellular applications for diagnosis or therapeutics, but also expanded the range of VHH as *in vivo* tools for disease treatment and functional genomics.

Supporting Information

Figure S1 Detection of homologous recombinant rate and diversity of *Camelus Bactrianus* VHH yeast library by PCR amplification and independent colonies calculation. A, 10^{-6} dilution plating of the cultured library indicated a total dimension (or complexity) of 2×10^{9} clones. B, 20 clones were randomly picked to determine the library functional diversity by PCR using universal primers T7 and 3′AD (Table 1). Meanwhile, sterile water and pGADT7-Rec vector templates were used as negative controls. All the clones have amplified the 400 bp VHH fragments (lane 1–20), while negative templates control haven't amplified any bands (lane N1, N2). M indicated the DL2000 DNA marker.
(DOCX)

Table S1 Comparison the screening procedures and time required between phage display and Y2H.
(DOCX)

Table S2 Comparison the advantages and disadvantages of VHH phage display and Y2H library.
(DOCX)

Table S3 The co-transformation results of yeast two-hybrid (refer to Figure 4).
(DOCX)

Author Contributions

Conceived and designed the experiments: XF ED ZY. Performed the experiments: XF XG SH DH PZ SY. Analyzed the data: XF ED ZY. Contributed reagents/materials/analysis tools: XW SZ RD ED ZY. Wrote the paper: XF ED ZY.

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