Construction of a Bactrian Camel-Derived Naive Phage Nanobody Display Library and Screening of Anti-GDH Nanobodies (Post-print)

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Abstract

Objective: To construct a phage display library of natural nanobodies as a platform for screening nanobodies against various antigens, and to validate the constructed library by screening GDH-targeting nanobodies using Clostridium difficile glutamate dehydrogenase (GDH) as antigen. Methods: Total RNA was extracted from the spleen of Bactrian camel using Oligo(dT) and reverse-transcribed. The complete repertoire of heavy chain variable region genes was obtained by nested PCR and cloned into the phagemid vector pCANTAB5E. The recombinant plasmids were transformed into E. coli TG1 by multiple electroporations to construct a primary phage antibody library, which was subsequently rescued with helper phage to generate a phage display library. The library capacity and diversity were analyzed and characterized. Meanwhile, the library was panned against GDH as the target antigen, the panning recovery rate was calculated, and monoclonal colonies from the third round of panning were identified by ELISA. Results: The insertion rate of the constructed natural phage nanobody library was approximately 95%. The amino acid homology of 9 randomly selected clones was 66.17%, demonstrating good diversity upon MEGA analysis. After rescue with helper phage, the titer of the phage display library reached 4$×$10^{12} CFU/ml. During three rounds of panning, the recovery rate increased progressively, indicating effective enrichment of phages. Positive clones were sequenced and analyzed, yielding two anti-GDH nanobody sequences. Conclusion: A natural phage nanobody display library derived from Bactrian camel was successfully constructed with good diversity, laying a foundation for subsequent screening of other target antigens. Meanwhile, two anti-GDH nanobody sequences were obtained, providing technical support for the development of diagnostic antibodies against Clostridium difficile glutamate dehydrogenase.

Full Text

Construction of a Camelid-Derived Natural Phage Display Nanobody Library and Screening for Anti-GDH Nanobodies

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Abstract

Objective: To construct a natural phage display nanobody library as a platform for screening nanobodies against various antigens, and to validate this library by screening for nanobodies targeting *Clostridium difficile* glutamate dehydrogenase (GDH). **Methods:** Total RNA was extracted from Bactrian camel spleen using Oligo dT and reverse-transcribed to obtain the complete repertoire of heavy-chain variable region genes through nested PCR. These genes were cloned into the phagemid pCANTAB5E vector and transformed into *E. coli* TG1 via multiple electroporations to construct a primary phage antibody library. Following rescue with helper phage, a phage display library was established and analyzed for capacity and diversity. The library was panned against GDH antigen, with recovery rates calculated and monoclonal phage from the third round of selection evaluated by ELISA. **Results:** The constructed natural phage nanobody library achieved an insertion rate of approximately 95%, with randomly selected clones showing 66.17% amino acid homology and good diversity as confirmed by MEGA analysis. After helper phage rescue, the phage display library titer reached 4$×10^{12}$ CFU/ml. Panning enrichment was effective, as evidenced by progressively increasing recovery rates across three rounds. Sequencing and analysis of positive clones yielded two distinct anti-GDH nanobody sequences. **Conclusion:** We successfully constructed a Bactrian camel-derived natural phage display nanobody library with satisfactory diversity, providing a foundation for subsequent screening against other target antigens. The two anti-GDH nanobody sequences obtained offer technical support for developing diagnostic antibodies against *C. difficile* glutamate dehydrogenase.

Keywords: nanobody; phage display technology; GDH

Introduction

Camelids possess a unique type of heavy-chain antibody that naturally lacks light chains, consisting only of a single heavy-chain variable domain and two conventional CH2 and CH3 regions [1]. The single-domain antibody derived from the variable region of these heavy-chain antibodies, designated VHH (variable domain of heavy-chain antibody), has a molecular weight of merely 15 kDa

—approximately one-tenth that of conventional IgG—with dimensions of 2.5 nm in diameter and 4 nm in length, hence the term "nanobody" (Nb) [2]. Nanobodies represent the smallest known antibodies with intact antigen-binding activity [3]. Their compact size confers strong antigen-binding capacity, while their stable structure and high hydrophilicity, combined with VHH sequences showing over 80% homology to human VH domains [4], result in low immunogenicity. These advantageous properties make nanobodies highly promising for disease diagnosis and therapy. Phage display technology enables fusion expression of inserted foreign genes with coat proteins, displaying them on the phage surface to retrieve the encoding genes. Consequently, constructing a large-capacity library with good diversity facilitates the acquisition of genetically engineered antibodies.

Clostridium difficile, a normal inhabitant of the human gut, can be present in healthy individuals. However, inappropriate antibiotic use and chemotherapy disrupt the intestinal microenvironment, leading to *C. difficile* overgrowth and secretion of toxins A and B, which cause *C. difficile* infection (CDI), including *C. difficile*-associated diarrhea and pseudomembranous colitis [5,6], with severe cases resulting in death. Glutamate dehydrogenase (GDH) is a transmembrane protein in *C. difficile* that is stably expressed and serves as a common surface antigen across different strains [7], with high-level production during early infection. ELISA or colloidal gold immunochromatography assays targeting this common surface antigen can serve as preliminary clinical screening tools [8]. This study constructed a Bactrian camel-derived natural phage display nanobody library and screened it against GDH to obtain specific nanobodies for diagnostic reagent development, while validating the library as a universal platform for subsequent genetically engineered antibody preparation and modification.

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Materials and Methods

1.1.1 Reagents and Instruments The GDH prokaryotic expression vector was constructed and stored in our laboratory. Other materials included plasmid mini-prep kits (Tiangen Biotech), TRIZOL reagent, RNA purification kits, reverse transcription kits (Tiangen Biotech), DNA gel extraction kits (OMEGA), ExTaq DNA polymerase, T4 DNA ligase (TaKaRa), restriction endonucleases Not I and Sif I (NEB), HRP-TMB substrate kits (CWBIO), yeast extract and peptone (Oxoid), skim milk (Coolaber), HRP-labeled mouse anti-M13 secondary antibody (Sino Biological), and analytical-grade chemical reagents. Major instruments comprised a PCR thermal cycler, gel imaging system, microplate

reader, and BioRad gene pulser.

1.1.2 Strains and Plasmids The phagemid pCANTAB5E vector was purchased from Changsha U-Bio Biotechnology Co., Ltd. *E. coli* TG1 and helper phage KM13 were kindly provided by the Chinese Academy of Sciences.

1.2.1 Spleen RNA Extraction and Reverse Transcription Camel spleen tissue from a healthy Bactrian camel (sourced from a slaughterhouse in Alxa Left Banner, Inner Mongolia) was retrieved from -80°C storage, ground in liquid nitrogen, and homogenized in TRIzol (1 ml per 50–100 mg tissue). Total RNA was extracted using chloroform and isopropanol, followed by mRNA purification with an mRNA purification kit. cDNA was synthesized using a reverse transcription kit with Oligo dT primers under the following conditions: 37°C for 30 min, 85°C for 5 s, and storage at 4°C.

1.2.2 VHH Gene Amplification and Vector Construction Nested PCR was employed to obtain the heavy-chain variable regions. First-round PCR used cDNA as template with primers CALL001 and CALL002 (Table 1) [9] to amplify a ~700 bp fragment under these conditions: 94°C for 30 s, 56°C for 30 s, 72°C for 45 s (32 cycles), and final extension at 72°C for 10 min. The product was gel-purified and quantified. Second-round PCR used 1 L of the purified 700 bp fragment as template with primers VHH-Forward and VHH-Reverse (Table 1) in a two-step protocol: 94°C for 40 s, 64°C for 40 s, 72°C for 40 s (5 cycles), followed by 94°C for 40 s, 68°C for 45 s, 72°C for 40 s (32 cycles), and final extension at 72°C for 10 min. The resulting ~400 bp VHH fragment was gel-verified, purified, quantified, and stored at -20°C. Both VHH fragments and pCANTAB5E vector were double-digested with Not I and Sif I, then ligated using T4 DNA ligase at 16°C for 4 h followed by overnight at 4°C. Ligation products were stored at -20°C.

Table 1. Nested PCR Primer Design

Primer	Sequence (5' -3')
CALL001	GTCCTGGCTGCTCTTCTACAAAG
CALL002	GGTACGTGCTGTTGAACTGTTCC
VHH-Forward	TCGCGGCCCAGCCGGCCCAGGTCCAACTGCAGGAGTCTGGGG
VHH-Reverse	ATAAGAATGCGGCCGCTGAGGAGACGGTGACCTGGGTCCCC

1.2.3 Electrocompetent Cell Preparation and Phage Library Construction TG1 electrocompetent cells were prepared using glycerol resuspension following published protocols [10] and stored in aliquots. Ten microliters of ligation product were electroporated into TG1 cells (2.5 kV, 25 F, 200 Ω) in five separate transformations. After electroporation, cells were recovered and 10 L of recovered culture was serially diluted and plated on SOBAG medium to

calculate transformation efficiency. The remaining culture was spread onto 20 SOBAG plates and incubated overnight at 37°C. The following day, bacterial lawns were scraped from each plate with 5 mL 2×YT medium, pooled, mixed with glycerol to a final concentration of 25%, aliquoted (1 mL per tube), and stored at -80°C.

1.2.4 Library Characterization Twenty random colonies from the transformation plates were selected for colony PCR to determine insertion efficiency. Ten positive clones were sequenced, and amino acid homology was analyzed using DNAMAN while diversity was assessed by MEGA phylogenetic tree analysis.

1.2.5 Helper Phage Amplification and Rescue Helper phage KM13 was amplified by serial dilution using the top agar method, with individual plaques picked for overnight culture. Phage particles were precipitated using PEG/NaCl, titered, and stored at -80°C. For library rescue, 1 mL of library stock was inoculated into 2×YT/Glu/Amp medium and grown to logarithmic phase before infection with helper phage at MOI=20:1. After 1 h infection, cells were pelleted and resuspended in 2×YT/Amp/Kana medium for overnight shaking at 30°C. The next day, phage particles were precipitated from the supernatant with 1/5 volume PEG/NaCl on ice, and the rescued VHH phage display library titer was determined and stored at -20°C for subsequent panning.

1.2.6 Phage Library Panning Recombinant GDH was expressed with IPTG induction in *E. coli* and purified via Ni-affinity chromatography. Microtiter plates were coated with 20 g/mL GDH overnight at 4°C and blocked with 2% PBSM. The phage library was pre-incubated with 2% PBSM at a 1:3 ratio before adding to the coated plates for 2 h binding. After washing 10 times with PBST, fresh log-phase TG1 cells were added to each well for 15 min infection. The bacterial suspension was removed, and 100 L of 0.2 M glycine (pH 2.7) was added per well for 10 min at 37°C to elute bound phage. Eluates were neutralized with 1 M Tris (pH 9.1) at a 1:0.2 ratio, mixed with TG1 cells (OD_{600}=0.5), and incubated at 37°C for 30 min. One hundred microliters of infected cells were serially diluted to determine output titer, while the remaining culture was plated on 2×YT/Amp/Glu plates overnight. The next day, bacterial lawns were scraped, and an appropriate volume was inoculated into 100 mL 2×YT/Amp/Glu, grown to log phase, infected with KM13 at MOI=20:1 for 30 min at 37°C, pelleted, and resuspended in 200 mL 2×YT/Amp/Kana for overnight shaking. Phage were precipitated with 1/5 volume PEG/NaCl, titered, and used for the next round of selection. This process was repeated for three total rounds.

1.2.7 Phage-ELISA Screening of Positive Clones From the third-round selection plates, 500 colonies were picked and cultured in 96-well deep plates (5 colonies per well) in 2×YT/Amp/Glu overnight. The next day, 50 L from

each well was transferred to a new plate, grown to log phase, infected with KM13 at MOI=20:1 (37°C static for 20 min, then shaking for 30 min), pelleted, and resuspended in 2×YT/Amp/Kana for overnight shaking. Supernatants were collected for indirect ELISA. Microtiter plates were coated with 10 g/mL GDH, blocked with 5% PBSM overnight, then incubated with pre-treated phage supernatants for 2 h. After washing, HRP-labeled anti-M13 antibody was added, followed by four washes and TMB substrate development. Absorbance values were recorded. The ten wells with highest OD values were selected, replated, and ten single colonies from each were cultured individually in 96-well deep plates. Phage monoclonal supernatants were prepared as described and subjected to Phage-ELISA. Positive clones (OD > 2× negative control) were sequenced.

Results

2.1 VHH Fragment PCR Amplification Total RNA was extracted and purified from camel spleen, reverse-transcribed to cDNA, and used as PCR template. First-round PCR with primers CALL001 and CALL002 yielded a ~700 bp fragment [Figure 1: see original paper]. The purified 700 bp product (1 L) served as template for second-round PCR using VHH-Forward and VHH-Reverse primers, which amplified the expected ~400 bp VHH fragment under optimized conditions [Figure 2: see original paper]. The 400 bp product was gel-purified, quantified, and stored at -20°C.

Figure 1. First-round PCR agarose gel electrophoresis. M: DNA marker DL1000; lanes 1–3: 700 bp PCR products.

Figure 2. Second-round PCR agarose gel electrophoresis. M: DNA marker DL1000; lanes 1–5: 400 bp PCR products.

2.2 Electroporation and Phage Library Construction The second-round PCR products and pCANTAB5E vector were double-digested with Sif I and Not I, gel-purified, and quantified. Vector and insert were ligated at a 1:3 molar ratio in a 10 L system overnight. Fifty microliters of ligation product were electroporated into *E. coli* TG1 in batches. Transformed cells were serially diluted to calculate library capacity, while the remainder was plated on SOBAG plates. Bacterial lawns were scraped and stored in 20% glycerol at -80°C. Colony counting revealed an initial library capacity of $2\$\times 10^{\{6\}}. After rescue with helper phage KM13 and PEG/NaCl precipitation, the phage display library titer reached 4\times 10^{\{12\}}\$$ CFU/ml, meeting construction requirements [Figure 3: see original paper].

Figure 3. Library titer determination by serial dilution. A: 10^{-2} dilution; B: 10^{-4} dilution; C: 10^{-6} dilution; D: 10^{-8} dilution.

2.3 Library Quality Assessment Twenty random colonies were selected for colony PCR to evaluate insertion efficiency. Nineteen of twenty clones showed the expected 400 bp band, indicating an insertion rate of ~95% [Figure 4: see original paper]. Ten positive clones were sequenced, revealing 66.17% amino acid

homology by DNAMAN analysis and good diversity by MEGA phylogenetic tree analysis. BLAST analysis confirmed all sequences as camelid heavy-chain antibodies [Figure 5: see original paper]. Amino acid alignment [Figure 6: see original paper] showed the four characteristic hydrophobic-to-hydrophilic mutations in FR2 (indicated by dashed lines) that enhance solubility and maintain structural stability.

Figure 4. Colony PCR analysis of insertion rate. M: DNA marker DL1000; lanes 1–20: randomly selected colonies.

Figure 5. MEGA phylogenetic tree analysis of VHH sequences.

Figure 6. Amino acid sequence alignment of nine random VHH clones.

2.4 Enrichment of GDH-Specific Phage GDH-coated 96-well plates were used to pan the phage display library for specific binders through three rounds of selection. Input and output titers were determined by serial dilution to calculate recovery rates and monitor enrichment. Effective enrichment was observed, with phage quantity increasing significantly across rounds .

Table 2. Selective Enrichment During Panning

Round	Input (CFU/mL)	Output (CFU/mL)	Recovery Rate
1	4$×10^{12}$	1.5$×10^{4}$	3.8$×10^{-9}$
2	3$×10^{9}$	7.0$×10^{4}$	2.3$×10^{-5}$
3	1$×10^{9}$	5.0$×10^{6}$	5.0$×10^{-3}$

2.5 Phage-ELISA Results From the third-round selection plates, 500 colonies were picked (5 per well) for helper phage rescue to generate polyclonal phage supernatants. Indirect Phage-ELISA identified wells with absorbance values exceeding twice the negative control [Figure 7: see original paper]. The ten highest OD_{450} wells were selected, replated, and ten single colonies from each were cultured individually for monoclonal phage production. Monoclonal supernatants were tested by Phage-ELISA, with positives defined as OD $> 2\times$ negative control [Figure 8: see original paper]. Ten high-affinity clones were sequenced, yielding two successful sequences (D1 and F1). BLAST analysis confirmed both as camelid VHH sequences with high homology but distinct sequences.

Figure 7. Phage-ELISA detection of polyclonal phage supernatants.

Figure 8. Phage-ELISA identification of monoclonal phage. (a) Monoclonal phage supernatant ELISA; (b) Indirect Phage-ELISA of positive colonies.

Discussion

Phage display technology has rapidly evolved into a powerful biotechnological tool widely applied in antibody engineering, protein engineering, peptide

screening, and drug discovery, with significant implications for clinical disease diagnosis and therapy. Compared with immune libraries, natural phage antibody libraries circumvent animal immunization, enabling direct screening of specific antibodies from the library and avoiding cumbersome immunization protocols. With adequate capacity to cover virtually all possible antibody variants, natural libraries can effectively capture specific binders. Constructing a phage display library and screening for anti-GDH nanobodies provides support for diagnostic reagent development and early detection of *C. difficile* infection. Clinically, monoclonal antibodies have limitations including large molecular weight, poor tissue penetration, slow serum clearance, and heterologous immunogenicity. Nanobodies offer superior characteristics including small size and high stability, making them promising for tumor diagnosis and therapy. For example, CXCR4 nanobodies can inhibit the CXCL4/CXCL12 axis to suppress tumor cell proliferation [11], and Groot' s group isolated specific anti-HIF-1α nanobodies from a non-immune camelid library that downregulated HIF-1 transcriptional activity by binding to its ODD domain [12].

During library construction, IMGT amino acid sequence analysis confirmed that all sequenced clones were camelid heavy-chain antibodies containing four FR regions and three CDR regions, consistent with nanobody structural features. The CDR3 region, which critically influences diversity, is longer than in conventional antibodies [13], forming stable exposed loops that can penetrate antigen clefts and enhance binding affinity. Camel VHH sequences show high homology to human VH domains, enabling humanization of the framework regions to create novel humanized genetically engineered antibodies targeting TNF-α, Her2, CD19, and other antigens for drug development.

Furthermore, CAR-T cell technology is widely employed in cancer immunotherapy research, with clinical trials exploring new CAR targets for hematological and solid tumors. This successfully constructed natural Bactrian camel phage display nanobody library, with its good diversity and capacity, serves as a universal screening platform for various target antigens. Future studies can utilize this nanobody library to isolate highly specific and affinity-matured single-chain antibodies for developing targeted CAR-T cell therapies, potentially improving therapeutic efficacy.

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Note: Figure translations are in progress. See original paper for figures.