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Development of naïve phage display VHH libraries from Indian camel

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ABSTRACT

The present study describes the construction of naïve camelid VHH single pot library from lymphocytes of non-immunized *Camelus dromedarius*. RNA extracted from 6 healthy female camels was pooled together and used for construction of library in phagemid vector. The VHH inserts were found in 50–70% of the phagemid vector (pMECS vector) cloned in *Escherichia coli* and the size of the libraries was estimated to be around 10^5–10^7cfu. Sequence analysis of VHHs revealed variations in the length of complementary determining regions and amino acid usage resulting in a variety of different paratopes. Further expression of VHH rescued from the library was optimized in prokaryotic system using pET32 expression system. Functional antibodies generated from this naïve library will be explored for affinity against various antigens in further studies.

Key words: Nanobody, Phagemid, Redundancy, Single pot library

Specific antibodies are the essential tools required to study gene functions, disease diagnosis and treatment; and are conventionally produced by *in-vitro* selection with specific binders by hybridoma technology (Gibbs 2005). These recombinant antibodies are selected through biopanning of recombinant bacteriophage. However, another unique variable heavy chain antibody fragments (VHHs) found only in camels, llamas, and shark (Muyldremans 2013) are now explored for diagnostic utility. These VHHs have distinct advantages over other recombinant fragments such as Fabs, and scFvs like smaller size, higher thermo-stability, solubility and tissue penetration (Hollinger and Hudson 2005). Variable regions of heavy chain antibodies (VHHs), also known as nanobodies, were discovered in IgG2 and IgG3 repertoire of serum in *Camelidae* and cartilaginous fishes as Nurse Sharks (Hamers-Casterman *et al.* 1993). These VHHs can be produced through easy one-step cloning as single polypeptide chain of size <15 kDa and high efficiency of relatively stable protein expression in bacteria.

The length of the CDR3 loop of VHH ranges from 10–24 residues unlike conventional VHs with average of 11.6 residues and this facilitates the penetration into active site clefts and cavities of the antigens. VHH could prove to be a potentially important tool especially in understanding viral pathogenesis and diagnosis because it consists of a convex H3 loop, which attributes in recognizing conformational epitopes rather than planer epitopes overran distinct advantage over the conventional antibodies (Delic *et al.* 2014). The VHHs have an additional advantage of being soluble and have high expression due to replacement of hydrophobic amino acids in conventional VH by hydrophilic (more polar and charged residues) in framework 2, viz. Val37Phe, Gly44Glu, Leu45Arg, Trp47Gly. The camel VHHs has mutation at position Ser11Leu which is less conserved in llamas further ascribes to its solubility with no inclusion bodies during recombinant antibody production.

Conventionally, a library is generated from hyperimmunized animals every time for production of specific recombinant antibodies against desired antigens which is redundant, costly, and time consuming process for production of specific VHHs for certain cases which arise due to repeated immunization thus lacking somatic maturation (Olichon and Marco 2012, Sabir *et al.* 2014). On the contrary, it could be better to establish a single pot library, which is more diverse in nature, therefore allowing us to identify binders for any potential antigen irrespective of its origin. In this study, naïve library from Indian camel (*Camelus dromedarius*) was developed and characterized using pMECS phagemid vector system.

MATERIALS AND METHODS

Selection of animals: Healthy female camels (6) of approximately 2–4 years of age maintained at NRC on Camel, Bikaner was selected for the study. Approximately 100 ml of blood samples were collected from each animal in heparinized vacutainer for separation of lymphocyte.

VHH gene amplification: Total RNA was extracted from

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the peripheral blood lymphocytes using Trizol and quantified by Nanodrop spectrophotometer. mRNA was isolated from the total RNA using mRNA purification kit. cDNA was synthesized from approximately 2 µg of mRNA from each camel in 25 µl reaction using reverse transcriptase enzyme and OligodT (12–18) primer. Variable VHH gene segment was amplified by nested PCR (Vincke *et al.* 2012). The amplified PCR products were gel purified as described above and stored at –20ºC till further use.

Library generation: The gel purified products of VHH obtained from the 6 individual camels were pooled in equimolar ratio and cloned into phagemid expression vector pMECS using DNA ligation kit as per the recommendations. The ligated product was chemically transformed into the competent *E. coli* TG1 cells. The positively transformed *E. coli* cells were selected by plating onto the LB agar plates containing ampicillin and glucose. The colonies appeared for the vector system were cultured in 2× YTG broth at 37°C for 3 h with shaking at 180 rpm.

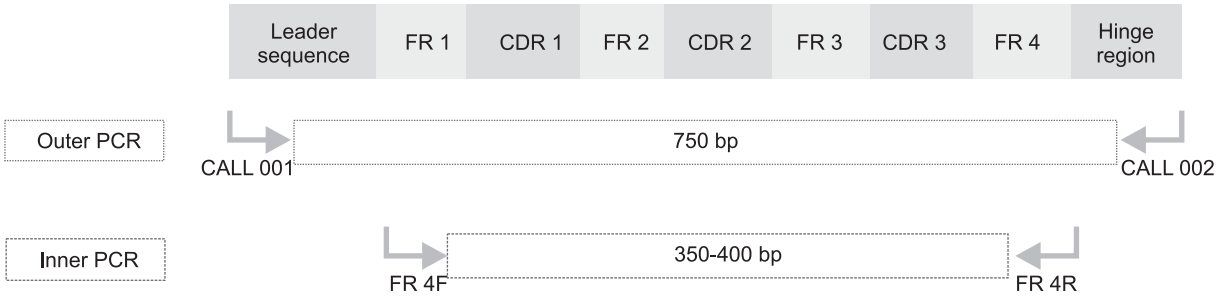
Library evaluation: The size of the library was quantified by estimating colony forming units (cfu) as per the standard procedure. The colonies were counted and cfu was calculated considering the dilution factor using the formula: CFU/ml = No. of colonies on each plate × 10 × dilution factor × volume of transformed cells. Further, colonies picked randomly from the library were screened for VHH by restriction digestion (*Pst*I and *Not*I) and colony PCR. Finally, nucleotide sequencing was carried out using VHH specific forward and reverse primers. The specificity of the nucleotide sequences was confirmed using the BLAST tool of NCBI. The deduced amino acid sequences were aligned to reference VHHs using Clustal W in order to detect variations within the CDR regions of the VHH.

Optimization of expression of VHH in prokaryotic system: Plasmid extracted from VHHs library was used as template for re-amplification of VHH gene. The amplified VHHs product were cloned into pET32 expression vector and into BL21 (DE3) pLysS *E.coli* cells. Trx-His tagged VHHs was expressed by induction with 1mM IPTG at 28ºC for 6 h. Expressed fused VHH was confirmed by 15% SDS-PAGE and western blot analysis. The blot was developed with anti-Histidine monoclonal antibody.

RESULTS AND DISCUSSION

Nanobodies found in camel are increasingly being explored for therapeutic and diagnostic purpose because of their smaller size, lower molecular weight and higher solubility. Development of a single pot library for generation of recombinant antibodies against any antigen will be more advantageous than library developed from immunized animals that is expensive and time consuming. In this study, a naïve library from *Camelus dromedarius* (Indian single humped camel) was developed and characterized. VHH genes from 6 healthy camels were pooled to increase the gene repertoire in the library. Four phagemid vector systems were used for development of the library considering the various requirements in the future.

Generation of library: VHHs library was constructed successfully using phagemid vector from VHHs gene segments obtained from 6 healthy camels as described in Fig. 1. The mRNA extracted from PBMC was subjected to PCR amplification of VHH segment. The amplicons size



Position	Target region	Primer name	Primer sequence	Product size
Outer PCR	Leader sequence to Hinge region	CALL001	5'GTCCTGGCTGCTCTTCTACAAGG3'	750 bp
		CALL002	5'GGTACGTGCTGTTGAACTGTTCC3'	
Inner PCR	FR 1 to FR 4	FR4F	5'GATGTGCAGCTGCAGACGAGTCTGGRGGAGG3'	350-400 bp
		FR4R	5'GGACTAGTGCGGCCGCCGTGGAGACGGTGACCTGGGT3'	

N.B.: *CDR*, Complementarity determining regions; *FR*, Framework region; Text underlined in primer sequence denotes in-built restriction enzyme sites, i.e. *Pst*I and *Not*I in P3 and P4 respectively

Fig. 1. Nested PCR strategy to amplify VHH gene.

of ~700 bp was obtained using outer primers and the second set of PCR with inner primers resulted in ~ 400 bp product corresponding to VHH. The size of the libraries was estimated to be around 10^5–10^7cfu. The functional dimension of the library was evaluated by colony PCR where a band of 700 bp consisting of 400 bp insert and 300 bp of pIII protein of M13K07 was observed. The colony PCR was performed on 50 colonies picked from each of the 4 plates with different vector systems. The VHH inserts were found in 50–70% of the phagemid vector pMECS vector cloned in *E. coli.*

Analysis of library diversity: The library diversity was established via HinfI digestion pattern of PCR inserts, which revealed a slightly different size of VHH fragments and capillary sequencing. The nucleotide sequences of VHHs generated in the study are available from GenBank under accession numbers KT883956-KT883962. The nucleotide blast analysis using the blast tool of NCBI showed several sequence hit with available VHH sequences mostly in the FR region. The deduced amino acid (aa) sequences were aligned with the reference sequences to analyze the library diversity (Fig. 2).

The VHH are classified into 4 sub families (VHH1-4) based on conservation of amino acids at certain positions (Harmsen *et al.* 2000). Careful analysis of amino acid sequence in this study revealed that, majority of (4 of the 7) sequences, viz. clones 2, 4, 5 and 6 belong to subfamily VHH4, 2 belong (clones 3 and 7) to subfamily VHH2 and 1 (clone 1) could not be classified in any of the defined subfamilies. Similarly, in a previous analysis, a large number of VHHs could not be classified into any subfamilies (Harmsen *et al.* 2000). The length of CDR1 varied from 7–8 aa in which, 6 of the 7 sequences had 7 aa length CDR1 and 1 sequence had 8 aa length CDR1. In CDR2, length varied between 7 (in 3 sequences) and 8 (in 4 sequences). All the 7 sequences showed variable length of CDR3, it was as small as 12 aa to as long as 28 aa. It is to be noted that the average length of CDR3 in heavy chain antibody is 16–18 aa. The CDR3 is considered to be the main contributor of antigen binding. It is also known that a longer loop length increases the paratope repertoire. All the positions in CDRs showed variation in one or more sequences. The hallmark substitutions established in the VHHs compared to the conventional VH at position 37 (Phe/Tyr), 44 (Glu), 45 (Arg/Cys) and 47 (Gly) in FR2 were found to be Phe, Glu, Arg and Gly/Leu, respectively in the Indian camel VHHs. These residues in conventional VHHs form a hydrophobic surface association with VL and normally changed to hydrophilic residues in VHHs studied so far including this analysis. These substitutions in the VHH are believed to contribute to the solubility behavior of VHH. At position 11, the characteristic Ser residue was found in all the sequences analyzed in our study. This substitution is considered as an adaptation to accommodate the absence of CH1 domain in camel VHH. The cysteine residue in FR1 at position 22 and FR3 at position 92 that form the characteristic disulfide bond was found fully conserved in all the 7 sequences analyzed in this study. Besides, 5 sequences except clone 1, 4 and 8, had cysteine in CDR1 at position 33 and 5 sequences except clone 2 and 5 had cysteine at variable position in CDR3. The disulfide bonds between these additional cysteines that cross-link the antigen binding loops are believed to have a role in increasing the paratope repertoire (Desmyter *et al.* 2001). Interestingly, the sequence of clone 1 and 4, which lacks cysteine in CDR1 had 2 cysteines in CDR3. None of the

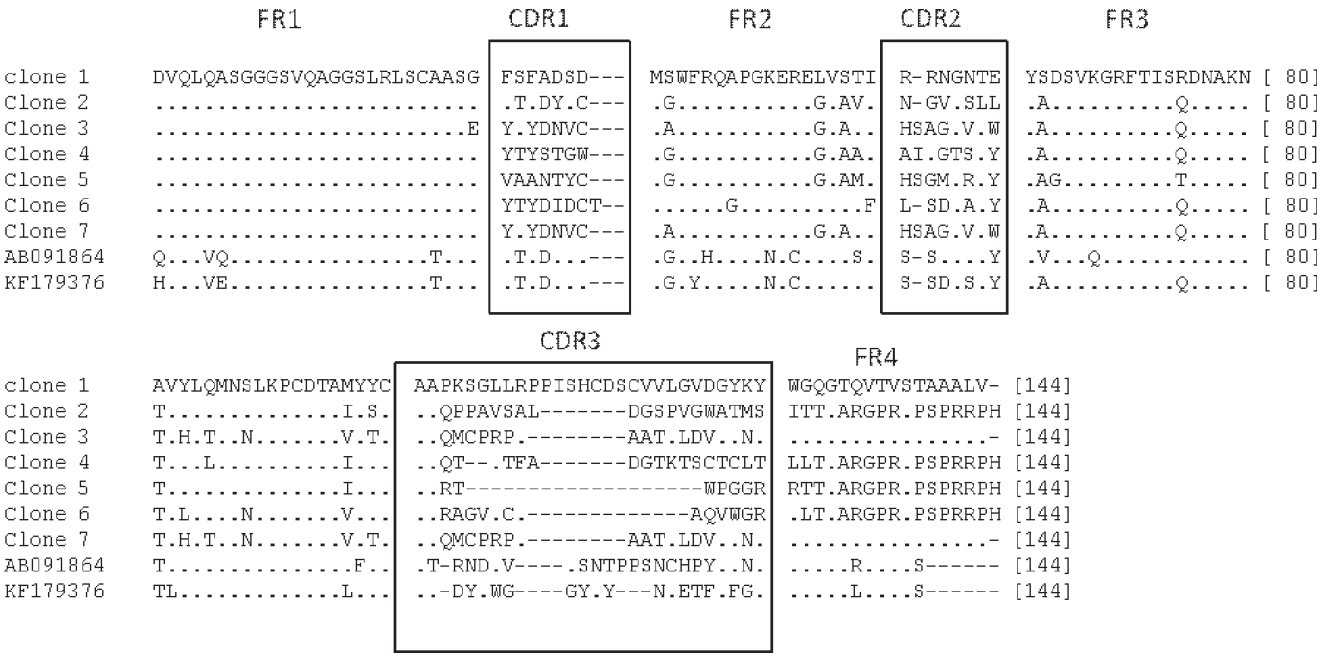


Fig. 2. Alignment of the deduced amino acids sequences of the VHH gene isolated from selected colonies of the naïve library of Indian camel. Four frameworks (FR) and three CDRs are indicated. Numbering was followed as per Kabat *et al.* (1991).

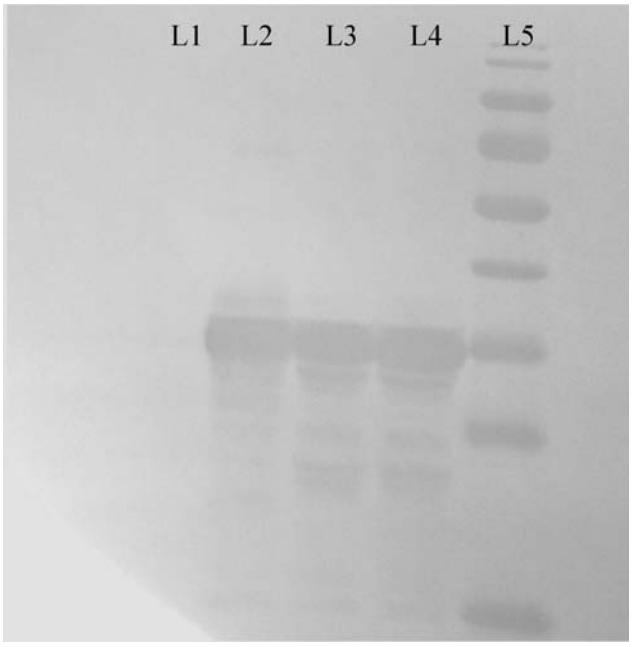


Fig. 3. SDS PAGE and western blot with anti-His Mab depicting expression of 32kDa VHHs along with his tag of pET32 vector. Lane 1, un-induced BL21(DE3)Plys S (6 h); lane 2, induced BL21(DE3)Plys S VH8 (6 h); lane 3, VH8 (5 h); lane 4, VH8 (4 h); lane 5, PAGE ruler pre-stained protein ladder.

sequences analyzed here had any cysteine in CDR2.

These primary naïve libraries with such vivid diversity shows possibility of identifying better binders for any potential antigen to which the dromederi has never been exposed. As in nature, the somatic hypermutation is cause of repertoire generation to deal with variety of antigens to which the body may or may not be exposed. In the similar fashion, after through study of the VHH sequence via site directed mutagenesis, some modifications can be done to CDR regions, the main player in antigen antibody interaction which leads to increased affinity to a particular antigen or may increase its capacity to bind variety of antigens (Monegal *et al.* 2009).

Expression of VHH in prokaryotic system: Expression of VHH rescued from the library was optimized in prokaryotic system using pET32 expression system. The constructs VHH-pET32 transformed in BL21(DE3)Plys S gave optimum yield of soluble VHH proteins on induction. Clear band was observed at 35 kDa that includes 15 kDa VHH tagged with TRX. The His-Trx fused VHH could readily be purified using metal affinity chromatography method and was confirmed by Western blotting using anti-Histidine monoclonal antibodies (Fig. 3). Though expression tag hinders refolding recombinant scFv antibody whereas under oxidizing environment, VHH has been demonstrated to regain its three dimensional structure and biological activity due to single chain even after expression in the cytoplasm of *E. coli* cells (Harmsen *et al.* 2000, Muyldermans 2013).

In conclusion, the naïve camelid VHH library constructed from present work can prove to be a valid useful biotechnical tool in order to retrieve potent antigen binders avoiding cumbersome immunization procedures against variety of different pathogens irrespective of their origins in stipulated time period. Thus, it is hypothesized that the established naïve library will suffice the need of neutralizing antibodies which have a pivotal role in disease diagnosis and treatment against various animal diseases irrespective of their origins.

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