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Development of a Novel Format of Stable Single Chain Antibodies against Alkaline Phosphatase as Therapeutic Agents against Immune Diseases

Pramathadhip Paul, Silpak Biswas and Ena Ray Banerjee*

Immunobiology and Regenerative Medicine Research Unit, Dept. of Zoology, University of Calcutta, 35, Ballygunge Circular Road, Kolkata

***Corresponding author:** Ena Ray Banerjee, Department of Zoology, Immunology and Regenerative Medicine Research Laboratory, University of Calcutta, 35, Ballygunge Circular Road, Kolkata, West Bengal, India, Tel: 91-33-24615445; E-mail: enaraybanerjee@gmail.com

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Abstract

Camelid antibodies are composed of two heavy chains but lack the CH1 domain. These antibodies contain a single variable domain (VHH) and two constant domains (CH1 and CH3). Alkaline phosphatase is one of the most extensively used immunodiagnostic reagents for medical and research application because of its homodimer formation. The aim of this study was to develop a novel stable single chain nanobody library against alkaline phosphatase that can have therapeutic and diagnostic use. Here, we are reporting first time the construction of recombinant antibody of phage display technology in India. After immunization with alkaline phosphatase enzyme and adjuvant in *Camelus dromedarius*, single domain heavy chain antibody was generated from V-D-J H gene arrangement in B-cell. We developed novel protocol for the production of stable single-domain antibodies in the camelid format. We believe that camelid antibodies will be a method of choice to control immunity related diseases and infectious disease.

Keywords: Camelid; Alkaline phosphatase; Heavy chain antibodies; Nanobody; Single-domain antibodies

Introduction

Recombinant antibody technology progressed rapidly during last decade, mainly because of their human therapeutic use. The vertebrate immune system generates billions antibody molecules. Of them, camelids have been found to possess structurally different kinds of antibodies. These camelid antibodies are composed of two heavy chains similar to conventional antibodies [1]. Contrary to the structure found in conventional antibodies these heavy-chain antibodies lack the CH1 domain [2]. Nanobodies are antibody-derived engineered therapeutic proteins. Nanobodies possess structural and functional properties of naturally occurring heavy chain antibodies. The nano-technology was originally developed after the discovery that camelidae (camels & llamas) possess fully functional antibodies without light chains. These antibodies contain a single variable domain (VHH) and two constant domains (CH2 and CH3) [3].

One of the reasons behind the interest shown by the industry in VHH is that the single domain allows production in microorganisms. The high levels of production and the subsequent purification is very efficient. These characteristics enable production of large quantities of VHHs with fewer expenses. To select the VHH with the desired properties from a large collection of VHH, phage display is performed [4]. Phage display uses bacteria and bacterial viruses, known as bacteriophage, to select antigen a specific antibody fragment that is VHH in our study. This technology combines the VHH displayed on bacteriophage particles to the DNA encoding this VHH, which is present in the phage. One of the strengths of this method is this coupling of phenotype to genotype. The performance of VHHs at high temperatures was one of the a most prominent advantages as compared to conventional antibodies and this is evident from the fact that they can withstand incubation of even two hours at high temperatures up to 90°C [5].

The applications using antibody technology are also increasing rapidly in the industry. In medicine and clinical science, antibodies are used as fusion proteins, to act as a carrier to target specific effector substances to a specific location, the so-called 'magic bullet' approach [6]. Previously, single-domain antibodies (sdAbs) able to bind small molecules such as caffeine and methotrexate, or toxins such as botulinum, cholera, ricin and others, and some viruses (rotavirus, HIV, Vaccinia and Marburg) have been isolated [7,8]

There are several advantages associated with using alkaline phosphatase (AP). AP is one of the most extensively used immunodiagnostic reagents for medical and research application because of its homodimer formation. AP has high resistance to inactivation, denaturation and degradation [9]. When AP and single chain variable fragment fused, its binding affinity to the specific antigen result is high [10]. Due to homodimer formation, AP exhibits high thermal stability.

Many single-domain antibodies have already proven useful for basic scientific research work and diagnostic tools. *In vivo* studies reported the biodistribution of single-domain antibodies for its deep penetration in to the dense tissues as well as rapid elimination through kidney [8].

The aim of this study was to develop a novel format stable single chain nanobody library against alkaline phosphatase without the disadvantages of conventional antibodies that can have therapeutic, diagnostic and cosmetics use as well as food supplement as nutraceuticals. We described here the methods of isolating camelid antibodies (VHH or Variable Heavy Chain antibodies) and generating an antibody repertoire for further downstream screening for specific uses.

Results

In this study we have described the production of camelid antibody. From 5 ml anti-coagulant blood we obtained 3.26 × 106 mononuclear cells and in the next round we obtained 200 µgm of RNA. Further, we have used oligonucleotide primers which annealed CH1, hinge and part of the CH2 axon. The product size was 700 bp. Next round PCR product, primers annealed to variable regions of the heavy chain antibodies, hinge and part of the CH2 axons, in which CH1 was absent. The size of the second PCR product was 400 bp. We verified the entire PCR product by agarose gel electrophoresis. Here we have used cloning vector, pGEMT to clone our antibody fragment. Screening of the positive clone was further verified by restriction enzyme digestion. Final result obtained by clone of expression vector like Phen4 vector. Helper phage infection was done successfully.

Lane 1 in Figure 1a showing 700 bp band which signifies isolated total m-RNA (concentration was 1213.5 ngm/µl) from B-lymphocyte generating c-DNA which was used as a template. From this result, we can conclude that oligonucleotide primers CALL001 and CALL002 were annealed at the CH2 region and at the framework 1 for VHH amplification, which is 700 bp.

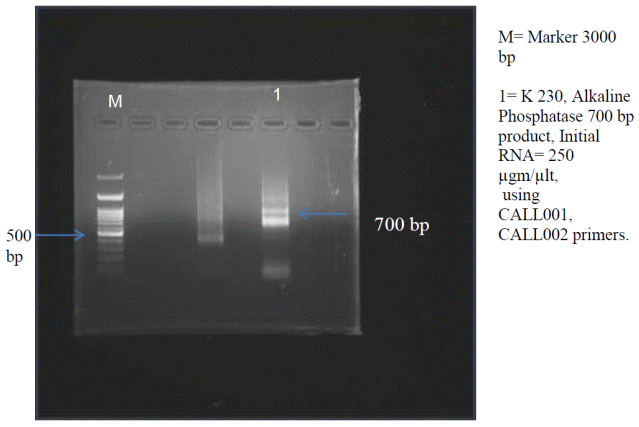


Figure 1a: 1st PCR Product of immunized alkaline phosphatase camelid blood. Isolated total RNA was used as a template for cDNA synthesis (Super Script II TM, Reverse Transcriptase, Invitrogen). The 1st PCR products of alkaline phosphatase encode the CH1, hinge, and part of the CH2 exons, using Call001 and Call002 primer. 5 µl PCR product was loaded on 1% agarose gel. Lane 1 indicates the PCR product (700 bp), Lane M indicates molecular weight marker (100-3000 bp). For image analysis gel doc (Bio Rad, software quantity one, version-4.6.3) was used.

In Figure 1b, lane 1 and Lane 2 signifies the smaller fragments of 400 bp which was purified from larger fragments 700 bp and second PCR was performed amplifying only the VHH genes of 400 bp using a pair of gene specific primer Pst1 and Not1. We can conclude that gene specific primer Pst1 and Not1 binds only the VHH gene which was 400 bp. In Figure 1c, lane 1 showing ligated pGEMT vector and 400 bp VHH gene which was digested with Pst1 and Not1 restriction enzymes. Lane 1 indicates 400 bp separated from pGEMT vector. Lane 2 to 6 indicates ligated pGEMT vector and 400 bp VHH gene digested by Pst1 restriction enzyme and therefore no 400 bp fragment was separated. Lane 7 signifies undigested product of ligated pGEMT vector and 400 bp VHH gene which was around 3.4 kb. Here, we showed that after digestion only we got the desire product 400 bp which can be further used for cloning in to TG1 bacteria. In Figure 1d, lane 1 showing 400 bp VHH gene of alkaline phosphatase product separated from pGEMT vector for gel elution. This 400 bp fragment can be further ligated to Phen4 plasmid vector to generate nanobody.

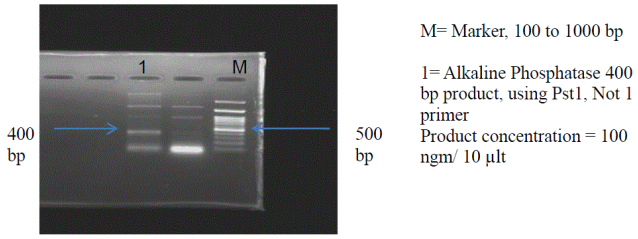


Figure 1b: 2nd PCR product of Alkaline Phosphatase from 1st PCR product. The 2nd PCR products of alkaline phosphatase encoded the variable regions of the heavy-chain antibodies (VHHs or Nanobodies), hinge, and part of the CH2 exons. PCR was done using Pst1, Not1 primers and visualized on 1% agarose gel. Lane 1 and 2 indicates PCR product with 400 bp of VHH gene. Lane M indicates molecular weight marker with 100-3000 bp.

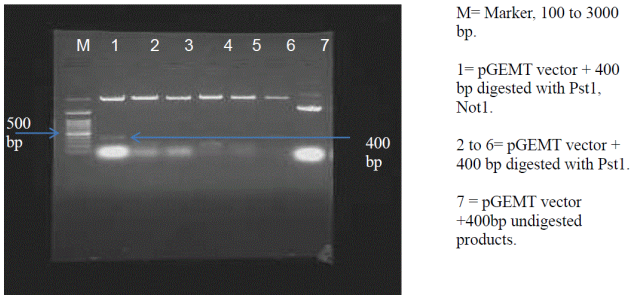
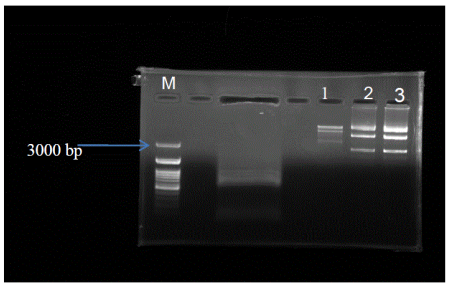


Figure 1c: pGEMT vector and 400 bp VHH gene digested with Pst1 and Not1 restriction enzymes. 400 bp VHH gene was ligated with pGEMT vector (Promega) and positive colonies were selected and were further grown on LB Ampicillin media. Isolated plasmid was digested with Pst1 and Not1 and visualized on 1% agarose gel. For image analysis gel doc was used.

Figure 1d: Alkaline phosphatase digested by Pst1 and Not1 and elute. Alkaline phosphatase 400 bp VHH fragment which was separated from pGEMT-400 ligated clone product by digestion of Pst1 and Not1, and the fragment was eluted and visualized on 1% agarose gel.

In Figure 2, lane 1, 2, 3 contained 1 µl purified plasmid sample which was visualized on 1% agarose gel which signifies their irregular shape, some of them were coiled and round form and the size was 4.5 kb.

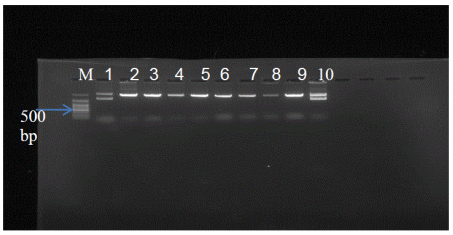


M= Marker 100 to 3000 bp.

1,2,3=Phen4 plasmid, 4500 bp

Figure 2: Phen4 plasmid isolation by alkaline solution-I, II, III. Phen4 plasmid was isolated by alkaline solution-I, II, III from the overnight bacterial culture. Phen4 plasmid was further purified using phenol-chloroform.

Lane1 of Figure 3 signifies undigested Phen4 plasmid which was 4.5 kb, of irregular shape. Lane 2 to 7 showed digested product of Phen4 with Pst1 enzyme which was observed as linear form. Lane 8 signifies Phen4 plasmid double digested with Pst1 and Not1 restriction enzyme which was observed as linear form. Lane 9 signifies Phen4 plasmid digested with only Not1 enzyme was observed as linear form as compared to undigested product in Lane 10.



M= Marker, 3000 bp

1= Phen4, uncut product.

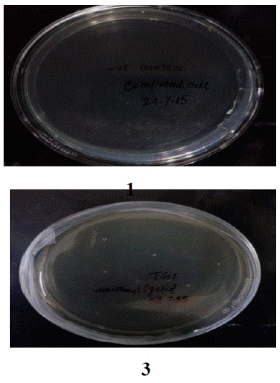
2 to 7 and 9= Phen4 digested product by Pst1, Not 1 enzyme.

8=Phen4 digested product with alkaline phosphate treated.

10= Uncut Phen4 product

Figure 3: Phen4 digested by Pst1, Not1 product. Phen4 plasmid was digested by Pst1, Not1 restriction enzymes sequentially. Pst1 digested Phen4 plasmid was checked by equal volume of Phen4 plasmid with Not1 restriction enzyme only, and was visualized on 1% agarose gel. M=Marker, 3000 bp, 1=Phen4, uncut product. 2 to 7 and 9=Phen4 digested product by Pst1, Not 1 enzyme. 8=Phen4 digested product with alkaline phosphate treated. 10=Uncut Phen4.

In Figure 4, Image 1 showing negative control plate contained only TG1 *E. coli* bacteria. The plate shows no colony due to the absence of ampicillin resistant gene. Image 2 showing positive control plate contained Phen4 vector, with ampicillin resistant gene present; whereas Image 3 signifies ligated plate contained VHH gene fragment and Phen4 vector in which ampicillin gene was present, therefore only clone colonies appeared. Figure 5 showing colony screening.



1

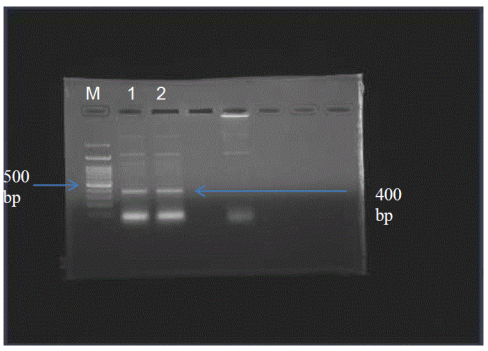
2

3

1= Negative control plate, containing TG1 bacteria only.
2= Positive control plate, containing Phen4 vector only.
3= Ligated colony, containing VHH and Phen4 ligated product.

Figure 4: Positive colony screening. After CaCl2 mediated transformation in TG1 *E. coli*, the cells were plated on selective agar plates containing ampicillin (100 µgm/ml) and 2% glucose and incubated overnight at 37°C.

Lane 1 to 7 of Figure 6 signifies positive clone with VHH gene insert, 700 bp products. Universal primer and GIII primers annealing at the gIII region of the Phen4 vector and therefore, amplifying right insert fragments i.e., VHH gene of alkaline phosphatase product. Lane 8 showing only Phen4 plasmid annealing with universal primer and GIII primer, therefore only 400 bp product was observed, which has no insert of VHH gene.

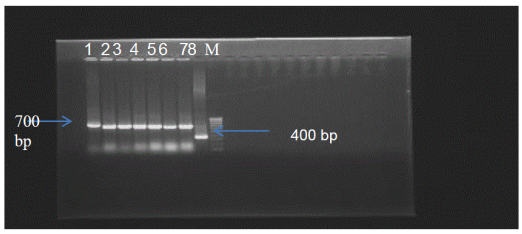


M= Marker, 3000 bp

1,2 = Ligated product digested and 400 bp

Figure 5: Digestion of ligated product by, Pst1 and Not 1, 400 bp product size. After colony screening, 5 ml clone culture kept overnight at 37°C. This plasmid was double digested and confirmed our desired fragment size present in the gel.

Recombinant plasmids were transferred in to *E. coli* bacteria TG1, and grown at 28°C by inducing 1 Mm isopropyl-D-thiogalactopyranoside (IPTG) for 10 hrs. The cell pellets were harvested to check the expression of VHH antibodies by SDS-PAGE (Figure 7a) compared with BSA concentration of 0.5 mg/ml and 1 mg/ml. Further, it was confirmed by Western blot (Figure 7b), in which sdAb with 6 Histidine tag binds to anti HA tag primary Ab followed by anti-rabbit IgG HRP coupled secondary antibody.

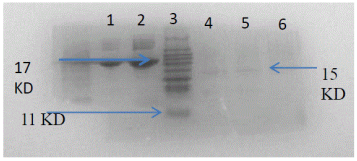


M= Marker 100 to 1200 bp

1 to 7= Positive clone of TG1, 700 bp, using universal primer, GIII primer.

8= Negative clone of TG1, 400 bp

Figure 6: Colony screening by PCR. Positive independent colonies with right insert size were identified by PCR, using universal primer and GIII primer. The PCR products from the vector with no insert and the vector with a full VHH sequence were about 400 and 700 base pairs respectively.

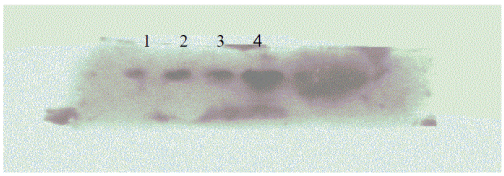


SDS-PAGE of the nanobody, lane 5 & 6 indicates 15 KD protein.

a.

1= BSA, 0.5 mg/ml
2= BSA, 1 mg/ml
3= Marker, 10 TO 245 KD
4= Alkaline Phosphatase Immunized Nanobody, Induced by IPTG, conc. 710 ugm/ml
5= Alkaline phosphatase Immunized nanobody, Induced by IPTG, 934ugm/ml

6= Uninduced protein, conc.360µgm/ml



1= Induced TG1, using rabbit anti HA primary antibody and anti-rabbit IgG HRP coupled secondary antibody, 5 ult load
2= Induced TG1, using rabbit anti HA primary antibody and anti-rabbit IgG HRP coupled secondary antibody, 10 ult load
3= Induced TG1, using rabbit anti HA primary antibody and anti-rabbit IgG HRP coupled secondary antibody, 15 ult load
4= Induced TG1, using rabbit anti HA primary antibody and anti-rabbit IgG HRP coupled secondary antibody, 20 ult load

b.

Figure 7: Expression of soluble antibodies detected by SDS-PAGE, Western Blot. Recombinant plasmids were transferred in to *E. coli* bacteria TG1, and grown at 28°C by inducing 1Mm isopropyl-D-thiogalactopyranoside (IPTG) for 10 hrs. The cell pellets were harvested to check the expression of VHH antibodies by SDS-PAGE (a) compared with BSA concentration of 0.5 mg/ml and 1mg/ml. Further, it was confirmed by Western Blot (b), in which sdAb with 6 Histidine tag binds to anti HA tag primary Ab followed by anti-rabbit IgG HRP coupled secondary antibody.

Discussion

Camelid antibodies form a novel concept in both immunology and biotechnology. Camelid antibodies have been shown to retain immunoglobulin functions. In addition, camelid antibodies are able to recognize other epitopes on antigens that cannot be accessible by 'classical' antibodies [11]. The number of reports on single-domain antibody applications clearly demonstrated that this class of antibodies represents an important tool for scientific research as well as biotechnological and therapeutic uses [8,12-15].

In this study, we are reporting first time the construction of recombinant antibody of phage display technology in India. Production of recombinant VHH phage display libraries from alkaline phosphatase immunized animals has been preferred tools for the isolation of high affinity specific binders [16,17]. Alkaline phosphatase is an enzyme which is present in blood stream. It is a marker which increases in case of liver diseases, abdominal pain, vomiting, nausea and cirrhosis. We have selected alkaline phosphatase enzyme because of its non-toxic nature, homodimer when it is fused with sdAbs. It is reported that alkaline phosphatase had great effects on the binding affinity of a single-chain variable fragment (scFv) [18-20].

After immunization with AP enzyme and adjuvant in *Camelus dromedarius*, single domain heavy chain antibody was generated from V-D-J-H gene arrangement in B-cell, lymphocyte separation was successfully done by using Histopaque 1077. RNA isolation was the key which gives the antigen specific small antibody, 15 KD size and 2.2 nm in diameter and 4 nm in height. Our first aim was to generate 700bp fragments and we have used cloning vector pGEMT for that purpose. The 1st PCR products of alkaline phosphatase encode the CH1, hinge, and part of the CH2 exons, using Call001 and Call002 primer. Next PCR products encode the variable regions of the heavy chain antibodies (VHH or Nanobodies), hinge and part of the CH2 exons (the CH1 is absent in the heavy chain antibodies).

Phage display technique was achieved by cloning the gene for the protein in a special type of M13 vector. This technique was carried out with a phage display library. This library was made up of many recombinant phages and each phage displaying a different protein. The advantages of phage display system are it is inexpensive, easy to handle and results in a variable protein within a very short period of time. The phage display system is simple, well understood and could be genetically manipulated. And also the culturing cost is minimal. *E. coli* scale up is fast, since doubling time is 20-30 min. Purification of the camelid antibody was easy as compared to other conventional antibody.

The development of polyclonal and monoclonal antibodies is time consuming [21]. Furthermore, animals (mostly mice) had to be injected and finally killed. For monoclonal antibodies, the selected cell lines had to be kept, grown and harvested for production, which was an expensive process.

After heavy chain antibody was produced by PCR, it was further ligated to pGEMT (Promega) vector for two reasons. First, isolated RNA from the camel peripheral blood was of very low volume. For the next round of PCR, it generated 700 bp and finally 400 bp fragments. When we digested the 400 bp heavy chain fragment, it required high concentration and high volume. pGEMT vector was the ideal one which could ligate our desired fragment and through plasmid isolation, we got large volume of the desired product. Second, when 400 bp product was double digested, it was very difficult to identify whether the product was really digested or not, especially in double digestion, because after PstI and NotI digestion, very few nucleotides were separated.

After Phen4 plasmid double digestion, product was treated with alkaline phosphatase enzyme because of the dephosphorylation. One of the main reasons of dephosphorylation was that, digested Phen4

fragments could be self-ligated. Therefore, during transformation it showed false positive results.

In case of RNA and cDNA isolation, the minimum reagent was added to get a better result, which was time consuming and cost effective product. During the plasmid isolation, lysis solution were prepared manually and used as minimal amount. Therefore, the production of sdAb fragments was the cheapest, easiest and most time saving techniques.

In summary, we believe that camelid antibodies will be a method of choice to control infectious disease agents. In this study, we developed novel protocol for the production of stable single-domain antibodies in the camelid format. Single-domain antibodies for their ability to bind small molecules will be a most potential therapeutic agent against immunity related diseases as well as other diseases even caused by infectious microorganisms. In our future study, we will try to understand the binding affinity using BIACORE and also try to ameliorate the complicity of asthma mouse model.

Experimental Procedures

Immunized protocol

I this study, immunized male animal was Kachchi and Jaisalmeri breed. The age of the immunized animal was three years. Higher dose applied every seven days and immunized for total 105 days. Alkaline phosphatase enzyme (SRL) 100 µgm was mixed with 1 ml of PBS and 1 ml of Freund adjuvant (Sigma). Total volume was 2 ml. Enzymes were mixed around 25-30 min by vigorous shaking. On the first day, 100 µgm enzyme was injected to the camel. After seven days, higher dose of 150 µgm enzyme and adjuvant mixture was injected subcutaneously. After 105 days of experiment animal was sacrificed and blood was collected from both immunized and control animal. Control animal was injected only with PBS.

Isolation of antigen-specific nanobodies

Separation of peripheral blood lymphocytes: 5 ml of anticoagulant fresh blood was taken and mixed with 0.9% Nacl solution (Nuclease free water) in a 15ml falcon tube. 2.5ml of Hisep 1077 (Sigma Aldrich) was added slowly in a 15 ml falcon tube. Blood mixture overlapped to the Hisep 1077. Care was taken to keep the blood mixture and Hisep in a separate layer. The mixture of blood was carefully centrifuged at 2800 rpm for 20 min at room temperature. We obtained three distinguished layer in which top layer was plasma, interphase appeared white ring which was peripheral blood lymphocyte and the bottom layer was Hisep. The interphase was collected to a new 1.5 ml eppendorf tube. Equal volume of freshly prepared PBS was added (1X with Nuclease free water) for wash. Centrifuged at 2200 rpm for 10 min at 4°C [9,10,22] and decanted the supernatant after centrifugation. Cell count was done with the help of Hemocytometer. Cell can be stored at -20°C in RNA later for long time without freezing and thawing [22-24].

RNA isolation from peripheral blood lymphocyte: Sample was taken from -20°C and kept at room temperature for 10 min. 250 µl of sample was taken and added to 750 µl of Trizol. Re-suspended the sample by pipetting several times. Incubated the sample for 5 min at room temperature. 200 µl Chloroform was added in 1 ml Trizol (Invitrogen) cap tube. Shaken vigorously or vortexed for 20-30 sec. Incubated at room temperature for 5 min. Centrifuged at 12,000 rpm for 15 min at 4°C. Only 300 µl sample (upper aqueous phase) was taken carefully and kept it in a new tube. Added 100% chilled alcohol and the volume made up to 1.5 ml. Sample kept at -20°C for further use. Next day, sample was centrifuged at 13,000 rpm for 10 min at 4°C. Supernatant was removed and the pellet was left. 400 µl 75% chilled alcohol was added to the pellet. The sample was inverted for 4-5 times and centrifuged at 9000 rpm, at 4°C for 5 min. The supernatant was discarded very carefully by pipetting. All the tubes were kept inside the laminar air flow for air drying and 20 µl of nuclease free water was added [25]. Eluted sample was treated with DNase I enzyme (Invitrogen). RNA sample was taken 1 µg (10 ul), DNase reaction buffer was added (10X) 2 µl, DNase I enzyme was added 1 ul, Nuclease free water was added 7 µl. Total volume was 20 µl. Incubated sample kept at room temperature for 15 min and then 1µl of 25 mM EDTA solution was added. The enzyme activity was inactivated at 65°C for exactly 10 min [25].

First-strand c-DNA synthesis: Total RNA sample was taken 8 µl, added Random Hexamer (Bioline) and mixed properly, incubated at 70°C for 10 min, 25°C for 15 min, and stored at 4°C. In this mixture, 6 µl strand buffer (Invitrogen, 5x), DTT (Invitrogen, 0.1 M) 3 µl, dNTPs (Taqara, 2.5 mM) mixture 6 µl, nuclease free water (Bioline) 1 µl, and reverse transcriptase (Invitrogen, 200 U/ult) 1ul were added. The PCR was set at 25°C for 10 min, 37°C for 60 min, 42°C for 60 min, 80°C for 15 min and finally stored at 4°C. Sample was stored at -20°C for long time storage without freezing and thawing [16].

First PCR reaction: Buffer was taken (10X)-2 µl, dNTPs (2.5 mM) 2 µl, Forward Primer (Call 001)-1 µl , Reverse Primer (Call 002)-1 µl , Taq Polymerase (Invitrogen, 5 U/ult) 0.5 µl , Nuclease free water added 11.5 µl. Total reaction volume was 20 µl. PCR temperature was set at 95°C for 5 min, 95°C for 45 Sec, 57°C for 45 sec, 72°C for 45 sec, 72°C for 10 min and 4°C for storage. PCR reaction was for 35 cycles. Each reaction product checked by 1% agarose gel. 5 µl sample was loaded in each well. PCR product was 700 bp [16].

Gel elute first PCR product: Prepared washing buffer-250 µl gel wash buffer was mixed properly with 1000 µl of ethanol. 700 bp product with the help of sharp knife was cut and kept it in an eppendorf tube. 100 µl gel binding buffer was added in the same tube and was kept it in the water bath. The temperature maintained at 55°C for 10 min. The gel solution was mixed continuously. The whole solution was poured in a fresh column and centrifuged at 12,000 rpm for 2 min 30 sec at 27°C. 300 µl gel binding buffer was added and centrifuged at 12,000 rpm, for 2 min 30 sec at 27°C. Mix buffer was discarded and 600 µl washing buffer was added. Centrifuged again at 12,000 rpm for 2 min 30 sec at 27°C. Centrifuged empty column at 12,000 rpm, for 2 min at 27°C. In a new column 20 µl elution buffer was added and centrifuged at 12,000 rpm, at 4°C, for 2 min 30 sec. Only liquid was taken into micro centrifuge tube and the sample was stored at -20°C.

Second PCR: 1.5 µl of sample was taken and 1.5 µl of forward primer pstI, 1.5 µl of NotI, 3µl of 10X reaction buffer, 3 µl of dNTPs, 1µl of Taq Polymerase enzyme, 18.5 µl of nuclease free water were added. Temperature was set at 95°C for 5 min, 95°C for 45 sec, 61°C for 45 sec, 72°C for 45 sec,72°C for 10 min and 4°C for storing [16]. 1% agarose gel was checked and 400 bp product was found. Total sample was loaded and elute 400 bp product followed by Himedia Purification kit.

Ligation reaction: 400 bp product was purified and ligated with Pgem®-T Easy vector (Pgem(R)-T Easy vector System I, Promega). 5.5 µl eluted second PCR product (12 ngm/ µl), vector 1 µl (50 ngm/µl), ligation buffer 7.5 µl, T4 ligation enzyme 1 µl were added. Total

reaction volume was 15 µl. Reaction mixture kept at 17°C, overnight in BOD incubator (Care was taken so that temperature distributed equally).

Competent cell preparation: 200 µl of DH5α bacteria culture was inoculated in 5 ml LB Broth. No antibiotic was added. Kept the tube in BOD shaker overnight for 37°C at 150 rpm. Turbidity of the bacterial culture was observed next day to confirm the growth. 0.1% bacterial culture was inoculated in to 50 ml LB broth in a 250 ml conical flask and kept in BOD shaker incubator at 150 rpm for 50-60 min. O.D at 600 nm was checked every 30 min. When O.D was 0.3, the flask was kept immediately in ice to stop further growth. Measured 50 ml culture in a centrifuge tube. Centrifuged at 2700 rpm, for 10 min, at 4°C. Supernatant was discarded and precipitate was dried with the help of paper towel used to drain out media. Chilled 0.1 M Cacl2 was added kept it in ice for 20 min. Centrifuged at 2,700 rpm, for 10 min at 4°C and dissolved the precipitate as much as required in 1.5 ml Nuclease free water. One eppendorf tube rack was taken, filled with some water and all competent cells tube kept in a rack, so that temperature equally distributed. Kept it at 4°C for overnight for transformation [16,25,26].

Transformation: Three competent cell tubes were taken. 3 tubes were labeled as ligated product, positive control and negative control. DNA was added all 3 competent cell tubes inside the hood. Ligated product was Pgem®-T Easy vector (Pgem(R)-T Easy vector System I, Promega) and desired gene product, Positive control was Pgem®-T Easy vector only, Negative control was nothing. Gently tapped for mixing. Kept in ice chilled bucket for 30 min. Heat shock maintained at 42°C. All eppendoff tubes closed with parafilm. Kept all the tubes at 42°C for exactly 90 sec. Tubes were kept in ice for 15 min. In each tube 900 µl LB broth was added for recovery of the cell. Kept it at 37°C for 1 hr in a BOD shaker, with 150 rpm. 200 µl transformed cells were taken and put on LB agar plate containing Ampicillin and thoroughly spreaded with the help of sterilized spreader. Kept it inside the laminar air flow for drying. Kept all the tubes inverted for overnight growing at 37°C in BOD. Next day colonies were observed and compared with positive and negative control [25].

Colony screening: Positive colonies were picked up by sterilized wooden tooth-pick. Individual Colony with tooth-pick was inoculated in to 6 ml LB broth with ampicillin and kept it at 37°C for overnight growth rpm in BOD shaker [16,27].

Plasmid isolate from the positive clone: Maxi preparation-(i) Alkaline solution I: 50 mM Glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0). Prepared solution-I from standard stocks in batches of 100 ml autoclave for 15 min, at 15 psi (1.05 kg/cm2) on liquid cycle, and store at 4°C. (ii) Alkaline solution II: 0.4 N NaOH (freshly diluted from 10 N stock), 2% (W/V) SDS. (iii) Alkaline Solution III: 5 M Potassium Acetate-60 ml, Glacial Acetic Acid-11.5 ml, Nuclease free water-28.5 ml. The resulting solution was 3M with respect to Potassium and 5 M with respect to Acetate. Stored the solution at 4°C and transferred it to an ice bucket just before use. (iv) STE Buffer: Sodium Chloride-Tris-EDTA Buffer, 10X-pH 8.0. 10X solution containing 100mM Tris-HCl, 10 mM EDTA and 1 M NaCl.

Cell harvested-5 ml of primary culture was inoculated at 37°C in BOD shaker at 160 rpm. Care was taken for decontamination. On the same day evening, turbidity for bacterial growth was checked. 0.5% inoculum was inoculated to 50 ml LB ampicillin medium. Kept it in BOD shaker at 160 rpm for overnight culture. Next day, 50 ml of culture media took in a sterilized centrifuge tube inside the laminar air flow and centrifuged the culture media at 4000 rpm for 6 min at 4°C temperature. Supernatant was discarded and took the pellet. 10 ml STE buffer was added. The tube was vortexed for 1 min so that precipitate dissolved and centrifuged at 9000 rpm for 6 min at 4°C. Only pellet was taken, 10 ml alkaline Solution-I was added and freshly prepared 1ml of Lysozyme was added (stock 10 mg/ml, Amresco) and then vortexed for 1 min. Then incubated for 10 min in an ice bucket. After incubation, 20 ml of freshly prepared alkaline solution-II was added and inverted several times for mixing. Tubes were kept in ice exactly for 5 min. 15 ml chilled solution-III was added. Reagents were mixed properly. Incubated for 30 min (maximum) in ice and centrifuged at 13,000 rpm for 15 min, at 4°C. Supernatant was taken by filter paper. Around 40 ml supernatant was taken. Added 0.7-0.8 volume of supernatant, 32 ml Isopropanol. Mixed by inverted position by 2-3 times. Incubated at room temperature for 20 min and centrifuged at 13,000 rpm for 15 min at room temperature. Supernatant was discarded. Only pellet was taken and added 5 ml 70% alcohol (used Marck 100% alcohol, added Nuclease free water). Vortexed the sample so that the pellet was partially dissolved. Centrifuged at 13,000 rpm for 15 min at 4°C. Alcohol was decanted and pellet was completely dried. Pellet was stored at -20°C for overnight. Next day, 500 µl nuclease free water was added and thoroughly mixed. 30 µl Rnase A (Invitrogen, 20 mg/ml) was added and kept in BOD incubator for 3 hrs at 37°C [25].

Digestion by pst1 and Not1: Plasmid was confirmed by electrophoresis run in 1% agarose gel. Product size was further confirmed by 100-1200 bp marker. Desire product digested by Pst1, Not1 enzyme. Prepared master mix of pst1 and Not1 enzyme each 1 µl (NEB, 20U/µl), BSA 1 µl, DNA 20 µl, NEB Buffer 3.5 µl, total volume was 30 µl. Incubated exactly for 45 min at 37°C in BOD. Immediately digested product kept at 4°C. For long time storage it was kept at -20°C. After incubation, digested product was run in 1% agarose gel. Digested 400 bp product was separated out from the pGEM-T vector. Desired fragment was further purified by Himedia purification kit.

Plasmid isolation (PHEN4 vector): maxi preparation (i) Alkaline solution I: 50 mM Glucose, 25 mM Tris-Cl (pH 8.0), and 10 mM EDTA (pH 8.0). Prepared solution-I from standard stocks in batches of ~100 ml autoclave for 15 min, at 15 psi (1.05 kg/cm^2) on liquid cycle, and stored at 4°C. (ii) Alkaline solution II: 0.4 N NaOH (freshly diluted from 10 N stock), 2% (W/V) SDS. (iii) Alkaline Solution III: 5 M Potassium Acetate-60 ml, Glacial Acetic Acid-11.5 ml, Nuclease free water-28.5 ml. The resulting solution was 3M with respect to Potassium and 5 M with respect to Acetate. Stored the solution at 4°C and transferred it to an ice bucket just before use. (iv) STE Buffer: Sodium Chloride-Tris-EDTA Buffer, 10X-pH 8.0. 10X solution containing 100 mM Tris-HCl, 10 mM EDTA and 1 M NaCl.

Cell harvested-5 ml primary culture was inoculated at 37°C in BOD shaker at 160 rpm. On that same day evening we have checked the turbidity for bacterial growth. 0.5% inoculum was inoculated to 50 ml of LB ampicillin medium and kept it in BOD shaker at 160 rpm for overnight culture. Next day, 50 ml of culture media was taken in a sterilized centrifuge tube inside the laminar air flow. Centrifuged the culture media at 4000 rpm for 6 min at 4°C temperature. Supernatant was discarded and only pellet was collected. 10 ml STE buffer was added. Vortexed the tube for 1 min to dissolve the precipitate and centrifuged at 9000 rpm for 6 min at 4°C. Only pellet was taken, 10 ml alkaline Solution-I and freshly prepared Lysozyme 1ml (stock 10 mg/ml, Amresco) was added, then vortexed for 1 min and incubated for 10 min in an ice bucket. After incubation, 20 ml freshly prepared Alkaline solution-II was added and mixed properly by inverting several times. Tubes were kept in ice exactly for 5 min and 15 ml chilled

solution-III was added. The reagent was mixed for three times and incubated for 30 min (maximum) in ice. Centrifuged at 13,000 rpm for 15 min at 4°C. Around 40 ml of supernatant was collected by filter paper. Added 0.7-0.8 volume of supernatant, 32 ml Isopropanol. Mixed by inverted position by 2-3 times and incubated at room temperature for 20 min. Centrifuged at 13,000 rpm for 15 min at room temperature and discarded the supernatant. Only pellet was collected and 5 ml of 70% Alcohol was added. Vortexed the sample to dissolve the pellet partially and centrifuged at 13,000 rpm for 15 min at 4°C. Decanted alcohol and pellet was completely dried. Pellet was stored at -20°C for overnight. Next day, 500 µl of Nuclease free water was added and thoroughly mixed up. Added 30 µl Rnase A (Invitrogen, 20 mg/ml). Kept in BOD incubator for 3 hrs at 37°C [25].

Plasmid purification by phenol-chloroform-isoamyl alcohol: 500 µl of sample was taken in an eppendorf tube and equal amount of saturated Phenol:Isoamyl alcohol:Chloroform-25:24:1 ratio was added. Tubes were vortexed vigorously at least 3 min and centrifuged at 13,000 rpm for 15 min at 4°C. There were three distinguished layer. Aqueous phase was taken. This was repeated twice. 3 M 0.1 volume of Sodium Acetate, pH 5.2 was added and incubated sample for 10 min at room temperature. 0.8 volume of Isopropanol was added and incubated for 20 min. Sample was Kept at -20°C for overnight. Next day centrifuged the sample 13,000 rpm, for 15 min at 4°C. Supernatant was decanted and 500 µl of 70% alcohol was added in to the pellet. The sample was vortexed and centrifuged at 13,000 rpm for 15 min. Alcohol was decanted and kept in laminar air flow for drying. Pellet should not dry completely. 500 µl of nuclease free water was added and dissolved the pellet. Checked in 1% agarose gel [25].

PHEN4 vector digested by PstI and NotI: 2 µl (2 µgm/µl) of DNA sample was taken, added PstI NEB enzyme, NEB Cut smart buffer-2.5 µl (10X), BSA-1 µl, and finally nuclease free water-18.5 µl was added. Total volume was 25 µl. Kept at 37°C inside the BOD incubator for exactly 15 min. Immediately after digestion, mixture was kept at 4°C. Prepared master mix for NotI enzymatic digestion. NotI enzyme 2 µl, BSA 1 µl, NEB buffer (10X)-3 µl were taken. 24 µl of nuclease free water was added. Total reaction volume was 30 µl and distributed in 6 eppendoff tubes, each tube contained 5 µl of master mix. 5 µl of previously digested product (PstI) was added in each tube and kept at 37°C in the BOD incubator for 30 min. Tubes were immediately kept at 4°C. 1 µl XhoI (NEB, 10 U/µl) was added in each tubes and kept it in the BOD incubator for another 30 min. Sample was then stored at -20°C [16,28].

Gel elute digested phen4 product: Prepared washing buffer-250 µl of gel wash buffer was mixed with 1000 µl of ethanol. Both reagents were mixed properly. Electrophoresis of PHEN4 digested product was done in 1% freshly prepared agarose gel. Around 4500 bp product with the help of sharp knife was cut and kept it in an eppendorf tube. 100 µl gel binding buffer was added in the same tube and kept it in the water bath at 55°C for 10 min. Gel solution was mixed continuously to dissolve it. All solution was poured in a fresh column and centrifuged at 12,000 rpm for 2 min 30 sec. at 27°C. 300 µl of gel binding buffer was added and centrifuged at 12,000 rpm, for 2 min 30 sec at 27°C. Mix buffer was discarded and 600 µl of washing buffer was added. Centrifuged again at 12,000 rpm for 2 min 30 sec at 27°C. Centrifuged empty column at 12,000 rpm, for 2 min at 27°C. 20 µl of elution buffer was added in a new column and centrifuged at 12,000 rpm, at 4°C, for 2 min 30 sec. Only liquid was collected in to micro centrifuge tube and sample was stored at -20°C.

Ligation reaction: Added eluted digested Phen4 Vector 1 µl (50 ngm/µl), 2nd PCR double digested product (around 12 ngm/µl) 3 µl, Ligation Buffer 5 µl, T4 Ligation enzyme 1 µl. Total reaction volume was 10 µl. Reaction mixture kept at 17°C, overnight in BOD. Next day, reaction mixture was taken and kept at -20°C [16].

Competent cell preparation of TG1: 200 µl of TG1 bacterial culture was inoculated in 5 ml of LB Broth. No antibiotic was added. Kept the tube in BOD shaker overnight at 37°C at150 rpm. Next day, turbidity of the bacterial culture was observed to confirm the growth. 0.1% bacterial culture was inoculated in to 50 ml of LB broth in a 250 ml conical flask. Kept in BOD shaker incubator at 150 rpm for 50-60 min. O.D at 600 nm was checked every 30 min. When O.D was 0.3, the flask was kept immediately in the ice to stop the further growth. Measured 50 ml of culture in a centrifuge tube and centrifuged at 2,700 rpm for 10 min at 4°C. Supernatant was discarded and precipitate was dried. Chilled 0.1 M CaCl2 was added and kept it in ice for 20 min. Centrifuged at 2700 rpm for 10 min at 4°C. Precipitate was dissolved as much as required in 1.5 ml nuclease free water. All competent cell tubes were kept in a rack to maintain the equal temperature and kept it at 4°C for overnight for transformation experiment [16,25,26].

Transformation: Three competent cell tubes were taken. Ligated product, positive control and negative control were labeled and DNA was added. Ligated product was Phen4 vector and desired gene product, positive control was Phen4 vector only, negative control was nothing. Gently tapped to mix and kept in ice chilled bucket for 30 min. Heat shock-maintained temperature exactly at 42°C. All eppendoff tubes were closed with parafilm and kept all the tubes at 42°C for exact 90 sec. Tubes were kept in ice for 15 min exactly and 900 µl of LB broth was added in each tube for recovery of the cell. Tubes were kept at 37°C for 1 hr in a BOD shaker, with 150 rpm. 200 µl of transformed cells were taken and inoculated on LB agar plate containing Ampicillin and spreaded thoroughly with the help of sterilized spreader. Kept it for some time for drying inside the laminar air flow. Kept all the plates at 37°C in the BOD incubator. Next day, colonies were observed and compared with the positive and negative control [25].

Colony screening: Ten colonies were picked up from the ligated plate as it is mentioned before. Individual colony was inoculated in to 6 ml LB broth and ampicillin mixture. Kept it at 37°C for overnight growth at 150 rpm in BOD shaker [16,27].

Colony PCR using GIII primer: PCR was performed on 10 independent colonies in order to determine the vector with the right insert size. Combined dNTPs (2.5 mM), universal reverse primer and GIII primer (20 uM), Taq DNA polymerase (Invitrogen 2.5 u/µl), PCR buffer and volume made up to 20 µl. Single colony was taken using sterile tooth pick. Cells were taken in to the PCR reaction by stirring the tooth pick in the reaction mix. PCR reaction was set for 35 cycle and the reaction temperature was 95°C for 5 min, 95°C for 45 secs, 57°C for 45 secs, 72°C for 45 secs, 72°C for 10 min and 4°C for storing the samples. Used 5 µl of each reaction for analytical gel electrophoresis on a 1% agarose gel in 1 × TAE buffer. DNA smart ladder was used as DNA molecular weight marker. The PCR product from the vector with a full VHH sequence were 700 base pairs [16,29].

Preparation of phagemids: 100 µl library stock was inoculated in to 100 ml of TY containing ampicillin (100 ugm/ml) and glucose (2%). Kept for growth at 37°C for 2-3 hrs with shaking at 150 rpm. Added 1012 pfu M13KO7 helper phage (NEB). Incubated for 30 min without shaking at room temperature. Centrifuged at 4000 rpm for 10 min.

Cells re-suspended in 300 ml 2 × TY containing ampicillin and kanamycin (70 µgm/ml). Incubated overnight at 37°C, with shaking at 150 rpm. Centrifuged at 8000 rpm, for 30 min at 4°C. Transferred supernatant containing phages to a fresh tube. Added 1/6th volume to 50 ml PEG/NaCl solution. Mixed well and placed in ice for 30 min. Centrifuged at 4000 rpm for 30 min at 4°C. Supernatant was removed and allowed the tubes to drain on paper tissue. Phage pellet was re-suspended in PBS to make it final volume 1 ml. Centrifuged at 13000 rpm for 5 min to remove bacterial cells, debris. Transferred supernatant to a fresh micro centrifuge tube [8,11,13].

Screening by ELISA: For recombinant phage clone, 96 well microplate was coated using 100 µgm/ml antigen. The plate was incubated for 1-2 hours at room temperature in a humidified container. Each well of the microplate was filled with 1X blocking buffer. The wells of the plate were dried with paper towel. 200 µl of the recombinant phage antibody supernatant was added to each antigen coated well, and incubated for 1-2 hours at room temperature. After washing and blotting, HRP/anti-M13 monoclonal conjugate (GE Healthcare) was added to each appropriate well and kept for incubation for 1 hour at room temperature. After washing, ABTS solution was added and reading was taken at ELISA plate reader at 405-415 nm. The value was three times higher than the negative control.

Expression of recombinant antibody: TG1 clone bacteria inoculated in to 10 ml of LB-Ampicillin media and incubated at 37°C till O.D reaches 0.6. IPTG 1 mM was added and kept overnight at 28°C. Periplasmic fraction of the bacteria was further confirmed by SDS PAGE and Western blot. Membrane fraction solubilized protein were transformed from 10% SDS-PAGE gel to Polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat dried milk at 4°C. The membrane was treated with Rabbit anti-HA antibody (Invitrogen) in 1:1000 ratio for overnight. Next day, secondary antibody was added and kept in shaker at room temperature for 2 hours. Visualization of AP-VHH specific bands was done by ECL Chemiluminescence (BioVision).

Conflict of Interest

The authors have declared that no conflict of interest exists.

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