SELECTION AND CHARACTERISATION OF PPRV-SPECIFIC SINGLE DOMAIN ANTIBODIES

Aswathy Joji

A dissertation submitted for the partial fulfilment of BS-MS Dual Degree in Science

Under the guidance of

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Indian Institute of Science Education and Research, Mohali

April 2019

Certificate of Examination

This is to certify that the dissertation titled “Selection and Characterization of PPRV specific Single Domain Antibodies” submitted by Miss. Aswathy Joji (MS14148) for the partial fulfilment of BS-MS dual degree programme of the institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report is accepted.

Dr Kausik Chattopadhyay

Dr Rajesh Ramachandran

Dr Sharvan Sehrawat
(Supervisor)

Dated: April 26, 2019

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr Sharvan Sehrawat at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgements of collaborative research and discussion. This thesis is a bonafide record of original work done by me and sources listed within have been detailed in the bibliography.

Ms. Aswathy Joji
(MS14148)

Dated: April 26, 2019

In my capacity as the supervisor of candidates project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sharvan Sehrawat
(Supervisor)

Dated: April 26, 2019

ACKNOWLEDGEMENT

Though the name adorning the cover belongs to me, I owe my solemn gratitude to a cohort of people, who contributed to this effort. This project is also a product of the support, inspiration and guidance I received from them.

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ADCC	Antibody Dependent Cell mediated Cytotoxicity
BCR	B Cell Receptor

BT	Biotinylation Tag
cAb	conventional Antibody
CD	Cluster of Differentiation
cDNA	Complementary DNA
CH_1	Constant domain of heavy chain
CDR	Complementarity Determining Region
CHO	Chinese Hamster Ovarian cells
CIP	Calf Intestine Phosphatase
CTL	Cytotoxic T Lymphocytes
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent assay
Fab	Antigen binding fragment
FP	Forward Primer
hcAb	heavy-chain Antibody
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1 thiogalactopyranoside
mAb	Monoclonal antibody
pAb	Polyclonal antibody
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
RP	Reverse Primer
ScFv	Single chain Fragment variable
sdAb	Single-domain antibody
SDS	Sodium dodecyl sulfate
TAE	Tris-Acetate EDTA
TCR	T Cell Receptor
TCS	Thrombin Cleavage Site

V_H	Variable domain of Heavy-chain
V_HH	Variable domain of Heavy-chain of Heavy-chain antibody
V_L	Variable domain of Light-chain

CHAPTER - I

PHAGE DISPLAY TECHNOLOGY FOR SELECTION AND CHARACTERISATION OF V_HH (SINGLE DOMAIN ANTIBODIES)

1.1 BACKGROUND

The ability to defend and enhance their fitness when in threat is an inherent biological behavior that account for the evolutionary success of the survived and the surviving. Employing the same logic, our immune system ensures our fitness. Upon encountering a foreign, altered self or pathogen derived immunogenic organic materials, the host engages its defense machinery to react resulting in either their removal or rendering them debilitated in order to ensure the constant surveillance and maintenance of normal bodily functioning. Higher order organisms possess an intricate system composed of cell-mediated and humoral responses delineated through the individual and integrated activity of innate and adaptive immune systems within them. Pathogens keep on evolving in-order to counter the impact of the antigen-specific memory cells by improvisation through mutation. T lymphocytes and B lymphocytes with their TCRs and BCRs distinguished by higher specificities initiate the cellular response via detection of complimenting antigen signature and elicit responses by the release of cytokines responsible for activation of phagocytes, CTLs, and the release of molecules (chemokines) signaling the migration of other cells for degradation or suppression of the antigen. B-cells are characterized by their ability to synthesize humoral immune responses via the production of antigen-specific antibodies on activation. Antibodies can be considered as powerful tools capable of causing antibody-dependent cell mediated cytotoxicity (ADCC)', 'complement activation', 'opsonization' to promote cellular death via apoptosis or necrosis. Subsequent

encounters with the same pathogen can elicit enhanced immune responses offered by the enriched population of memory B cells and T cells.

1.1.1 Conventional Antibodies (cAbs)

Generally, the cAbs are dimers of heterodimers of a 'heavy-chain' polypeptide and a 'light-chain' polypeptide each with an N terminal variable region and a C terminal constant region and have an approximate molecular weight of 150 kDa. Most cAbs possess two domains in the light chain and four domains in the heavy chain and the chains are held together with disulfide bonds between cysteine residues. They are characterized by their high specificity towards a particular signature epitope offered by the complimenting action of V_L and V_H; their small size, ability to elicit strong responses *in-vitro* as well as *in-vivo*, clonability etc. They are modified into further efficient formats employing recombinant DNA technology for therapeutic and diagnostic applications. The specificity of the BCR and the specificity of the cAbs produced by the same B lymphocyte will be identical.

1.1.2 Heavy Chain Antibodies (hcAbs)

A special class of antibodies distinguished by the absence of light chains and C_H1 domain (MW~90kDa) in comparison to the conventional antibodies, found in the serum of cartilaginous fishes (0.1-1.0 mg/ml) and Camelidae family (~10-20 mg/ml)[1,2,3] along with cAbs. The variable domain of hcAbs responsible for its antigen specificity is termed as V_HH (Variable domain of Heavy chain of Heavy-chain antibody). The smallest unique fragment of these antibodies equipped to detect the complimenting antigen with high affinity can be termed as a 'nanobody'[4]. If it is composed of a single domain of the antibody, the nanobody can be termed as a single domain antibody (sdAb). The Camelid V_HH (~15kDa) is such a sdAb with very promising applications with reference to its experimentally verified and quantified physicochemical properties[4].

1.1.3 Camelid V_HH

In comparison to cAbs, camelid V_HHs are so compact that they are able to detect even the sites

defined to be cryptic for canonical mAb or pAb. Evolved 50 million years ago, with high solubility and stability offered by the hydrophilicity of the regions corresponding to the V_H-V_L interface, V_HH replaces V_H domain of cAb in camelids to form hcAbs that are devoid of light chains. They can be cloned, modified and expressed in various host systems like *E. coli*, yeast, mammalian system and recently also reported in plants with highest antibody yields reported in bacterial hosts[1,5]. Though the genetic diversity of the sequence encoding the polypeptide is amplified by VDJ recombination, the contribution towards diversity in antigen specificity against the peptide by CDR3 is comparatively higher than that of CDR1 and CDR2 as the DNA sequence that encode CDR3 is generated during the B-cell development while the sequences of others are encrypted as in the germline. Camelid V_HH has its 3-28 amino acids longer CDR3 while the human V_H domain has 8-15 amino acids[6,7]. The elongated CDR3 loops fold over onto the side of V_HH corresponding to the V_H interacting with the V_L in human IgG and this elevates the potential of the efficient V_HH binding in comparison to the binding of the V_H-V_L complex to the target as described in the structural analysis of co-crystallised V_HH nanobodies specific for PD-L1 and CD38[8,9].

Table1.1: Advantages of V_HH over conventional full-length antibodies[10].

	ADVANTAGES OF V_HH
1.	Single domain nature enables coherent genetic manipulation.
2.	No decrease in library diversity as it occurs due to the random reshuffling of V_H-V_L domains in the cAb library.
3.	High physicochemical stability. V_HHs are stable at temperatures ranging from 50^{o}C to 65^{o}C and long range of pHs . Shark V_HHs are highly stable in Urea rich shark blood[11].
4.	High solubility offered by the hydrophilicity of the amino acid composition at sites corresponding to the V_H-V_L interface of cAb.

5.	Small size enables rapid tissue penetration and quick clearance.
6.	Small size and single domain nature enable proper refolding in foreign hosts and hence well expressed with lower aggregation.
7.	Small size and flexible, elongated CDR3 loop assist efficacious detection of cryptic sites inaccessible to cAb. This property might offer better antiviral properties.
8.	Ease in the generation of stable multivalent complexes due to the relaxed choice of linker sequences, and reduced complexity offered by single domain format.
9.	Facile production of oligo-clonal products as the events of V_H-V_L as the mispairing is eliminated.
10.	Immunogenicity in a degenerate individual is low as it lacks F_c and humanized recombinant V_HH has shown ADCC in in-*vitro* assays.[12]

Table1.2: Disadvantages of V_HH over cAbs

	DISADVANTAGES OF V_HH
1.	Poor activator of the complement system.
2.	Poor agglutinin
3.	Poor response towards bacterial and fungal epitopes.
4.	No reported ability to induce ADCC in-*vivo.*

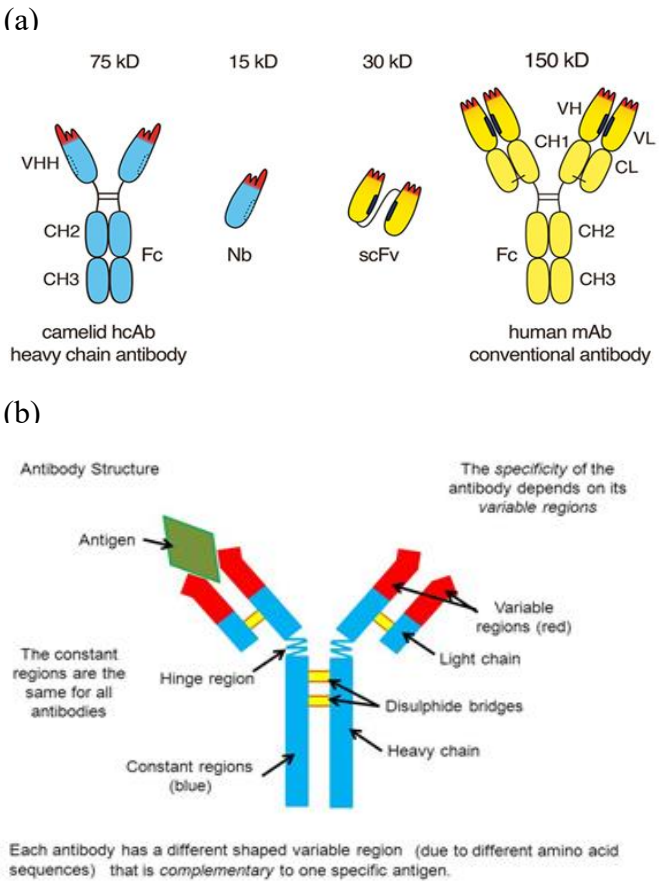


Figure 1.1.1: (a) **camelid hcAb and cAbs**: Camelid hcAbs are dimers made of 2 heavy chains held together by disulfide bonds. Each monomer would have 1 variable domain and 2 constant domains. cAbs are multimers made of 2 heavy chains and 2 light chains held together by the means of a disulfide bond. Heavy chains are made of 1 variable domain (V_H) and 3 constant domains *viz.* C_H1, C_H2 and C_H3, of which C_H1 is joined to C_H2 via a linker peptide in the hinge region. Light chains are composed of a variable domain (V_L) and a constant domain (C_L)[42]. (c) **Domains of cAbs:** Variable domains are composed of FR1, FR2, FR3 and FR4 with intervening complementarity determining regions CDR1, CDR2, and CDR3. HCAbs lack light chain as well as the C_H1 domain. CDR3 loop in V_HH is longer and more flexible than that in cAbs[43].

1.1.4 Deriving tools and machineries employing V_HH

The capability of V_HH to maintain stability and potency in harsh conditions; easier recognition of antigenic sites, rapid penetration, ease of humanization enabled by small size; cost-effectiveness; low agglutination; high solubility etc. offer opportunities to invest efforts in designing and commercializing these fastest developing class of therapeutic proteins. Although V_HHs are favourable for applications involving high stability such as detectors in immunoaffinity purification[13], use as anti-dandruff shampoo[12], applications in the development of biosensors[13] etc., we focused to select clones of V_HH that can be used for therapeutic application or in

immunodiagnosis employing phage display technology in which V_HHs are expressed in conjugation with a phage coat protein and could be screened to select and characterize V_HHs of a particular antigen specificity.

1.1.5 Phage display technology

Phage display technology was first used and demonstrated by George P Smith in 1985 in which Fabs specific for a particular antigen of interest was selected using phages equipped with scFv on their phage coat, based on their affinity towards the antigen. Later, clonal selection of antibody fragments was performed in various host cell-lines. Phages are viruses, which can infect bacteria. Generally, the bacteriophages used in generating phage display are filamentous bacteriophages, the most common being members of Ff-phage family (M13page, Fd-phage, Fl phage), which can infect the standard recombinant DNA host cells, *E. coli*, containing F-conjugative plasmid (TG1 strain of *E. coli)* and can generate a titer up to 10^{13} phages per ml from bacterial cultures. The generation of the first phage display library was reported during the 1990s [15, 16, 17, 18], which later on underwent numerous improvements for its varied application.

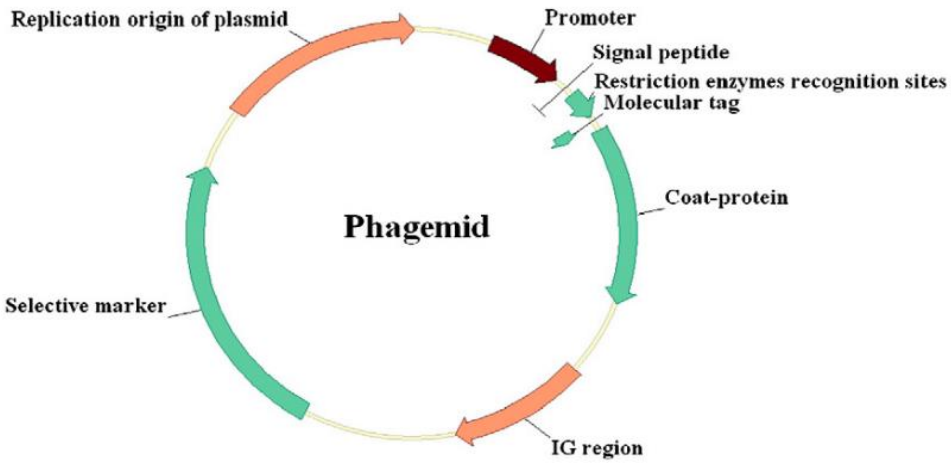


Figure1.1.2.: Phagemid vector map[44].

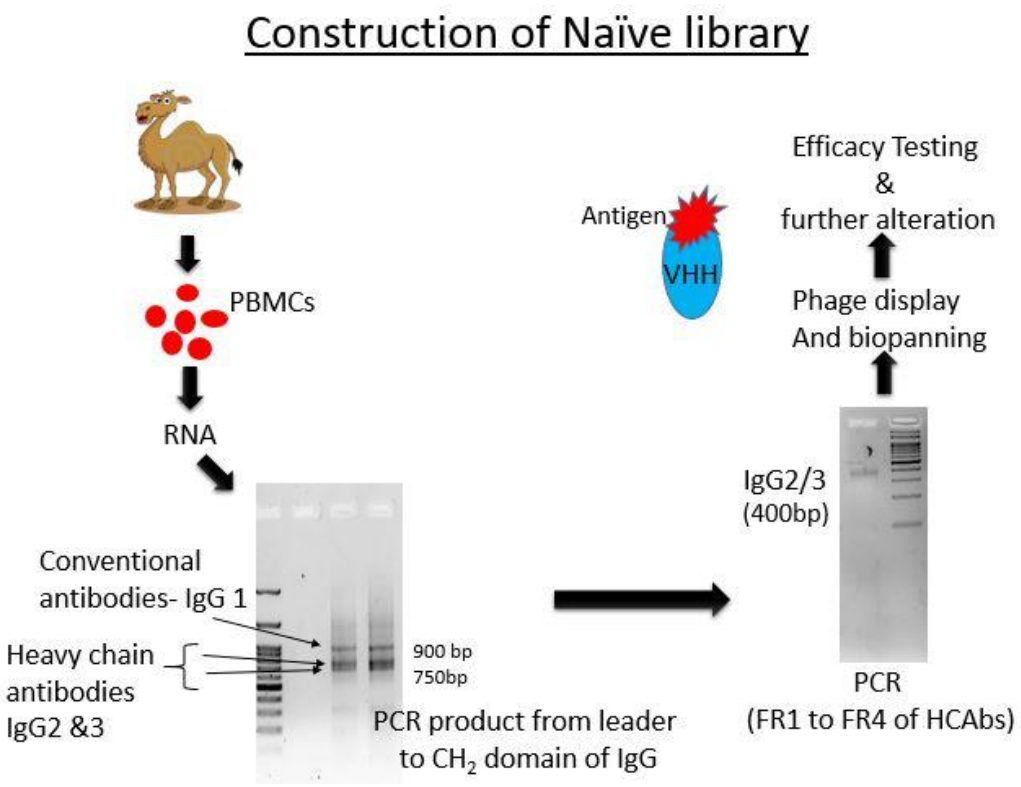


Figure1.1.3: Schematic of construction of phage library, biopanning and screening. (Courtesy: Manpreet Kaur)

The mRNAs was isolated from the lymphocytes/PBMCs that were separated from the peripheral blood of camels. The mRNA was subjected to RT-PCR using degenerate primer in order to obtain the cDNAs. Two-step PCR was performed to obtain the amplified sequence encoding V_HH. The sequences were customized by incorporating selected restriction sites via site-directed mutagenesis at the either end for downstream cloning steps. The V_HHs were initially cloned into a phagemid vector downstream to the genomic sequence for pIII coat protein with an amber stop codon in between. The modified phagemids were transformed into TG1 strain of *E. coli* and induced protein expression after co-infection with M13KO7 helper phages resulting in the amplification of phages displaying V_HH in conjugation with their pIII coat protein (phage display). This library of phages displaying V_HH on their surface was stored in -80^oC. The library was revived and used for biopanning in which phages binding the antigen of interest with low affinity or not at all were removed through multiple steps of washings with buffer containing non-ionic detergents. Those phages with higher affinity were eluted using a low pH organic solvent. Multiple rounds of

biopanning were performed to increase the titer of phages with higher specificity in the phage pool. The eluted phage pool was used for infecting TG1 (*E. coli)* cells, the presence of full-length V_HH at genomic level was determined using colony PCR and further confirmed using double digestion. The selected clones were used for phage ELISA in which the amplified phages obtained by co-infection of M13KO7 with the TG1 cell cultures containing selected phagemids were used as to interact with the antigen of interest. The V_HH sequences from clones, which exhibited higher affinity on phage ELISA were selected and cloned into an expression vector, induced for the protein expression, purified, refolded and characterised various biophysical tools and techniques. The selected V_HHs thus obtained could be further customized for various diagnostic and therapeutic applications.

Biopanning can be optimized based on the type of antigen. For example; in the case of membrane proteins, it is difficult to immobilise the protein on a plastic surface due to its hydrophobic nature and hence whole cell biopanning is preferred.

CHAPTER - II

VECTOR MODIFICATION

2.1 ABSTRACT

The resurgence of V_HH single domain antibodies is considered as a breakthrough in the field of antibody engineering as it broadens the scope to develop cost-effective, globally applicable therapeutic proteins that can be custom modified for use in the field of diagnosis, research and treatment. The V_HH from the phagemids selected on the basis of phage ELISA can be customized by insertion of tags that serve as the retrieval handles if these binders are used as detecting agents. Therefore, the experiments were planned to modify the expression vector, pET22b(+) containing V_HH with a 6X(HIS). A thrombin cleavage site and an additional sequence for biotinylation were inserted downstream to V_HH but upstream to 6x(HIS). The vector thus modified was labeled as BNT for expressing protein that can be used for biotin-streptavidin based pulldown assays, Ni-NTA sensor based binding kinetics of V_HH with the specific antigen, generation of tetrameric versions and for using in immunohistochemistry as well as in-*vivo* administrations.

2.2 INTRODUCTION

The technological advancements and simplicity in manipulating the expression systems account for the success of molecular cloning, in faster construction and iteration of the multitude of clones exploiting various genotypes and their encrypted phenotypes. Molecular cloning enables insertion, deletion, mutation, expression of swappable genes etc. within a vector or genomic DNA employing the basic trail starting with, the generation and isolation of insert and vector or the target DNA. This is followed by the purification of digested vector and insert having sites for appropriate

restriction enzymes, their assembly under optimal conditions for ligation and transformation of the modified target DNA into an apt host based on the downstream application. The flawless clones can then be confirmed through colony PCR using specific primer sets, restriction digestion with suitable controls and ultimately through the sequencing procedures.

Generally, the DNA sequence encompassing the V_HHs from the selected phagemids on Phage ELISA is subcloned into pET-22b for the protein expression for HIS-tag based purification of the protein. These V_HHs are expressed with a PelB sequence at the N terminal and a 6x(HIS) tag at the C-terminal. Proteins with the PelB leader sequence would be directed to the cell's periplasm and the tag is removed by signal peptidase *in vivo*. On the other hand, many of the protein antigens have either inherent polyhistidine patches in their tertiary structure or linear poly-histidine sequences or both which would, in turn, interfere with the 6x(HIS)-tag based pull-down assays as both the interacting partner (antigen) and V_HH (the binder) would interact with the Ni-NTA beads or Ni-NTA sensors used in Bio-layer interferometry (BLI). Hence, for the removal of 6x(HIS) tag, I introduced a thrombin cleavage site (TCS) upstream to the tag and downstream to V_HH along with a restriction site (*Nhe-1*) towards the N terminal of TCS. A biotinylation tag (BT) was then cloned in between the V_HH and *Nhe1* site that could be used for the pulldown assays and affinty measurement studies using BLI upon removal the 6x(HIS) tag. The activity of the sites was analyzed at the protein level. The biotinylation of V_HH was further confirmed by western blotting using streptavidin-HRP conjugate.

2.2.1 Thrombin Cleavage site (TCS)

Thrombin is a serine protease, widely exploited as a biochemical tool, which can recognise the consensus sequence Leu-Val-Pro-Arg-Gly-Ser and can cleave the junction of Arg-Gly. TCS has been extensively incorporated in between fusion proteins for enabling splitting of the fusion product post-purification. The enzyme is expressed and purified from the bovine plasma. The recombinant thrombin has also been produced in transfected Chinese Hamster Ovarian (CHO) cells in-*vitro*[19]. TCS was incorporated in the vector between the V_HH and 6x(HIS)-tag for the removal of the tag.

2.2.2 Biotinylation Tag (BT)

The biotinylation tag, Pro-Leu-Gly-Gly-Ile-Phe-Glu-Ala-Met-**Lys**-Met-Glu-Leu-Arg-Asp (the biotinylation tag in Vector BNT) act as a recognition site for BirA ligase alias *E.coli* biotin holoenzyme synthetase. The enzyme catalyses the covalent binding of biotin to specific lysine in the active site of the enzyme in the presence of ATP. Biotin, a 244 Dalton vitamin molecule, exhibits an extraordinary binding affinity (more than $1x10^{-14}$) for avidin and streptavidin. Biotin and avidin interaction is rapid and once the bond is established it can survive up to 3M guanidine-hydrochloride and extremes of pH. The biotinylated molecules are efficiently probed or purified with avidin or streptavidin conjugated to reporter molecules, such as peroxidases or phosphatases, or immobilized on agarose beads. So this tag was introduced downstream to V_HH, concurrently upstream to TCS in order to obtain V_HH without 6x(HIS)-tag which can be biotinylated and used for biotin-based pulldown assay, Ni-NTA sensor based BLI and for tetramer generation.

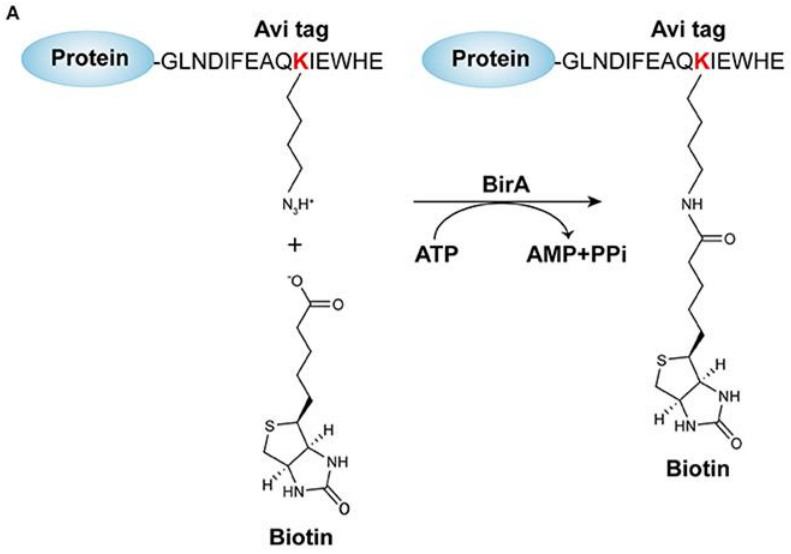
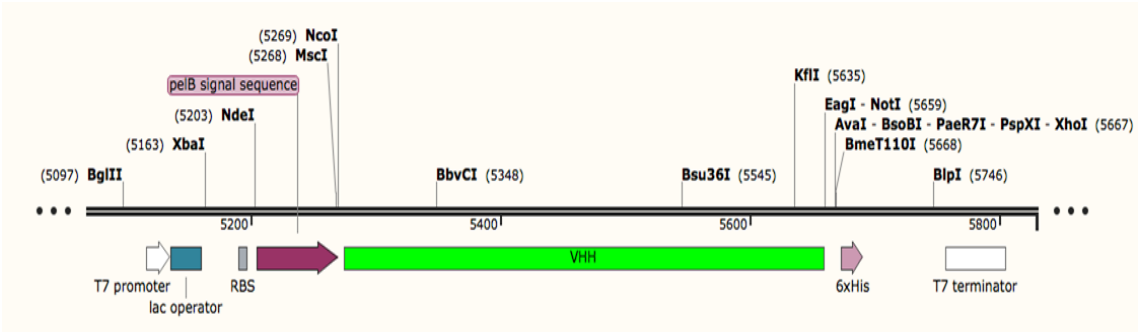


Figure 2.2.1: Biotinylation of Avi-Tag which is another variant of biotinylation tag at Lysine residue in the sequence, under the catalytic activity of Bir A Ligase through dephosphorylation of ATP[20].

2.2.3 Strategy

The vector E (Figure: 2.2) was modified with two consecutive sets of molecular cloning steps to obtain Vector BNT (B-biotinylation tag, N-Nhe-I restriction site, T-Thrombin Cleavage site) (Figure: 2.2). The insert for the first cloning was generated by 2 sets of PCR, with V_HH specific forward primer, 'target insert' specific reverse primers for each step and Vector E as the template. The insert 5'-*Nco*1-V_HH-*Not* 1--Nhe1-- Thrombin cleavage site---Xho1-3' was cloned into Vector E in between *Nco*1 and *Xho*1 sites to obtain Vector NT. The insert for second cloning, 5'-overhang-*Nhe* 1-biotinylation tag-*Not* 1-V_HH- 3' was generated by 3 sets of PCR with V_HH specific forward primer, 'target-insert' specific reverse primers and the vector NT as template (Figure:2.2). The activity of the inserted tags in the protein product was confirmed through PAGE (The elimination of 6x(HIS)-tag and western blotting using streptavidin HRP

(a)



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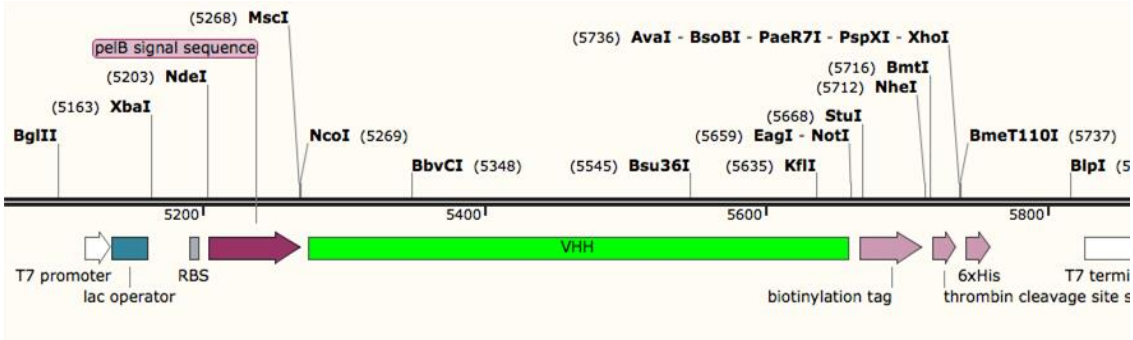

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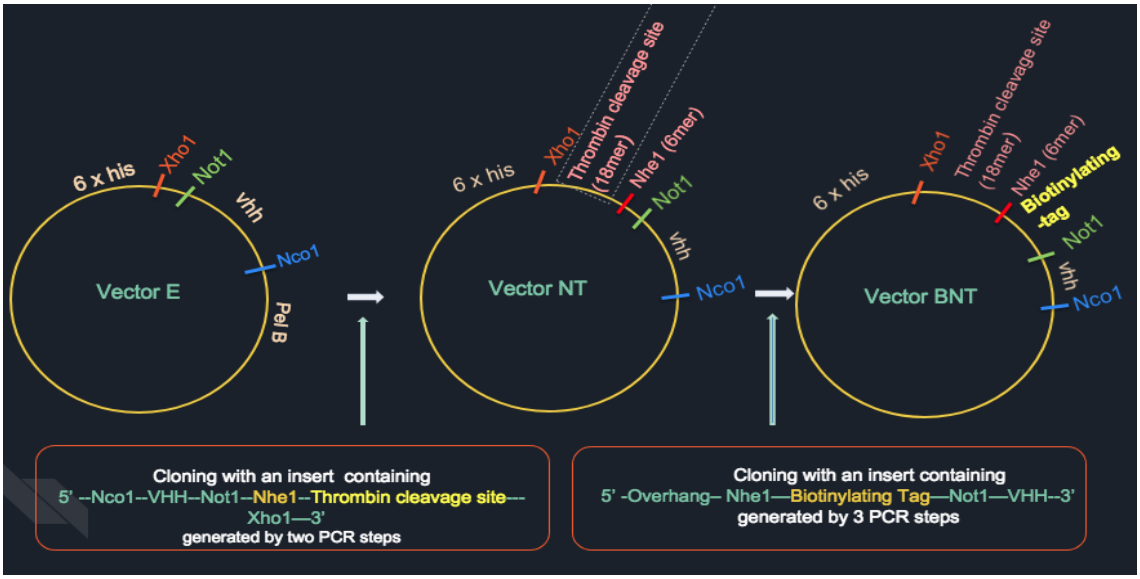


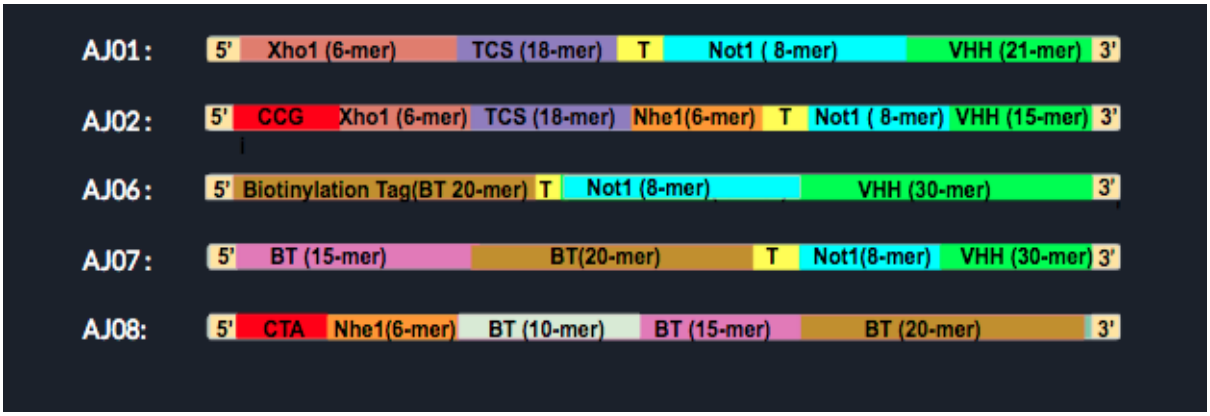
Figure 2.2.2: (a) V_HH in pET22b (5829bp) (b) V_HH- BT-*Nhe*1-TCS in pET22b (5898bp). The genomic map of V_HH in Vector E and Vector BNT, respectively is demonstrated. **(c) The schematic of Vector modification performed to generate Vector BNT.**

2.3 MATERIALS AND METHODS

CLONING of BIOTINYLATION TAG AND THROMBIN CLEAVAGE SITE to pET-22b(+)

2.3.1 PRIMER DESIGNING

In order to modify the original vector, five degenerate reverse primers were designed using Snapgene 4.2 and the annealing temperature, Gibbs free energy to assess homodimers, heterodimers and hairpin formation were analysed and corrected using Oligo Analyzer Tool by Oligo IDT. The primers designed in the color coding scheme are given below:



AJ01:CTCGAGACTACCTCTTGGTACTAGTGCGGCCGCTGTGGAGACGGTGACCTGGGT

AJ02:CCGCTCGAGACTACCTCTTGGTACTAGGCTAGCTGCGGCCGCTGTGGAGACGGTGAC

AJ06:TCAAAAATACCACCTAATGGTGCGGCCGCTGTGGAGACGGTGACCTGGGTCCCCTGG

AJ07:TCCATCTTCATAGCTTCAAAAATACCACCTAATGGTGCGGCCGCTGT

AJ08:CTAGCTAGCATCGCGCAGTTCCATCTTCATAGCTTCAAAAATACCACCTAATGGT

T_m value of the primers was determined by performing gradient PCR in T_m ranging from 62 to 68° **C** using pET-22b with Vector E as template.

The PCR mixture was prepared as follows:

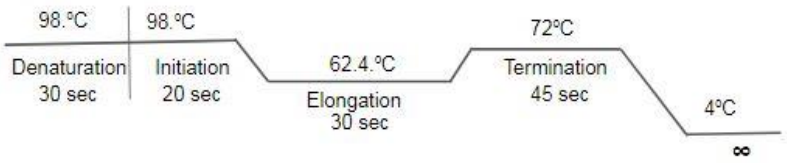
Forward primer (100 nM) - 5nM

Reverse primer (100 nM) - 5nM

dNTPs (25 µM) - 0.5µM

HF polymerase (1 unit/µl) - 0.01 unit/µl

HF Buffer	(10x)	- 1x
$MgCl_2$	(2.5 mM)	- 0.05mM
MilliQ water		- to make up the volume
Template		- 1µg



The PCR products were run on 1% agarose gel at 100V and constant current for 30 minutes with 1Kb DNA ladder in 10x TAE.

Agarose Gel Electrophoresis:

1% Agarose gel (100mL):

SeaKem Agarose	- 100mg
TAE	- 1x
ddH_2O	- 100mL

Heated in microwave for 2 minutes, allowed to cool, 0.005% Ethidium Bromide was added before pouring to the casting tray

50x TAE

EDTA disodium salt	- 50mM
Glacial acetic acid	- 1M
Tris Base	- 2M

2.3.2 CLONING OF THROMBIN CLEAVAGE SITE TO V_HH-E

Plasmid isolation of V_HH-E

Plasmid (pET-22b(+)-V_HH-E) was isolated from 5 ml of primary culture in LB-Amp grown for 12-16 hours using Macherey Nagel Nucleospin plasmid isolation kit. The concentration of the plasmid was determined using nanodrop.

Preparation of LB media **:** 2.5%LB in ddH_2O

Preparation of LB-Agar plates **:** 2.5%LB, 1.5%bacteriological grade Agar in ddH_2O

Amplification of insert 1

The amplicon 5'--*Nco*1--V_HH--Not1--Thrombin cleavage site--Xho1--3' obtained on PCR of V_HH-E using MKS23 (FP) and AJ01 (RP) was gel purified by cutting the DNA band of ~450bp from 1% Agarose gel after gel electrophoresis using 'Invitrogen PCR and Gel purification kit'. The purified PCR product #1 was used as the template for second PCR using MKS23 (FP) and AJ02 (RP). The concentration of the gel purified PCR product was determined using nanodrop.

Restriction Digestion of Vector and Insert

Double digestion reactions were set up for ~1µg of Vector and insert DNA for 3 hours at 37ºC using *Nco*1-HF and *Xho*1-HF. The reaction was set up as follows:

DNA - > 0.9µg

Restriction enzyme (20U/µl) - 3 units for 1µg of DNA

Cutsmart Buffer (10X) - 1x

MilliQ water is added to make up the reaction to the volume based on the downstream requirement. Generally we setup 50µl reaction.)

The digestion reaction was stopped by incubating the reaction at 65ºC for 20 minutes. Vector was treated with CIP (1U for 1µg of Vector) at 37ºC for 1 hour. The digested product was gel purified and stored in -20ºC. Under circumstances where the concentration of insert was low, purification of the digested insert was performed through isopropanol ethanol precipitation method as gel purification can retain only 10% of the total product.

Purification by isopropanol-ethanol based precipitation

One reaction volume of chilled molecular grade isopropanol and 1/10th of the reaction volume of 3M Sodium acetate were mixed with the reaction mixture containing DNA, centrifuged at 4ºC, 14000 rpm for 20 minutes. The supernatant was discarded carefully and washed the pellet with 1 reaction volume of chilled ethanol and again spun at 14,000 rpm for 20 minutes. The supernatant

was discarded. The pellet was air dried and dissolved in minimum amount of MilliQ water to estimate its concentration using nanodrop diluted as per requirement.

Ligation Reaction

The amount of insert to be used for ligation reactions of different insert to vector ratios were calculated using the equation:

Mass of insert DNA (μg) = Desired insert to vector ratio X mass of vector DNA X (length of insert/length of target vector)

The reaction was set up as follows:

Vector	- (100ng)
Insert	- (as per calculation)
Ligase Buffer (10X)	- 1X
T4 DNA ligase (400000U/μl)	- 1μl

The reaction was incubated at 25^0C for 2.5 hours with a control reaction with digested vector alone (without insert).

Transformation

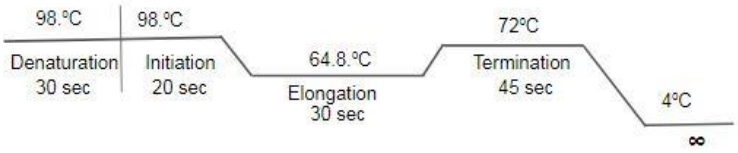
The ligation mixture along with competent cells Top1 strain of E.coli maintained in -80ºC were incubated on ice for 15 minutes for the cells to thaw. 10μl of the reaction mixture was added to 30μl of competent cells and were incubated on ice for 15 minutes. Then a heat-shock was given at 42ºC for 50 seconds and incubated on the ice again for 5 minutes. 1ml LB was added to the cells and incubated at 37ºC for 40 minutes on shaking. The cells were pelleted by centrifuging the culture at 4500 rpm for 5 minutes. The cells were dissolved in 150μl of LB and plated on LB-Amp and incubated at 37ºC for 10-12 hours

Confirmation of clone by PCR and Double digestion

A few of the colonies from the test and control plate were randomly selected and some cells collected from the colonies using a micropipette tip, were streaked on another plate. The bacterial cells thus selected were dissolved in 10μl of PCR mixture prepared with insert specific primer to

set up PCR with control reaction with V_HH-E as the template with T7 promoter and terminator specific primers.

PCR temperature settings



The PCR products obtained were resolved on 3% agarose gel to visualise the shift in the amplicon size. The plasmids were isolated from the primary cultures of some of the PCR positive clones and V_HH-E in LB-Amp grown for 12-16 hours at 37°C. Double digestion reaction with *Nco*1-HF and *Nhe1*-HF was performed for 3 hours at 37°C and the digested products were analysed by 3% agarose gel.

2.3.3 CLONING OF BIOTINYLATION TAG TO VECTOR NT

The amplicon obtained from the reaction mixture involving Vector NT using MKS23 (FP) and AJ06 (RP) was gel purified and the product was used as the template for the next PCR with MKS23 and AJ07 (RP). The third PCR was performed with the gel purified amplicon of second PCR as the template with MKS23 and AJ08 (RP) in order to obtain the insert with BT. The gel purified PCR product 3 and Vector NT were double digested with *Nhe*1-HF and *Nco1*-HF, Ligation, transformed into Top1 cells and confirmed for the insert by colony PCR, digestion and sequencing.

2.3.4 PROTEIN EXPRESSION AND EXTRACTION

Selected clones with positive PCR, digestion and sequencing results were transformed into *E. coli* origami strain and the expression were induced by adding 1mM IPTG to the 100mL secondary culture of the clone at OD_{600} = 0.4. Before adding IPTG 1ml uninduced cultures were used as control. The culture was further grown at 37°C for 6-8 hours and centrifuged at 11000 rpm for 10 minutes at 4°C to obtain the pellet. The pellets was stored at -20°C until use. 0.3 OD of uninduced culture and induced culture were taken in 2 microcentrifuge tubes and spun at 7000 rpm for 3 minutes at RT. The pellets were dissolved in 20µl of 1x SDS running buffer and 1x SDS non-reducing dye and boiled at 95°C for 7 minutes. The supernatants thus obtained after centrifugation

were resolved on SDS-PAGE at 100V, constant current. The gels were stained with Coomassie brilliant blue stain and the excess of stain was removed by destaining solution. The gel images were obtained in Bio-RAD Gel-Doc. The materials used are prepared as given below:

12% SDS resolving gel (1 gel)

ddH_2O	- 1.65 mL
Acrylamide (30%)	- 2.00mL
1.5M Tris, pH = 8.8	- 1.25mL
SDS (10%)	- 50.0µl
APS (10%)	- 50.0µl
TEMED	- 2.50µl

5% SDS stacking gel (1 gel)

dd Water	- 1.7mL
Acrylamide (30%)	- 415µl
1M, Tris, pH=8.0	- 315µl
SDS (10%)	- 25µl
APS (10%)	- 25µl
TEMED	- 2.5µl

1X SDS running buffer

Tris-base	- 30g
Glycine	- 144g
SDS	- 10g

Dissolve the salts in 1L water

30% Acrylamide

Acrylamide	- 29g
NN'methylbisacrylamide	- 1g

The above salts were dissolved in 60 mL ddH_2O and the final volume was made upto 100 mL

Staining solution **:** Methanol (45%), dd water (45%), Acetic Acid (10%), CBBR250 (3g/L)

Destaining solution **:** Methanol (40%), water (50%), Acetic acid (10%)

The induced pellet was resuspended in 10mL resuspension and 100µl of PMSF (100mM in isopropanol) by vortexing, added with 2µg of lysozyme and, the samples was incubated at 4^{o}C for 2-3 hours on rotaspin (10rpm). Thereafter, the sonication was performed at 40% power with 15 second pulse and 15 second interval for 30 minutes, followed by centrifugation at 5000 rpm for 1 hour at 4^{o}C. The supernatants and the pellets were collected and separately stored at at -20^{o}C.

HIS-tag based purification of V_HH using Ni-NTA beads

1mL Ni-NTA beads from Qiagen were used for purification column. The beads and the column were equilibrated by passing 20ml of Tris-NaCl buffer. The supernatant was added to the beads and incubated the column at RT on rota-spin (5 rpm) for 10 minutes, then the flowthrough was collected in a falcon. This was followed by passing 30ml of wash buffer, first 4ml of the buffer was collected in microcentriguge tubes and the remaining flow-through was discarded. Another wash was performed the same way with wash buffer 2 and first 4 mL flowthrough was collected and stored. Then 5ml of elution buffer was added to the beads and incubated at RT for 10 minutes and collected 1 ml each of elutions in separate vials. SDS- PAGE on 12% resolving gel was performed with 20µl each of washes, elutions, supernatant and pellet in order to confirm the purity and to roughly estimate the concentration of protein of interest.

Tris-Nacl Buffer **:** Tris-15mM, NaCl-50mM, pH=8.0.
Resuspension buffer **:** 5mM Imidazole in Tris-NaCl Buffer, pH=8.0
Wash Buffer 1 **:** 20mM Imidazole in Tris-NaCl Buffer, pH=8.0
Wash Buffer 2 **:** 40mM Imidazole in Tris-NaCl Buffer, pH=8.0
Elution buffer **:** 400mM Imidazole in Tris-NaCl Buffer, pH=8.0

Concentration of protein with Buffer Exchange

The protein obtained on elutions were concentrated using already equilibrated 3KDa Amicon Ultra Centrifugal filter units after diluting in Tris-NaCl buffer and centrifuging at 8000 rpm at 4^{o}C.

2.3.5 BIOTINYLATION

The supernatant concentrated more than 4 times its initial concentration using 3kDa Amicon filter was biotinylated by setting up 50µl reaction as follows:

Bir A Ligase -7.5µg*
Buffer A -1X
Buffer B -1X
D Biotin -500µM
PMSF -1mM
Protein -10nM**
MilliQ - to make up the final volume

* Lab prepared Bir A Ligase was used in amounts prescribed based on the prior determination of efficiency.

** Concentrated supernatant obtained on lysis of induced pellet was used instead of purified protein.

Confirmation of Biotinylation with Western Blot using HRP conjugated Streptavidin

30µl each of biotinylated supernatant, biotinylated H2Kb, pre-biotinylated H2Kb, and un-biotinylated control H2Kb was loaded on 12% SDS gel after heating the samples at 95ºC for 5 minutes along with 1X non-reducing dye and electrophoresis was performed in 10X SDS running buffer. Concurrently, a PVDF membrane of 0.2 µm pore size was cut, recharged with 100% Methanol for 1 minute, washed the membrane with TBST for 3 minutes and was placed over a stack of filter papers soaked in 0.1%TBST arranged over the -ve electrode of the Bio-Rad Semi-Dry Blot Transfer apparatus. The gel was transferred to ddH_2O, then to 1% TBST and placed it over the PVDF membrane devoid of air bubbles in the blot transfer apparatus. Equal number of filter papers drenched in TBST were stacked over the PVDF membrane and performed the transfer at 90mA for 30 minutes. The blot was blocked overnight, with 5% skim milk at 4ºC on shaking. 3 washes were give using 0.1%TBST with intermittent 5 minutes incubation on shaking at RT. The blot was then incubated with 10 mL of 1:5000 dilution of HRP-conjugated streptavidin prepared in 0.1% TBST. The blot was washed 3 times as done after the blocking step. The image was

processed in x-ray film using after treating with substrate (TMB substrate, BD biosciences). TBST used for the procedure was prepared as below:

10X TBS

Tris -198 mM

NaCl - 1.5 M

0.1%TBST was prepared by dissolution of 0.1% Tween 20 in 1X TBS.

2.4 RESULTS AND DISCUSSION

2.4.1 CLONING OF BIOTINYLATION TAG AND THROMBIN CLEAVAGE SITE TO VECTOR E

The tags were cloned into a backbone vector, Vector E containing V_HH -E in Pet22b. Initially the clone was confirmed by digesting the vector with degenerate combinations of restriction enzymes (Figure 2.4.1a). A digested product of size approximately 450 bp was obtained on digestion with Nco1 and Xho1 and this testified the presence of template sequence in the vector.

The PCR product#1 obtained on the amplification of Vector E using MKS23 and AJ01 (Figure 2.4.1b) was gel purified and used as the template for PCR#2 with MKS23 and AJ02. The gel purified PCR product#2 and Vector E were digested with Nco1 and Xho1 (Figure 2.4.1c), gel purified, ligated and transformed into E.coli TOP10 cells. The clones were confirmed by colony PCR and double digestion assay (Figure 2.4.1d, e).

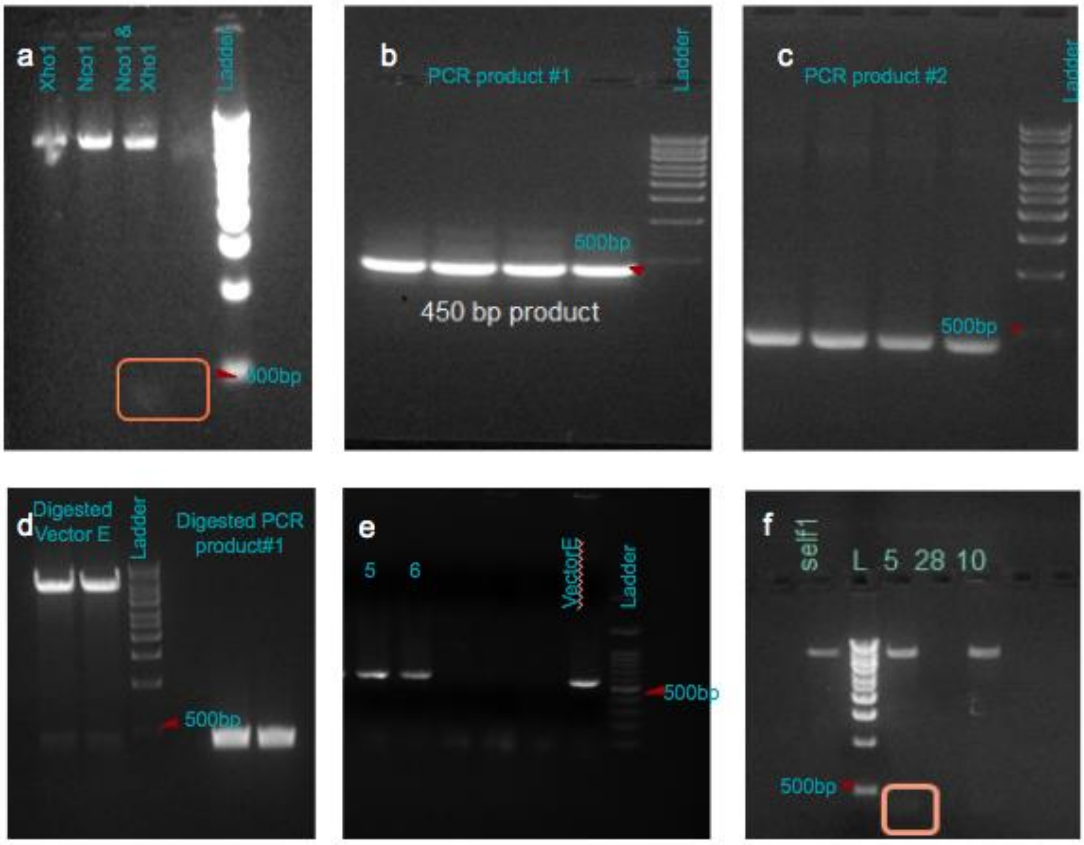


Figure 2.4.1: (a) **Confirmation of backbone vector (Vector E) through digestion assay with vector specific restriction enzymes Nco1 and Xho1.** A band of ~450bp was obtained on double digestion with Nco1 and Xho1. (b) **Large scale amplification of PCR product#1.** The amplicon (~450bp) was generated from a 150µl PCR reaction, with Vector E as template, MKS23 (FP), AJ01 (RP) and T_m=62.4. (c) **Large scale amplification of PCR product#2.** The amplicon (~450bp) was generated from a 150µl PCR reaction, with PCR product#1 as template, MKS23 (FP), AJ02 (RP) and T_m=62.4. (d) **Vector E and gel purified PCR product#2 double digested with Xho1-HF and Nco1-HF.** (e) **Colony PCR of Vector NT** (f) **Confirmation of clone by double digestion assay with Nhe1 and Nco1.**

Similarly, the insert with biotinylation tag was generated by 3 steps of PCR with Vector NT as template, MKS23 used as forward primer and AJ06, AJ07 and AJ08 used as reverse primers for PCR#1, PCR#2 and PCR#3 respectively. The final product was cloned in Vector NT, in between Nco1 and Nhe1 recognition sites and the clone was confirmed as done for Vector NT (Figure 2.4.2

a,b,c,d). Sequences of both Vector NT and Vector BNT were further confirmed by sequencing.

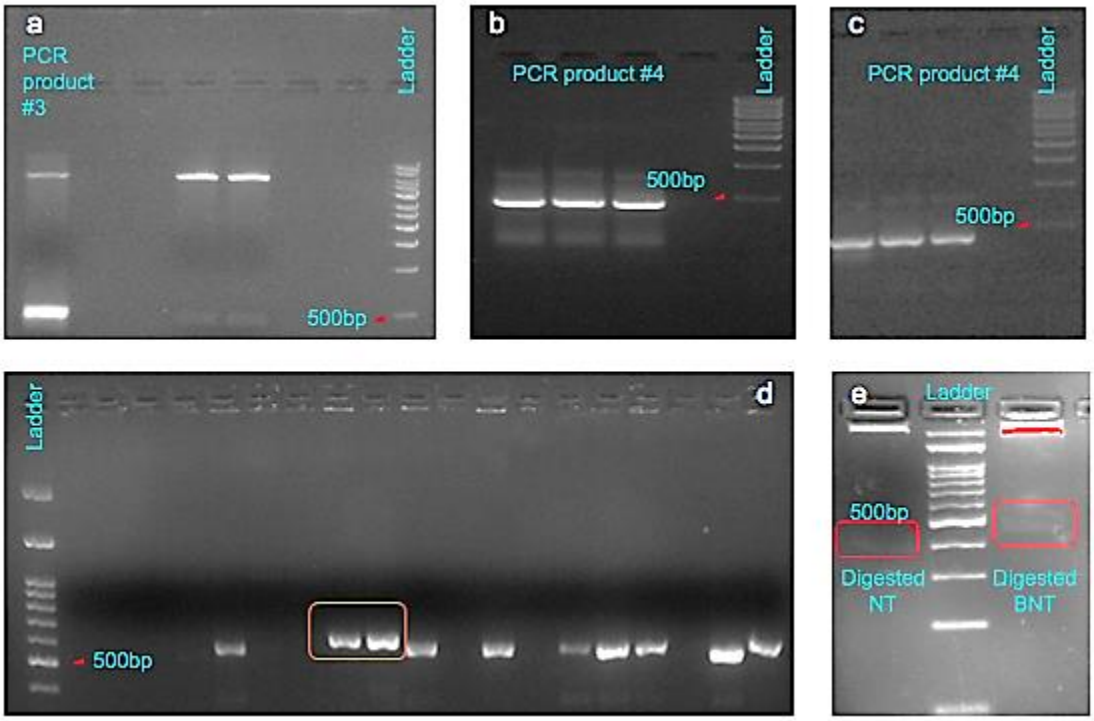


Figure 2.4.2: (a) **Large scale amplification of PCR product#3.** The amplicon (~450bp) was generated from a 150µl PCR reaction, with Vector NT as template, MKS23 (FP), AJ06 (RP) and T_m=62.4. (b) **Large scale amplification of PCR product#4.** The amplicon (~450bp) was generated from a 150µl PCR reaction, with PCR product#3 as template, MKS23 (FP), AJ07 (RP) and T_m=62.4. (c) **Large scale amplification of PCR product#5.** The amplicon (~450bp) was generated from a 150µl PCR reaction, with PCR product#3 as template, MKS23 (FP), AJ08 (RP) and T_m=62.4. (d) **Colony PCR of Vector NT.** (e) **Vector NT and Vector BNT double digested with Nhe1-HF and Nco1-HF.**

2.4.2 PROTEIN EXTRACTION AND BIOTINYLATION

Vector BNT was transformed into *E.coli* Origami cells and protein expression was induced. The cells were lysed and the secreted proteins were collected in the lysis supernatant. On His Tag based purification, we could not segregate V_HH from other supernatant proteins. It could be because of the cleavage of TCS by other serine proteases of E.coli origin. Hence, instead of purifying the

protein, the supernatant was concentrated upto 10 times with simultaneous buffer exchange in order to grade down the imidazole concentration in elution buffer from 400mM to 12.5mM (Figure 2.4.3a,b). The concentrated supernatant and a control protein (H2Kb with biotinylation tag) was biotinylated for 6 hours and the activity of the tag was confirmed by western blot with HRP conjugated streptavidin. A signal of approximately 15 kDa size was obtained in the biotinylated concentrated supernatant as it was obtained at 25 kDa size for H2Kb. Surprisingly, the un-biotinylated control protein was also giving signal testifying a probability for in-*vivo* biotinylation of the protein (Figure 2.4.3c).

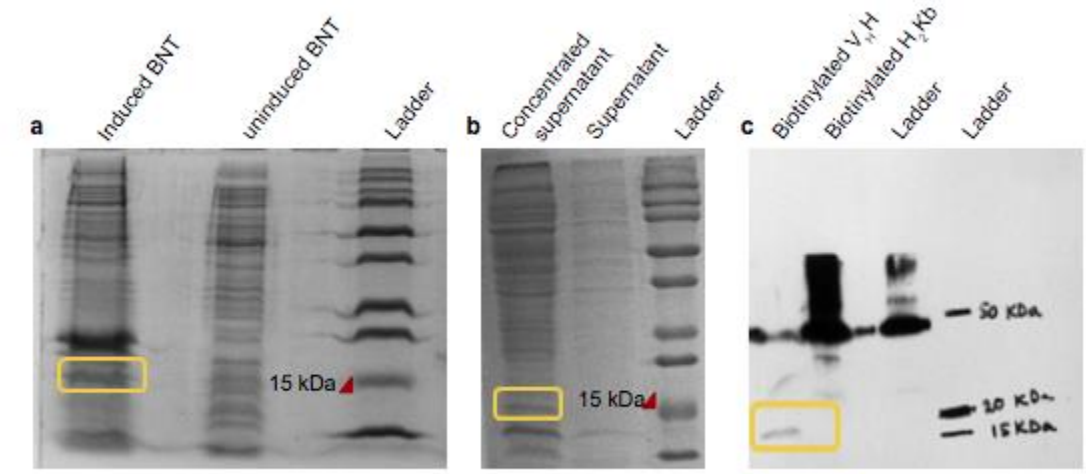


Figure 2.4.3: (a) **Confirmation of protein induction**, (b) **Intensity of V_HH in 20µl of concentrated supernatant,** (c) **Confirmation of biotinylation of V_HH with western blot using streptavidin conjugated HRP.**

2.4.3 FUTURE DIRECTIONS

The activity of TCS should be checked and quantified. The question, whether the failure in HIS-Tag based purification of V_HH was a consequence of activity of endogenous bacterial serine proteases on the TCS should be addressed. Further more useful vector constructs can be designed with tags enabling detection (eg. GFP tag) or oligomerization (eg. LPETG site) of V_HH s.

2.5 CONCLUSION

A vector construct facilitating the downstream biophysical characterisation of the selected V_HH s was generated successfully and was confirmed by sequencing. The activity of biotinylation tag was established through western blot of the biotinylated protein using HRP conjugated streptavidin. The failure of the recombinant protein product to get segregated on incubation with Ni-NTA beads indicates a probable activity of bacterial, endogenous serine proteases, which should be tested and confirmed.

CHAPTER - III

SELECTION AND CHARACTERISATION OF PPRV SPECIFIC V_HH S

3.1 ABSTRACT

A phage library containing phages displaying V_HH s obtained from the whole blood of a naive Indian Bactrian Camel was generated and maintained in the lab. Peste des petit ruminant virus (PPRV), the causative organism of goat plague is a highly contagious pathogen which infects ruminants like sheep and goat. Commercially available PPRV specific monoclonal antibodies are expensive and has limited scope in terms of diagnostic and therapeutic applications. So we performed biopanning for PPRV live attenuated form of virus and 2 V_HH s were selected after phage ELISA for protein level analysis.

3.2 INTRODUCTION

Phage display technology has been guiding the field of antibody science towards a new horizon of hopes and opportunities from past few decades. Selection and characterisation of V_HH s employing phage display system has transformed the process into a less complex methodology facilitating the screening of nanobodies of higher affinity. Morbilliviruses cause clinically and economically important diseases of humans, domestic and wild animals. They cause significant morbidity and very high mortality which reaches up to 50% in small ruminants (*peste-des-petits ruminants*) resulting in massive economic losses. The PPRV infection leads to immunosuppression of the host and results in fatal secondary infections. In PPRV, the level and mechanism of immunosuppression is not fully understood and the available information is meager. It is also not possible to distinguish

vaccinated from those recovered from natural infection. Therefore a timely detection of such viral infections using a test employable in field conditions would be critical. This would help put mitigating strategies in place. Single domain antibodies that can be selected from camelid VHH phage display library could come handy for developing such a test. Furthermore these antibodies can be expressed as intrabodies to decipher viral trafficking events in the cells thereby providing targets for anti-viral approaches. A phage library containing phages displaying V_HHs was obtained earlier in the lab and used for the selection of PPRV specific binders. Commercially available PPRV specific monoclonal antibodies are expensive and have limited scope in terms of their diagnostic and therapeutic applications. Cheap and efficient antibodies are inevitable for research and therapeutics. So we decided to screen the phage library for PPRV specific single domain antibodies.

3.2.1 PPRV

Peste des petit ruminant virus is a *Mobilivirus* belonging to the family of *Paramyxoviridae,* responsible for the contagious viral disease termed as 'Goat plague', has reported mortality rates as high as 90% and could usually be 50%. Being in a society dependent on livestocks for livelihood, we aimed to select sdAbs for PPRV antigens so that in the time to come an efficient robust diagnostic assay could be developed. This virus has a -ve-strand RNA genome enclosed in a ribonucleocapsid enriched with nucleoprotein N (Figure 3.2.1).

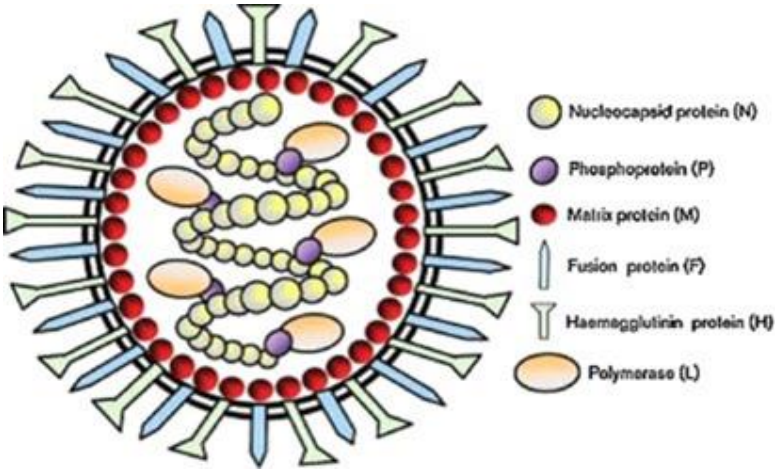


Figure 3.2.1 Schematic diagram of PPR Virion (adapted from Banyard *et al*.,, 2010). F (Fusion protein) and H (Hemagglutinin-Neuraminidase) proteins are viral coat proteins. M protein is the matrix protein and L protein is the RNA-dependent RNA polymerase. The genomic DNA is enclosed in ribonucleoprotein (RNP) composed of nucleoprotein (N protein) along with phosphoprotein (P protein) and large protein (L protein)[24].

3.2.2 Major proteins of PPRV

PPRV genome encodes six structural proteins namely the nucleocapsid protein (N), the phosphoprotein (P), the fusion protein (F), the matrix protein (M), the hemagglutinin - neuraminidase protein (HN), the large protein (L) and two non-structural proteins (C and V) in the order of 3'-N-P/C/V-M-F-HN-L-5' [21].

	Structural protein (weight)	Location	Sequence similarity among mobiliviruses	Features
1.	N protein (58 kDa)	RNP complex	67% - 74%[24]	Major protein produced in highest amount in *Mobiliviruses*[22]. Major component of Ribonucleoprotein (RNP) complex[23].
2.	P protein* (60 kDa)	RNP complex	Least conserved[25]	Component of RNP complex. Cofactor of RNA dependent RNA polymerase[26].
3.	M protein (38 kDa)	Matrix	91%[27]	Major component of matrix. Role in progeny viral particles'

				formation and budding of virus from plasma membrane[28].
4.	F protein (59.137 kDa)	Viral surface	_____	Fusion protein enabling fusion into host cell. It has 4 domains. Hemolysin property[29]
5.	H protein (67 kDa)	Viral surface	Less conserved[30]	Mediates viral attachment to host cell surface via its hydrophobic domains. Induce agglutination of RBC[32]. Downregulation of complement pathway[31].
6.	L protein (247.3 kDa)	RNP complex	70.7% with RPV	Produced in the smallest amount[33].

Table 3.2.1: Structural proteins of PPRV

*Variation in the level of phosphorylation might result in varied molecular weights in SDS gel.

PPRV also have 2 major non-structural proteins, protein C and V of sizes 20 kDa and 32.28 kDa respectively. C protein mediates effective virus replication in the peripheral blood cells[34], modulation of RdRp activity[37], virulence determination[35], RNA synthesis[36], and blocking induction of type I interferons[38]. V protein has 2 domains of which N terminal domain is involved in interaction with STAT1[39] and the C - terminal domain is involved interactions with JAK1 and Tyk2[39]. There are also reports on inhibition of interferon signaling by the C-terminal domain of PPRV C protein.

3.2.3 Background of the project

Phages obtained after 2 rounds of biopanning with live-attenuated PPRV resuspended in PBS as

antigen, was maintained in the lab. As biopanning was performed with complete form of virus, if the phages selected for their affinity, is exhibiting the affinity because of their specificity towards a particular epitope, the probable epitope can be one of the coat proteins, protein F or protein H. I used these phages for screening and selecting PPRV- specific V_HH s. These V_HH s can be engineered for trafficking the localisation of virus at various stages of infection. The observations can be correlated with Measles virus infection, which belong to the same genus as PPRV and is less explored due to various ethics issues.

3.2.4 Strategy

The selected phages obtained after second round of biopanning were utilized for infecting TG1 (*E. coli)* cells possessing F-conjugative plasmid, which would enable the transmission of phage genome to the cell. Spreading the infected cells on media containing ampicillin facilitates differential amplification of cells with phagemids. But there can be phagemids with either 'no' V_HH, 'truncated' V_HH or 'intact' V_HH in conjugation with pIII protein. Those phages with intact V_HH s could be selected through colony PCR and double digestion approaches. The phages with intact V_HH s or even the truncated products can be used for Phage ELISA in which the phages displaying the V_HH s obtained on phage amplification employing selected phagemid bearing TG1 clones, were used as the source of the primary antibody against PPRV. Thus, TG1 cells have a mutation resulting in the continuation of translation at amber codons, which enables the synthesis of V_HH-pIII fusion product. Those phages which provides signal greater than the background signals produced by control phages are selected for protein expression. The selected V_HH s then can be subcloned into Vector BNT and transformed into *E. coli* Origami cells for inducing the protein expression. The protein can then be purified either from inclusion bodies or from the periplasm and analysed for its affinity and further characterization.

3.2.5 GPR114

G-protein Coupled Receptor 114 is an orphan adhesion GPCR which is expressed on immune cells like dendritic cells, T cells etc. GPR 97 and GPR56, both belonging to the same family have already established roles in immune responses. Commercially available antibodies against

GPR114 are limited and insufficiently characterized and therefore not suitable for downstream use. So I made an attempt to screen some sdAb against GPR114 from the V_HH library.

3.3 MATERIALS AND METHODS

PHAGE AMPLIFICATION

$1x10^{10}$ M13KO7 helper phages were added to 1L of TG1 secondary culture, already prepared by inoculation of 1% TG1 primary culture in 2XYT media lacking antibiotics; once the secondary culture attained an OD_{600} of 0.4. The culture were incubated at room temperature for 30 minutes to 1 hour in static condition so that the phages could infect the TG1 cells. Then kanamycin was added to a final concentration of 50μg/ml in order to screen infected TG1 cells and the culture was left on overnight shaking at 37ºC.

PURIFICATION OF PHAGE

Autoclaved PEG/NaCl was added to the supernatant obtained by centrifuging the culture at 5000 rpm at 4ºC for 45 minutes, maintaining PEG/NaCl to the supernatant ratio 1:6. Then the mixture was incubated on ice for 45 minutes to 1 hour. Phages thus precipitated were pelleted by centrifuging the mixture at 4000 rpm at 4^0C for 30 minutes in a swing bucket rotor. The supernatant was discarded and the pellet was dried by inverting falcons on tissue papers. The pellet was resuspended in 200μl of PBS. 10μl of the resuspended phages were dissolved in 990μl of 10 mM PBS and the OD values were measured to obtain an estimate of amplified phages. An OD_{260} =1 implies 33 μg/mL of ssDNA 1 ml aliquots of $1x10^{12}$ phages were prepared and stored in -80ºC.

REVIVAL OF LIBRARY

The stock of TG1 cells transformed with phagemids containing sequences of V_HH was maintained in -80^0C with a diversity of 1x10^10 cells/ml of library stock. 1ml of the library stock was resuspended in 500 mL 2XYT media containing 100μg/mL of ampicillin and 1% glucose followed by incubation at 37ºC for 2-3 hours. The cells were then centrifuged at 2000 rpm for 10-20 minutes.

The pellet was resuspended in in 500 mL of 2XYT-Amp and incubated till an OD_{600} of 0.4 was attained. M13KO7 phages were then added to the culture maintaining phage to bacteria ratio 1:20 and incubation was done for 1 hour at 37°C in static condition. Kanamycin was added to a final concentration of 50µg/mL and the incubation was continued while shaking for 12-14 hours. The purification of phages displaying V_HH was performed subsequently.

CLEANING OF PHAGE LIBRARY

Phages were retrieved after incubation of revived phage library (100µl/well) in '96 well flat bottom plates at RT / 37°C for 1 hour for eliminating phages with affinity to polystyrene if any.

3.3.1 BIOPANNING

Day#1: Coating of antigen on plates

100µl of antigen diluted in PBS was aliquoted into 5 wells (5µg/well) of 96 well round bottom plates, 100µl of PBS was aliquoted in 5 other wells (uncoated control) and the plate was incubated in 4°C overnight.

Day#2: Screening of phages from revived library

The wells were washed 3 times by adding 200µl of 0.05% of PBST in each well with incubations of 5 minutes in PBST before each wash, dried further by tapping the plates on tissue paper. The blocking of the unoccupied sites of the surface of wells was done for 2-3 hours at RT in static condition with 200µl/well of 5%BSA prepared in 0.05%PBST. Then three further washing were performed, 100µl of cleaned phages (titer~1 X 10^{11}) was aliquoted into each well and the plate was incubated at RT for 1 hour in static condition. The wells were then washed vigorously 20 times with 0.2% PBST without incubation to remove loosely bound phages. Freshly prepared TEA solution (70µl of TAE mixed with 5 mL of fresh MilliQ water) (pH=10, light sensitive) was added to each well and incubated at RT in dark for 30mins for elution. The low pH was neutralised by adding Tris-HCl Buffer (pH 7.4) in 100µl/well volume with vigorous pipetting. The eluted phages from test and

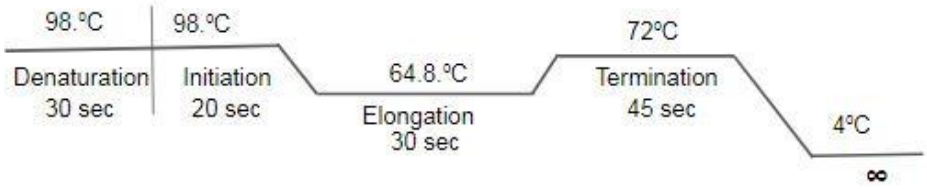
control wells were collected separately and stored at -80ºC.

3.3.2 INFECTION OF TG1 (E. coil) CELLS WITH SCREENED PHAGES

Dilutions of test and control phages ranging from 10^{-1} to 10^{-8} were prepared in 100µl PBS aliquots and 10µl of each dilution of phages was transferred to 90µl aliquots of TG1 secondary culture (at OD_{600} =0.4) in 16 wells of 96 well round bottomed plates in order to obtain TG1 cells infected with phage. This way 8 dilutions each for test and control phages ranging from 10^{-2} to 10^{-9} were obtained. The cultures with phages were incubated at RT for 45 minutes to 1 hour in static condition and were then plated on 2XYT-Amp agar plates keeping TG1 secondary culture as a negative control. The plates were incubated at 37ºC for 10-14 hours to obtain colonies.

3.3.3 SCREENING THROUGH COLONY PCR AND DIGESTION

Colony PCR was performed using the insert-specific primers MKS22 (Reverse Primer) and MKS23 (Forward Primer) to determine the presence of V_HH sequence in the phagemid using 10µl reactions per colony.
PCR temperature settings are provided below.



Gel electrophoresis of the PCR products was done using a 1% Agarose gel along with 100bp ladder (BR Biochem). Phagemid isolation was done from 5ml primary cultures of the PCR positive clones using plasmid isolation kit. Further confirmation of clones was done by subjecting phagemids to double digestion. The digestions were performed using Nco1-HF and Not1-HF (3U each for 1µg of DNA) along with Cutsmart buffer from NE Biolabs for 3 hours. The reaction was analyzed by gel electrophoresis of the undigested vector as control using a 3%

agarose gel.

3.3.4 PHAGE ELISA

Phage Amplification for ELISA

150µl of secondary cultures of PCR and digestion positive clones obtained by biopanning were set up inoculating 1% of the primary culture to 148.5µl of TG1 bacteria in 2XYT-Amp media in separate wells of the 96 well round bottomed plates. The cultures were incubated at 37^{o}C for 2-3 hours while shaking to attain an OD_{600} of 0.4. 1 X 10^{10} M13KO7 phages were then added to the secondary culture and a further incubation at RT for 30-45 minutes in static condition was performed. 1mM IPTG and 50µg/µl kanamycin was added to such cultures at 37^{o}C and 12-16 hours later the culture was stored in -80^{o}C. Prior to phage ELISA, the plates were thawed on ice and centrifuged at 4^{o}C, 4000rpm for 10 minutes.

Phage ELISA

ELISA plate were coated with 5µg antigen per well and incubated at 4^{o}C overnight. The wells were then washed three times with intermittent incubation for 5 minutes using 0.05% PBST (200µl/well) followed by tap drying inverted plates on tissue papers. The blocking was performed with 5% BSA prepared in 0.05% PBST for 2-3 hours. 50µl of the supernatant of the secondary culture containing amplified phages were aliquoted into designated wells (in triplicates) after 3 further washes with intermittent incubation. Different dilutions of M13KO7 ranging from 10^{-2} to 10^{-6} in PBS maintained as a control. 50µl each of PBS was poured into other control wells and the plate were incubated for 45 minutes - 1 hour at RT in a static state. Unbound phages were removed by 3 washes with 5 minutes intermittent incubation using 0.2% PBST. Mouse anti-phage gp-VII monoclonal antibody prepared in 1:10000 dilution using 0.05% PBST in 50µl/well was used as the primary antibody. Following the incubation with primary antibody for 1:30 hours and 3 washes with intermittent incubations using 0.05% PBST, the wells were treated with 1:20, 000 dilution of goat anti-mouse HRP-conjugated secondary antibody prepared in 0.05%PBST (50µl/well) for

1:30 minutes at RT. After 3 washings 100µl of the substrate was added. The substrate was prepared by mixing the 2 TMB reagents in 1:1 ratio in dark. The plates were incubated at RT for 2-5 minutes until color development. The reaction was stopped by addition of 50µl of 1M orthophosphoric acid. The variations in absorbance within wells were measure in GenTek ELISA plate reader at 450nm wavelength.

3.3.5 PROTEIN-LEVEL ANALYSIS

Cloning of phage ELISA positive V_HH sequences to BNT vector

The V_HH sequences that were positive by phage ELISA were amplified from the phagemids obtained from their primary cultures, using MKS23 (FP) and MKS22 (RP). The amplicon was gel purified, digested using Nco1-HF and Not1-HF and purified by isopropanol-ethanol precipitation followed by ligation of the double digested (Nco1-HF and Not1-HF), gel purified BNT vector in a 1:7 ligation reaction. The ligated product was then transformed into Top10 competent *E. coli* cells and the clones were confirmed by colony PCR and double digestion.

Protein Expression and Induction

The vectors isolated from the primary cultures of clones with phage ELISA positive V_HH in BNT vectors were transformed into origami competent cells and protein expression was induced in 500mL secondary culture for 12-14 hours. Induction was confirmed by resolving equivalent OD values of cultures from induced and uninduced culture using PAGE. Induced bacterial pellets were stored in -20^oC.

Purification of protein from inclusion bodies

The induced pellet was resuspended in 20mL Lysis Buffer-A and was sonicated for 8 cycles with 40% power and pulses and breaks of 1 minute each. The lysed cells were pelleted at 10000 rpm for 15 minutes at 4^oC and resuspended in 20mL of Wash Buffer A by vortexing followed by sonication, pelleting and resuspension of the pellet in 20mL of Wash Buffer B under same conditions. An additional wash was given using 20mL of Wash Buffer B. The pellet of the lysed

cell was resuspended in 25mL of denaturation buffer and incubated at RT for 15 - 24 hours on rotaspin with 10rpm speed. The suspension was spun at 5000rpm in RT for 20 minutes. The supernatant was passed through Ni-NTA beads already equilibrated with Wash buffer (Denaturation buffer + 40mM Imidazole) four times and the flow-through was collected and stored. Similarly, the beads were washed with 50mL of Wash Buffer and the flow-through was collected. The protein was eluted after incubating the beads in 5mL of Elution Buffer (Denaturation Buffer + 400mM Imidazole). The flow through, pellet, wash and elutions were loaded on 12% SDS-gel and PAGE was performed to confirm the elution of protein.

Lysis Buffer (pH = 8.0**)**

Tris base	-100mM
Na-EDTA	-10mM

Wash Buffer A (pH = 8.0**)**

Tris base	-100mM
Na-EDTA	-10mM
NaCl	-1M

Wash Buffer B (pH = 8.0**)**

Tris base	-100mM
Na-EDTA	-10
Triton X 100	- 1% (v/v)

Denaturation Buffer (pH = 8.0**)**

NaH_2PO_4	-100mM
Tris-base	-10mM
Urea	- 8M

Western blot at small scale with supernatant of lysed cells.

The supernatants of the 20mL secondary cultures induced at OD_{600}=0.4 and grown at 37ºC for additional 6 hours, were collected after sonication with lysis buffer as done for purification of

protein from inclusion bodies. The 20mL supernatant was concentrated to 1mL, biotinylated, mixed with BSA (final concentration 2%) and used instead of primary antibody in western blot with PPRV. ~20µg of live attenuated PPRV was loaded into 6 wells and ~0.5µg of biotinylated H2-K^b was loaded as a control in 12 % SDS gel. The proteins were transferred to PVDF membrane in Bio-Rad semi-dry blot transfer apparatus. 1:5000 dilution of Streptavidin conjugated with HRP was used to detect bound biotinylated protein (V_HH). The images were later processed in x-ray films at different intervals of exposure in hypercassette.

Determination of protein concentration by Bradford Assay

The concentration of eluted protein was determined using Bradford Assay. 200µl of Bradford's reagent was mixed to 5µl each of eluted protein and BSA solutions of known concentrations in dark. The mixtures were incubated at RT for 5 mins and their absorbances were measured at 595 nm.

Protein refolding

The elutions were dissolved in equal volume of guanidine solution and aliquots of 1.66 mL were prepared and stored in -80^{o}C. ~12mg protein can be refolded with 250mL of Refolding buffer. ~0.83mg each of the protein dissolved in guanidine solution was rapidly diluted in pre-chilled 50 mL of refolding buffer maintained at 4^{o}C on continuous stirring. Upto 2.5mg of protein was rapidly diluted in the same buffer with intermittent 8 hour intervals and incubated the system in aforementioned conditions for 24 hours more. The protein was concentrated with 3kDa amicon ultra centrifuge tubes. Concentration and purity of the protein was estimated based on PAGE.

Guanidine solution (pH = 8.0)

Guanidine-HCl	- 3M
Na-Acetate	-10mM
Na EDTA	-10mM

Refolding Buffer (pH = 4.2)

Tris-base	-20mM
Na EDTA	-1mM

Reduced glutathione	-1 mM
Oxidised glutathione	-0.1 mM
L- Arginine	-400 mM

Protein in the refolding buffer after the prescribed treatment were collected and concentrated to 2 mL in 30 kDa Amicon ultra centrifuge filters. The concentrated protein was injected into AKTA-pure HPLC apparatus in Tris-NaCl (20mM-Tris, 40mM-NaCl) buffer for chromatographic separation of V_HH.

3.4 RESULTS & DISCUSSIONS

3.4.1 Selection and characterisation of PPRV specific sdAbs

3.4.1.1 Screening of phages obtained on biopanning

The test and control phages obtained after second round biopanning were used for infecting TG1 cells. TG1 cells have an F-plasmid within them, which enable the uptake of the phagemids from the phages, while their 'amber mutation' facilitates the synthesis of V_HH fusion protein. On plating the cultures infected with serial dilutions of phages (10^{-1} to 10^{-8}), we obtained 156 colonies from the test cultures and 7 colonies from the control culture (Table 3.4.1). Almost 10 fold difference was obtained in the number of clones with phagemid.

Dilution of phages	**Test (cfu)**	**Control (cfu)**
.01	**131**	**4**
.001	**19**	**2**
.0001	**4**	**1**
.00001	**2**	**0**

Table 3.4.1: Colonies obtained on infecting TG1 cells with phages selected on biopanning

Colony PCR was performed to check for the presence of V_HH in the phagemids using FP: MKS23, RP: MKS22 with T_m 64.8^oC. On screening the entire 156 clones, 6 clones were identified to be possessing V_HH sequences ranging between 400-500bp (Figure: 3.4.1a,b). On checking with double digestion assay with Nco1-HF and Not1-HF, it was observed that 5 of them were digestion positive (Figure: 3.4.1 c).

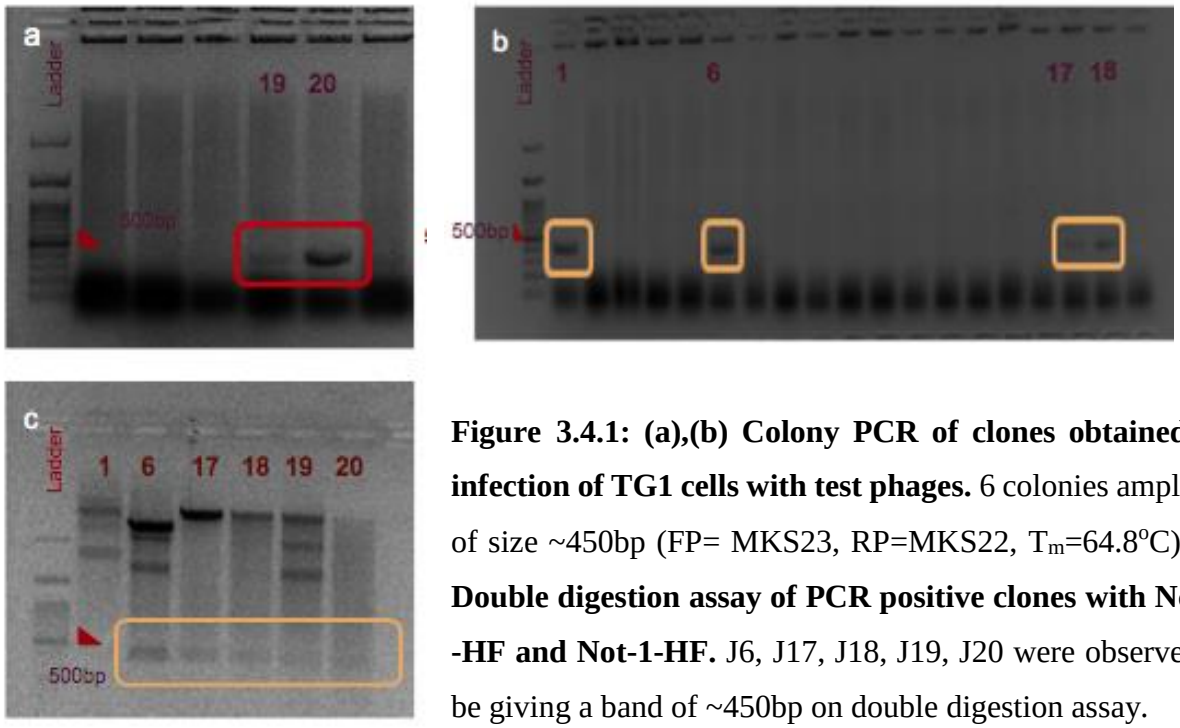


Figure 3.4.1: (a),(b) Colony PCR of clones obtained on infection of TG1 cells with test phages. 6 colonies amplicon of size ~450bp (FP= MKS23, RP=MKS22, T_m=64.8^{o}C). **(c) Double digestion assay of PCR positive clones with Nco-1 -HF and Not-1-HF.** J6, J17, J18, J19, J20 were observed to be giving a band of ~450bp on double digestion assay.

3.4.1.2 Phage ELISA

The affinity of the V_HHs towards the antigen of interest were analyzed by performing phage ELISA (Figure 3.4.2). Culture supernatants containing monoclonal antibodies against protein N and protein H of PPRV were utilized as a positive control and it ensured the presence of virus on the coated surface. Different dilutions of M13KO7 were used as a control to quantify the affinity of phages displaying selected V_HHs with respect to the inherent stickiness of phages. In addition to the selected 5 clones (J6, J17, J18, J19, J20), phages were amplified from 4 other clones (Y8, Y16, Y24 and Y32) obtained on previous round of biopanning for phage ELISA (Appendix 1). It was observed that clones J6 and J20 were giving higher affinities than wild type phages, though it failed to produce a 2-fold higher binding as it is generally considered as a baseline interaction for selecting the clones for further analysis at molecular and protein level in cases where there a many clones to be screened through phage ELISA. Although, phages displaying V_HH : Y16 and Y24 did exhibit a higher affinity, on performing colony PCR they were generating amplicons of size greater than 1 Kbp. It could be due to the accumulation of few mutations and the issue can be addressed by modifying the vector with insertion of restriction sites through site-directed mutagenesis, if

there are no better restriction sites on either ends of the V_HH sequence which could be employed for the subcloning of the sequence to an expression vector.

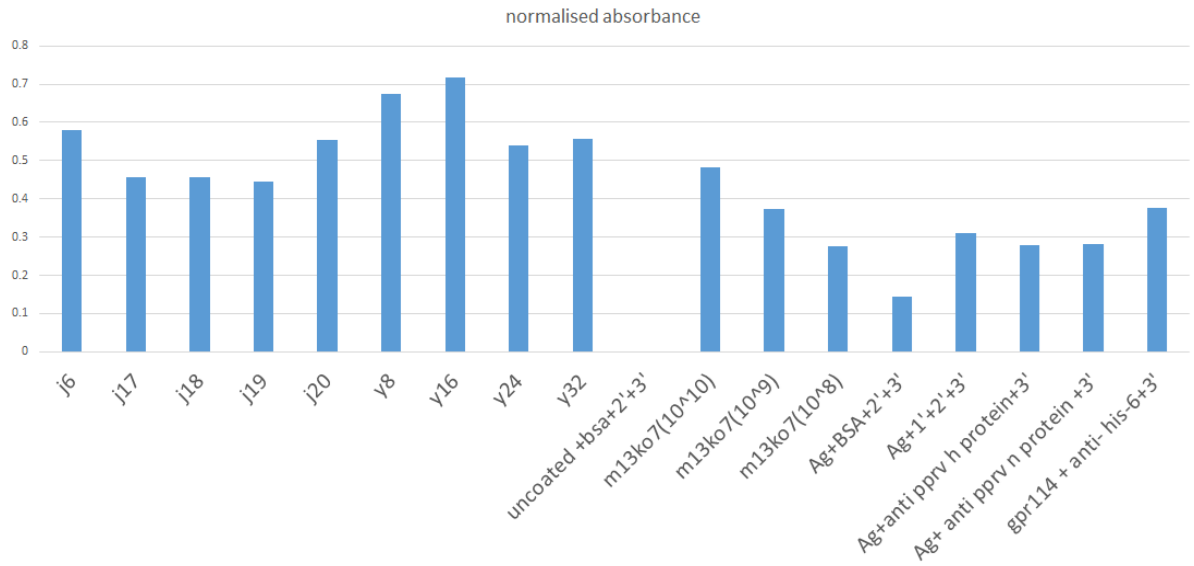


Figure 3.4.2: Phage ELISA of clones selected after colony PCR and double digestion assay. Phage ELISA was performed J6,J17, J18, J19, J20 with Y8, Y16, Y24, and Y32 as a positive control, different dilutions of wild phages as negative control and other combinations of various reagents used as other controls. Uncoated well treated with BSA, anti- M13 gp8 protein (against phage coat protein) and goat anti-mouse as secondary antibody was taken as blank.

3.4.1.3 Subcloning of selected V_HH s to Vector BNT

All the 5 clones were subcloned into Vector BNT (Figure 2.2) in between Nco1 and Not1 recognition site. CIP treatment was given to the digested vectors to avoid self-ligation. No colonies were observed in the negative control LB-Amp plate showing successful inhibition of self-ligation by CIP.

On spreading the transformed cells on LB-amp plates, number of colonies obtained are as noted below:

J06 = 83 J17 = 130 J20 = 153

J17 = 97 J18 = 80 Control*= 0

*control = cells transformed with ligation reaction with digested vector treated with CIP and no insert

The clones were confirmed through colony PCR [FP: MKS23; RP: AJ08 (AJ08 is used to confirm that biotinylation tag is present in the vector)] and double digestion assay with Nco1-HF and Nhe1-HF (Figure 3.4.3 a,b). The plasmids were isolated from primary cultures of clones with V_HH s J20 and J6 in Vector BNT and transformed into *E. coli* Origami cells for the protein expression**.**

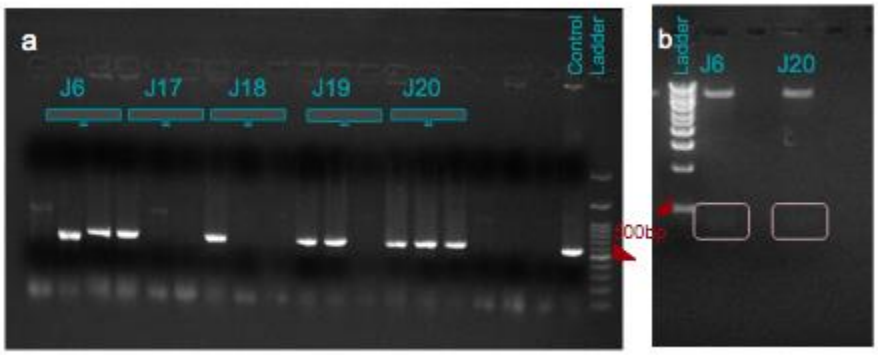


Figure 3.4.3: (a) Colony PCR of clones obtained on spreading the cells on LB-Amp post-transformation. Amplicons of size ~50bp greater than that of control (Vector NT) were considered as positive clones (FP=T7 promoter, RP= T7 terminator and T_m=64.8^{o}C.) **(b) Confirmation of clones by double digestion assay.** Double digestion of ~1µg of plasmids with Nco-1-HF and Not-1 HF generated ~450bp long digested product confirming the presence of V_HH.

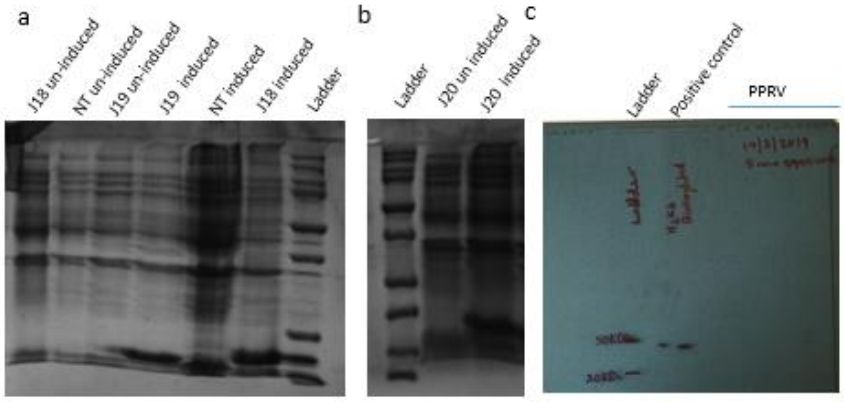
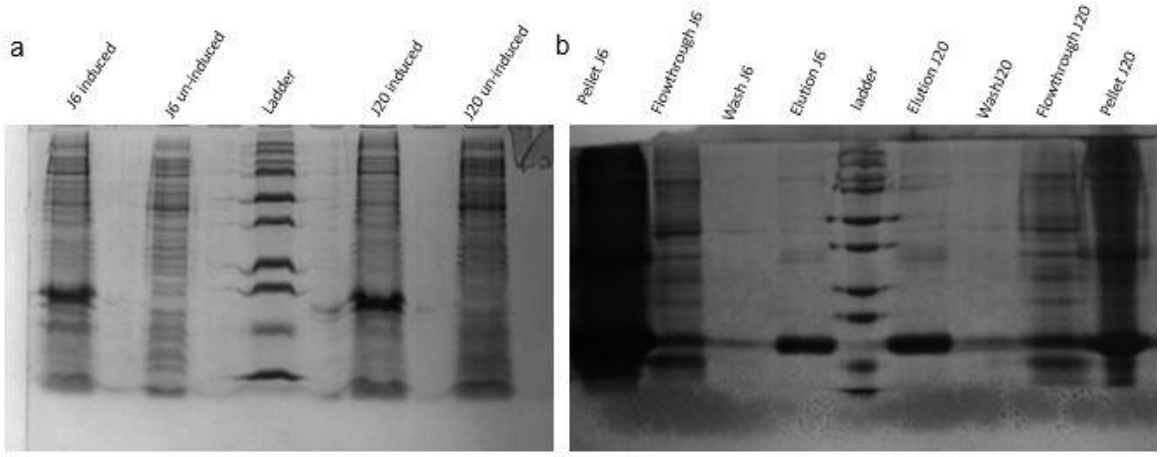


Figure 3.4.4: (a), (b) Induction of protein expression in NT, J18, J19 and J20; (c) Western Blot of PPRV with biotinylated supernatant of cell lysates. SDS lysed heat killed PPRV has an intense band around 65kDa, but no biotinylated component of the cell lysate showed binding affinity to the proteins.

3.4.1.4 Protein purification from inclusion bodies

According to the observations during analysis of activity of Vector BNT, it was observed that 6XHIS-Tag of the recombinant protein was getting cleaved by cytoplasmic proteases. When proteins are expressed at high level, they will form aggregates known as inclusion bodies. When proteins are purified from inclusion bodies, the probability for the same to be cleaved by cytoplasmic proteases becomes minimal. So the protein expression was induced, cells were lysed and were purified from the inclusion bodies using Ni-NTA beads (Fig 3.4.5 a,b).

The protein concentration was estimated to be approximately 2.5mg/mL. Protein was treated as per protocol for refolding, concentrated, and injected to AKTA pure HPLC for column chromatography based separation of refolded and misfolded protein. But the fractions collected were of low absorbance at 260 nm indicating that they were misfolded (Figure 3.4.5 c). It was observed that the properly refolded V_HHs would get eluted in the fractions ranging from 55mL - 70mL and misfolded fractions of V_HH were collected from the elutions from 90mL - 110mL. The pI of the protein J6 was calculated to be 6.8. The refolding buffer should be having a 2 units of pH difference from the pI of the protein. The refolding of the proteins should be optimized.



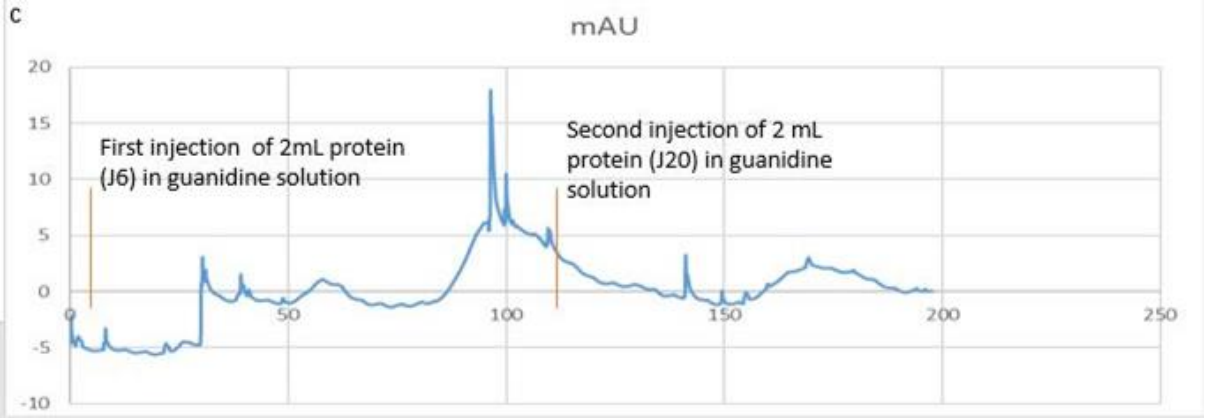


Figure 3.4.5: (a) Induction of protein expression in J6 and J20. (b) J6 and J20 purified from inclusion bodies. (c) Chromatogram of denatured proteins (J6 and J20) treated in refolding conditions.

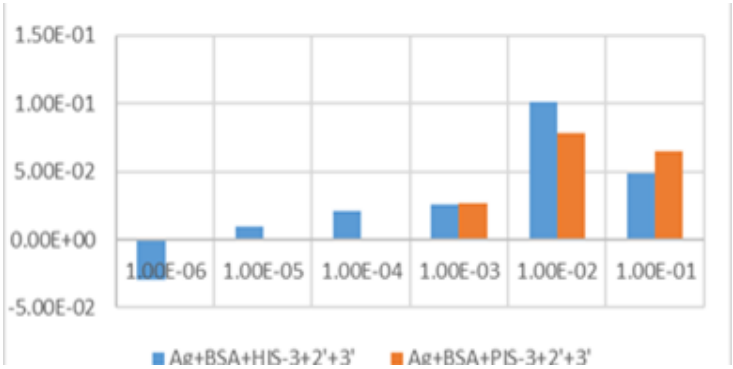
3.4.1.5 Future directions

The proteins should be refolded, and their affinity at protein level can be characterized. These V_HHs, if having fair specificity can be custom modified and utilized for viral trafficking, FACS staining, immunohistochemistry etc.

3.4.2 Screening of GPR114 specific single domain antibodies

3.4.2.1 ELISA of GPR114 with Camel Pre-immune serum and Hyper-immune serum

Although an elevation was estimated in the absorbance signal on ELISA of GPR114 different sera samples but the variation is not statistically significant (Figure 3.4.6). It could be due to variation in response of animals. Still, there would be sufficient antibodies against the epitopes in GPR114 in the serum, which from the genetic level would have copied during the generation of the phage library and reproduced on the phage surface through phage display, which could be screened through biopanning.



sample	absorbance
ag+BSA+2+3 (blank)	0.3516667

Figure 3.4.6: The normalized absorbance obtained on ELISA of different dilutions of GPR114 with different sera samples.

3.4.2.2 Biopanning of GPR114

The phages were eluted from test and control plates after biopanning and were used for infecting TG1 cells at 10^{-2} dilution (Table 3.4.2). Though, 2 clones (J5 and J19) were observed to be having V_HH on colony PCR (FP: MKS22 RP: MKS23, Tm= 64.8^oC) (Figure 3.4.6 a), they were found to be having truncated V_HH on double digestion with Nco1-HF and Not1-HF (Figure 3.4.7 a,b).

Dilutions	Test	Control
0.01	lawn	Lawn
.0.001	~800	~630
0.0001	138	120
0.00001	14	13

Dilutions	Test	Control
0.01	20	13
0.001	2	3

Table 3.4.2: Number of colonies obtained after first and second round of biopanning in different dilutions of phages.

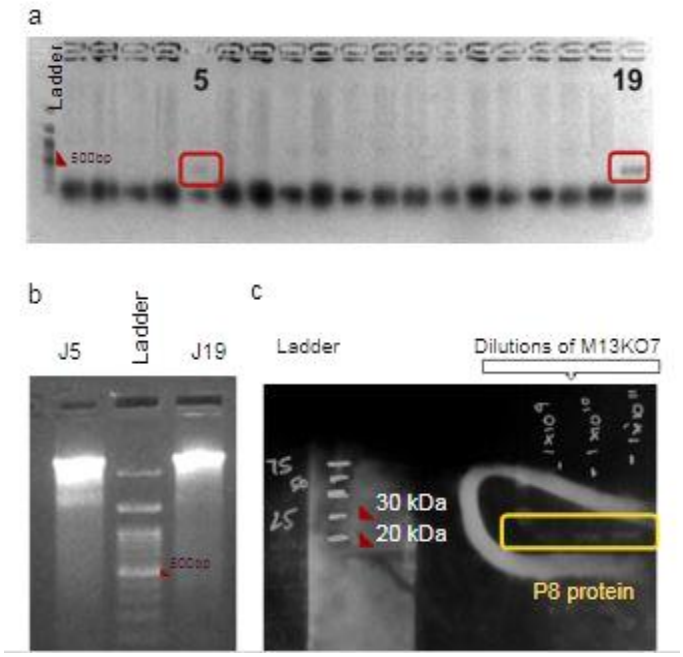


Figure 3.4.7: Colony PCR of clones obtained on infection of TG1 cells with test phages eluted after bio panning of GPR114. J5 and J19 generated amplicons of size ~450bp. **(b) Double digestion of plasmids of J5 and J19 with Nco-1 HF and Not-1 HF.** No digested product was obtained**. (c) Western blot of different dilutions of M13KO7 probed with 1µg of refolded GPR114.** GPR 114 is binding to P8 protein in the blot (~25kDa).

It was observed that GPR114 is also showing affinity towards P8 protein on the surface of and some other proteins of wild type phages (Figure: 3.4.6 c) (on performing western blot of phages with GPR114) which might interfere with efficiency of biopanning and phage ELISA. An extensive screening should be performed to obtain V_HH specific for GPR114.

3.5 CONCLUSION

The test and control phages eluted after biopanning of PPRV were used for infecting TG1 cells in order to screen the phagemids possessing V_HH. Five (J6, J17, J18, J19, J20) out of six PCR positive clones generated digested products of sizes equivalent to V_HH. J6 and J20 were selected for protein level characterization of their affinity towards PPRV based on the elevated absorbance values exhibited by the respective clones with respect to the values produced by the wild phages in Phage ELISA. All the 5 V_HHs were sub cloned into Vector BNT for the ease of downstream characterization of the proteins. The clones were confirmed through colony PCR and double digestion assay. The modified vector was transformed into origami cells and protein expression was induced. The western blot of PPRV probed with biotinylated cell lysate supernatants obtained from the lysis of the cells from secondary culture induced with 1mM IPTG at OD_{600}=0.4 and grown at 37^oC for 6 additional hours failed to show any affinity. As the activity of bacterial serine proteases would cleave off the 6XHis tag of completely refolded V_HH, while in cytoplasm; protein purification based on 6XHis tag is feasible only from inclusion bodies. The refolding conditions of thus purified J6 and J20 proteins should be optimized and further characterization can be performed. The proteins were induced in large scale and purified from inclusion bodies. Extensive screening should be done for selecting V_HHs from the phages obtained on biopanning of GPR114.

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Appendix I

Phage ELISA of PPRV with the selected V_HHs

sample	i	ii	iii	average
j6	0.875	0.613	0.85	0.779333333
j17	0.679	0.488	0.802	0.656333333
j18	0.769	0.461	0.74	0.656666667
j19	0.7	0.516	0.716	0.644
j20	0.832	0.641	0.787	0.753333333
y8	0.979	0.793	0.852	0.874666667
y16	1.407	0.691	0.652	0.916666667
y24	3.008	0.73	0.752	0.741
y32	0.567	0.876	0.831	0.758
uncoated +bsa+2'+3'	0.183	0.193	0.225	0.200333333
uncoated+bsa+1'+2'+3'	0.493	0.322	0.58	0.465
m13ko7(10^10)	0.523	0.738	0.791	0.684
m13ko7(10^9)	0.44	0.507	0.771	0.572666667
m13ko7(10^8)	0.397	0.44	0.594	0.477
Ag+BSA+2'+3'	0.394	0.321	0.316	0.343666667
Ag+1'+2'+3'	0.494	0.4	0.642	0.512
Ag+anti-PPRV H protein+3'	0.487	0.413	0.536	0.478666667
Ag+ anti-PPRV N protein +3'	0.543	0.357	0.544	0.481333333
gpr114 + anti- his-6+3'	0.533	0.575	0.622	0.576666667
sample	normalised absorbance			
j6	0.57900033			
j17	0.45600033			
j18	0.45633367			
j19	0.443667			
j20	0.55300033			
y8	0.67433367			
y16	0.71633367			
y24	0.540667			
y32	0.557667			
uncoated +bsa+2'+3'	0			
m13ko7(10^10)	0.483667			
m13ko7(10^9)	0.37233367			
m13ko7(10^8)	0.276667			
Ag+BSA+2'+3'	0.14333367			
Ag+1'+2'+3'	0.311667			

Ag+ anti- PPRV H protein+3'	0.27833367			
Ag+ anti- PPRV N protein +3'	0.28100033			
gpr114 + anti- HIS Tag+3'	0.37633367			

Ag = Antigen (live attenuated PPRV)

1’ = selected V_HHs

2’ = Mouse anti-HIS Tag

3’ = Anti-mouse raised in goat

BSA = Bovine Serum Albumin

Appendix II

ELISA for GPR144 with Hyper immune serum (HIS) and Pre-immune serum (PIS)

Ag+BSA+HIS-3+2'+3'	1	2	3	average	normalising with blank
1.00E-01	0.418	0.375	0.409	0.400666667	0.048999667
1.00E-02	0.356	0.504	0.499	0.453	0.101333
1.00E-03	0.332	0.425	0.376	0.377666667	0.025999667
1.00E-04	0.317	0.439	0.363	0.373	0.021333
1.00E-05	0.344	0.385	0.355	0.361333333	0.009666333
1.00E-06	0.305	0.347	0.313	0.321666667	-0.030000333
Ag+BSA+PIS-3+2'+3'					
1.00E-01	0.417	0.422	0.41	0.416333333	0.064666333
1.00E-02	0.474	0.462	0.354	0.43	0.078333
1.00E-03	0.383	0.384	0.367	0.378	0.026333
no antigen +BSA+1'+2'+3'	0.338	0.315	0.347	0.333333333	
ag +BSA+1’	0.312	0.393	0.32	0.341666667	
ag+BSA+1’+2’	0.323	0.351	0.302	0.325333333	
ag+BSA+2’+3’ (blank)	0.359	0.357	0.339	0.351666667	
ag+BSA+1’+3’	0.342	0.365	0.348	0.351666667	
ag+BSA+3’	0.306	0.332	0.334	0.324	
Ag+BSA	0.314	0.323	0.334	0.323666667	
Ag	0.218	0.463	0.398	0.359666667	

Ag = Antigen (GPR114)

1’ = HIS

2’ = Mouse Anti-hyper immune serum

3’ = Anti-mouse raised in goat

BSA = Bovine Serum Albumin