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Selection of Single Chain Human Monoclonal Antibody (scFv) Against Rabies virus by Phage Display Technology

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Abstract— **Human monoclonal antibodies against Rabies virus were selected from non-immunized human scFv library (YAMO-I library) and immunized library (Yamo-Rb library) by using phage display technology. The biopanning was performed for 2-5 rounds by using two types of inactivated rabies vaccines as targets. These are purified vero cell rabies vaccine (PVRV) and purified chick embryo cell vaccine (PCEC). A total of 14 positive clones from various method of biopanning that can bind to rabies, i.e.; IRA7c, IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c, IYF5c, IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v and IVB4cv, were isolated and their genes were sequenced. The ELISA result showed that the positive clones always bind strongly to the targets that were used for biopanning; however some clones can cross-react to the related virus. These selected scFv antibodies will be tested for neutralization activities *in vitro* in the next step.**

Index Terms—**Phage display, Rabies virus, single-chain fragments(scFv), human monoclonal antibody.**

I. Introduction

Rabies is a fatal zoonotic disease that is transmitted by both wild and domestic animals. Globally, it is estimated that at least 55,000 people die of rabies and there are about 10 millions people who receive post-exposure vaccination annually. Currently available rabies immune globulin (RIG) for clinical use are Equine Rabies Immunoglobulin (ERIG) and Human Rabies Immunoglobulin (HRIG). However, RIG was produced in limited amounts [1]. Moreover, HRIG is too costly, not easily available, and suffered from potential disadvantages, such as limited capacity, batch-to-batch variation, and possible contamination with blood borne adventitious agents. ERIG also has drawbacks of animal origin that carries a risk of occasional adverse reaction, including anaphylaxis, especially after second exposure [2, 3]. Thus, utilization of Phage display technology for the production of human antibody specific to rabies virus is attractive and suitable alternative strategy for the prevention, treatment and diagnostic of rabies. Phage display technology has been shown to be a powerful method for the generation of antibody *in vitro* by mimicking the selection strategies of the immune system [4]. In phage display, antibody fragments are expressed as fusions to capsid proteins presented on the surface of the filamentous bacteriophage particles, which are approximately 7 nm wide by 900-2000 nm in length. Therefore this system provides direct linkage between the antibody genotype (DNA sequence in phage particle) and its phenotype (affinity and specificity of phage-displayed antibody).

In this study, Phage display single chain fragments of variation (scFv) antibody libraries were used to select single chain human monoclonal antibodies (scFv) against rabies virus. scFv is a popular format in the recombinant antibody technology because it can be cloned and manipulated as individual polypeptide and efficiently displayed on the surface of bacteriophage (phage). Even if scFvs are significantly smaller then full-length human antibodies IgGs (25 vs 150 kDa). They can still bind their respective antigens tightly (i.e. with dissociation constants of 5 uM to 10 nM) and represent structurally minimized version of full-length human antibodies IgGs. Moreover, when compared to fragment of antigen binding (Fabs) which are ~50 kDa, scFvs which generally resistant and aggregation have twice smaller than Fabs [5]. Therefore scFv antibody is an attractive nanomaterial for both diagnostic and therapeutic purposes.

II. Meterials and Methods

Materials

Two types of phage display libraries, i.e., Non-immunized (YAMO-I) [4] and immunized libraries (Yamo-Rb) were constructed in our laboratory. Both libraries were constructed using antibody genes isolated from the peripheral blood of human donors. The YAMO-I library was constructed from 140 non-immunized (Naïve) donors; whereas, Yamo-Rb library was constructed from four human donors immunized with PVRV (VeroRab, Pitman-more/ W138-153-3M strain, Sanofi-Pasteur, Lyon, France) or PCEC (LEP-Flury strain, Rabipur, Chiron, India).

Selection of human scFv phage library on inactivated rabies virus (Biopanning)

Selection was performed using inactivated rabies vaccines (PVRV or/and PCEC) as targets. Two to five rounds of selection were carried out. Maxisorp Immuno tube (Nunc, Denmark) was pre-coated with 0.35-1.4 IU of inactivated rabies virus, at 37 ^{o}C for 3 hours following by 4 ^{o}C overnight, in 100 mM $NaHCO_3$, pH 8.5. After that, the immuno tube was

stabilized with 5% w/v sucrose, 0.3 % w/v BSA, and 50 mM $NaHCO_3$ for 45 min. Then, the tube was washed three times with phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and blocked with PBS containing skimmed milk (2%, w/v, MPBS) for 1 hour. For each round of biopanning, the phage library (~10^{10} phages) was incubated with pre-coated inactivated rabies virus in 4%, w/v, MPBS for 2 hours at room temperature. Unbound phage was washed away with PBS supplemented with 0.05% (v/v) Tween 20 (PBST) and with PBS. Phage antibody against rabies viruses was recovered with 1x trypsin buffer and 0.2M glycine HCl, pH 2.0. The eluted phage was infected into *E. coli* to obtain individual phage clones as previously described [6]. For each round of selection, specificities of individual phage scFv clones were identified by enzyme-linked immunosorbent assay (ELISA) [6].

Monoclonal Phage ELISA

Single colony of each round of biopanning was randomly picked and cultured in 96-deep well plate followed by super-infection by KM13 helper phage [4]. Phage supernatants were collected after centrifugation and subjected to ELISA for screening of monoclonal anti-rabies virus phage-scFv. The Immuno 96 microWell™ plate (Nunc, Denmark) was immobilized with inactivated rabies at 37 °C for 3 hours following by 4 °C overnight in 100 mM $NaHCO_3$ and 2% skim milk in 100 ul PBS buffer as a control. The phage supernatant was added to Immuno 96 microWell™ plate (Nunc, Denmark) and the binding phage-scFv was detected with HRP-conjugated anti-M13 antibody (1:5000). The color of the reaction was developed with ABTS reagent (Fluka). The reaction was quantified by measuring the absorbance at 405 nm.

DNA sequence and analysis

After the positive cloned are confirm by ELISA twice. Each clone of positive Phagemid DNA was extracted using DNA miniprep kit (Qiagen). The restriction fragment analysis was performed by using *Bst*NI. The selected positive clone with variable restriction pattern were confirmed by automated DNA sequencing (Macrogen, Korea) and analyzed with Igblast software (http://www.ncbi.nlm.nih.gov/igblast/) and the sequence alignment of the scFv antibodies was done using CLUSTALW 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

III. Results and Discussions

A total of 14 positive clones from various method of biopanning were isolated. These are IRA7c, IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c, IYF5c, IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v and IVB4cv. After DNA sequence analysis, we found that clones IVB4cv and IRA7c were identical. These two clones were isolated from the same library, i.e., Yamo-Rb by using different biopanning method. Clone IVB4cv was obtained from the fourth round of biopanning using a combination of both PCEC and PVRV as targets; whereas clone IRA7c was from the first round of panning using PCEC as a target. Clones IIIRC2c, IRC3c, IYC11c, IYC12c, IYD1c and IYF5c were selected by using PCEC as a target; while IIRD5v, IIYB5v, IIYG4v, IIYE5v, IIYG8v, IIYD4v were selected by using PVRV as a target. The ELISA results in Fig. 1 showed that the positive clones always bind strongly to the target that was used for biopanning. Nine clones were obtained from naïve library (YAMO-I) and four clones were from immunized library (Yamo-Rb). Five clones which are IRA7c, IIIRC2c, IIYG4v, IIYE5v, and IVB4cv showed cross-reactivity to both PCEC and PVRV targets.

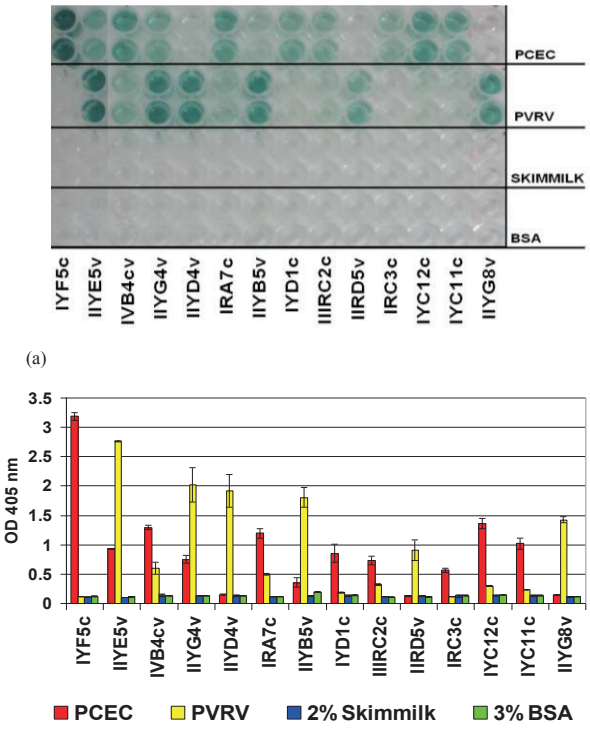

Fig. 1. (a) ELISA plate showing positive reactions (green colour) with PCEC or PVRV and negative reaction (clear) with 2% skim milk and 3 % BSA
(b) ELISA signal at O.D. 405 nm

The *Bst*NI fingerprinting analysis of 14 positive clones was shown in Fig. 2. In this figure pMOD (empty vector) was digested to compare with the positive scFv clones (pMOD+scFv gene).

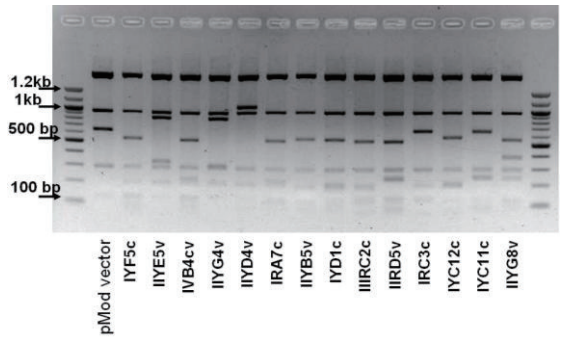

Fig. 2. Restriction fragment analysis by *Bst*NI

After the positive cloned were confirmed by automated DNA sequencing. The DNA sequence of each scFv clone was analyzed with Igblast and the sequence alignment of the 14 scFv antibodies was done using CLUSTALW software. Fig. 3 showed the origin of germline and family of all the isolated V_H and V_L segments; whereas, Fig. 4 illustrates the amino acid sequence alignment of all positive clones. It is interesting to note that clone IRC3c, which consist only V_L, could still bind to the target, even if the signal is quit low when compared to other positive clones. There have been many reports on the binding of VHH, which is a single-domain antibody (sdAb) that consists only of V_H domain from camelidae [5, 7, 8]. Our data is the first report on the binding of single V_L domain of human antibody.

No.	Name		Germline	Amino acid difference from germline	Family
1	IRA7c	VH	IGHV3-33*01	18	VH3
		VL	IGLV2-14*02	7	VL2
2	IIIRC2c	VH	IGHV4-39*07	19	VH4
		VL	IGLV2-14*01	8	VL2
3	IRC3c	VH			
		VL	IGLV2-14*01	3	VL2
4	IYC11c	VH	IGHV1-2*02	7	VH1
		VL	IGLV3-21*03	0	VL3
5	IYC12c	VH	IGHV3-23*01	6	VH3
		VL	IGLV1-44*01	13	VL1
6	IYD1c	VH	IGHV3-23*01	6	VH3
		VL	IGLV3-19*01	8	VL3
7	IYF5c	VH	IGHV1-18*01	0	VH1
		VL	IGLV1-47*01	2	VL1
8	IIRD5v	VH	IGHV1-2*02	15	VH1
		VL	IGLV2-14*01	5	VL2
9	IIYB5v	VH	IGHV1-69*01	1	VH1
		VL	IGLV1-44*01	3	VL1
10	IIYG4v	VH	IGHV6-1*01	0	VH6
		VL	IGLV6-57*01	11	VL6
11	IIYE5v	VH	IGHV6-1*01	5	VH6
		VL	IGLV6-57*01	11	VL6
12	IIYG8v	VH	IGHV3-9*01	1	VH3
		VL	IGLV1-44*01	8	VL1
13	IIYD4v	VH	IGHV3-30-3*01	2	VH3
		VL	IGLV6-57*01	1	VL6
14	IVB4cv	VH	IGHV3-33*01	18	VH3
		VL	IGLV2-14*02	7	VL2

Fig. 3. Germlines and Families of V_H and V_L segments of all clones.

Fig. 4 showed the amino acid sequence alignment of all isolated scFv clones. The TAG amber stop codons were found in clone IIYB5v, IIYD4v and IYD1c. These codons are translated as glutamine instead of stop codon in *E. coli* suppressor strain such as DH5alpha or TG1; therefore, the functional antibody can be displayed on filamentous phage. The complementary determining regions (CDRs) of the heavy and light chain of the antibody (V_H/CDR1, V_H/CDR2, V_H/CDR3, V_L/CDR1, V_L/CDR2 and V_L/CDR3) and the linker sequence were indicated. Amino acid sequence analysis revealed that all isolated positive clones have only the lambda type of the light chain (V_L) from family 1, 2, 3, and 6; while variable heavy chains (V_H) were from family 1, 3, 4 and 6. No clone containing the kappa type of the light chain (V_K) was isolated.

IV. Conclusions

In conclusion, 13 unique phage-displayed anti-rabies virus scFv antibodies were successfully selected from naïve library (YAMO-I) and immunized (Yamo-Rb) human scFv antibody libraries. All antibodies possessed variable heavy chains (V_H) from family 1, 3, 4 and 6 and only lambda light chains (V_L) from family 1, 2, 3, and 6. One clone consisted of only V_L fragment. These selected phage-scFv clones will be further engineered to generate soluble scFv nanobodies and tested for neutralization activities *in vitro* in the next step.

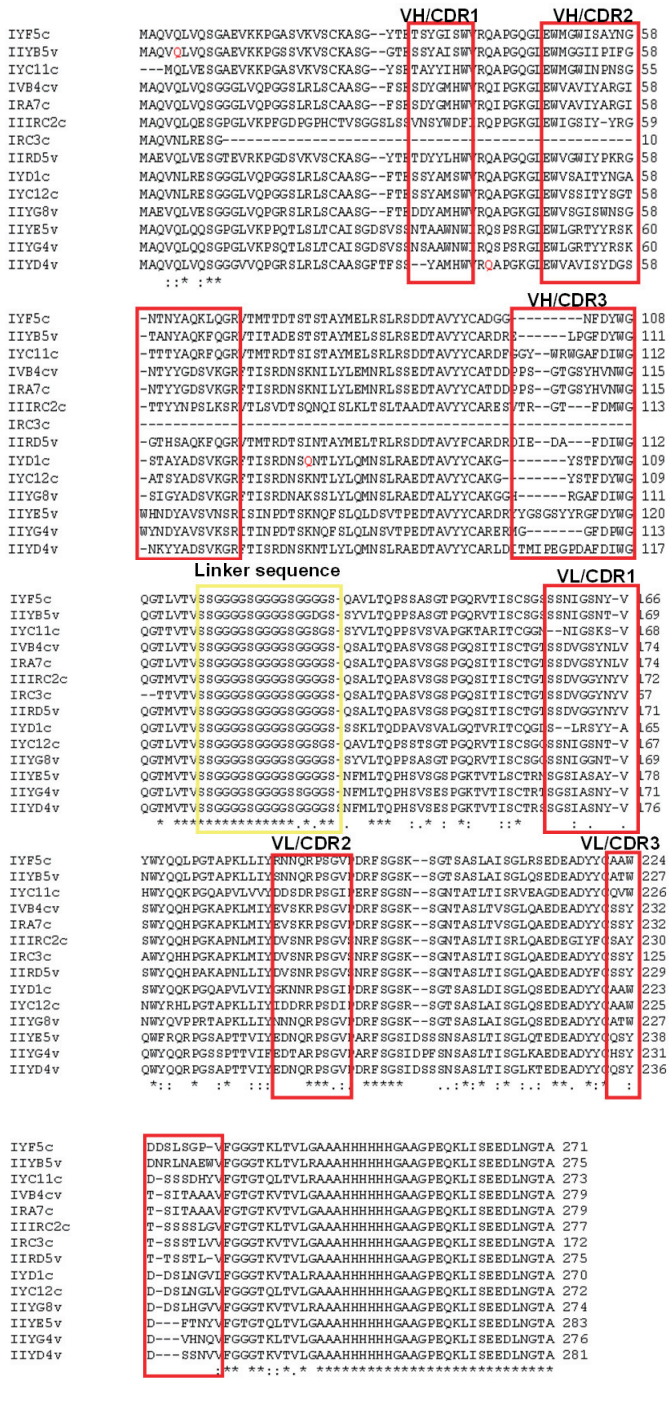

Fig. 4 Amino acid sequence alignments of all positive clones are shown. The GC-rich sequence that links V_H and V_L segments is indicated. The three complementary determining regions (CDRs) are marked with box. These scFv antibodies are linked with 6xHis and Myc epitope at the C-terminal. Red Q is glutamine that is translated from an amber stop codon in suppressor *E. coli* strains.

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