Thesis for the degree of Master of Science

Construction of cloudy catshark (*Scyliorhinus torazame*) variable new antigen receptor (vNAR) library using phage display and application for antigen detection

by

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Phage display를 이용한 두툽상어의 variable new antigen receptor (vNAR) library 구축 및 항원 검출 활용

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by
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Abstract

Monoclonal antibodies (mAbs) have been widely applied as diagnostic and therapeutic platforms. However, these conventional mAbs have shown several shortcomings, such as their large size and difficult to store. To overcome the problems of the mAbs, second-generation antibodies based on the single-chain fragment variable (scFv) have been developed but, unstability and lacking avidity are the shortcomings of scFV antibodies. Nowadays, the Immunoglobulin New Antigen Receptor (IgNAR) founded in sharks is considered as an attractive candidate for applicable antibody technologies because of several advantageous properties such as cryptic antigen recognition domain structure, small molecular size, fast secretion, stability.

Phage display is a rapid, cost-effective technology to display the antibody, so we constructed the phage library display using the vNAR gene from cloudy catshark (Scyliorhinus torazame) with the phage display platform. Furthermore, we performed screening about Lysozyme A to check the operation of constructed phage library and did another screening about VHSV to confirm the applicability of specific pathogens.

Based on the results of screening about Lysozyme A and VHSV, we confirmed that the generated phage library display was working well and checked the applicability to a specific antigen. However, a present study about the VHSV is incomplete so, it is necessary to find more VHSV positive clones through the ELISA assay, and further studies using VHSV positive clones such as neutralization assay, diagnostic tests about VHSV infected cells and tissues.

Consequently, it is necessary to perform further studies, but the constructed phage library display can be applied to specific antigens such as other Rhabdovirus, therapeutic and diagnostic, and will enable rapid, cost-effective production of antibodies with specificity.

Phage display를 이용한 두툽상어의 variable new antigen receptor (vNAR) library 구축 및 항원 검출 활용

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초록

Monoclonal Antibody는 진단과 치료의 목적으로 널리 접목, 사용되고 있는 기술에 해당한다. 하지만 monoclonla Ab가 가진 큰 크기와 보관상의 문제가 존재하였고, 이러한 단점을 극복하기 위하여 등장한 것이 ScFV (Single Chain Fragment Variable)이다. ScFv의 경우 그 크기는 작지만 안정성이 떨어지고 항원항체간 결합력이 낮다는 단점이 존재한다. 그리하여 현재는 1990년대에 상어에서 발견된 비정형의 IgNAR 구조를 구성하는 1개의 varaiable region인 vNAR이 가진 극한의 pH, 온도 조건에서 보이는 안정성, 높은 다양성 및 빠른 분비속도와 같은 이점을 토대로 매력적인 항체 연구 후보로 떠오르고 있다.

본 연구에서는 항체를 display할 수 있는 다양한 방식 중에서 단기간에, 그리고 비용 절약적으로 제작할 수 있는 phage library display 방식을 채택하고, 두툽상어에서 증폭한 vNAR 유전자를 이용하여 library를 구축하고자 실험을 진행하였다. 더 나아가 구축된 library가 작동하는지 확인하

기 위하여 Lysozyme A에 대한 screening을 진행하였고, 해당 결과를 바탕으로 특이 항원에 접목 가능성을 보기 위하여 VHSV (Viral Haemorrhagic Septicemia Virus)에 대한 screening 또한 이번 연구에서 진행하였다.

Lysozyme A에 대한 screening을 진행함에 있어 “Yin-Yang” method를 참고한 방식을 통해 최대한 non-specific binder를 제거하고 positive selected phage clone을 찾기 위하여 3회의 bio-panning을 실시했다. 실시한 bio-panning 결과를 무작위로 선정한 96개의 clone으로 실시한 ELISA assay를 통해 확인하여 4개의 positive clone을 찾을 수 있어 구축한 library가 잘 작동함을 확인하였다.

나아가 구축된 library가 특이 항원에 접목될 수 있는지 확인하기 위하여 선정한 VHSV에 대해서도 동일한 과정을 거쳐 확인한 결과, 우리는 3번 진행한 ELISA assay에서 2개의 clone을 찾을 수 있었기 때문에 특이 항원에 대한 구축된 library가 적용될 가능성이 있음을 확인하였다.

그러나, 이번 연구에서 수행한 VHSV 관련 결과는 충분하지 않기 때문에 ELISA asasy를 반복하여 더 많은 VHSV positive clone을 찾고, 해당 clone을 위한 western blot을 통해 찾은 clone들이 target하는 virus particle을 찾아야 한다. 또한, 치료 목적으로 사용할 수 있는지 확인하기 위한 neutralization assay, 진단 목적으로서 찾은 clone이 VHSV에 감염된 세포나 조직에서도 virus particle을 잡을 수 있는지에 대하여 확인하는 추가 연구가 필요한 것으로 보인다.

추가 연구를 통해 찾은 clone들이 제대로 작동하는 것을 확인한다면, phage display 방식을 통해 구축한 library가 VHSV 이외의 다른 Rhabdovirus에 적용할 수 있을 것으로 보이며, 더 나아가 여러 병원체에 적용하여 치료 및 진단 분야에서 그 기능을 할 수 있는 항체를 빠르고 비용 절약적으로 생성할 수 있을 것으로 보인다.

I. Introduction

Monoclonal antibodies (mAbs) have been widely applicated as diagnostic and therapeutic platforms for diverse diseases, and many of them have been clinically used in human medicine (Ritz et al. 1981; Waldmann 1991) The conventional first generation mAbs are based on immunoglobulin G (IgG), and are composed of two heavy chains that are connected to two light chains by disulfide bonds (Hochman, Inbar, and Givol 1973). However, these conventional mAbs have shown several shortcomings due to their large size, such as the possibility to have immunogenicity in therapeutic applications and the limitation in detection targets in diagnostic applications (Salvador, Vilaplana, and Marco 2019). To overcome the size problem of the first generation mAbs, second generation antibodies based on the single-chain fragment variable (scFv) in which the heavy chain variable region is joined with the light chain variable region by a peptide linker have been developed and demonstrated to have a high potential as diagnostic and therapeutic tools (Willuda et al. 1999). Although the size became much smaller compared to the conventional mAbs, unstability and lacking avidity are the shortcomings of scFV antibodies (Pillay and Muyldermans 2021).

In the 1990s, antibodies with un-conventional structures called heavy-chain only antibodies (HcAbs) were detected in camelids and sharks, which spurred the development of third generation antibodies (A. Greenberg, D. Avila, M. Hughes 1995). Nowadays, the Immunoglobulin New Antigen Receptor (IgNAR) founded in sharks is

considered as an attractive candidate for applicable antibody technologies because of several advantageous properties of IgNAR compared to conventional mAbs, such as cryptic antigen recognition domain structure, small molecular size, fast secretion, stability about thermal, pH, and chemical environment (Müller et al. 2012; Juma et al. 2021; Flajnik 2016)

Mature IgNAR is organized with homodimers of clusters which one variable new antigen receptor (vNAR) and five constant new antigen receptors (cNAR) (Bojalil et al. 2013). Each cluster is formed with one variable (V), joining (J), constant (C) segment, and three Diversity (D) segments, and properties of IgNAR originated from their unique vNAR cluster structure (Stanfield et al. 2007). The vNAR cluster consists of CDR (Complementary Determining region) 1, 3, and HV (Hypervariable loops) 2, 4, and because CDR 2 region offset during evolution, vNAR possesses a longer CDR3 length and small molecular size of 12-15 kDa (Rumfelt et al. 2001; Cabanillas-Bernal et al. 2019). Especially the CDR3 region is a factor involved in most antigen-binding activities, and the CDR3 region obtains stability through the disulfide-bond derived from cysteine residue in the framework region (Nuttall et al. 2003; Diaz, Greenberg, and Flajnik 1998). Moreover, the vNAR does not have a light chain and CDR 2 region related to diversification, yet, the vNAR obtains the diversity by recombination of the VDDDJ segment that is concerned with the RAG (Recombinase Activating Gene), and RSS (Recombination Signal Sequence) and antigen-induced somatic hypermutation (Cabanillas-Bernal et al. 2019;

Zielonka et al. 2015; Barelle, Gill, and Charlton 2009).

The vNAR have been classified into five isotypes based on the number and distribution of non-canonical cysteine in each vNAR domain and the pattern of a disulfide bond (Kovalenko et al. 2013).

Type I vNAR, found in Nurse sharks (Ginglymostoma cirratum), has cysteine residue in the FR 2, 4 regions and possesses loop shape CDR3 derived from two pairs of disulfide bonds (Diaz et al. 2002; Feng et al. 2018; Stanfield et al. 2004). Type II vNAR found in most common sharks has cysteine residue in the CDR 1, 3 and possesses the protrude CDR3 structure derived from the lack of a cysteine residue in the CDR3 anchor. (English, Hong, and Ho 2020; Cheong et al. 2020; Kovalenko et al. 2013). Type III vNAR, found in newborn and immature sharks, has non-variable tryptophan (Trp) residue in the CDR1 and a short length of CDR3 structure, so the type III vNAR did not show sufficient diversity (Diaz et al. 2002; Liu et al. 2007). Type IV vNAR has only two canonical cysteine residues in their domain and, different from the other type of vNAR, the type IV vNAR does not have non-canonical cysteine residue, so type IV vNAR has more flexibility CDR3 structure. If the Type IV vNAR contained Tryptophan residue in the CDR1 region, that vNAR is called Type IIb vNAR. (Kovalenko et al. 2013; Kovaleva et al. 2014; Cheong et al. 2020; Juma et al. 2021). Type V vNAR has non-canonical cysteine residue in CDR3 and organize with disulfide bonds derived from cysteine residue in FR2, 4, and CDR1 regions (Cabanillas-Bernal et al. 2019).

Various platforms, including hybridoma cell, ribosomal display, mRNA display, phage display, have been used to display monoclonal antibodies and antibody-like proteins. Especially the phage displays have the advantage of a short production period and are cost-effective compared to the other platforms (Tsuruta, dos, and Moro 2018). The phage display technology is manufactured and utilized through phenotypes displayed the fragment into the surface protein of phage and genotypes that code foreign genes into the front of phage coat protein gene (Bazan, Całkosiński, and Gamian 2012). Nowadays, diagnosis of several diseases, vaccines and, enzymatic inhibition research using antibody technologies advanced due to the development of the phage display technique, and several studies have been conducted due to the various properties of vNAR and the applicability of vNAR to molecular imaging, diagnosis, and therapeutic applications (Deutscher 2010; Ubah et al. 2018).

In this study, we constructed the vNAR library using obtained sequence from the Cloudy catshark (*Scyliorhinus torazame*) and designed an artificial 16 NNK CDR3 primer. Also, we performed the bio-panning and ELISA assay to find the Hen-egg white Lysozyme A (Lysozyme A) positive clones for checking the operation of constructed phage library.

The VHSV (Viral Haemorrhagic Speticemia Virus) belongs to the *Novirhabdovirus* genus, *Rhabdoviridae* family, and negative-stranded RNA virus (Baillon et al. 2020). The genome of VHSV consisted of N

(Nucleocapsid), P (Phosphoprotein), M (Matrix protein), G (Glycoprotein), NV (Non-virion protein), L (Polymerase) and, it organized in order of 3'-N-P-M-G_NV_L-5' (Kong et al. 2019). Moreover, the VHSV classifying into four genotypes and several subgroups following their genetic variabilities in G and N protein (Einer-Jensen et al. 2004). Genotype I was isolated from European countries, Genotype II isolated from the Baltic Sea, Genotype III isolated from Norway, Genotype IV isolated from North America and Asian county (Snow et al. 2004). Moreover, the VHSV listed on OIE (World Organization for Animal Health) notifiable disease because of the severe economic damage in the aquaculture industry derived from high mortality in various species of cultured and wild fishes and geographical distributions (Skall, Olesen, and Mellergaard 2005)

Nowadays, various research of VHSV like Western-blot assay using purified monoclonal antibodies purifying from adjuvant mixed virus injected mice hybridoma cells (Kong et al. 2019) and neutralization assay of the VHSV N protein-specific monoclonal antibodies from myeloma cell is now in progress (Lorenzo, Estepa, and Coll 1996). Those researches were conducted with mammalian and cell experiments to purify mAb, so it seems to have several weaknesses, a long production period, and high-cost consumption (Falkenberg 1998).

Consequently, we performed a bio-panning and ELISA assay to find the VHSV positive phage with high specificity clones using a rapid, cost-effectively constructed phage library and secured the

sequence for further studies about the therapeutic and diagnostic application that, one of the prime purposes of this study.

II. Materials & Methods

1. Construction of Phagemid

1.1. cDNA precipitation and cloning vNAR region

To synthesized cDNA, we extracted the total RNA from leukocytes following procedure below. The peripheral blood was sampled from the caudal vein of two cloudy catsharks (40cm) using a heparinized syringe (50U/ml). The sampled blood was subsequently diluted with DMEM (Dulbecco's Modified Eagle's Medium, WELGENE) as 1:1 ratio and layered onto a 10ml of the 50% Percoll gradient solution. The blood sample was centrifuged at 5000rpm for 30mins at 4℃. Afterward, the leukocytes layer was collected and washed twice with PBS by centrifugation at 1500rpm from 10min at 4℃.

Total RNA was extracted from the leukocytes using a hybrid-R kit following the manufactures instruction. The 1ug RNA and Random hexamer primer (Elpis-biotech) was used to synthesized cDNA. The vNAR region was amplified with RT-PCR (reverse transcription PCR) and nested PCR using the PCR primer set which, was designed by aligning several sharks vNAR sequences in NCBI data base. Amplified vNAR fragment was checked through the electrophoresis and purified products using an Expin^{TM} Gel SV kit (Geneall) and was cloned into T easy vector (Promega).

Cloned vNAR sequence was analyzed by ABI 3730xl System (Macrogen sequencing service), and the framework region and type of purified vNAR were specified based on alignment data with predicted vNAR sequence from the other sharks in the NCBI database and NCBI conserved domain database. Moreover, the new primer pairs for the preparation of the phagemid insert were designed based on those data.

Table 1. Primers to cloning vNAR region

Name	Nucleotide sequence (5' to 3')	Application
Sto_IgNAR_F	TCGCTGACCATCAACTGYGCC	RT-PCR
Sto_cNAR1_R2	CACTTGACAGCTGTACGCAGAACC	RT-PCR
Sto_IgNAR_F	TCGCTGACCATCAACTGYGCC	Nested PCR
Sto_cNAR1_R1	CCGCTGATCAGACAAAGTAGCTGTA	Nested PCR

Note : primers were designed by aligning the predicted vNAR sequence of another shark in NCBI data base

1.2. Preparation of Phagemid insert

The cDNA was synthesized using total RNA from the spleen, peripheral leukocytes. Subsequently, the 1st PCR (95℃ 3min / 95℃ 30sec, 60℃ 30sec, 72℃ 30sec X 30cycle / 72℃ 7min) was performed using a synthesized cDNA template and primer pair described below to amplifying the vNAR region. Afterward, the PCR result was confirmed through the electrophoresis and purified the PCR sample using a Expin^{TM} Gel SV kit.

The artificial randomized CDR3 region was added to 1st PCR results through the 2nd PCR (95℃ 3min / 95℃ 30sec, 60℃ 30sec, 72℃ 45sec X 30cycle / 72℃ 7min) using a primer pair containing 16 NNK sequence. Moreover, the PCR result was also confirmed and purified as the previous method.

The purified vNAR products were cloned into T easy vector and analyzed the sequence. The vNAR single clone, which has in-frame (not containing stop codon and frameshifting), was selected based on the sequencing results. The cloned vector was prepared through the Exprep^{TM} Plasmid SV Mini kit (Geneall) as another 2nd PCR template.

The 3rd PCR (95℃ 3min / 95℃ 30sec, 60℃ 30sec, 72℃ 45sec X 30cycle / 72℃ 7min) was performed to add a SfiI restriction site in the 5' and 3' end of the 2nd PCR products. Moreover, the PCR results were purified and confirmed.

All the processes were repeated for the prepare phagemid insert

again. The purification step in the repeated process, was conducted through the Expin^{TM} PCR SV kit (Geneall).

Table 2. Primers for phagemid insert PCR

Name	Nucleotide sequence (5' to 3')	Application
IgNAR_new_F	GAAAGAGTGGAGCAAACACCACG	1st PCR
IgNAR_new_R	TCTGGCGTTCACCGTTAGCAT	1st PCR
IgNAR_new_F	GAAAGAGTGGAGCAAACACCACG	2nd PCR
IgNAR_randomized_CDR3_for_type_IV_R	TCTGGCGTTCACCGTTAGCATGGTGCCAGATCCGTAMNNMNNMNNMNNMNNNMNNMNNMNNMNNMNNMNNMNNMNNMNNMNNMNNMNNAGTTCTGCAGATGTAGGTGCCACCGTC	2nd PCR
IgNAR_new_F_sfiI_extension	GGTTTCGCTACCGTGGCCCAGGCGGCCGAAAGAGTGGAGCAAACACCACGTTATGC	3rd PCR
IgNAR_new_R_sfiI_extension	ATATGGTGCTGGCCGGCCTGGCCTCTGGCGTTCACCGTTAGCAT	3rd PCR
pComb_F_new	CCGATTCATTAATGCAGCTGGCAC	Sequencing
pComb_R_new	CACGTTTTCATCGGCATTTTCGG	Sequencing

(underline : SfiI restriction site)

1.3. Construction of Phagemid

The phagemid was constructed with a pComb3xss vector and phagemid insert. The pComb3xss was amplified following the manufacturer protocol of the Exprep™ Plasmid SV Mini kit (Geneall), and then prepared pComb3xss vector and phagemid insert was digested by SfiI restriction enzyme (New England Biolabs) at 50′c for overnight.

And then, all the enzymatic restriction reaction result was checked through the electrophoresis, and the restricted phagemid insert (352bp) and pComb3xss vector (3319bp) were purified as a previous method described in the phagemid insert preparation step. The enzyme ligation was conducted with 1ul of the T4 DNA ligase (Enzynomics) at a ratio of 1000ng: 400ng (pComb : Insert) overnight at 16℃. The ligation mixture was purified through the ExpinTM PCR SV kit and eluted ligated phagemid in nuclease-free water.

2. Construction of the Phage display ligation

2.1. Electrocompetent cell

To produce the *E. coli* ER2738 (Lucigen) electrocompetent cells, we picked one ER2738 colony into 20ml Luria-Bertani broth containing tetracycline (15mg/ml) (LB-tet) from an LB-Tet plate.

On the next day, 6ml overnight cultures were inoculated to LB broth and incubated at 37℃, 210rpm, until the OD600 value was 0.45~0.55. And then, the cultures were aliquoted to centrifuge bottles and centrifuged at 4℃, 6000rpm for 10mins.

The bacterial cell pellets were washed with deionized distilled water three times to remove the salt. After the last washing, all the supernatants were discarded and resuspended the bacterial cell pellet to 600ul of autoclaved 10% glycerol and aliquoted 50ul into a microcentrifuge tube (Axyzen) and stored at -80℃ until use.

2.2. Electroporation

The electroporation was performed with 50ul of electro-competent *E. coli* ER2738 and 1ug of the ligated phagemid. The electrocompetent cell was mixed with a phagemid vector, transferred to a 0.2㎝ gap electro-cuvette, and tapped to settle down to the bottom of the electro-cuvette. Then, closed the lid of an electro-cuvette, electroporation was performed at 2500V, 25㎳ using Eporator® (Eppendorf). After electroporation, all the bacterial cells were transferred to a microcentrifuge tube with 810ul of SOC media and recovered the bacterial cells at 37℃ for 1hrs 30mins.

The recovered bacteria were collected in one 50 ml conical tube and mixed with 1/3 volume of glycerol to store. And then, isolated the 100ul from the conical tube and 10-fold diluted with LB broth until the proper dilution level and spread the 100ul of diluted into LB plate containing Ampicillin (100mg/ml) (ALB), and incubated 37'c for overnight and the rest of the bacterial cells were stored at -80℃ until the phage rescue. The next day, counted the colony number and calculated the bacterial library size following the CFU calculation formula.

2.3. Rescue of the phage

The bacterial library stock stored at -80℃ was thawed at room temperature and aliquoted the same volume into two 50ml conical tubes (Hyundai-micro), and filled up to reach 40ml with ALB broth. Then, centrifuged the conical tube at 20℃, 3000rpm for 10 mins to remove glycerol from stock and repeated the centrifugation to remove extra glycerol from the library bacterial stock.

The bacterial pellet was resuspended in 30ml of 2XTY-AG (2XTY containing ampicillin (100mg/ml) and 2% glucose) and then inoculated to a 600ml of 2XTY-AG, and incubated at 210rpm, 37℃ until the OD600 value was 0.5~0.55.

After the incubation, inoculated an M13K07 helper phage (New England Biolabs) corresponding to 20 times the library size and incubated at RT for 30mins with stationary and performed at 210rpm, 37℃ for 30min with shaking.

The bacterial cultures were collected through the centrifugation at 4℃, 4500rpm for 10mins and the collected bacterial pellet was resuspended to 2XTY-AKI (2XTY containing ampicillin (100mg/ml), kanamycin (50mg/ml), and 0.1M IPTG) and incubated at 210rpm, 27℃ for overnight to induced the phage.

And then, the overnight cultures were centrifuged at 4℃, 6000rpm for 20mins, and all the collected supernatants were transferred to a 50ml conical tube, and added the supernatant 1/5 volume of PEG/NaCl

solution and mixed well through several times of inverting.

The 1st phage precipitation was conducted at 4℃ for 1hrs and then centrifuged at 4℃, 8000rpm for 15mins. After centrifugation, the supernatants were discarded, and the remaining phage pellet was resuspended in TBS buffer.

The 2nd phage precipitation was conducted to remove the extra bacterial cell from the phage solution. 1st phage precipitation solution was centrifuged at 4℃, 6000rpm for 10mins, and the supernatants were transferred to a new 50ml conical tube. Same with 1st precipitation, supernatant 1/5 volume of PEG/NaCl solution was added and mixed well. After 1hrs, centrifuged at 4℃, 8000rpm for 15mins, and then, the phage pellet was resuspended in TBS buffer and added the same volume of glycerol and stored at －20℃.

2.4. Phage Tittering

To titer the phage eluant, we picked one *E. coli* ER2738 colony into 4m LB-tet from an LB-Tet plate.

The next day, inoculated 100ul of overnight cultures to 10ml LB broth and incubated at 37℃, 210rpm, until the OD600 value was 0.5~0.55. Simultaneously, prepared the melted top agar using a microwave and adjusted the LB plate temperature to RT.

After the incubation, aliquoted 200ul of bacterial cultures to the micro-centrifuge tube and inoculated 10ul of phage properly diluted in TBS. Simultaneously, aliquoted the 3ml of top agar adjusted to RT to 5ml round tube and mixed with phage inoculated bacterial cultures using the 700ul of top agar.

All the mixture was poured into an LB plate and spread, and the plate was incubated at 37℃ overnight. The next day, counted the formed plaque on the LB plate and calculated the titer following the formula described below.

$$Phage\ Titer\,(PFU/ml) = \frac{Average\ of\ plaques}{Dilution\ factor\ X\ Inoculation\ volume\,(ml)}$$

3. Screening of Lysozyme A positive clone

3.1. “Yin-Yang” based bio-panning

Before every round of positive selection panning, negative selection panning was performed to remove BSA binding phage corresponding to the blocking reagent. The BSA (Bioshop) was dissolved in PBS to a final concentration of 3% and aliquoted 100ul to each well of Flat bottom 96 well (Corning). The 96 well microplate was put into an airtight container and coated at 4℃ overnight with agitation.

And then, discarded the coating solution and, each well was washed five times with washing solution (TBS containing 0.1% Tween-20), and then 100ul of blocking solution (3% BSA in TBS containing 0.05% Tween-20) was added to each well and blocked well at 4℃ for 1hrs. After the blocking, rinsed each well five times and inoculated 100ul of $1x10^{12}$ PFU/ml phage solution diluted in TBS and incubated at RT for 1hrs. After incubation, supernatant of each well containing unbound phage (negatively selected phage) was used to positive selection panning.

To screen the Lysozyme A positive clones, the Hen egg-white Lysozyme A was dissolved in PBS to a final concentration of 50㎍/㎖ and aliquoted 100㎕ of Lysozyme A solution to each well of Flat bottom 96 well then the 96 well microplate was put into an airtight container and coated at 4℃ overnight with agitation.

And then, discarded the Lysozyme A solution and washed it five

times with 100ul of washing solution, and blocked the wells with 100ul of blocking solution at 4℃ for 1hrs. After the blocking, rinsed each well five times and inoculated the negatively selected phage to each well, and the plate was incubated at RT for 1hrs.

After incubation, rinsed each well ten times with 100ul of washing solution, immediately the phage was eluted using 100㎕ elution buffer (Glycine-HCl, pH 2.2) at RT for 15mins. The phage eluant was neutralized with 16ul of neutralization buffer (Tris-HCl, pH 9.5) and collected into a 50ml conical tube. The phage eluant was stored at 4℃ until used for the phage amplification.

3.2. Phage amplification

To amplify the phage eluant, we picked one *E. coli* ER2738 colony into 4m LB-tet from an LB-Tet plate.

The next day, inoculated 250ul overnight cultures to 25㎖ LB broth, and incubated at 37℃, 210rpm until the OD600 value 0.5~0.6. Then, aliquoted 10ml of cultures to two 50ml conical tubes and inoculated the same volume of phage eluant to each conical tube and incubated at 210rpm, 37℃ for 1hrs. After the incubation, added 15ul ampicillin stock (100mg/ml) to each conical tube and 50ul of 2x1011 PFU/ml M13K07 helper phage diluted in TBS. The further procedure after helper phage infection was performed with the same method as the phage rescue step.

3.3. Phage Panning (further round)

The further round of negative selection panning was progressed with the same concentration of BSA and used TBS containing 0.5% Tween-20 (0.5% TBST) as a washing solution.

The 2nd round of positive panning was progressed using the 15ug/ml of Lysozyme A solution concentration and, the washing step proceeded with 0.5% TBST. Moreover, the 3rd round of positive panning was performed using the 5ug/ml of Lysozyme A solution and used 0.5% TBST in the washing step.

4. Confirmation of the Lysozyme A selected phage clones

4.1. Colony forming assay and single clone phage amplification

To obrain the bacterial clones, we picked one *E. coli* ER2738 colony into 4m LB-tet from an LB-Tet plate.

The next day, inoculated 100㎕ overnight cultures to 10㎖ LB broth and incubated at 37℃, 210rpm until the OD600 value 0.5~0.6. After the incubation, aliquoted 50ul of bacterial cultures to a microcentrifuge tube, and inoculated 10ul of properly diluted phage solution, and incubated the bacterial at 37℃, 210rpm for 1hrs. Then, spread the cultures into an ALB plate and incubated the plate at 37℃ overnight.

Followed by, picked a single colony in the ALB plate into 800ul of 2XTY-A broth (2XTY containing ampicillin (100mg/ml), and incubated at 37℃, 210rpm for 5hrs. After incubation, inoculated 50㎕ of 2x1011 PFU/ml M13K07 helper phages to each microcentrifuge tube, and further incubation was performed at 37℃, 210rpm for 2hrs. Then, centrifuged the bacterial cultures at 4℃, 3000rpm for 10min immediately discarded the supernatant. Afterward, resuspended the bacterial pellet into 800ul of 2XTY-AKI broth and induced the single clone phages through incubation at 37℃, 210rpm for overnight.

And then, centrifuged all the cultures at 4℃, 8000rpm for 10mins, and transferred the supernatant to a new microcentrifuge tube, and

used the supernatant was directly for a single clone phage Enzyme-Linked Immunosorbent Assay (ELISA) assay.

4.2. Single clone Phage Enzyme-Linked Immunosorbent assay (ELISA)

The Lysozyme A was dissolved in PBS to a final concentration of 10ug/ml and aliquoted 100ul diluted to each well of flat bottom 96 well plate. The plate was put into an airtight container and coated at 4℃ overnight with agitating.

And then, discarded the coating solution and, each well was washed three times with washing solution (TBS containing 0.05% Tween-20), and then 100ul of blocking solution was added to each well and blocked well at RT for 1hrs 30mins. After blocking, rinsed each well three times using 100ul of ELISA washing solution and inoculated 100ul of the single clone phage solution, and incubated at RT for 1hrs 30mins.

After the blocking, rinsed each well three times using 100ul of ELISA washing solution and inoculated the 100ul of Anti-M13 HRP conjugated Ab diluted at 1:1000 ratio in Ab buffered solution (TBS containing 0.5% BSA and 0.05% Tween-20) and incubated at RT for 1hrs 30mins. Afterward, rinsed each well three times using 100ul of ELISA washing solution and observed the OD405nm (Optical Density) following manufacturer protocol of 1-step ABTS solution and through Optima L-100XP.

Simultaneously, a Control ELISA assay of about 10ug/ml of Lysozyme A and the same concentration of BSA was performed using

rescued phage and 3rd round panned phage.

4.3. Sequence analysis of selected clones

The phagemid of the single clone phages colony that was observed more than twice OD405nm of other single clone phages in ELISA assay was extracted following the manufacturer protocol of the Exprep^{TM} Plasmid SV Mini kit and analyzed the phagemid sequence through ABI 3730xl System.

5. Prepartion of target antigen

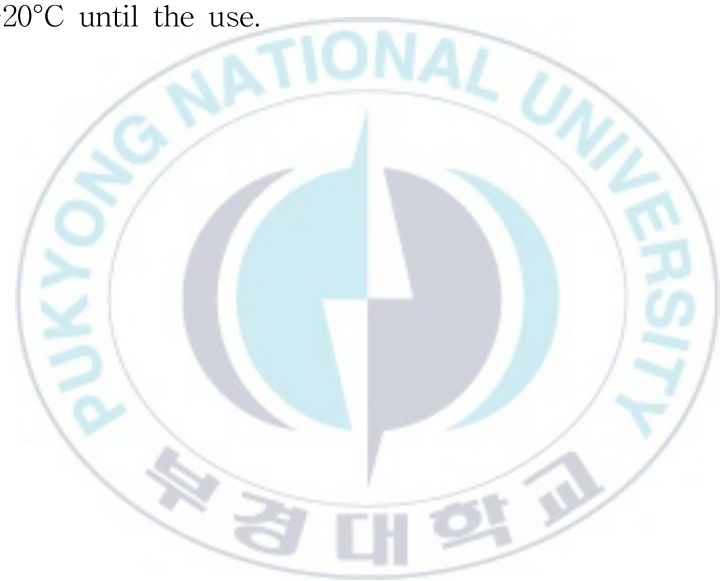
5.1. Cells and Virus

VHSV KJ2008 strain was propagated with EPC cell (Epithelioma Papulosum Cyprini). The EPC cell was cultured at 28°C with an L-15 medium containing 10% FBS (WELGENE) and 1% antibiotic -antimycotic solution (Sigma-Aldrich) (culture media). Before the VHSV amplification, the attached EPC cell was washed with DPBS and detached with 2ml of Trypsin-EDTA solution (WELGENE) for 5min. The detached cells were neutralized with the same volume of culture media, dispensed to a microcentrifuge tube, and centrifuged at 4°C, 3000rpm for 5mins. After the centrifugation, discarded the supernatant and resuspended cell pellet in culture media. Counted the cell number after the trypan blue (bio-solution) staining, and then the $1x10^7$ cells were seeded to a new T-75 flask (SPL), fulfill the flask up to 10ml using culture media, and cultured at 28'c for 2~3days. When the cells became a monolayer (80%), cell culture temperature was gradually adjusted the temperature from 25°C to 20°C and 20°C to 15°C for one day.

The next day, washed the cells with an L-15 medium, inoculated the 500ul of VHSV KJ2008 stock, and fulfilled the L-15 media containing 2% FBS, 1% antibiotic-antimycotic solution up to 10ml and cultured at 15°C until the complete CPE appeared. When the complete CPE appeared, the flask was frozen at -20°C and next day, thawed in RT. After repeated 2~3 times of this process, centrifuged at 20°C,

4000rpm for 10mins, gathered the supernatants and filtered with a 0.45um syringe filter.

The gathered VHSV KJ2008 stock by repeating those processes was thawed at RT, 10 ml of virus stock was layered to an ultracentrifuge tube containing 20% sucrose solution of 10 ml and centrifuged at 4°C, 20000rpm for 1hrs. After the ultracentrifugation resuspended the remaining pellet into 6ml of PBS then stored the virus stock at -20°C until the use.



5.2. Virus Titration

Virus titer was calculated through a plaque-forming assay. Before the plaque assay, the EPC cell was seeded in 35-dish, cultured at 28°C for 2~3days, and then adjusted the temperature to 15'c. Then the cells were washed with L-15 medium, fulfilled 1.8ml of L-15 containing 2% FBS, 1% Antibiotic-Antimycotic, inoculated the virus properly 10-fold diluted in L-15 medium, then incubated at 15°C for 2h. During the 2h incubation, the plaque media (L-15 medium containing 0.7% agarose) was prepared and cooled the medium to below 60°C to add the 1% Antibiotic-Antimycotic solution. After 2h, carefully removed the supernatant, poured 2ml of the prepared medium, and cultured at 15°C until the appropriate plaque appeared.

When the virus plaque appropriately appeared in the bottom of 35-dish, 10% formalin solution (Junsei) was added and fixed for 2h. Afterward, removed the hardened medium, stained the 35-dish using 1ml of crystal violet solution (Biosolution) for 5~10mins, and calculated virus titer based on the number of formed plaques and formula described below.

$$Phage\ Titer\,(PFU/ml) = \frac{Average\ of\ plaques}{Dilution\ factor\ X\ Inoculation\ volume\,(ml)}$$

6. Screening of VHSV positive clone

6.1. Phage Panning (1st round)

The negatively selected phages were prepared through the same method with Lysozyme A step.

The VHSV was diluted in PBS to a final concentration of $5x10^6$ PFU/ml and aliquoted 100ul diluted to each well of flat bottom 96 well plate. The plate was put into an airtight container and coated at 4℃ overnight with agitating.

And then, discarded the VHSV solution and washed each well five times with 100ul of washing solution, and blocked the wells with 100ul of blocking solution at 4℃ for 1hrs. After the blocking, rinsed each well five times and inoculated the negatively selected phage to each well, and the plate was incubated at RT for 1hrs.

After incubation, eluted the phage using 100ul of elution buffer, neutralized with 16ul of neutralization buffer, and eluted phage was amplified as the same method of the Lysozyme A step.

6.2. Phage Panning (further round)

The further round of negative selection panning was progressed with the same BSA concentration as the previous round, and a 2nd round negative selection panning was performed with 100ul of diluted phage solution at a concentration of $3x10^{11}$ PFU/ml. Also, the 3rd round negative selection panning was performed with 100ul of diluted phage solution at a concentration of $2x10^{11}$ PFU/ml.

A further round of positive selection bio-panning was progressed with the same VHSV concentration and methods as the previous step. However, in the 2nd round of positive selection panning, rinsed ten times with 0.5% TBST before the phage elution, and 3rd round rinsed fifteen times with 0.5% TBST before the phage elution.

7. Confirmation of the VHSV selected phage clones

7.1. Single clone Phage ELISA

ELISA assay was performed with 100ul of single clone phage gained from single clone bacterial colony from colony-forming assay, and 100ul of virus stock diluted at a concentration of $1x10^6$ PFU/ml in PBS. The blocking was conducted with 100ul of blocking solution at RT for 1hrs 30mins, 100ul of ELISA washing solution was used in every washing step. Moreover, the 100ul of Anti-M13 HRP conjugated Ab diluted at 1:1000 ratio in Ab buffered solution was used at RT for 1hrs 30mins. The fluorescence was formed through the manufacturer protocol of a 1-step ABTS solution and observed through Optima L-100XP.

To check the reproducibility, a single clone phages colony that was observed more than twice OD405nm of other single clone phages in ELISA assay was amplified. Then, 100ul of phage solution diluted at a concentration of $1x10^9$ PFU/ml in TBS was used to another ELISA assay that was conducted with the same methods to find definitive positive phage clones.

7.2. Sequence analysis of selected clones

The phagemid of the single clone phages colony that showed reproducibility in ELISA assay was extracted following the manufacturer protocol of the Exprep^{TM} Plasmid SV Mini kit and analyzed the phagemid sequence through ABI 3730xl System.

Ⅲ. Results

1. Construction of Phagemid

The cDNA was synthesized using total RNA extracted from spleen and peripheral leukocytes, and the vNAR fragment (Fig. 1A) was amplified through RT-PCR and nested PCR. Based on the result of NCBI blast analysis, it was confirmed that the cloudy catshark vNAR sequence has similarities to other sharks (Table. 3A), and the type of vNAR corresponded to type IV (Fig. 2). Moreover, the framework domain (Table. 3B) was specified through the NCBI conserved domain research analysis (Fig. 2) and alignment data with other sharks.

The vNAR fragment possessing artificial CDR3 (Fig. 1B) was amplified through the 2nd PCR, and based on sequence analysis (Fig. 3), found vNAR fragment containing intact protein residue and nucleotide sequence so, use the founded vNAR fragment to further round of PCR. Also, obtained the vNAR fragment containing the SfiI restriction enzyme site (Fig. 1C) through the 3rd round PCR. And then, the phagemid (Fig. 4A) was constructed through ligation of enzymatic restricted vNAR fragment and pComb3xss vector, and the result of ligation was confirmed through an increase in the size of the bound phagemid compared to the pComb3xss vector limited in gel electrophoresis (Fig. 4B).

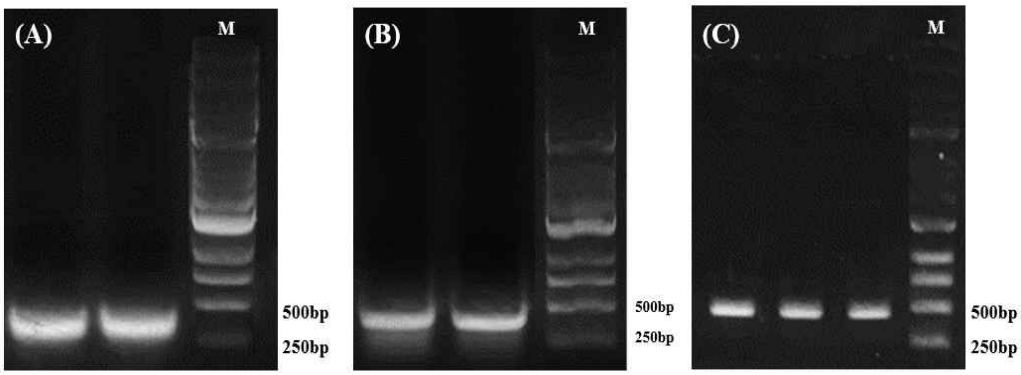


Figure 1. Results of each round PCR (A) Nested PCR (331bp) to amplifying vNAR fragment. (B) 2nd PCR (339bp) to synthesize the artificial 16 amino acid residue in CDR3. (C) 3rd PCR (390bp) to construct SfiI restriction enzyme site.

(A)

Name	Match to NCBI data base			
	Query Cover(%)	Identity(%)	Accession No.	Scientific name
Obtained vNAR	99	55.36	AF096019.1	*Ginglymostoma cirratum*
	99	57.14	AF096075.1	
	98	55.56	AY114849.1	
	98	57.41	AY114845.1	
	98	55.56	AY114874.1	
	98	55.56	AY114850.1	

(B)

Name	Accession No.	Range	E-value
Obtained vNAR	cd00099	3-107	1.86e-20

Table 3. Results of obtained vNAR sequence through the NCBI data base (A) NCBI Basic Local Alignment Search Tool (Blast) result (B) NCBI Conserved Domain Search result

Figure 2. Type classification and specification of framework region (underline : cysteine residue)

1 - GAAAGAGTGGAGCAAACACCACGTTATGCAACAAAATATATC

44 - GGCGAATCGCTGACCATCAACTGTGCCTACATTGACAGCAGC

87 - TATCCTCTGACCAGTACCTATTGGTATCGGACAGAACTGGGA

130 - TCAACGGACAAAGAAAGCATATCAATTGGTGGAAGATATGCT

173 - GTATCAGTCAACAAGAGAGCAAAAACGTTTTCTCTGCGAATC

215 - GGGGATCTGAGAGTTGAGGACAGTGGCACCTACATCTGCAG

256 - AACTGGGTCGCCGCCGGCGCCGGCTGTGTCGCATGGGCGGT

298 - CGTCGAGGTCTTACGGATCTGGCACCATGCTAACGGTGAACG

CCAGA

Figure 3. Sequence of selected clone for PCR template (underline : Artificial synthesized CDR3 region)

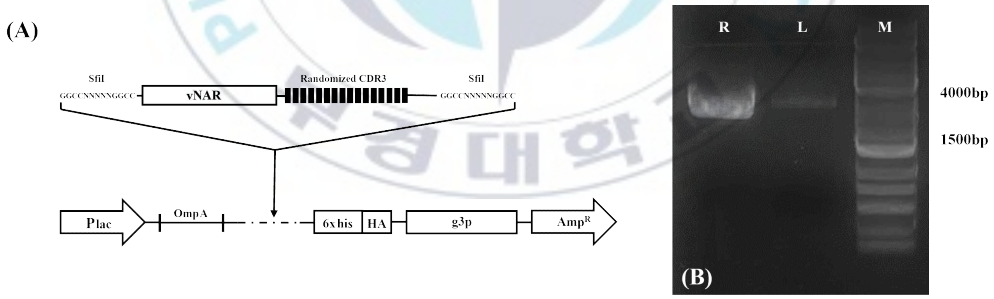


Figure 4. Phagemid construction result (A) Phagemid constriction design. (B) results of enzymatic ligation. R lane is SfiI restricted pComb3xss vector, the L lane is enzymatic ligated phagemid vector. and the M lane is DNA size marker.

2. Construction of library

The phagemid was transformed to *E. coli* ER2738 strain through electroporation repeatedly. The individual size of the constructed and gathered bacterial library was $7.4x10^9$ CFU/ml, and then the vNAR library was displayed on the M13 bacteriophage surface using fusion with gene III fragments.

Based on library sequence analysis, frame-shifting occurred in fourteen clones of analyzed sixty clones (23.3%) and nucleotide sequence deletion in seven clones of analyzed clones (11.6%). So, only thirty-nine clones contained in-frame artificially 16 NNK CDR3 sequence and length (65%) but, eighteen clones out of those clones possessed amber stop codon (TAG) in the CDR3 region (30%). Consequently, the size of the bacterial library, which corresponded to functional phage, was calculated at $4.8x10^9$ CFU/ml (Table. 4), and bacterial library size that had the potential to express functional, soluble form out of functional clones was calculated at $2.6x10^9$ CFU/ml.

The phage titer constructed with the collected bacterial library was calculated at $2.5x10^{12}$ PFU/ml through plaque assay (Fig. 5).

Trial	Efficiency (CFU/ml)	Trial	Efficiency (CFU/ml)	Total* (CFU/ml)	Calculated** (CFU/ml)
1	$3.2x10^6$	13	$4x10^6$		
2	$2.5x10^6$	14	$1.3x10^7$		
3	$2x10^6$	15	$3.2x10^7$		
4	$1.3x10^6$	16	$1.1x10^7$		
5	$1.3x10^6$	17	$1.4x10^7$		
6	$5.4x10^6$	18	$4x10^7$	$7.4x10^9$	$4.8x10^9$
7	$1x10^6$	19	$1.4x10^7$		
8	$4.9x10^6$	20	$1.9x10^7$		
9	$1.8x10^6$	21	$2.4x10^7$		
10	$1.5x10^6$	22	$1.6x10^7$		
11	$5.7x10^6$	23	$5x10^7$		
12	$2x10^6$	24	$3.5x10^7$		

* Total (CFU/ml) means gathered bacterial library size.
** Calculated (CFU/ml) means calculated the sequence analysis results.

Table 4. Efficiency of each electroporation and calculated size of bacterial library.

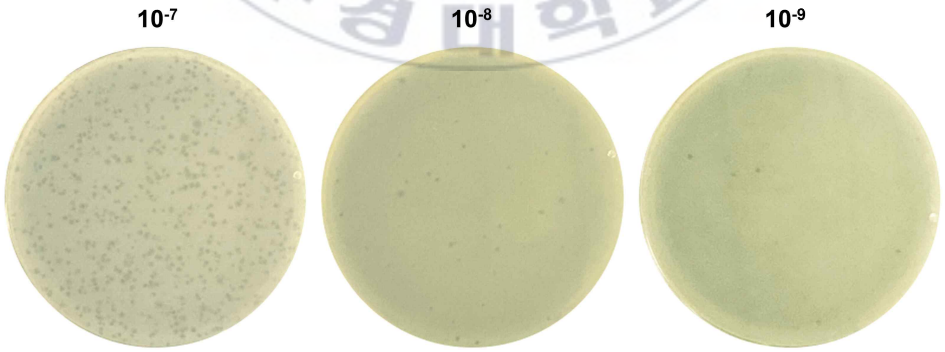


Figure 5. Phage plaque in LB agar plate.

3. Screening of Lysozyme A positive clone

The eluted phage gained after three rounds of bio-panning about Lysozyme A was amplified through re-infection to *E. coli* ER2738, and the titer was calculated through plaque assay. The amplified phage titer was calculated to 3.8×10^{11} PFU/ml in 1st round bio-panning, 1.6×10^{11} PFU/ml in 2nd round bio-panning, and 9×10^{10} PFU/ml in 3rd round bio-panning.

4. Confirmation of the Lysozyme A selected phage clones

The ELISA assay using ninety-six single clone phage was conducted, and based on the OD405nm data, confirmed that only four clones (#14, #49, #58, and #96) corresponded to Lysozmye A positive clones. The observed OD405nm absorbance was 1.584 in #14, 0.806 in #49, 1.788 in #58, 1.174 in #96 (Fig. 6A). Moreover, sequence analysis data of obtained clones (Fig. 7) shows that #14 and #58 clones contained in-frame sequence, but the same sequence, and #49 and #96 clones contained intact sequence but possessed the amber stop codon in the CDR3 region.

Through, control ELISA confirmed that the Lysozyme A specific binding phage population was increased through the three rounds of bio-panning yet, confirmed that the BSA binding phage population was also increased despite the "Yin-Yang" based bio-panning (Fig. 6B).

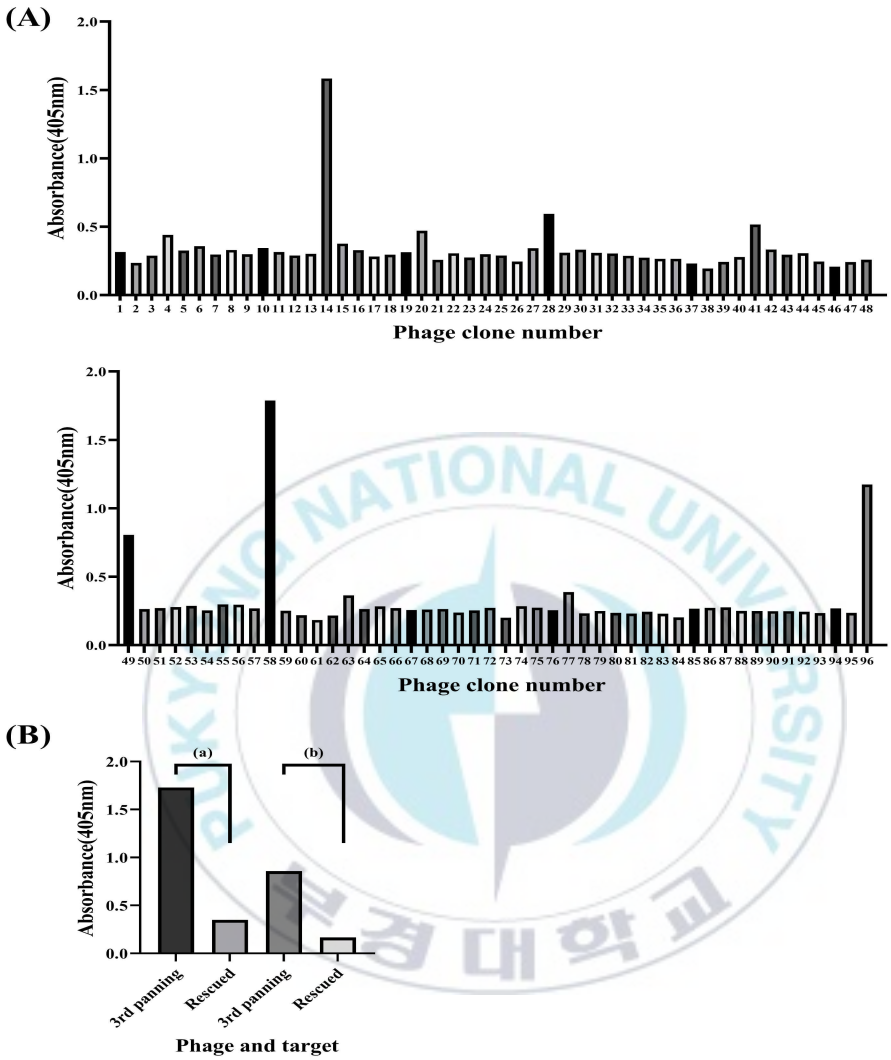


Figure 6. Results of Lysozyme A ELISA assay. (A) Randomly selected ninety-six single phage clones ELISA results. (B) Result of control ELISA, (a) is results about 10ug/ml Lysozyme A, and (b) is results about 10ug/ml BSA.

Figure 7. Sequence data of Lysozyme A positive clones.

5. Screening of VHSV positive clone

The titer of ultracentrifuged VHSV KJ2008 strian was $6x10^7$ PFU/ml.

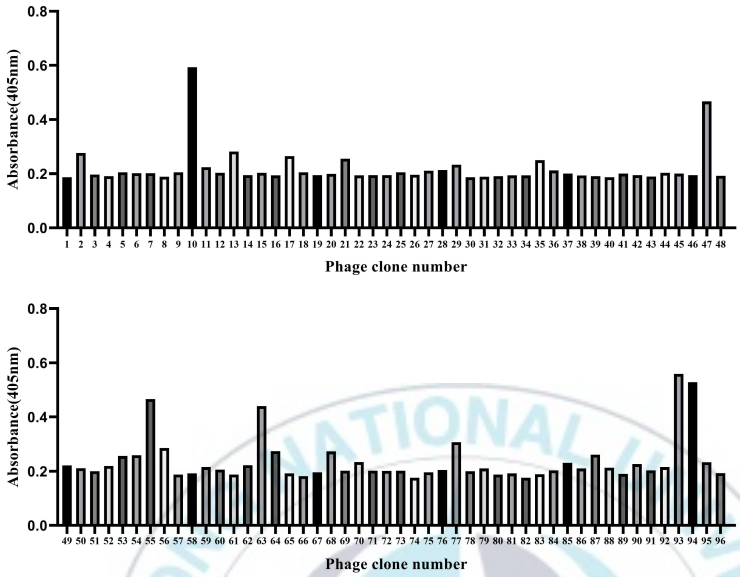
The eluted phage gained after three rounds of bio-panning about VHSV KJ2008 strain was amplified through re-infection to *E. coli* ER2738, and the titer was calculated through plaque assay. The amplified phage titer was calculated to $9.6x10^{11}$ PFU/ml in 1st round bio-panning, $6.4x10^{11}$ PFU/ml in 2nd round bio-panning, and $8.8x10^{10}$ PFU/ml in 3rd round bio-panning.

6. Confirmation of the VHSV selected phage clones

The three round of ELISA assay using ninety-six single clone phage was conducted, and based on the OD405nm data, confirmed that only eight clones (#94 in 1st ELISA, #42, #60, and #70 in 2nd ELISA, #55, #66, #91, and #92 in 3rd ELISA) expected to VHSV positive clones. The observed OD405nm absorbance was 0.529 in #94, 0.809 in #42, 0.402 in #60, 0.409 in #70, 0.315 in 55, 0.485 in 66, 0.488 in 91, 0.944 in 92 (Fig. 8). Moreover, in the ELISA assay result to confirm the reproducibility, only two clones (#42, #92) showed reproducibility, and the observed OD405nm absorbance was 1.568 in #42, 1.892 in #92 (Fig. 9), so those two clones were confirmed as VHSV positive clones.

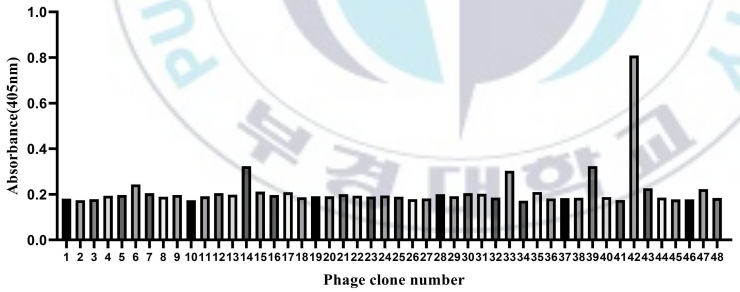
The sequence analysis data of obtained clones (Fig. 7) showed that #42 and #92 clones contained in-frame sequences and did not contain an amber stop codon in the CDR3 region (Fig. 10).

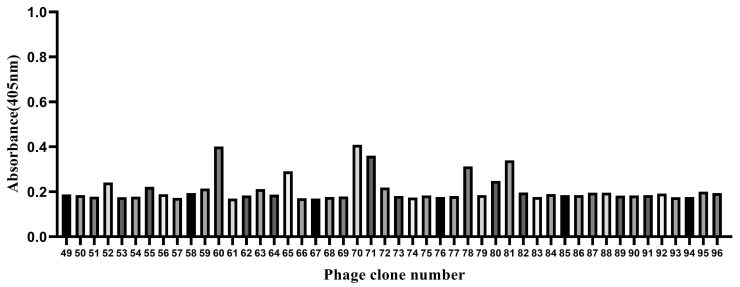
(A)


Absorbance(405nm)
0.0
0.2
0.4
0.6
0.8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
Phage clone number

Absorbance(405nm)
0.0
0.2
0.4
0.6
0.8
49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96
Phage clone number

(B)


Absorbance(405nm)
0.0
0.2
0.4
0.6
0.8
1.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
Phage clone number


Absorbance(405nm)
0.0
0.2
0.4
0.6
0.8
1.0
49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96
Phage clone number

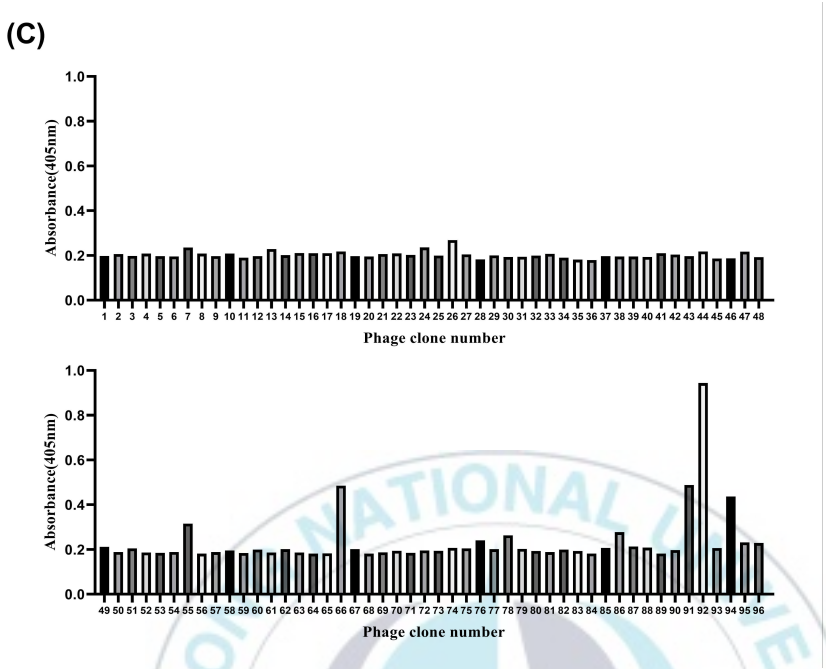


Figure 8. Results of VHSV ELISA assay. (A) 1st trial ELISA OD405nm absorbance results. (B) 2nd trial ELISA OD405nm absorbance results. (C) 3rd trial ELISA OD405nm absorbance results.

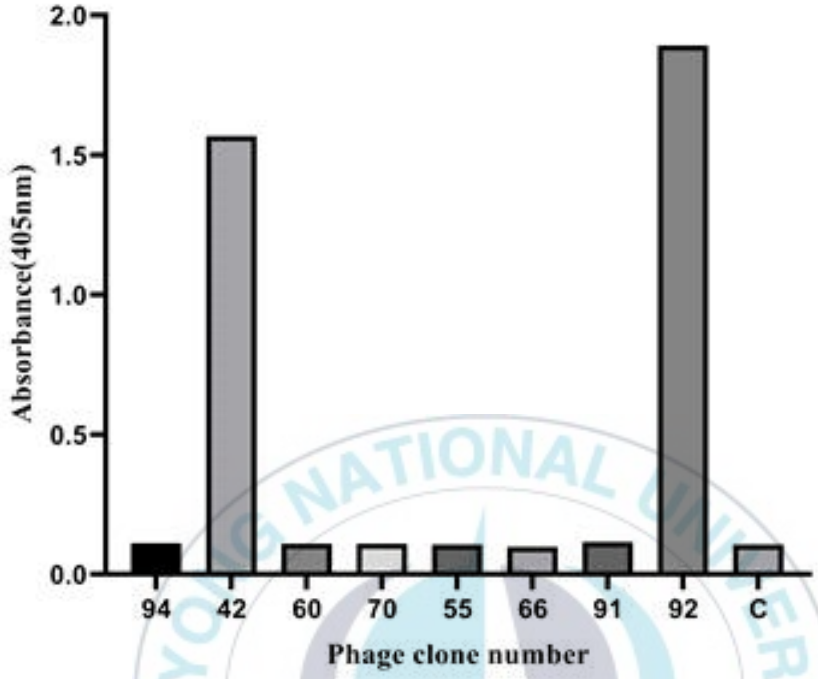


Figure 9. Results of reproducibility check ELISA assay. C lane is ELISA assay using rescued phage about VHSV KJ 2008 strain.

Figure 10. Sequence data of VHSV positive clones.

Ⅳ. Discussion

In this study, we construced the vNAR phage library display from two cloudy catsharks. The vNAR fragment was amplified using total RNA extracted from peripheral leukocytes but, vNAR fragments were also obtained from total RNA from the spleen so, we used total RNA extracted from both peripheral leukocytes and spleen. To grant the diversity to amplified vNAR fragment, we synthesized the artificial CDR3 region that possesses 16 amino-acid residues which, exists at a high rate (Cabanillas-Bernal et al. 2019), instead of the immunization method that takes several months to find sufficient antigen (Leow et al. 2018). Because the classified vNAR fragment type was type IV and the structure that does not contain cysteine residue in CDR3 shows more flexibility in antigen recognition than contained one or more cysteine residue in CDR3 (Zielonka et al. 2015), we designed an artificial CDR3 synthesizing primer with only 16 of NNK sequence. In the early stage of phagemid insert preparation, we synthesized the artificial CDR3 region into non-fixed vNAR fragments and analyzed the sequence (data not shown). Based on the sequence analysis result, the artificial CDR3 was synthesized well but occurred many nucleotide deletions in the framework region. So, we chose and used the specific clone that has the in-frame sequence as a phagemid inserts preparation PCR template.

To find the high specific clones through the antibody library system, construction of the large size of libraries is crucial (Maynard et al, 2000) and, the library size used in other studies was $3.7x10^7$ to

3x10^9 individual clones (Ohtani et al. 2013; Shao, Secombes, and Porter 2007; Moutel et al. 2016) so, we repeated the electroporation to generate enough libraries.

The size of calculated library size was $7.4x10^9$ CFU/ml. The 35% of those clones had nucleotide deletion and frame-shifting derived from 3′ to 5′ exonuclease activities of the Taq polymerase and, PCR error. 30% of the rest clones had an amber stop codon (TAG) derived from the character of the NNK sequence that can code the 20 amino-acid and amber stop codon in the CDR3 region. So, only 30% of clones ($2.2x10^9$ CFU/ml) was corresponded to be expressed functional, soluble form. In this way, constructed bacterial size is sufficient for antigen recognition assay, but the proportion of clones with the potential to be expressed in a functional, soluble form appeared to be slightly lower than 35% to 45% shown in other studies (Dooley, Flajnik, and Porter 2003; Ohtani et al. 2013). Those two studies used the PCR, the same as our method, to construct the phagemid so, if we optimize the PCR condition in phagemid insert preparation, it seems possible to improve the proportion of the functional, soluble form bacterial clones.

In most studies, they performed two to three rounds of bio-panning to find specific clones (Lee et al. 2003) so, we did three rounds of bio-panning to find Lysozyme A, VHSV positive clones. Moreover, to eliminate the negative binder as much as possible, we performed the “Yin-Yang” method in every bio-panning (Coelho et al. 2015). However, we confirmed that the BSA binding phage population

increased as the positively selected phage population increased. So, we could not eliminate the BSA binders absolutely through the "Yin-Yang" method.

In the results of the ELISA assay about Lysozyme A to confirm the operating of constructed phage library and VHSV to check the potential to specific antigen application, we could not find many positive clones. The reason why can not found many positive clones is the generated incomplete phage in the phage re-infection procedure (Bruin et al. 1999) and increased non-specific binders (Lim, Woo, and Lim 2019). Especially in the VHSV procedure, remaining cell debris, FBS components despite being dissolved in PBS after ultracentrifugation, and the irregular shape of virus particles generated by ultracentrifugation (Sugita et al. 2011) disturb the bio-panning procedure so, it was difficult to find many positive clones. Nevertheless, we found two VHSV positive clones and acquired the sequence of those clones.

A present study about the VHSV is incomplete so, it is necessary to find more VHSV positive clones through the ELISA assay. Furthermore, we confirm the target virus particle through the western blot assay, perform the neutralization assay for checking the potential of therapeutic application, and additionally should check the VHSV recognition ability through applicating to VHSV infected cells or tissues.

Consequently, we constructed the vNAR phage display library possessing several properties such as structural stability, small size, fast secretion, and high antigen recognition ability through the bacterial

platform that is rapid and cost-effective. Also, based on the results of the studies and if we confirm the results of further studies, phage library display will be applied to diagnostic and therapeutic assay and several pathogens, not only the other Rhabdoviruses.

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