Selection of single-chain antibody variable fragment (scFv) against feline immunoglobulin G for biosensor applications

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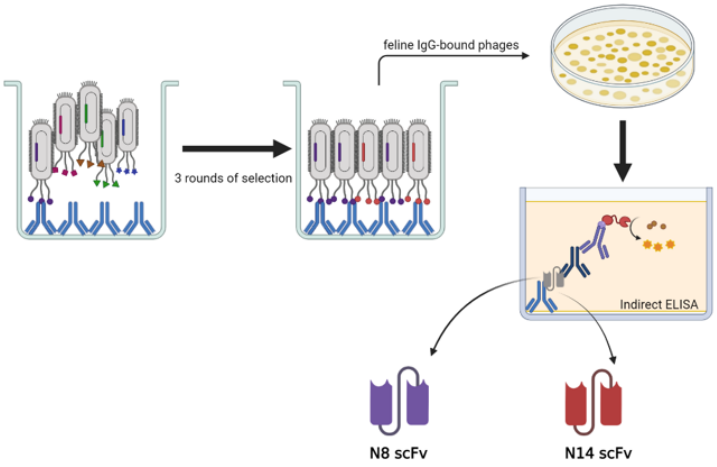
Abstract: Feline infectious disease is one of the most commonly health problems and leading to cause of death for over decades such as Toxoplasmosis, Feline leukemia virus (FeLV) disease, especially Feline immunodeficiency virus (FIV) disease. The early of diagnosis is essentials to increase the chance for successful of treatment. Measurement of IgG are generally considered of an individual's immune status for particular pathogen. In addition, the antibodies specific to feline IgG is an essential component for develop the detection kit. However, conventional monoclonal antibodies have been concerned in term of time-and cost-consuming production, animal requirement, and unstable under harsh conditions. Currently, recombinant antibody fragment technology becomes an effective strategy to rapidly produce binder molecules such as single-chain variable fragment (scFv) that are time-and cost-effective, batch consistency, and pilot scale production. Herein, this study aimed to select feline IgG-bound scFv by using phage display technology. Three rounds of biopanning was done against purified feline IgG. Out of the soluble 8 scFv clones were subjected to determine the binding ability against the target by indirect ELISA. N8 and N14 clones elicited the highest binding capacity against the purified feline IgG. The results from PCR and western blot analysis were done to reveal the expected molecular size in term of DNA (~1000 bp) and protein (~29 kDa). Taken together, this study successfully selected the feline IgG-bound scFv that could be further engineered, purification and development for FIV detection kit.

Graphical abstract:



Keywords: feline IgG; phage display; single-chain variable fragments (scFv)

1. Introduction

Feline immunodeficiency virus (FIV) disease has become the most commonly health problem and cause of death in domestic cats leading to increasing annual mortality rate. The FIV-infected cats may not show any symptoms for years. Early diagnosis has been concerned to follow disease progression. However, lack of rapid detection leads disease progression leading to transmission the other cats and death [1]. Development of rapid detection kit is challenging. IgG level is usually used as a target for feline infectious detection that have widely developed as a commercial detection kit in the market worldwide[2]. Monoclonal antibody mostly has been used to detect/determine the IgG level. Unfortunately, the production of monoclonal antibody has some limitations such as their large size (~ 150 kDa), instability as well as time-and cost-consuming production leading to increasing the cost of the kit. To overcome this concerned, phage display has become an effective strategy to rapidly produce binder molecules such as single-chain variable fragment (scFv) that are time-and cost-effective and mainly animal host dependent.

scFv is a small fragment of antibody (~ 25-30 kDa) that consists of variable regions of heavy (VH) and light (VL) chains, which are joined together by a flexible peptide linker because of scFv are significantly smaller than full-length antibodies they can bind to target tightly, easily expressed in bacterial system and more stable than antibodies, this molecule have been reported for development in feline detection kit such as feline calicivirus disease and also another class of Ig which are IgE [3, 4].

In this study, to develop feline detection kit, phage display scFv antibody libraries were used to select scFv against feline IgG. The selected scFv can utilized and decrease production cost of detection kit.

2. Materials and Methods

2.1 Materials

Yamo-I phage display library was kindly provided by Prof. Montarop Yamabhai laboratory as described previously.[5] *E.coli* TG1, HB2151 and KM13 helper phage was obtained from the Tomlinson libraries and was propagated as described in the MRC phage display protocols.[6]

2.2 Biopanning of feline IgG-bound scFv by using phage display library

Biopanning process was performed by using purified F IgG (Bethyl, US) as a target. A 96 wells microtiter plate (Thermo Fisher Scientific, US) was coated with the target and incubated at 4 °C for 16-18 h. The excess of the target was removed by washing with 0.05% PBST. The coated well was then blocked with 5% BSA at 37 °C for 1 h. At the indicated time, the well was washed with 0.05% PBST. Yamo-I phage display library (~10^9 pfu/mL) was added and incubated at 37 °C for 2 h. After phage enrichment, unbound phages was eliminated by extensive washing. All bald phages were removed by trypsin digestion. The feline IgG-bound phages were eluted by adding 0.2 M glycine solution pH 2.2 and neutralizing with 1 M Tris-HCl pH 9.2. For phage titter determination, the eluted phages were diluted with 10-fold serial dilution. The diluted phages was incubated with mid-log phase *E.coli* TG1 for phage infection. The infected *E.coli* TG1 was grown on LB agar containing 100 μg/mL ampicillin. For phages propagation, superinfection or phage rescue was performed by incubating KM13 helper phages with *E.coli* TG1 harboring phagemid. The supernatant of KM13 helper phage-infected *E.coli* TG1 was precipitated with PEG precipitation. The precipitated phages were suspended and subjected to the next round. Biopanning was done in three rounds following the procedure as described above.

2.3 Binding Screening of feline IgG bound-scFv clones by indirect enzyme-linked immunosorbent assay

To prepare soluble feline IgG bound-scFv, the randomly picked *E.coli* TG1 harboring phagemid of the third round biopanning were rescued by KM13 helper phages and precipitated by PEG precipitation method following the procedure as described above. The individual phages were performed for infection of non-suppressor *E.coli* HB2151 strain. Briefly, mid-log phase of *E.coli* HB2151 was infected with the individual phage clones. The infected *E.coli* HB2151 were grown for soluble scFv production under IPTG induction. The supernatant of the IPTG-induced *E.coli* HB2151 were collected for binding screening. Indirect ELISA was performed. The purified feline IgG was immobilized on a 96-well microtiter plate with PBS buffer pH 7.4 at 4°C for 16-18 h and BSA was used as a control. At the time, the excess of immobilized antigens was removed by washing and blocking with 5% skimmed milk at 4°C for 1 h. The supernatant containing soluble scFv was added to antigen-immobilized well and incubated at 4°C for 16-18 h. For detection, hexahistidine tag antibody (1:5000, Thermo Fisher Scientific, US) and HRP conjugated goat-anti mouse (1:5000, Thermo Fisher Scientific, US) were performed as a primary and a secondary antibody, respectively. The color of the reaction was developed with TMB substrate (Life Technologies, US). The reaction was quantified by measuring the absorbance at 450 nm (A_{450}). scFv clones that exhibited A_{450} of feline IgG 2-fold higher than A_{450} of BSA were chosen for the further analysis.

2.4 scFv polymerase chain reaction and Western blotting

For polymerase chain reaction, phagemid extraction of positive scFv clones were done by using GF-1 plasmid DNA extraction kit (Vivantis, Malaysia) and were used as a DNA template. The forward and reverse primer were LMB3 (5′- CAG GAA ACA GCT ATG AC-3′) and pHEN seq (5′- CTA TGC GGC CCC ATT CA-3′), respectively. The PCR reaction consisted of Dream Tap buffer (10X, 5.0 µL), dNTP (2.5 mM, 5.0 µL) (Thermo Fisher Scientific, USA), forward primer (5 µM, 5.0 µL), reverse primer (5 µM, 5.0 µL) and TaqDNA polymerase (Thermo Fisher Scientific, USA) (1.0 U/mL, 0.2 µL). For PCR amplification, PCR sample was first denatured at 95°C for 10 min. Subsequently, 40 cycles of denaturation (95°C/30 sec), annealing (60°C/30 sec), and extension (72°C/10 sec) were performed. The PCR product was analyzed by gel electrophoresis (1% agarose gel).

For expression screening, western blot analysis was performed. The pellet of each ELISA positive clones was harvested and separated with 12% SDS-PAGE and transferred to nitrocellulose membrane. The transferred membrane was blocked with 5% skimmed milk at 4°C for 1 h. After washing, the expression of scFv was visualized by hexahistidine tag antibody (1:5000, Thermo Fisher Scientific, US) and incubated at 4°C for 16-18 h. After washing, HRP conjugated goat-anti mouse (1:5000, Thermo Fisher Scientific, US) was used for probing. Finally, the membrane was detected by LumiFlash™ chemiluminescent substrate (Enegenesis biomedical, Taiwan).

2.5 scFv-bound feline IgG structure modelling

scFv clone N8 amd N14 were done plasmid extraction and determined DNA sequence. After that the sequence was translated by using expasy translate tool (https://web.expasy.org/translate) and the amino acid sequences were used for construct protein structure by using swiss model webserver (https://swissmodel.expasy.org/) and CDR regions were determined by the international ImMunoGeneTics information system® (http://www.imgt.org/)

3. Results

3.1. Feline IgG-bound phage enrichment by using phage display library

To select scFv against feline IgG, naïve Yamo-I phage display library were used in biopanning procedure. 10^9 pfu/mL of the library was used as an input titer. After each round of biopanning, phage enrichment rate (output/input phage concentration) was calculated to determine binding capacity of each round biopanning under gradually decreasing feline IgG concentration. As anticipated, the phage enrichment rate was gradually increased even decreasing the target. The highest phage enrichment rate was shown in the third round biopanning, approximately 10-fold (Fig.1). After the third round of biopanning, the sixty-five colonies were randomly picked for expression screening. The individual phages were propagated for *E.coli* HB2151 infection. The infected *E.coli* HB2151 colonies were picked for expression screening. 8 out of 65 clones were expressed under IPTG induction. The scFv-expressed *E.coli* HB2151 clones were subjected to the further analysis.

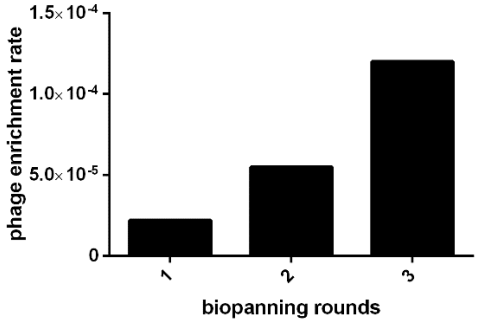


Figure 1. Enrichment of feline IgG-bound phages for three rounds of biopanning.

3.2 Soluble feline IgG-bound ScFv binding determining by Indirect ELISA

The scFv-expressed *E.coli* HB2151 clones were grown and expressed under the described condition above. Accordingly, the scFv gene was constructed in-frame with secreting signal peptide. The supernatant of scFv-expressed *E.coli* HB2151 clones were collected for binding determination. For indirect ELISA, the feline IgG and BSA were separately immobilized in a 96-microtiter well as a target and an antigen control, respectively. Surprisingly, N8 and N14 demonstrated the highest binding capacity among the other clones and the signals are also 2-fold higher than antigen control (Fig.2). Taken together, N8 and N14 would be promising clones for feline IgG detection.

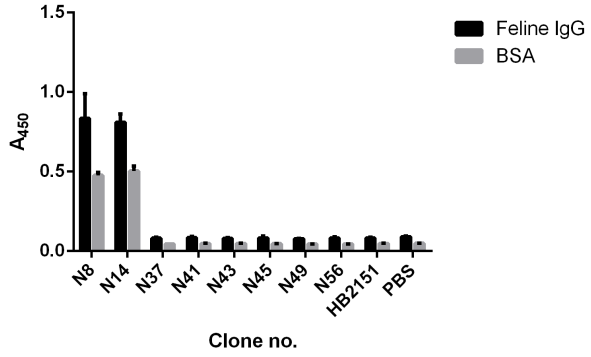
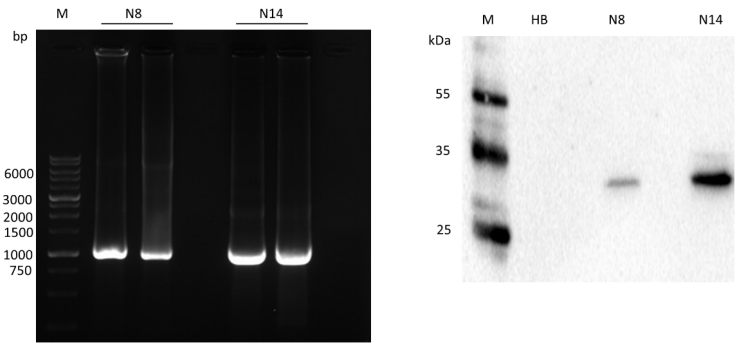


Figure 2. Screening of feline IgG-bound scFv were performed using indirect ELISA. BSA as antigen control, HB2151 and PBS as negative control. N8 and N4 clones showed All data are expressed as the mean±SD of triplicated.

3.3 Biochemical characterization of the feline IgG-bound scFv

Molecular sizes of N8 and N14 were confirmed by PCR and western analysis. Vector-specific primers were used to amplify the scFv gene. Under the PCR specific condition, the expected size was shown in ~1000 bp on agarose gel in both N8 and N14 (Fig.3A). Moreover, western blot results were also confirmed the molecular weight of scFv (~29kDa) (Fig.3B), which *E.coli HB2151* lysate did not express any relevant protein bands. Taken together, N8 and N14 were successfully expressed in bacterial system. However, large-scale production would be further optimized before purification process.

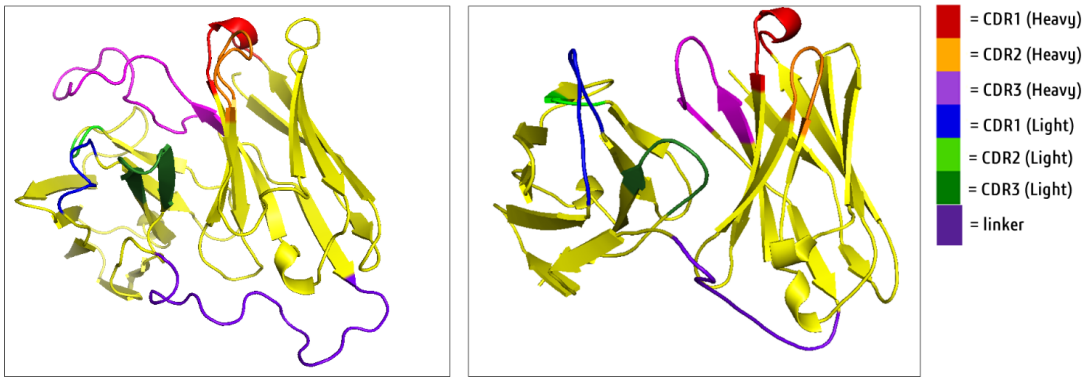


(**a**) (**b**)

Figure 3. Determination of scFv positive clone. (a) PCR amplification with scFv-specific primer, positive bands were 1000 bp. (b) Western blot analysis of expressed scFv in E.coli, the protein band were 29 kDa; M in (a) DNA ladder bp; M in (b) protein marker in kDa; HB in (b) HB2151 as a negative control; N8 and N14 are scFv clones.

3.4 scFv-bound feline IgG protein modeling

3D structure of scFv-bound feline IgG was successfully built by Swiss model. 3D structure of N8 (Fig. 4a) and N14 (Fig. 4b) was showed the structure of scFv that consisted of variable region of heavy chain and light chain are joined together by a flexible peptide linker.



(a) (b)

Figure 4. 3D structure of scFv-bound feline IgG . (a) N8 3D structure (b) N8 3D structure. Red indicated CDR1 of heavy chain, orange indicated CDR2 of heavy chain, purple indicated CDR3 of heavy chain, blue indicated CDR1 of light chain, light green indicated CDR2 of light chain, dark green indicated CDR3 of light chain and purple indicated peptide linker.

4. Discussion

Domestic animals, especially cats have annually been reported with increasing the mortality rate from infectious diseases. However, the early diagnosis and development of rapid detection kits are still challenging. IgG is one of antibody class that are produced by immune cells after infection that would be a promising target for biosensor development. Herein, enrichment selection of single-chain variable fragment (scfv) was performed to select feline IgG-bound scFv for biosensor development.

For decades, phage display technology has demonstrated its advantage over conventional hybridoma technology for monoclonal antibody production. An antibody fragment format becomes a current interesting molecule for biosensor development. Introducing scFv gene to phage display technology elicits a powerful and robust strategy to select the binder molecule against various targets within a couple week [7-9]. After the successful three round biopanning, increasing phage enrichment revealed the sixty-five clones that were able to bind to the target. N8 and N14 were the potential scFv candidates that exhibited the highest binding capacity over the others and antigen control. Moreover, PCR and western blot results indicated that N8 and N14 elicited scFv character in term of DNA and molecular weight [10]. After sequencing, deduced amino acid sequences of N8 and N14 also confirmed the scFV structure components. These selected scFv would be valuable tool for biosensor application.

5. Conclusions

In this study, 3 rounds of selection with biopaning procedure was performed by using purified feline IgG as a target. Total 65 scFv clones were randomly picked for expression screening and 8 clones were selected for determined the binding affinities by indirect ELISA. N8 and N14 had the binding potential to feline IgG. The result from PCR and western blot analysis also confirmed a characteristic which are contains expected size (~ 1000 bp) and 29 kDa of scFv.

These selected scFv clones will be further engineered, purification and development for feline detection kit.

Author Contributions: Conceptualization, N.R., M.Y., LT and K.C.; methodology, N.R. and M.Y.; validation, N.R. and L.T.; formal analysis, N.R.; investigation, L.T. and K.C.; data curation, N.R.; writing—original draft preparation, N.R.; writing—review and editing, L.T. and K.C.; supervision, K.C.; project administration, M.Y., L.T. and K.C; funding acquisition, K.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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