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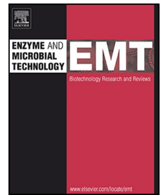

# Development of high-affinity single chain Fv against foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) is caused by the FMD virus (FMDV) and results in severe economic losses in livestock farming. For rapid FMD diagnostic and therapeutic purposes, an effective antibody against FMDV is needed. Here, we developed a high-affinity antibody against FMDV by FACS-based high throughput screening of a random library. With the FITC-conjugated VP1 epitope of FMDV and high-speed FACS sorting, we screened the synthetic antibody (scFv) library in which antibody variants are displayed in the periplasm of *Escherichia coli*. After three rounds of sorting, we isolated one antibody fragment (#138-scFv) against the VP1 epitope of FMDV. Next, to improve its affinity, a mutation library of #138-scFV was constructed by error-prone PCR and screened by FACS. After three rounds of sorting, we isolated one antibody (AM-32 scFv), which has a higher binding affinity ($K_D$ = 42.7 nM) than that of the original #138-scFv. We also confirmed that it specifically binds to whole inactivated FMDV.

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## 1. Introduction

Foot-and-mouth disease (FMD) is one of the most devastating diseases in wild and domestic cloven-hoofed animals [1,2]. In 2011, a FMD outbreak in Korea consisting of 3.5 million infected livestock, especially swine and cattle, caused huge economical loss [3]. The FMD virus (FMDV), which causes FMD, is a picornavirus, the prototypic member of the genus *Aphthovirus*, and is classified with seven distinct serotypes [O, A, C, Asia 1, SAT1, SAT2, and SAT3] by antigenic diversity [4]. Serotype-O is the most prevalent and occurs worldwide, including in Korea [5]. To rapidly and effectively control FMD, we must identify FMDV infection at an early stage, which requires the development of rapid and reliable diagnostic systems. Several diagnostic methods, including ELISA and PCR, have been used, and the antigen-detecting ELISA with an antigen-specific antibody is currently preferred [6]. In these ELISA-based methods, the essential and key component is the antibody, which has a strong binding affinity and high specificity to FMDV serotypes.

Biotechnologies for antibody engineering allow the development of various forms of antibodies for diagnostic and therapeutic usage [7]. One of these techniques focuses on minimizing the size of the antibody, resulting in various antibody fragments that induce passive immunity and these can be used for biosensors, drug targeting, and drug delivery systems [8,9]. These antibodies, including monovalent antibody fragments (Fab), single-chain variable fragments (scFv), and single domains, are well established as an alternative to conventional monoclonal antibodies such as immunoglobulin G (IgG). Conventional monoclonal antibodies are fully structured and complex proteins, are mostly produced in mammalian hosts, and are relatively expensive. In contrast, recombinant small antibody fragments can be economically produced on a large scale from bacterial expression systems and the antibody properties (affinity, specificity) can be easily engineered [7,10]. Additionally, antibody fragments like scFv and Fab can be used to detect various antigens such as haptens, proteins, and whole pathogens [11].

FMDV has an icosahedral shell composed of 60 copies of each of four structural proteins (VP1–VP4), and among four proteins, VP1 is highly immunogenic and has been proposed to play a major role in serotype specificity and eliciting an immune response [1,4,12]. The G–H loop (amino acids 140–160 at the C terminus) of VP1 is a major antigenic site and is responsible for the induction of protective neutralizing antibodies [13]. Therefore, VP1 epitope could be a useful target for an antibody specific to FMDV. Here, we sought to isolate an scFv specific to the VP1 epitope of serotype-O FMDV by screening a synthetic antibody library constructed in *E. coli*. For the screening of library, we used a FITC-conjugated VP1 epitope

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of FMDV and a FACS-based high-throughput screening, which we previously established [14]. We improved the affinity of the isolated antibody by an affinity maturation process and determined its specific binding to inactivated FMDV.

## 2. Materials and methods

### 2.1. Bacterial strains

The *E. coli* strains and plasmids used are listed in Table 1. *E. coli* Jude-1 [15] was used for the library construction and screening. *E. coli* MG1655 was used for the production and purification of the isolated antibodies (scFv).

### 2.2. Error-prone PCR

For the affinity maturation, random library was constructed by error-prone PCR protocol [16]. Briefly, 10 μL of 10 × PCR buffer (without $MgCl_2$, Takara Bio Inc., Shiga, Japan) was combined with dNTP mix (2.0 μL of 10 mM dGTP, 3.5 uL of 10 mM dATP, 4.0 μL of 10 mM dCTP, 1.4 μL of 100 mM dTTP), 10 μL of both 5 mM $MnCl_2$ and 25 mM $MgCl_2$, and 10 ng of DNA (#138-scFv). Polymerization was performed by low-fidelity rTaq DNA polymerase (Takara Co., Japan) with the primers Hv-Amp (5′-GATTGTTATTACTCGCGG) and Lgt-Amp (5′-GGCCGCGAATTCG). The PCR products were digested with *Eco*RI and *Nco*I and ligated into pMoPac16, which contains a pelB signal peptide for the periplasmic production of the scFv. The ligated DNA was transformed into *E. coli* Jude-1 by electroporation at 2.5 kV using a Gene Pulser (Bio-Rad Co., Hercules, CA, USA). After electroporation, cells were plated on SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) agar plates containing ampicillin (100 μg/mL) and incubated overnight at 37 °C.

### 2.3. Library screening by FACS

For the isolation of a primary antibody against FMDV, we used FACS to screen the previously constructed human synthetic scFv antibody library [14]. We inoculated 10 mL of Luria–Bertani (LB) media containing 2% (w/v) glucose and chloramphenicol (35 μg/mL) with the synthetic scFv antibody library. After growth overnight at 37 °C, 200 μL of the culture was transferred to 10 mL of fresh LB media. When cell density reached an $OD_{600}$ of 0.6–0.7, 1 mM isopropyl-$\beta$-d-thiogalactopyranoside (IPTG) was added. Four hours after induction, cells were harvested by centrifugation (6000 rpm, 4 °C, 10 min), and the cell pellets were washed two times with 10 mM Tris–HCl (pH 8.0) and resuspended with STE solution (0.5 M sucrose, 10 mM Tris–HCl, 10 mM EDTA, pH 8.0). After incubation at 37 °C for 30 min, cells were collected by centrifugation and resuspended in Solution A (0.5 M sucrose, 20 mM $MgCl_2$, 10 mM MOPS, pH 6.8) containing 1 mg/mL of hen egg lysozyme (Sigma–Aldrich, ST. Louis, MO, USA). After incubation at 125 rpm for 15 min at 37 °C, the spheroplasts were washed twice with 1 mL of phosphate buffered saline (PBS, 21.5 mM $Na_2HPO_4$, 685 mM NaCl, 13.5 mM KCl, 7 mM $KH_2PO_4$) pH 72. The resuspended cells were incubated with 5 μM FITC-conjugated VP1 epitope probe for 1 h at room termperature. The cells were then washed twice with 10 mM Tris–HCl (pH 8.0) and the fluorescent probe-labeled cells were sorted using a high-speed flow cytometer (MoFlo XDP, Beckman Coulter, Miami, FL, USA). After sorting in each round, the scFv genes were amplified from the sorted cells by PCR. After digestion with *Eco*RI and *Nco*I restriction enzymes, the scFv genes were cloned into pMoPac1 and transformed into *E. coli* Jude-1 for the next round screening.

### 2.4. Screening of affinity-maturated scFv candidates by FACS

After cultivation as described above, the cells were harvested by centrifugation (6000 rpm, 4 °C, 10 min). For labeling with fluorescent probes, cell pellets were resuspended in 5× PBS buffer. The resuspended cells were incubated with 5 μM of FITC-conjugated VP1 epitope peptide at 25 °C for 1 h. The cells were washed twice with 5× PBS, and the fluorescent probe-labeled cells were sorted using a high-speed flow cytometer. All *E. coli* cells sorted in each round of screening were immediately reused for the next round of FACS sorting without regeneration. The sorting was repeated until the highly fluorescent population was enriched. To obtain the required sample volume, 500 μL of sheath buffer was added to the sorted samples during each round. After the final sorting, the scFv genes were amplified from the sorted cells by PCR with primers, Assembly-F (5′-GATTGTTATTACTCGCGG) and Assembly-R (5′-GGCCGCGAATTCG). After digestion with *Eco*RI and *Nco*I, the amplified scFv genes were cloned into pMoPac16 and transformed into *E. coli* MG1655 for further analysis of the single clones.

### 2.5. Purification of the antibody fragment

After growth in a shake flask, cells were harvested by centrifugation (6000 rpm, 4 °C, 10 min), and cell pellets were washed twice with binding buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 7.0). Crude extracts of the cells were prepared by sonication (20 min, 50% pulse, 20% amplitude) and the extracts were centrifuged (10,000 rpm, 4 °C, 10 min) to yield soluble lysates. The soluble lysates were purified with Talon metal-affinity resin (Clontech, Mountain View, CA, USA). The resin was washed twice with 10 mL of washing buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 7.0), and the attached scFvs were eluted using elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 150 mM imidazole, pH 7.0). The purified antibody fragments were stored at 4 °C for further analysis.

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

The GST-fused VP1 epitope [17] was mixed with 0.05 M carbonate-bicarbonate coating buffer, pH 9.6 to a final concentration of 2 μM. Then 100 μL of antigen solution (GST-fused VP1 epitope) was loaded onto a 96-well ELISA plate and incubated for 2 h at 37 °C. Subsequently, each well was washed four times with PBS-T (PBS with 0.05% Tween-20 at pH 7.2) and blocked with 100 μL 5% skim milk in PBS-T. The plate was incubated for 1 h at 37 °C. After washing with PBS-T four times, each scFv analyte (soluble lysate or purified sample) was loaded onto the plate, and the plate was incubated for 2 h at room temperature. Each well was washed four times with PBS-T, after which monoclonal anti-6xHis tag antibody conjugated to horseradish peroxidase (HRP) (1:5000; Sigma–Aldrich) was added and the plates were incubated at room temperature for 1 h. Finally, the wells were washed with PBS-T, and tetramethylbenzidine (TMB) peroxidase substrate was added for the colorimetric detection of bound scFv clones. The reaction was arrested by adding 2 M $H_2SO_4$ stop solution. The absorbance was measured at 450 nm using a TECAN Infinite M200 Pro ELISA plate reader (Tecan Group Ltd., Männedorf, Switzerland).

The reactivity of scFv antibodies against inactivated serotype-O FMDV were analyzed by indirect ELISA. In this analysis, a serotype-O FMDV Manisa strain-coated plate (Prionics AG, Schlieren-Zurich, Switzerland) was used. The serially diluted scFvs were added to each well of the plate. After incubation at room temperature for 1 h, plates were washed four times with PBS-T and incubated with a HRP-conjugated anti-6xHis tag antibody (1:5,000) (Sigma–Aldrich) at room temperature for 1 h. Plates were washed four times with PBS-T and 100 μL TMB peroxidase substrate was added to initiate the peroxidase reaction. To stop the reaction, 50 μL of 2 M $H_2SO_4$

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