Thesis progression

Thesis title: Development of fluorescence-labeled broadly reactive flavivirus recombinant antibody

Thesis progression title: Construction and Characterization of rEGFP/4G2 scFv antibody in mammalian expression system

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Date: 16 July 7, 2024

1. Introduction

Dengue virus (DENV), which causes dengue, is the most significant re-emerging arboviral illness in tropical and subtropical regions including Southeast Asia. Mosquitoes, especially Aedes aegypti and Aedes albopictus, are the main vectors for the transmission of the dengue virus between humans. More than 3.9 billion individuals are at risk due to the disease's endemic prevalence in over 100 countries. An estimated 390 million DENV infections occur each year, with 96 million of them being symptomatic, ranging from mild dengue fever with or without warning signs to severe dengue with plasma leakage, which can cause shock, bleeding, and/or organ damage. Dengue is a positive single-stranded RNA virus within the flaviviridae family and has four distinct serotypes consisting of DENV-1, DENV-2, DENV-3, and DENV-4, which cause several conditions, including undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). Dengue virus genome has a length of approximately 11 kb which contains a single open reading frame (ORF) encoding three structural proteins (capsid (C), membrane (M), envelope (E)) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Kularatne & Dalugama, 2022).

The standard detection method for the dengue virus is reverse transcription-polymerase chain reaction (RT-PCR) in serum or plasma samples which is the common method for virus detection. Virus identification is mostly performed by immunofluorescence assay (IFA) using dengue-specific monoclonal antibodies labeled with a fluorochrome (Henchal 1983, Gubler 1984). This technique allows the visualization of a specific protein or antigen by a specific antibody conjugated with fluorescent dye. The advantage of this assay is that the

molecules can be observed directly with high specificity and sensitivity. Rather than fluorochrome, antibodies can be labeled by various labeling for detection such as an enzyme, chemical, and nanoparticle. There are several techniques that use antibody labeling including western blot, enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunocytochemistry, and immunohistochemistry.

Mouse anti-flavivirus envelope protein antibody (4G2) is one of the common monoclonal antibodies used in the method for dengue detection. It has been shown to recognize the envelope (E) protein of the dengue virus, west nile virus, japanese encephalitis virus, and zika virus (Aubry. Et al., 2016). The 4G2 antibody binds to the fusion loop at the extremity of domain II of the E protein and prevents syncytia formation (Summers. et al., 1989). The epitope is highly conserved amongst flaviviridae and has been functionally analyzed in detail (Crill & Chang, 2004). Therefore, 4G2 is very useful in terms of flavivirus detection. Nowadays, recombinant techniques have become popular for protein production. A single-chain fragment variable (scFv) is a recombinant antibody, that is generated by a fusion of the variable heavy (VH) and variable light chains (VL) of immunoglobulins by a short polypeptide linker (Wang, R et al., 2013). Each VH and VL domain contains three complementarity-determining regions (CDRs), responsible for antigen binding. The advantages of scFv production including low molecular weight and immunogenicity, large-scale expression system, superior tissue penetrating, and genetic modification capacity make it can be used as an antibody format for other diagnostics as well as therapeutic purposes. Additionally, cost-effectiveness is well described in the production of scFv which can increase production capacity to meet demand.

In this study, we develop the fluorescence-labeled broadly reactive flavivirus recombinant antibody and characterize the potential of recombinant antibody fusing fluorescent proteins for dengue virus detection.

2. Objective

2.1 To construct recombinant 4G2 scFv antibody specific to dengue envelope protein.

2.2 To express and characterize the recombinant 4G2 scFv antibody for dengue envelope protein.

2.3 To conjugate and validate recombinant 4G2 scFv antibody for dengue virus detection.

3. Materials and methods

3.1 PCR amplification of EGFP and 4G2 scFv genes

The EGFP from pIRES2/EGFP and 4G2 scFv-pFLAG-CMV-3 plasmid DNA were used as a template for EGFP/4G2 scFv gene amplification by using a specific primer as shown in Table 1. Briefly, the PCR reaction was performed by using PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Japan) in a 50 µl mixture containing 1.5 µl of each forward and reverse primer (10 µM), DNA template up to 10 ng, 4 µl dNTP mixture (2.5 mM each), 10 µl 5X PrimeSTAR GXL buffer (Mg^{2+} plus), and 1 µl PrimeSTAR GXL DNA polymerase (125 U/50 µl). The amplification conditions comprise an initial denaturation at 98°C for 30 sec, followed by 35 PCR cycles of 98°C for 10 sec, 60°C for 15 sec, 68°C for 1 min, and a final extension step of 68°C for 10 min. The amplified products of EGFP/4G2 scFv gene was analyzed by using 1% agarose gel electrophoresis and the amplified products was purified by using the PCR clean-up (Takara Bio Inc., Japan).

Table 1. Specific primers for EGFP/4G2 scFv amplification.

Primer ID	Sequence 5' → 3'	Purpose
P1_EGFP	CGAAAGCTTGTGAGCAAGGGCGAGGAGCTGTTC	Amplified EGFP
P2_EGFP	GCTGCCACCTCCACCGCTACCGCCGCCTCCCTTGTACAGCTCGTCC	
P3_scFv	GGTGGAGGTGGCAGCCAGCTGCAACAGTCTGGACCTGAGCTG	Amplified scFv
P4_scFv	GCGCGGCCGCGGTACCTATTTCCAGCTTGGTC	

3.2 Overlap PCR reaction of EGFP fusion with 4G2 scFv genes

The EGFP and 4G2 scFv fragments from the previous step were used as a template for the fusion full-length EGFP/4G2 scFv gene. This PCR reaction does not use any primers and relies on the overlapping sequences generated in the previous step for primer extension. The overlap PCR reactions were carried out in a volume of 50 µl. The mixture containing DNA template up to 250 ng, 4 µl dNTP mixture (2.5 mM each), 10 µl 5X PrimeSTAR GXL buffer (Mg^{2+} plus), and 1 µl PrimeSTAR GXL DNA polymerase (125 U/50 µl). The amplification conditions comprise 35 PCR cycles of 98°C for 10 sec, 60°C for 15 sec, 68°C for 1 min, and a final extension step of 68°C for 10 min. The amplified products of EGFP/4G2 scFv gene was

analyzed by using 1% agarose gel electrophoresis and the amplified products was purified by using the PCR clean-up (Takara Bio Inc., Japan).

3.3 PCR amplification of full-length EGFP/4G2 scFv genes

The final PCR step is to amplify the full-length EGFP/4G2 scFv fusion product for cloning into expression vector. The EGFP/4G2 scFv from the previous step was used as a template for amplification by using a specific primer as shown in Table 1 (P1 and P4 primers). Briefly, the PCR reaction was performed by using PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Japan) in a 50 µl mixture containing 1.5 µl of each forward and reverse primer (10 µM), DNA template up to 10 ng, 4 µl dNTP mixture (2.5 mM each), 10 µl 5X PrimeSTAR GXL buffer (Mg^{2+} plus), and 1 µl PrimeSTAR GXL DNA polymerase (125 U/50 µl). The amplification conditions comprise an initial denaturation at 98°C for 30 sec, followed by 35 PCR cycles of 98°C for 10 sec, 60°C for 15 sec, 68°C for 1 min, and a final extension step of 68°C for 10 min. The amplified products of full-length EGFP/4G2 scFv gene was analyzed by using 1% agarose gel electrophoresis and the amplified products was purified by using the PCR clean-up (Takara Bio Inc., Japan).

3.4 Construction of recombinant EGFP/4G2 scFv antibody

The recombinant EGFP/4G2 scFv fragment was ligated into the pFLAG-CMV-3 (CMV-3) expression vector at *Hind*III and *Kpn*I restriction sites. The ligation products were transformed into *E. coli* DH5**α** competent cells and the plasmid of the ampicillin-positive clones was confirmed by colony PCR and sequencing analysis.

3.5 Expression and characterization of rEGFP/4G2 scFv antibody

The EGFP/scFv-pFLAG-CMV-3 plasmid was transfected into a HEK293T cell. One day before transfection 3.5×10^6 cells/ml HEK293T cells were seeded into a 100 mm dish plate culture up to the confluency reached 70%. The HEK293T cells were transfected with 4 µg of EGFP/4G2 scFv-pFLAG-CMV-3 plasmid by using Lipofectamine™ 2000 Reagent (ThermoFisher Scientific) according to the manufacturer's protocol. Briefly, plasmids were mixed with transfectant reagent and incubated at room temperature for 5 min. The transfected cells were grown at 37°C, 5% CO_2 with 5 ml 2% FBS DMEM medium for 4 h. Then, 5 ml 2% FBS DMEM medium was added into a 100 mm dish plate culture and incubated at 37°C, 5% CO_2 for 24 h before replacing the media with serum free-DMEM medium and continuing culture

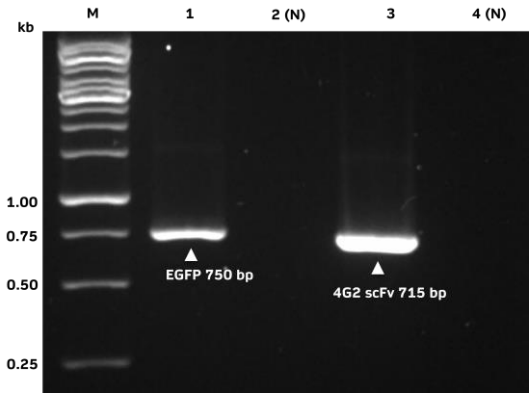
for 5 days. Then transfected cells were observed under the microscope. The pellet and supernatant of transfected cell culture were harvested by centrifugation at 4°C, 12,000 g for 10 min. The expressed EGFP/4G2 scFv protein were observed under the fluorescence microscope and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis by using mouse anti-FLAG-HRP antibody (1:5000).

4. Results

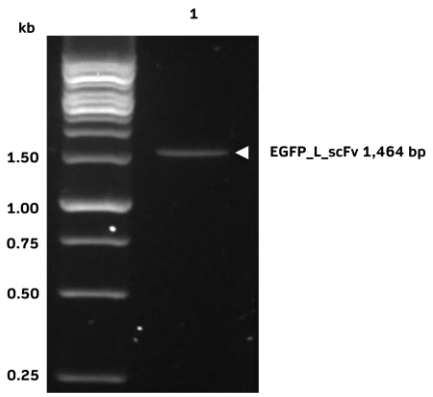
4.1 The EGFP/4G2 scFv gene amplification

The pIRES2/EGFP and 4G2 scFv-pFLAG-CMV-3 plasmids were used as a template for EGFP and 4G2 scFv genes amplification. The full length of EGFP and 4G2 scFv genes at 750 and 714 bp respectively as shown in Figure 1 (a). The PCR products of these two genes were used as a template for fusion of full-length EGFP/4G2 scFv gene by overlap PCR reaction. The fusion EGFP/4G2 scFv fragment at approximately 1,464 bp as shown in Figure 1b. The EGFP/4G2 scFv from the overlap PCR reaction step was used as a template for amplifying the full-length EGFP/4G2 scFv fusion product for cloning into expression vector by using a specific primer (P1 and P4) and the full-length were observed at approximately 1,464 (Figure 1c).

(a)



(b)



(c)

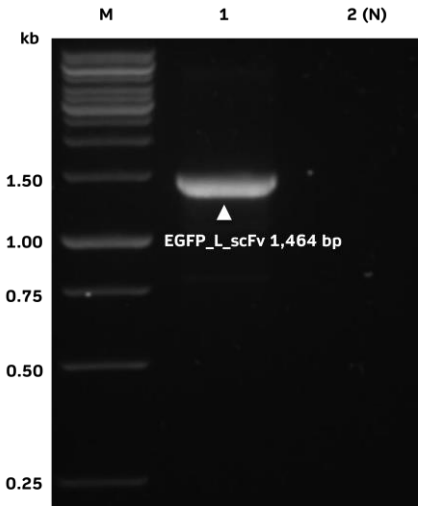


Figure 1. Electrophoretic analysis of a) PCR product by using specific primer sets (P1-P4): Lane 1 and 2: EGFP and 4G2 scFv fragments, b) Fusion product of full-length EGFP/4G2 scFv gene by overlap PCR reaction and c) PCR product of full-length EGFP/4G2 scFv gene.

4.1 Construction of rEGFP/4G2 scFv antibody

The PCR product of EGFP/4G2 scFv fragments were digested with *Hind*III and *Kpn*I restriction enzymes. After that, the digested product was ligated with T4 DNA ligase and cloned into the pFLAG-CMV-3 (CMV-3) expression vector. The positive clones were selected by colony PCR as shown in Figure 2 and confirmed the existence of the plasmid by sequencing. Sequence homology analysis was analyzed by Macrogen Korea. The sequence of rEGFP/4G2 scFv-CMV-3 positive clone showed 100% identity to the *Mus musculus* antibody 4G2 immunoglobulin G2a heavy chain mRNA, complete cds (accession KJ438785.1), *Mus musculus* antibody 4G2 immunoglobulin kappa light chain mRNA, complete cds (accession KJ438784.1), and Synthetic construct green fluorescent protein gene, partial cds (accession OP866271.1) as shown in Table 2.

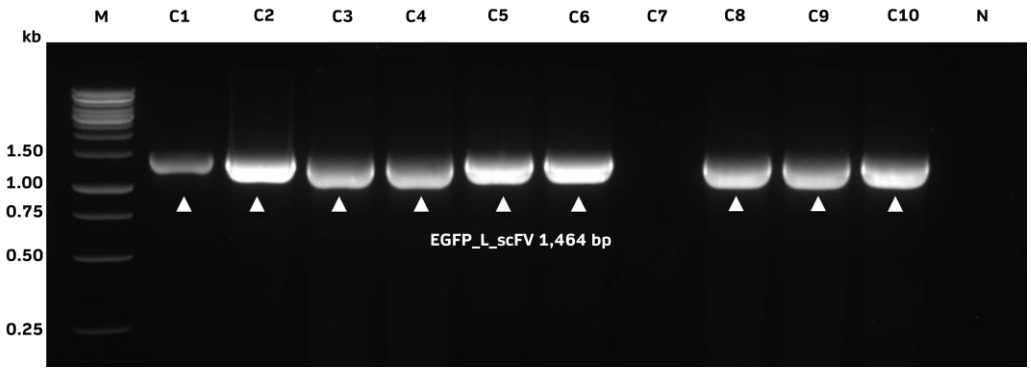


Figure 2. Electrophoretic analysis of ligated product EGFP/4G2 scFv fragment by colony PCR.

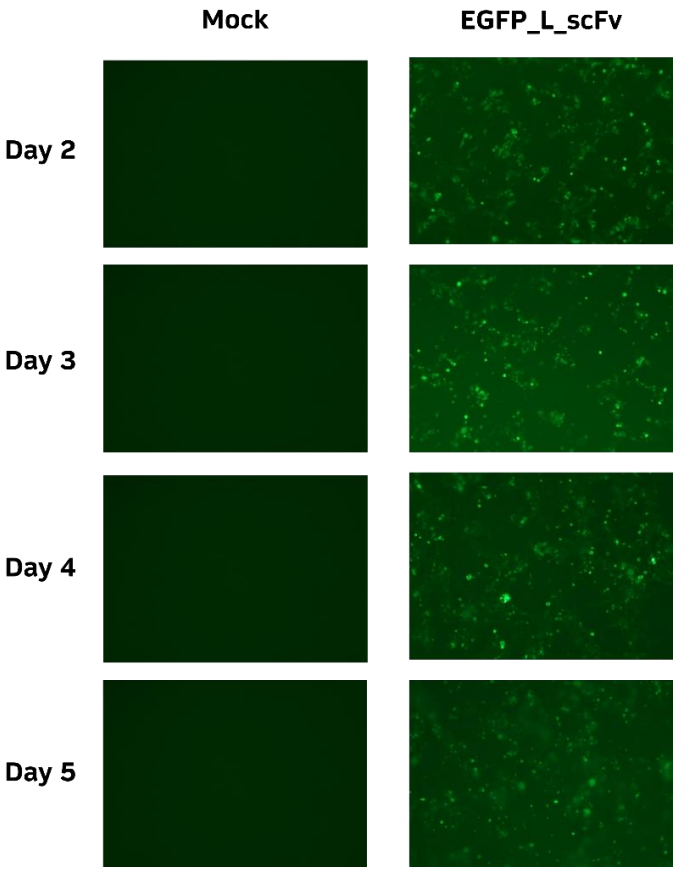
Table 2. Sequence homology analysis by BLAST NCBI.

Description	Per. Identity	Accession number
Mus musculus antibody 4G2 immunoglobulin G2a heavy chain mRNA, complete cds	100	KJ438785.1
Mus musculus antibody 4G2 immunoglobulin kappa light chain mRNA, complete cds	100	KJ438784.1
Synthetic construct green fluorescent protein gene, partial cds	100	OP866271.1

4.2 Expression of recombinant 4G2 scFv antibody in HEK293T cells

The rEGFP/4G2 scFv-CMV-3 plasmid was transfected into a HEK293T cell by using Lipofectamine™ 2000 Reagent (ThermoFisher Scientific, Germany). After transfection, the rEGFP/4G2 scFv antibody was observed under a fluorescence microscope to assess the expression of EGFP as shown in Figure 3a. The rEGFP/4G2 scFv antibody specific to dengue E protein was successfully expressed in HEK293T cells in a soluble form. The expressed proteins were collected from supernatants of transfected cell culture and negative control protein from days 1-5 post-transfection. Lysate was a cell pellet of EGFP/4G2 scFv and negative control protein from day 4 and 5 post-transfection. The express protein was visualized by SDS-PAGE and western blot before purification. Western blot result showed the expected band of the EGFP/4G2 scFv protein at ~53 kDa in supernatant start from day 4 and the higher intensity band was found in day 4 post-transfection (Figure 3b, lane 4). The protein was detected in cell lysate on day 5 post-transfection (Figure 3b, lane 6). Therefore, the expressed protein was presented in soluble form. However, the SDS-PAGE result did not show the band intensity at the expected size of the EGFP/4G2 scFv protein as shown in Figure 3c.

(a)



(b) (c)

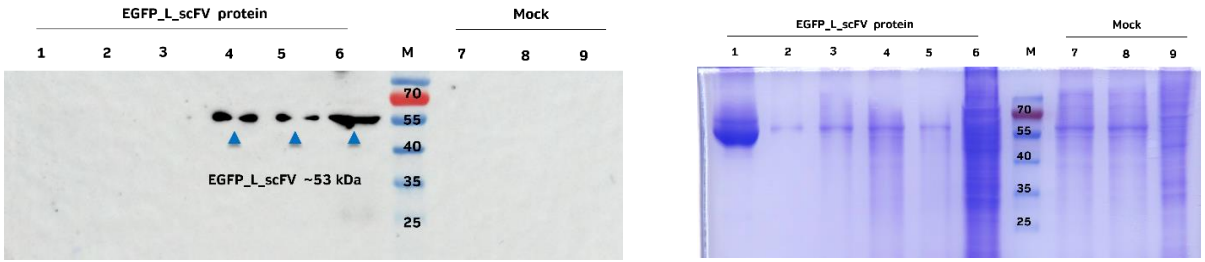


Figure 3. a) The expression of rEGFP/4G2 scFv protein under fluorescence microscope, b) Western blot analysis, and c) SDS-PAGE. Lane M: Standard molecular weight marker, Lane 1-5 and 7-8: Supernatant of rEGFP/4G2 scFv protein. Lanes 6 and 9: Lysate of rEGFP/4G2 scFv protein and mock. Western bot was detected by anti-FLAG HRP antibody.

5. Conclusion

The rEGFP/4G2 scFv-CMV3 plasmid was successfully constructed and expressed in a mammalian cell system by transient expression. The expressed protein can be secreted as a soluble form into the culture medium in optimal condition on day 4 post-transfection. The purification and characterization of the binding activity and specificity of the construct 4G2 scFv will be further investigated. This 4G2 scFv will be used for the development of dengue virus detection method.

6. References

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