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Discovering Dengue Virus Antibody Using Phage Display Technology

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ABSTRACT: Dengue virus (DENV) is a type of flavivirus transmitted globally by the Aedes aegypti mosquito and is the causal agent of Dengue fever. Due to global warming and rising temperatures worldwide, the rate of exposure to DENV is now reaching unprecedented levels, with over 400 million people getting infected each year. Despite worldwide attention to DENV, there is not currently an effective treatment or vaccine. Here, we focused on developing an antibody for the NS1 protein of the DENV, which enables earlier detection of disease and could further decrease the rate of death. Our study utilized a method more time-saving and cost-effective than the traditional method of extracting antibodies from the blood or B-cells of infected animals that show immunity: Phage Display. This technique allowed us to derive monoclonal antibodies (mAbs) from the genome library and select DENV antibodies that bind to antigen proteins. In this study, we used Phage Display to select ninety-six potential antibodies from three phases of panning, which then went through indirect ELISA screening for optical density (OD) confirmation (OD > 1.5). Sixteen of the high optical density antibodies were sent to a DNA sequencing analysis facility where fifteen of them were revealed to be the same 1G1 strain. By understanding how antibodies are discovered using phage display technology, we hope to advance the field with reevaluation of current vaccines and the development of novel ones.

INTRODUCTION

Dengue is a mosquito-borne viral infection mostly mediated by Aedes albopictus. The virus has 4 distinct serotypes (DENV 1-4), which are members of the Flaviviridae family, genus flavivirus. Dengue Virus (DENV), namely DENV 1-4, spreads remarkably well in tropical regions where high temperatures are maintained all year long. The global temperature increase has resulted in a higher number of tropical and subtropical regions. Previously safe regions have become targets of the DENV, increasing the number of people at risk [1]. These opinions are supported by statistics

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gathered by well-known organizations such as the Centers for Disease Control and Prevention (CDC). Their statistics reveal a dramatic increase in the incidence rate of Dengue in recent decades with over 400 million infections, 100 million people showing symptoms, and 22,000 deaths each year [2]. While 22,000 deaths alone are not a statistic to be ignored, we must also watch out for the symptoms of Dengue fever. Most people who are infected by DENV recover within 2 -7 days, accompanied by mild symptoms including nausea, vomiting, and rash. However, severe dengue can result in severe internal bleeding and organ impairment [2].

In order to prevent such symptoms and casualties, it is imperative to diagnose the disease quickly and precisely. Currently, most diagnoses is based on reverse-transcription PCR (RT-PCR), a technique that enables the identification of a patient's serotype of DENV. In addition to diagnosing, we must also find a cure or vaccine for Dengue. The vaccine, Dengvaxia, is currently in use; however, in 2017, the manufacturer announced that even those who have received the vaccine may be at risk of developing severe dengue if they become infected after being vaccinated [3]. As such, finding a more effective vaccine or treatment against DENV has become more important than ever. Conventional methods of vaccine development require the injection of the virus's antigens into experimental animals such as mice and rabbits. Scientists wait for the animals to develop an immune reaction and then extract either the animal's antibodies against the virus from their blood or B cells, which develop the antibodies [4]. Due to the duration of the antibody development in the animal's immune system, this method is time-consuming (usually about 6 months when using rats). Furthermore, we cannot disregard the ethical implications of animal testing. Therefore, in our study, we offer an alternative –Phage Display technology– in the discovery of an antibody for the non-structural protein 1 (NS1) antigen, a biomarker for DENV.

Phage Display technology refers to a bioengineering technique that allows in vitro selection of monoclonal antibodies (mAbs) by displaying peptides or antibodies on the surface of bacteriophages. Since the whole process occurs outside a living organism in a short timeframe, it is more ethical and time-effective compared to conventional methods [5]. The procedure consists of two main parts: antibody discovery and antibody production. The antibody discovery phase begins with the construction of the phage display library. Once the library is created, the panning process begins, which exposes the library to the target protein - the NS1 protein. Unbound phages are washed away, and those that show affinity for the protein are recovered by elution. The recovered phages are used to infect new host cells for amplification. Repeated panning in the Phage Display technique allows for the selection of only those that maintain a high affinity for the protein, resulting in high specificity of the antibodies. Once the final panning is complete, bounded antibodies go through ELISA screening with a secondary antibody containing an anti-HA tag. Only those with Optical Density (OD) values over 1.5 are considered valuable. The plasmid of these antibodies is separated and sent to sequencing analysis. The antibody production phase began with the cloning of eligible colonies. Consistent human antibody fragments produced through the Phage Display technique can be re-cloned into IgG expression vectors for the generation of antibodies in the human body. Because it maintains compatibility with the human immune system, it should be highly efficacious and safer compared to the conventional method.

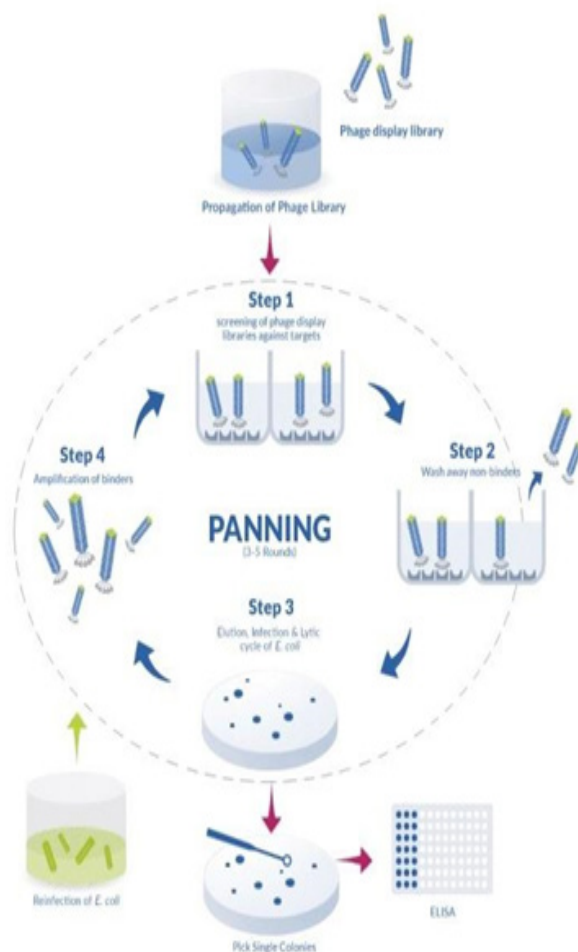

Figure 1. Phage Display Cycle [6]

Over the course of this project, we focused on developing an antibody for the Earth-threatening Dengue virus with a more productive 'Phage Display' technique. Discovering the specific sequence of antibodies for the NS-1 protein of Dengue Virus has the potential of manufacturing helpful vaccines or treatments.

METHODS

1. Antigen coating, panning, rescue

Immune tubes containing a coating buffer and protein were prepared prior to the experiment. The panning and rescuing steps were executed three times each.

Phages and a 3% skim milk 1XPBS solution were added to the tubes. After washing and eluting, the solution was transferred to a tube containing Tris-Cl and bacterial strain TG1. In order to estimate the number of infected bacteria, the cultured bacteria were smeared on a 90mm LB agar plate containing Ampicillin. The remaining infected bacteria were centrifuged and smeared on a 150mm LB agar plate before overnight incubation.

After counting the number of bacteria grown in the 90mm plate, a culture medium was added to the 150mm plate. The mixture was then inoculated in a SB-ampicillin culture medium. Helper phage and Kanamycin were also added before overnight incubation.

To prepare for the third and final panning step, the final medium was centrifuged and added to a tube containing 5XPEG. The supernatant was then filtered and used for panning and rescuing.

2. Seeding and induction

The bacteria grown in the 90mm plate were counted. Single colony bacteria were picked from the 90mm plate and seeded into a 96 well plate containing the SB-ampicillin culture medium. After overnight incubation, the single colony bacteria stock was transferred to another 96 well plate containing the SB-ampicillin culture medium and placed in the deep freezer. IPTG, a molecular mimic of allolactose, was added before overnight incubation.

3. Screening and seeding

To prepare for screening, periplasmic sup was collected. The incubated single colony bacteria were centrifuged. After removing the supernatant, 1XTES buffer was added to the bacteria pallet, creating a concentration difference. This was kept in ice to extract periplasm

using osmosis. After a 0.2XTES buffer was added and kept in ice, the mixture was centrifuged to collect the periplasmic sup from E.coli in a form of supernatant.

In order to prepare ELISA screening, the protein-coated 96 well plate was blocked with 3% skim milk. 3 columns of the plate were coated with protein and skim milk, while another 3 columns were blocked by skim milk only (for the control). The periplasmic sup was added to each well (6 columns). After 3 times of washing with a wash buffer, a TMB substrate solution was added. TMB reaction was stopped by adding sulfuric acid, and the result was compared with the control. The optical density (O.D) of each well was recorded with an ELISA reader (450nm). The stocks of the wells with positive results (1.5 < O.D) were transferred into different 15mL tubes.

4. DNA prep and Sequencing

The incubated stocks were transferred to 1.5ml tubes, which were then centrifuged. The pallet was filtered and resuspended with P1 buffer (resuspension buffer) [7]. P2 buffer (lysis buffer) was added and mixed by flipping the tubes upside down several times, and it was repeated with P3 buffer (neutralization buffer) [7]. After centrifuging the mixture, the supernatant was transferred into miniprep tube columns. With these columns, the mixture was centrifuged 5 times (first: the mixture itself; second: with EW buffer; third: with PW buffer; fourth: the resultant mixture; fifth: with new tube columns and DW buffer). In order to sequence the DNA collected, they were quantified with a TAKE3 device. These samples were sent to Macrogen and sequenced. Using the online website, Expasy, the sequenced DNA was translated into an amino acid sequence. The website gave several amino acid sequences depending on the starting base to be read as a codon. The frame containing the linker (GQSS … GGGG) and H6 tag was chosen as the correct sequence.

RESULTS

Titering Results from Panning and Seeding in 90mm plate

To estimate the number of colonies after the 3rd panning, we counted the number of single colonies in the 90mm plate. Table 1 shows the number of colonies counted on each plate (total 4). We divided the section of the plate into eighths and counted each section. Assuming each section maintains a similar number of colonies, we multiplied the number of colonies counted in each section by 8.

Analyzing the ELISA Result

In order to test the presence of targeted antibodies, we performed ELISA screening on the samples of periplasmic sup. The result of the ELISA test was presented in tables 2A to 2D, each with separate columns for antibody and for 3% skim milk. We compared the columns for antibody to the columns for 3% skim milk which were controls.

After the analysis of the data from the ELISA reader (450nm wavelength), we collected several positive results (blue and yellow cells from Table 2) with an anomaly (C6 in Table 2A). Each table is performed by different people. Stocks with a number greater than 0.2 are highlighted in blue with the gradient (darker blue means the value is greater). Blue samples indicate positive results which means the antibodies are present. 8 yellow stocks were randomly selected for sequencing.The anomaly could have resulted from contamination during pipetting or unsuccessful washing. Based on these results, we randomly chose 8 stocks that gave positive results (Table 2) to quantify the DNA of antibodies before sequencing. 8 randomly selected stocks are colored in yellow in Tables 2A to 2D.

Table 1. Titering Results of Four Plates After the 3rd Panning Process

Plate number	Number of Colonies	Number of Colonies in 1μL	Number of Colonies in 10ml
1	496 x 2	1*10^3	1*10^7
2	712 x 8	5.7*10^3	5.7*10^7
3	457 x 8	3.6*10^3	3.6*10^7
4	537 x 8	4.3*10^3	4.3*10^7

Table 2. Cultured antibody ELISA Test Result under 450nm wavelength

A)

	Antibody			3% Skim Milk		
	1	2	3	4	5	6
A	0.049	0.669	0.787	0.043	0.044	0.043
B	0.043	1.577	1.386	0.044	0.045	0.045
C	0.876	1.691	2.086	0.046	0.047	0.081
D	0.077	1.862	0.76	0.043	0.043	0.045
E	0.046	0.135	2.039	0.044	0.043	0.045
F	2.989	1.39	1.833	0.043	0.043	0.045
G	2.023	1.895	0.1	0.043	0.047	0.043
H	0.044	2.309	2.893	0.053	0.045	0.044

B)

	Antibody			3% Skim Milk		
	1	2	3	4	5	6
A	0.054	1.515	0.416	0.045	0.046	0.044
B	1.453	0.045	0.051	0.044	0.067	0.044
C	0.052	0.046	0.043	0.045	0.05	0.044
D	0.046	1.695	0.042	0.051	0.046	0.082
E	0.305	1.459	0.045	0.044	0.043	0.042
F	0.043	0.044	0.043	0.044	0.045	0.043
G	0.044	0.047	0.044	0.044	0.044	0.047
H	0.045	0.043	0.043	0.045	0.048	0.046

C)

	Antibody			3% Skim Milk		
	1	2	3	4	5	6
A	0.062	2.262	1.941	0.044	0.043	0.049
B	0.05	0.044	0.046	0.047	0.044	0.047
C	0.393	0.045	1.758	0.045	0.045	0.044
D	0.047	0.78	0.2797	0.047	0.046	0.045
E	1.869	0.115	0.049	0.046	0.065	0.045
F	0.225	0.049	1.456	0.044	0.044	0.047
G	0.045	0.046	0.056	0.046	0.046	0.049
H	2.684	0.055	0.064	0.046	0.045	0.048

D)

	Antibody			3% Skim Milk		
	1	2	3	4	5	6
A	0.043	0.043	0.491	0.045	0.042	0.043
B	0.045	0.044	0.778	0.044	0.043	0.043
C	0.044	0.044	0.043	0.044	0.045	0.043
D	0.045	0.389	0.043	0.043	0.046	0.044
E	2.034	0.045	0.25	0.043	0.043	0.044
F	2.152	0.046	0.044	0.044	0.045	0.044
G	0.044	0.044	1.964	0.043	0.044	0.043
H	0.043	0.044	1.997	0.044	0.043	0.044

DNA Quantification before Sequencing

The stocks that have absorbance ratios of 260 nm/280 nm above 1.8~2 were considered to have sufficient amounts of DNA for successful sequencing [8]. With the Take3 micro-volume plate, the absorbance ratio (260nm/280nm) of each sample was collected (Table 3). Samples were randomly selected (8 stocks which are yellow cells in Figure 2). The highlighted number indicates the concentration of DNA.

There were variances in the value of the DNA quantities per microliter. It was expected to have no variance as the samples were prepared with the same method and the environment. These variances may indicate that the samples were not pure or the sequencing was unsuccessful. However, because the observances at 260 nm were greater than those at 280 nm, resulting in high DNA to protein ratios (greater than 2), we assumed that the DNA samples were sufficiently pure to be sequenced.

Analyzing the Sequencing Results

After the DNA quantification, we requested Humanizing Genomics Macrogen to sequence the 8 samples. The purpose of single chain Fragment variable (scFv) sequencing was to understand the different types and positions of amino acids within the created antibody. After obtaining the plasmid of the single colony antibodies, each DNA strand was sequenced using the 5'-, 3'-oligonucleotide of a scFv. The final order of the scFv was determined after reading the sequence from both directions and combining the common regions. (ABI3730XL, Sanger-method sequencing, Phred Score ≥20). In order to analyze the results of the sequencing, we used an ExPASY(https://web.expasy.org/translate/) that translated the DNA sequence in our raw data (Figure 2) into corresponding amino acids. From the results, we determined the accurate reading frame (Figure 3) by identifying the H6 tag and the linker. Within the scFv, there is a light chain variable region followed by a linker and a heavy chain variable region. As heavy and light chains of the antibodies were linked with linker protein (gggssggggs) at heavy chain 6, the presence of the linker and H6 tag on the amino acid sequence indicates that we have successfully discovered our targeted antibody.

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>H210802-040_M16_1G1_pcd-forward.ab1     1512
GGACTAAAATTAATGAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCT
GGTTTCGCTACCGTGGCCCAGGCGGCCCTGACTCAGCCGTCCTCGGTGTC
AGCAAACCTGGGAGAAACCGTCAAGATAACCTGCTCGGGGAGTAGTGGTG
GCTATGGCTGGTATCAGCAGAAGTCACCTGGCAGTGCCCCTGTCACTCTG
ATCTATGACAACACCAACAGACCCTCGGACATCCCTTCACGATTCTCCGG
TTCCAAATCCGGCTCCACAGCCACATTAACCATCACTGGGGTCCAAGCCG
AGGACGAGGCTATCTATTTCTGTGGTGGCTACGACAGCAGTAGTGATAGT
GGTATATTTGGGGCCGGGACAACCCTGACCGTCCTAGGTCAGTCCTCTAG
ATCTTCCGGCGGTGGTGGCAGCTCCGGTGGTGGCGGTTCCGCCGTGACGT
TGGACGAGTCCGGGGGCGGCCTCCAGACGCCCGGAGGAGCGCTCAGCCTC
GTCTGCAAGGCCTCCGGGTTCGACTTCAGCAGTTATGAGATGCAGTGGGT
GCGACAGGCGCCCGGCAAAGGGTTGGAGTTCGTCGCTGGTATTGGCAACA
CTGGTAGTAGCACAGCATACGGGGCGGCGGTGAAGGGCCGTGCCACCATC
TCGAGGGACAACGGGCAGAGCACAGTGAGGCTGCAGCTGAACAACCTCAG
GGCTGAGGACACCGGCACCTACTACTGCGCCAAAACTACTGGTAGTGGCT
ATTGTGATATTTTTACTGATTACAGTTGTTGGGGTTATAGTACTGGTAAC
ATCGACGCATGGGGCCACGGGACCGAAGTCATCGTCTCCTCCACTAGTGG
CCAGGCCGGCCAGCACCATCACCATCACCATGGCGCATACCCGTACGACG
TTCCGGACTACGCTTCTTAGGAGGGTGGTGGCTCTGAGGGTGGCGGTTCT
GAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTGGCTCTGGTTCCGG
TGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCG
AAAATGCCGATGAAAACGTGCTACAGTCTGACGCTAAAGGCAAACTTGAT
TCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTCATTGGTGACGTT
TCCGGCCTTGCTAAGGGAATGGTGCTACGGGTGATTTGCTGGCTCTAATT
CCCAATGGCTCAAGTCGGGGACGGGATAATTCCCTTTAAGAAAAATTCCG
CAAATTACCTCCCCCCCCCAACGGTGAAGGCCCCCTTTTCTTTGGGCGGG
AAACATAGAATTTTTTTGTGGGAAAAAAAAACTTTTCCGGGGGTTTGTTT
TTTTTTTTTGCCCCTTTTTTTTTTTTTCTTTCAAACACCGCAAAAAAGAAA
CACAAAAAATATAAACGCCCGCCCGCGGGGAGCGGGGGGGAAAGATTTAT
TTTTTTTTTTTTTTTTTTCTTTTTTTCTTTCTCTCCTCCCCCCCACCCACC
CAACAGACGCAT
```

Figure 2. Raw DNA Sequence

Table 3. DNA Quantification Results of 8 Antibody Stocks Randomly Selected

	2	3	
		SPL1	Well ID
		0.063	260
A		0.031	280
		2.055	260/280
		63.015	ng/µl
	SPL2	SPL3	Well ID
	0.055	0.158	260
B	0.025	0.069	280
	2.18	2.283	260/280
	55.241	**157.537**	ng/µl
	SPL4	SPL5	Well ID
	0.033	0.178	260
C	0.019	0.085	280
	1.703	2.103	260/280
	33.008	**178.005**	ng/µl
	SPL6	SPL7	Well ID
	0.163	0.133	260
D	0.076	0.064	280
	2.159	2.078	260/280
	163.393	**133.356**	ng/µl
	SPL8		Well ID
	0.116		260
E	0.053		280
	2.182		260/280
	116.458		ng/µl

Figure 3. Accurate Translated Reading Frame

DISCUSSION

We demonstrate here the successful discovery of an antibody for the non-structural protein 1 (NS1) antigen, a biomarker of DENV. Our discovery employed Phage Display Technology, which enabled in vitro selection of monoclonal antibodies by presenting antibodies on the surface of bacteriophages.

At large, our results indicate that discovered antibodies are highly specific to the targeted NS1 antigen. scFv sequencing confirmed our accuracy with the identified H6 tag and linker on our Amino acid sequence. The high specificity of the antibody was derived from the ELISA technique and the quantity of DNA in the cultured colony was verified through DNA quantification prior to sequencing. Clones that had an OD value above 1.5 in the ELISA technique were only allowed in cultivation and colonies that maintained an absorbance ratio (260nm/280nm UV light) above 2 were considered pure enough to be tested.

Due to an increasing number of DENV patients concurrent with global warming, the necessity for therapeutics to overcome this crisis is on the rise. The ultimate goal of our study was to aid the development of effective vaccines or treatments for DENV; thus, enabling earlier detection of the disease and further lowering the rate of death.

Clearly, further studies are warranted to elucidate the immune parameters that could suggest a solution to overcome the limitation of the current vaccine (Dengvaxia). Unlike the current vaccine, the novel vaccine derived from our NS1 Protein antibody is expected to significantly lessen the possibility of re-infection of Dengue Variants. In prospective studies, the sequence of the antibody should be investigated further to figure out the adequate sequence of DNA that could potentially neutralize the targeted antigen. We thereby anticipate yielding efficient antibody therapeutic from the antigen-specific neutralizing sequence.

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Competing Interests
The authors declare no competing financial and non-financial interests.

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ABBREVIATIONS

DENV- Dengue Virus
1G1- Histone H3 Antibody
mAbs- Monoclonal Antibodies
NS1- Nonstructural protein 1
scFv- Single-chain Variable Fragment
OD- Optical Density
ELISA- Enzyme-linked Immunoassay
IPTG- Isopropyl β-D-1-thiogalactopyranoside

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