Spring 5-2017

Production of Antibodies for Specific Detection of Dengue Virus-2 By Surface Enhanced Raman Spectroscopy

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Production of antibodies for specific detection of dengue virus-2 by surface enhanced Raman spectroscopy

By

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A Prospectus of
a Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
Bachelor of Science
in the Department of Biological Sciences

December 2016

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Abstract

Dengue Virus (DENV) is a flavivirus spread by mosquitoes that are endemic in tropic and subtropic climates. It is the causative pathogen of Dengue Fever, Dengue Hemorrhagic Fever, and Dengue Shock Syndrome. Besides mosquito transmission, DENV can also be transmitted through blood transfusion. Due to the high expense and expertise needed to run tests like ELISA and Polymerase Chain Reaction (PCR) based-assays for DENV detection, a rapid, sensitive and cost effective gold nanoparticles mediated surface-enhanced Raman spectroscopy (SERS) assay has been developed as an alternative assay to detect DENV. In this project, I successfully produced a monoclonal antibody that specifically binds to DENV-2 from the HB46-ATCC cell line. Using HiTrap™ Protein G HP columns, the antibodies were purified and concentrated by SpinX centrifuge filters. Bradford assay was used to measure antibody concentration, SDS-PAGE was used to detect possible protein contamination, and immunostaining confirmed antibody specificity for DENV 2. The SERS results show that this antibody can be used for specifically detection of DENV-2.

Acknowledgements

I would like to acknowledge and thank my thesis advisor, Dr. Fengwei Bai, for his unwavering patience and support in my three years at the University of Southern Mississippi. I would not have been able to complete this thesis without the innumerable opportunities you have given me while doing this project.

I would also like to thank Amber Paul for her many hours of editing and revision, and for teaching me how to write like a scientist and not an English minor. This thesis would not have been completed without your guidance and critiques during the entire research process.

Finally, I would like to thank the Honors College for giving me the opportunity to follow my passion for science and research. The support of the faculty and community at the University of Southern Mississippi will be forever remembered.

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Chapter 1: Introduction

Dengue virus (DENV) is a mosquito-borne virus that can cause dengue fever (DF) and dengue hemorrhagic fever (DHF), which are two types of diseases that are prevalent in tropical and sub-tropical regions, such as South America, Southeast Asia and Africa[13]. The World Health Organization (WHO) has estimated DENV infects nearly fifty to one hundred million people each year, which has increased thirty-fold within last fifty years[13]. While other reports have estimated over three hundred million people are indeed infected with DENV every year[3,20]. Nevertheless, these DENV incidences suggest an important need for development of effective antiviral therapeutics and vaccines against DENV infection that are currently lacking.

DENV primarily spreads to humans by an infected mosquito bite. However, there are also many reported cases of the virus being transmitted through blood transfusion [5,13,19]. Despite the astounding prevalence of DENV infections, there is currently no blood screening measure implemented for DENV, which is partially due to a lack of sensitive, rapid, and cost effective assays[14,25]. Surface enhanced Raman spectroscopy (SERS) utilizes a highly specific method of analysis that can identify and differentiate small biological molecules, including bacteria and cancer cells[4,6]. Briefly, SERS identifies biological fingerprints that are enhanced in the presence of a metal nanoparticle surface, which makes this a promising alternative to traditional blood screening tools, such as ELISA and quantitative polymerase chain reaction (qPCR)-based assays of viral detection. The hypothesis of this study is that specific DENV antibodies can be used in SERS-based detection of DENVs. Thus, it can provide a sensitive and cost effective tool to detect DENVs in blood and blood products.

Chapter 2: Literature Review

The field of microbiology aims to catalogue and research all forms of bacteria, archaea, fungi and viruses[28]. Viruses have been classified as non-living organisms that contain genomic information in the form of nucleic acid, either RNA or DNA, encased in a protein shell called a nucleocapsid[1]. Viruses rely on the internal machinery of host cells to replicate their nucleic acids, package their genomes, and release them from infected cells to infect neighboring cells, thus propagating infection[1]. Viruses can be enveloped, meaning they have an outer membrane surrounding the nucleocapsid, or they can be naked with no outer lipid layer[1], and have multiple morphologies that range from the small circular picornaviruses (30 nm) to large mimivirus (750 nm)[1].

To begin the replication cycle, a RNA virus usually firstly infects a cell by binding to surface cell membrane receptors, inducing viral uptake into the cell by multiple mechanisms, including direct viral envelope to cell membrane fusion, or by receptor-mediated endocytosis[1]. The viral RNA genome is released into the cellular cytosol and the virus begins to replicate its genome using the available host replication machinery[1]. After all necessary proteins are available to be assembled, and adequate viral genome copies are produced, new infectious viral particles are formed and released from the host cell [1].

DENV belongs to the genus of flavivirus that consists of a group of positive-sense, single stranded, enveloped, RNA viruses with approximately fifty nanometers in diameter[1]. The infection cycle of DENV is initiated through a bite of a female mosquito taking a blood meal from an infected animal reservoir host[19]. The infected mosquito can then transmit the virus to another reservoir, and the cycle is continued (Figure 1)[19,21].

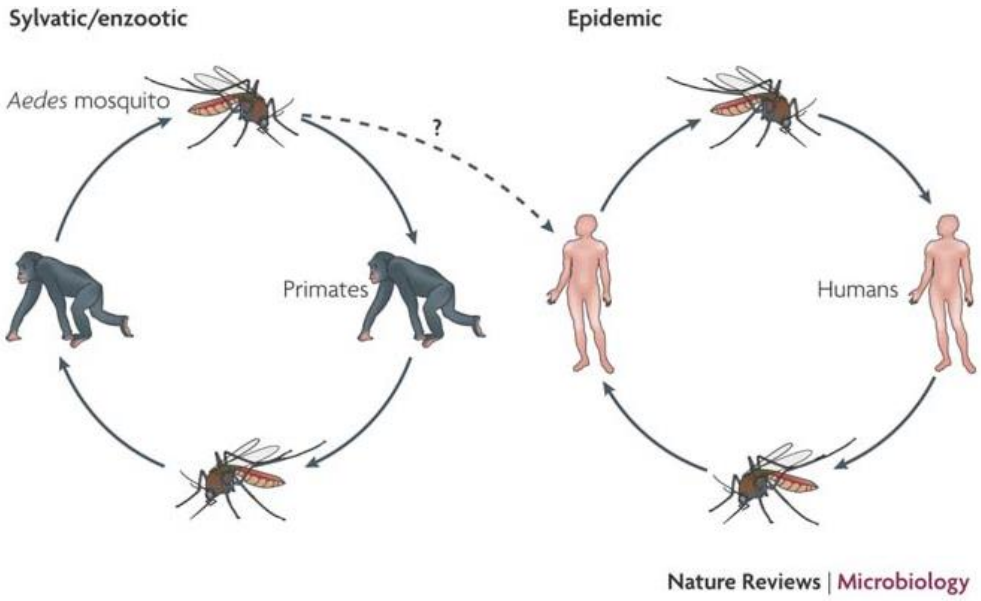

Figure 1. **Transmission cycle of DENV.** DENV is transmitted between zoonotic reservoirs (primates) to hosts (humans) via the vector female mosquito. Indicated there are two cyclic transmission patterns with DENV, a sylvatic/enzootic cycle and an epidemic cycle of viral transmission, whereby DENV may have jumped to the enzootic cycle to the epidemic cycle through the mosquito vector contracting the virus from an infected primate, and transmitting it to a human host. *Image adapted from Susan Jones, 2007*.[21]

DENV is a reemerging flavivirus that causes significant morbidity worldwide[19]. In 2013, it was estimated that 390 million people were infected with DENV, with 96 million showing clinical or subclinical symptoms of diseases[3]. This is compared to previous estimates of 80 to 100 million DENV cases in 1988[19]. This means that in a span of 25 years, the estimated burden of infection has tripled, especially in areas where vector of DENV transmission, *Aedes aegypti* resides[7,19], therefore highlighting DENV as a global public health concern[1].

DENV can cause three different diseases, ranging from mild Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) to severe Dengue Shock Syndrome (DSS)[3,15,16], however, 75.4% of reported infections are asymptomatic[4]. DF consists of flu-like symptoms, including headache, body pain, high fever, myalgia, and a rash, while DHF, along with the symptoms of DF, is accompanied by the increased permeability of blood vessels, which can lead to severe

internal bleeding and substantial water loss. Furthermore, DSS is characterized by extreme blood and water loss resulting in subsequent shock, which could be fatal if not treated immediately[17]. Although DF is commonly observed during primary infection of DENV, incidence of DHF and DSS increases during primary or secondary infection with other DENV serotypes[17].

Although the immune response against DENV is not completely understood, it can be categorized into two parts: primary infection and secondary infection, with the latter involving antibody-dependent enhancement (ADE). There are four known DENV serotypes (DENV1-4) that originated in monkeys in Africa or Southeast Asia approximately 100-800 years ago[34]**.** Upon primary infection by DENV, the human immune system undergoes both cell-mediated and humoral immune responses. The virus is first taken up in skin-resident Langerhans cells and macrophages of the innate immune system and viral antigens are displayed on major histocompatibility complex (MHC) molecules, which present viral antigen peptides to T cells to mediate the cellular immune response[14]. This includes lysis of viral-infected cells via CD8 T cells, natural killer cells, and macrophages[14]. Humoral immunity is initiated against DENV by B-lymphocytes involvement in binding or engulfment of viral antigens, followed by communication with macrophages and helper T cells, which differentiated the B cell into a DENV-specific antibody secreting plasma cell, releasing immunoglobulins, such as IgM, IgG and IgA to generate immune complexes for subsequent infection[14]. Immunoglobulins (antibodies) are proteins that consist of two heavy and two light protein chains bonded together by disulfide bonds (Figure 2)[7]. Each antibody has a conserved constant region (Fragment constant, Fc) and a variable region (Fragment antigen-binding, Fab), which is specific to the challenging antigen, on both the heavy and light chains[7,14]. The antigen binds to the Fab region,

forming an immune complex to aid in viral clearance and protection against subsequent infections against the same virus[7,14].

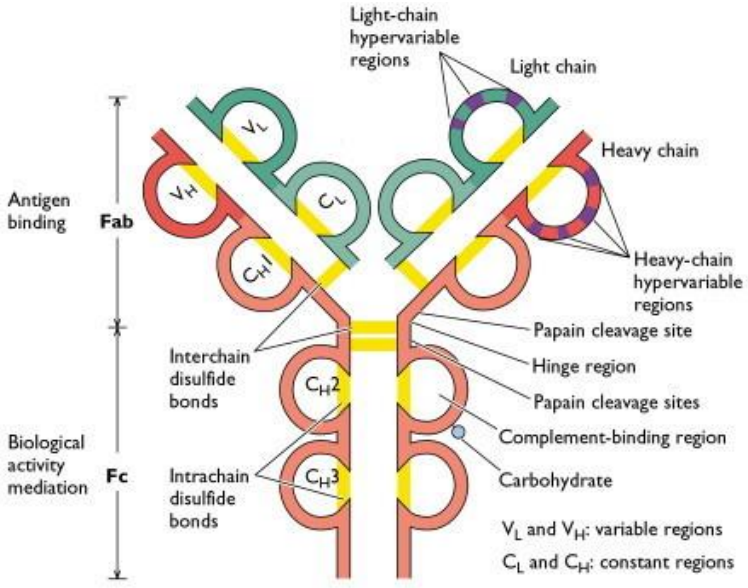

Figure 2. Construction of an antibody. Image represents an immunoglobulin G (IgG), that contains two heavy chains (pink/red) that contain and fragment constant (Fc) region, and two light chains (green). Fragment antigen binding (Fab) regions are indicated on both heavy and light chains containing hypervariable regions. Both chains are connected via disulfide bridges (yellow). *Image adapted from Flint, 2000.*[6]

Once the primary infection is cleared, some of CD8 T cells and B cells become memory cells resulting in lifelong immunity against the particular viral infection[14]. In case of DENV infections, the lifelong immunity is only specific against one type of DENV. Therefore, if an individual is exposed to a different DENV serotype during a secondary infection, the antibodies, such as IgG, against the first serotype don't fully neutralize the second DENV, but instead binding to Fc-receptors on immune cells particular monocytes and macrophages paradoxically enhancing infection leading to DHS/DSS, which is called as ADE[22]. In addition, anti-DENV IgG, IgM and complement C3 receptors have also been described to aid in ADE formation, as well[22]. Moreover, increased mononuclear cell activity releases a "cytokine storm," whereby an

over production and release of cytokines and chemokines that can lead to non-specific inflammation, which can result in increase of epithelial cell permeability contributing to vascular leakage and internal bleeding during in DHF and DSS[17,22]. Reports indicated that ADE increases risk for DHF and DSS, so there is a critical need for highly specific and rapid identification of DENV serotypes in donated blood before transfusion[15,17,26].

The presence of virus in the blood (viremia) increases during the incubation phase, peaks during the symptomatic phase, and slowly decreases as the immune system clears DENV from the body[10]. DENV viremia has been detected in asymptomatic patients five to seven days post symptoms in ranges of 3 to 7.4 x 10^{13} plaque forming units (PFU)[17-20]. This poses a severe threat in the context of donated blood and organs. Within the past ten to fifteen years, increasing reports have identified DENV transmission through blood transfusions and organ transplantations within endemic areas[5,12,18,20]. Due to variability of DENV symptoms, blood and organ donors may have unknowingly DENV infection [23]. In a study surveying donations in Puerto Rico, the results of polymerase chain reaction (PCR/qPCR) analysis of blood showed 56 viremic donations per 77,000 yearly donations collected[20]. In addition, studies in Australia, Brazil, and Honduras also showed similar results, indicating the global need for DENV blood screening[19].

There have been some assays performed to measure DENV in blood donations, including through DENV NS1 Ag screening, whereby anti-DENV antibodies in the blood implies previous infection[26]. However, this assay is not very specific and cannot differentiate acute from previous DENV infection, thus not useful to screen blood samples for viral particles[26]. The current gold standard for DENV detection in blood is through PCR, a highly specific viral genome detection assay[13]. Two PCR methods are currently performed, including nested PCR and traditional PCR.

While these assays are both serotype specific and highly sensitive, detecting as little as 10 PFU per ml[12], both processes take at a few hours to perform respectively[12-13]. PCR also requires non-portable, specialized equipment, trained technicians and costly reagents.

Surface-enhanced Raman spectroscopy (SERS) is a type of vibrational spectroscopy that uses gold nanoparticles (AuNP) to enhance a Raman signal to multiple powers of magnitude[23]. AuNPs are conjugated to a molecule-specific probe and excited by the Raman signal which relays a "molecular fingerprint" of the small biological molecule called the "analyte" [23]. Dr. Fengwei Bai's group has determined a flaviviral antibody (4G2)-AuNP complex that can bind to the DENV-2 and forms a specific DENV fingerprint using SERS[10] (Figure 3). SERS could be an alternative to the PCR based assays of DENV detection in blood as it is cheap, easy, and rapid to perform, taking as little as ten seconds per spectra sample[10]. Therefore, the full assay takes around "30 minutes from viral incubation with AuNP-4G2 to SERS sensing"[23]. Additionally, it is a very sensitive assay as it can detect as few as 10 PFU per 1 ml of sample, which is comparable to most standard assay, like PCR[12,23].

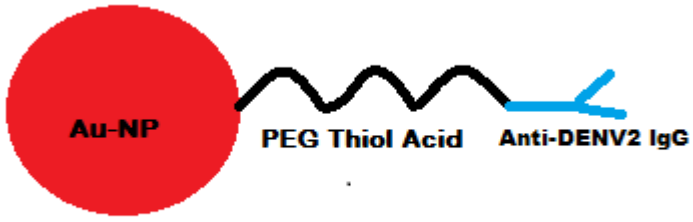

Figure 3. Proposed schematic of Bio-conjugation of anti-DENV2 IgG (HB46) to gold nanoparticles (AuNP). AuNPs are incubated with polyethylene glycol (PEG) and is bound via Au-Sulfur interaction. Purified HB46 IgG js then incubated with the AuNP-PEG via -COOH end binding to the thiol (S) acid. Estimates of approximately 100 IgGs can be bound to 1 AuNP *(Paul et al., 2014)*[24].

Early experimental issues using SERS was due to these non-specific reactions with biological materials; since then, several studies have focused on this issue, and have found that binding a specific "bio-recognition agent" to the nanoparticle can resolve these issues tremedously[6,11,23]. To do this, the majority of groups used a complex of thiolated polyethylene glycol (HS-PEG), which works to bind the AuNP surface to sulfur, stabilizing the nanoparticles[4,6,11,23]. Dr. Bai's group has used flavivirus specific antibodies to serve as the recognition agent, showing that antibody-antigen reactions could be used to make a DENV-specific SERS assay[23]. This method depends upon the antigen-antibody binding in the Fab region of the antibody to excite the now unstable magnetic fields when exposed to the SERS signal[4,6,11,23]. The movement of the bonds in the magnetic field are measured, resulting the spectra readout[4,6,9,23]. Without this complex, the antibody-PEG-AuNPs are stable and do not move, failing to provide the magnetic field that is utilized in SERS fingerprinting[4,6,9,23]. These results not only show that the SERS assay is very specific, but also the assay is very sensitive to detect as little as 10 PFU per ml (PFU/ml) DENV concentration[23]. Using antigen-specific antibodies ensures the specificity of the SERS complex by preventing multiple antigens from binding to the AuNP complex[21] and is especially useful for differential DENV serotype testing, which can be utilized in diagnostics. Moreover, this study also tested the reproducibility of the SERS spectra, and found that there was minimum shift between the peaks during multiple runs (Figure 4)[23]. Using this study as guidance, the purpose of this thesis is to produce specific DENV-2 antibodies (HB46) for the use of SERS applications, such as detection of specific DENV serotypes in blood donations and/or be used in diagnostics.

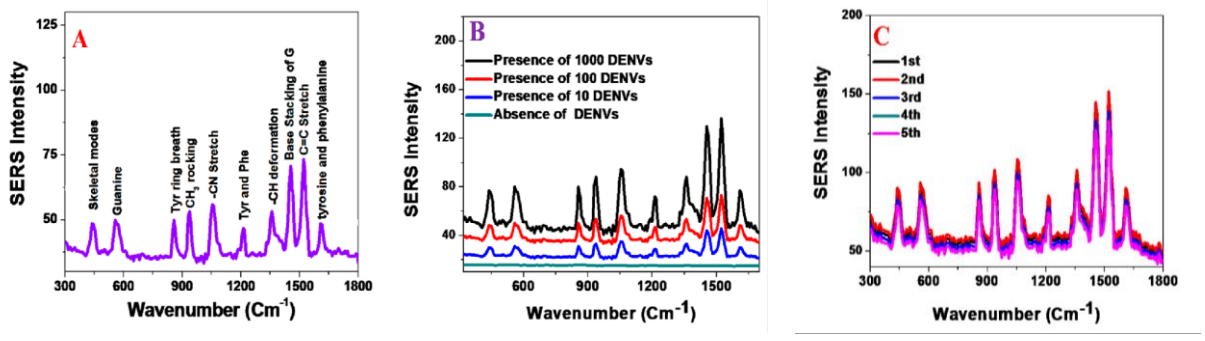

Figure 4. SERS viral vingerprints using AuNP-anti-flavivirus (4G2) antibodies. A. SERS viral fingerprint of DENV-2 indicates multiple surface-functional groups; **B.** Viral dilutions of DENV-2 followed by SERS assay indicates DENV-2 fingerprint is sensitive to detected 10 DENV-2 per 1 ml of sample; **C.** SERS DENV-2 fingerprint is reproduced after multiple runs (n=5). *Image adapted from Paul et al., 2015*[23].

Chapter Three: Methods and Materials

3.1. Experimental outline overview. Briefly, specific DENV-2 antibodies (HB46) were produced in mouse cell culture and these antibodies were then purified and concentrated to be subsequently conjugated to AuNPs to be used for SERS analyses in future studies. For this, DENV-2 antibodies were generated, purified and concentrated in a biosafety level (BSL) II facility at the University of Southern Mississippi (USM), while conjugation of these purified DENV-2 antibodies to AuNPs (AuNP-IgG) will be performed at Jackson State University (JSU) under the direction of Dr. Paresh Ray. For this, AuNP-HB46 will be cultured in the presence of various DENV1-4 dilutions in buffer by trained personnel at USM, and analyzed by SERS at JSU. Collaboration between institutes is instrumental to the future success of these experiments.

3.2. Isolation and Purification of DENV-2 antibodies. DENV-2 antibodies were produced through cell culture under an aseptic techniques and a BSLII cabinet, which involved growing an adherent cell line of HB46 (purchased from American Type Cell Culture, 3H5-1) in optimal growth conditions. HB46 are a mouse hybridoma cell line that has fused myeloma cells with B lymphocytes isolated from the spleens of mice that were immunized with DENV-2 antigens. Under serum free media conditions, HB46 cells divided and secreted DENV-2 antibodies into the media until a confluence (concentration) of cells that covered approximately ninety to ninety-five percent of the cell culture plate was reached and the media turned a yellow-orange color (low pH). This color change signifies expenditure of nutrients and the presence of increased metabolites. To re-culture the cells and to collect the antibodies in media, adherent cells were removed by scraping the bottom of the culture flask, were transferred to a 50 ml conical tube and were centrifuged at 2500 revolutions per minute (rpm) for 5 minutes to pellet the viable cells,

while secreted antibodies remained in the supernatant. The supernatant containing DENV-2 antibodies were isolated and stored at -20°C, and 10 ml of fresh, serum free media was added to the pelleted cells. The cells were then re-cultured at $1x10^6$ cells/ml and were incubated at 5% CO_2 in 37°C.

After about one liter of supernatant was collected, DENV-2 antibodies were concentrated and purified. Purification was performed using HiTrap™Protein G HP columns (GE Healthcare), which are specifically designed to bind to IgG antibodies in supernatant due to the charge of the molecules. All other media components passed through the column. Briefly, supernatant was centrifuged (1500 rpm for 5 minutes) to remove any particulates that may have formed during a freeze-thawing cycle, followed by dialyzation with phosphate buffered saline (PBS) to adjust the supernatant to a neutral pH (pH~7). The columns were attached to a peristaltic pump apparatus and rinsed through with PBS and 20 mM sodium phosphate (pH~7) (used to aid in the binding of IgG proteins within the column). A peristaltic pump is a tool used for antibody purification procedures as the rate of flow of supernatant can be tightly controlled through a relatively slow process, whereby supernatant would be introduced into the column at about a milliliter per minute. When 100 ml of medium was passed through the column (containing IgG) was eluted with 0.1 M glycine (pH~2) solution. Glycine at a pH of 2 was used, as this low pH is able to break the disulfide bonds of the antibodies (Figure 2), briefly, to elute them out of the column. Buffer neutralization is quickly achieved as the eluted solution was mixed with 500 mM of Tris-HCL (pH~8), which re-associated the broken antibody subunits. Elutes were stored at 4°C until concentration steps were performed.

3.3. Concentration of purified DENV-2 antibodies. After being stored, elutes were concentrated using a Corning Spin-X UF® concentrator column, which consists of a filter subunit (100 kD mw) attached to a flow-through receptacle underneath. The spin column was first prepped with 5ml of 20 mM sodium phosphate and incubated for 5 minutes. Purified elute (10 ml) was then added to the spin column and was centrifuged at 1500 rpm for 2 minutes (3x), with the replacement of flow-through (~5 ml) back to the filter subunit with each individual spin, followed by an additional spin at 2500 rpm for 15 minutes to ensure maximum concentration was reached. The remaining liquid in the top of the concentrator contained DENV-2 antibodies, which did not filter through due to their size (150 kD), were mixed with 40% glycerol to a final concentration of 1 mg per ml and were stored at -20°C in 100 µl aliquots.

3.4. Determining the concentration of purified DENV-2 antibodies. DENV-2 antibody concentrations were measured using a Bradford assay and a mouse IgG standard (1.2 mg/ml) as a control. Briefly, the Bradford assay consists of using a Bradford dye (Bio-Rad) that changes color from brown to blue in the presence of protein detection. This color change is in direct relation to the concentration of protein in the sample, which can be measured by spectroscopy **(**Bio-Rad SmartSpec^{TM} 3000). Unknown HB46 IgG samples were then compared to a standard mouse IgG control and concentration of the unknown HB46 IgG was determine by equation 1.

3.5. Determining purity of DENV-2 antibodies. To confirm our DENV-2 antibodies are pure, and do not contain proteins, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the antibodies. Briefly, 10% SDS pre-made casted gels, purchased from Bio-Rad, were used for protein separation. DENV-2 antibodies ran in parallel

with a mouse IgG standard control (0.6 mg/ml). Antibodies were treated with β-Mercaptoethanol (β-ME), which is a reducing agent that aids in breaking the disulfide bonds between the heavy and light chains of the antibody, identifying the heavy and light chain sizes (50 kD and 25 kD, respectively). A kaleidoscope protein ladder (Bio-Rad) was used, and the antibodies were serially diluted from 1.3 mg/ml to 0.013 mg/ml for SDS-gel detection.

To prepare the apparatus for SDS-PAGE, the pre-made casted gel was placed into an electrophoresis chamber, with 1x Running Buffer (25mM Tris, 192 mM glycine, 0.1% SDS, 10x Bio-Rad) was added. 10 µl of each IgG sample was mixed with 2x Laemmli sample buffer (Bio-Rad) and 15% β-ME and were boiled for 10 minutes. The antibodies were then added to each of the pre-cast wells, along with a standard protein ladder, and ran for about 1 hr at 200 A/60 V, or until the running buffer front was at the bottom of the gel. The gel was then was rinsed with double distilled water (H_2O) for five minutes (2x), then treated with a fixation solution (40% methanol, 10% acetic acid) for five minutes. After fixation, the gel was treated with a non-specific protein staining solution (0.025%(w/v) Coomassie Blue G-250, 10% acetic acid) for 15 to 30 min while gently shaking on a Stovall "The Bell Dancer" Orbital Platform shaker. The gel was then destained with the fixation solution and imaged on a Bio-Rad ChemiDoc^{TM} MP imager in the Mississippi INBRE facility.

3.6. DENV-2 Immunoassay. To determine if DENV-2 antibodies successfully detect DENV-2 antigens, immunoassays were performed. For this, Vero cells, an African green monkey kidney epithelial cell line that is fast growing and anchorage dependent, were infected with DENV-2 at multiple viral dilutions (stock = $3.5x10^6$ PFU/ml) to determine sensitivity of DENV-2 antigen detection. Briefly, Vero cells were cultured in Dulbecco's Modified Eagles Medium (DMEM)

supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37° C and plated at a concentration of $1x10^6$ cells/ml for 24 hr. Following incubation, cells were infected with DENV-2 virus (ATCC), by certified personnel for 1 hr for viral adsorbtion and penetration into the cells, followed by a wash with DMEM+10% and addition of the overlay media (DMEM+2%FBS and 1% Methyl Cellulose) for 4 days to allow for viral amplification and foci formation. After 4 days, cells were washed in PBS, and fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were then washed two times with PBS, and incubated with purified DENV-2 antibodies (1:100) overnight at 4°C. Secondary anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP) was then added (1:1000) for 2 hr at room temperature, and colorimetric detection was produced by the addition of True Blue Peroxidase substrate for focal forming unit (FFU) imaging. Images were taken with a ChemiDoc™ MP imager.

Chapter Four: Results

4.1. DENV-2 antibody concentration measurements

DENV-2 antibodies were collected from mouse HB46 cells and were purified using HiTrap™ Protein columns as described in the methods. Cells were then concentrated using Corning Spin-X UF® concentrator as described in the methods and their concentrations were determined using a Bradford assay (Table 1). A Bradford assay detects non-specific protein concentration in samples through colorimetric detection using spectroscopy. Absorbance at a wavelength of 595 nm was measured for each unknown concentration sample and a known standard IgG sample was recorded. 40% glycercol was added to each sample for -20°C storage conditions, then absorbance was measured a second time. Total sample concentration was determined by the formula in equation 1. These results indicate that the concentration technique was reproducible; as there was an expected loss of concentration after the addition of glycerol. However, because the final concentrations maintained about 1.1 mg/ml, the process was successful.

Experiment	Control BR (AU)	Unknown BR (AU)	[IgG] (mg/ml)	Post 40% glycerol Control BR (AU)	Post 40% glycerol Unknown BR (AU)	Final [IgG] (mg/ml)
1	0.398	0.571	1.72	0.422	0.395	1.12
2	0.709	1.008	1.7	0.667	0.765	1.4
3	0.709	1.008	1.7	0.667	0.718	1.3

Table 1. Bradford assay concentration calculations Multiple experiments (n=3) were perform to collect more than 1 mg/ml of purified antibody. A Bradford assay was performed to determine the concentration of the purified HB46 IgG in each example indicated above. Sample concentration calculations of DENV-2 antibody concentration were determined using equation 1, below. Control denotes standard IgG, BR denotes Bradford readout at an absorbance of 595 nm, AU denote arbitrary units.

$$= \left[\frac{Unknown\ sample\ absorbance_{595}}{Standard\ sample\ absorbance_{595}}\right] X\ Concentration\ of\ Standard\ IgG\ \left(\frac{mg}{ml}\right)$$

Equation 1. The equation used to convert Bradford spectrometry at an absorbance of 595 nm to concentration (mg/ml). Determining the concentration of unknown samples was measured by using the standard absorbance measurement (wavelength of 595 nm) and standard concentration and the above formula.

4.2. DENV-2 antibody identification by SDS-PAGE

Following antibody Bradford concentration determination, samples were subjected to SDS-PAGE identification to confirm the samples contained antibodies, and no other contaminates present. Purified and concentrated HB46 samples were subjected to reducing β-ME conditions to dissociate the heavy and light chains of the antibodies and were separated using an SDS-PAGE. Heavy (50 kD) and light (25 kD) chains in lane 3 of a HB46 sample were compared to a lane 2 standard IgG sample control, which indicated similar identification banding patterns, without additional bands observed in HB46 samples, suggesting HB46 samples were pure IgG. The Kaleidoscope Bio-Rad ladder is observed and compared 0.6 mg/ml IgG standard to 1.3 mg/ml sample of concentrated antibody (Figure 5).

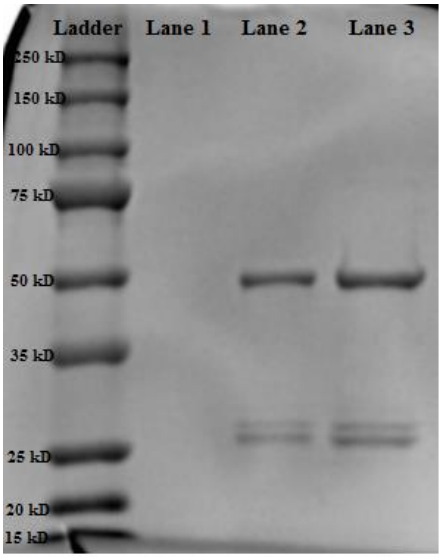

Figure 5. SDS-PAGE for IgG identification. Banding patterns of IgGs in order from left to right: the BioRad Kaleidoscope™ Ladder (10 kD to 250 kD); Lane 1, (blank) β-ME treated buffer with PBS without IgG, Lane 2, 0.6 mg/ml mouse IgG standard control treated with β-ME and Lane 3, 1.3 mg/ml HB46 IgG treated with β-ME.

4.3. Verification of DENV-2 antibody specificity to DENV-2 by an immunostaining assay

To confirm antibody specificity against DENV-2, immunostaining assays were performed using differential DENV (1-4) types. Comparisons were made with anti-flavivirus antibody (4G2, ATCC 3H5-1), which confirmed protocol reproducibility, while HB46 isolated antibodies were used to determine specificity to DENV-2 (Figure 6).

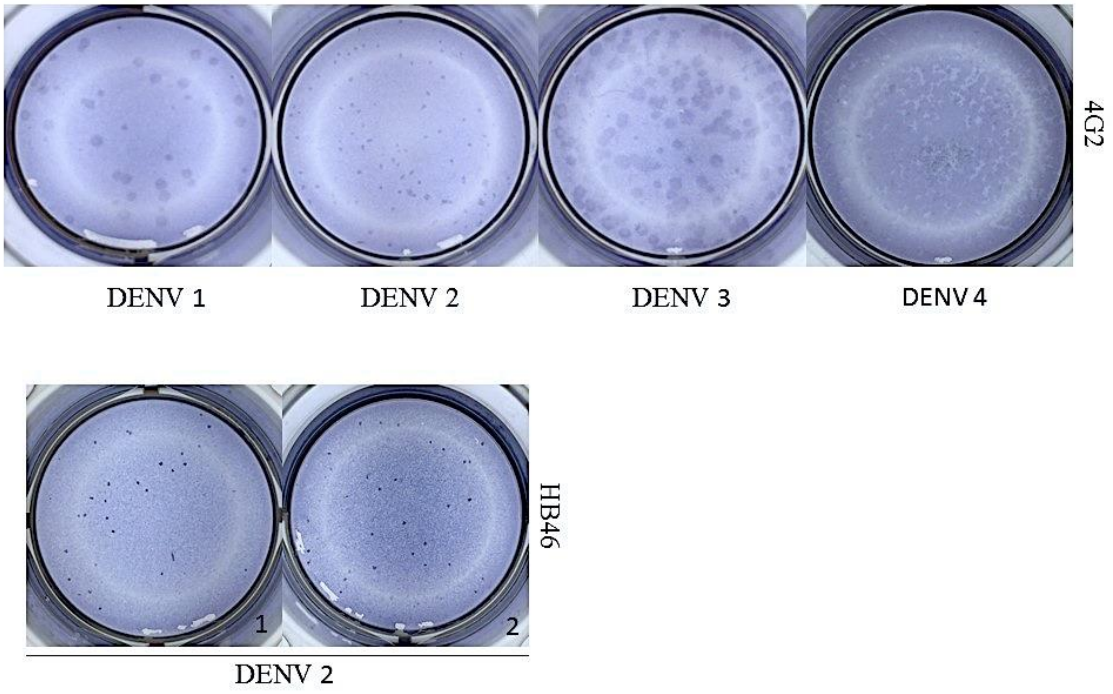

Figure 6. Comparison of HB46 IgG specificity with 4G2. A. To confirm DENV-2 antibodies were specific to DENV-2, a FFU assay of all four serotypes of DENV (labeled above) was performed with flavivirus-specific antibodies (4G2). **B.** A FFU assay was performed with DENV-2 serotype and HB46 (DENV-2 specific) in duplicate.

An additional FFU assay was performed to determine HB46 antibody sensitivity. For this, DENV-2 was serially diluted, followed by an immunostaining assay using HB46 antibodies

(0.013 mg/ml) (Figure 7.) Counted FFU are indicated in Table 2, which indicated HB46 could successfully resolve a 1000x-diluted sample of DENV-2.

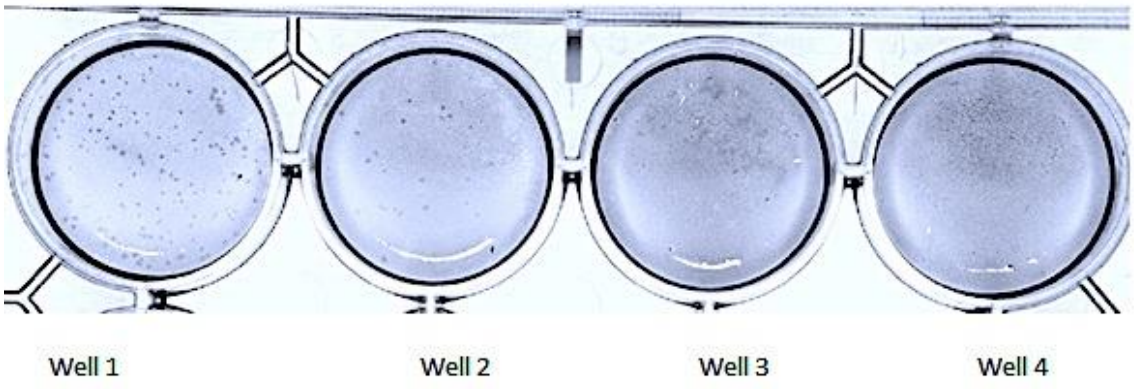

Figure 7. HB46 is highly sensitive to detection of DENV2. DENV2 dilution series and immunostaining images are represented, from left to right: well 1, 1/10 dilution; well 2, 1/100 dilution; well 3, 1/1000 dilution; well 4, no virus control. The wells were performed in triplicate, with best representation image reported. FFU are indicated as dark circles where HB46 antibodies have bound to the DENV-2.

Dilution Factor	No. of Foci per well
10^1	143
10^2	28
10^3	10

Table 2. Sensitivity of HB46 detection of DENV-2. Counted foci per dilution factor indicated reduced foci with increased DENV-2 dilution (10^1, 1/10 dilution, 10^2, 1/100 dilution, and 10^3, 1/1000 dilution of virus) and HB46 could detect as little as 1000x-diluted sample using an immunostaining assay.

Chapter Five: Discussion

The overall goal of this study is to produce and purify DENV-2 specific antibodies for future SERS applications. In brief, DENV-2 antibodies (HB46) were produced in cell culture, purified and concentrated, and tested for DENV-2 specificity in FFU assay. In future studies, these specific antibodies will be conjugated to AuNPs, incubated with DENV-2, and subjected to SERS analysis. The SERS assay will provide a sensitive, selective and rapid DENV-2 fingerprint detection for diagnosis and blood screening.

The HB46 cell line was grown to confluence to produce antibodies against DENV-2. Serum free media was an optimal growth medium to use for these cultures as antibodies that are grown in serum would produce non-specific SERS fingerprint spectra[4]. This is because serum contains multiple proteins, including non-specific antibodies, resulting in protein contamination, which affects downstream SERS signaling. Once enough medium was produced, antibodies were collected in columns that were used to purify and collect the IgG specific antibodies. HiTrap™ columns utilize G proteins bound to a biocompatible polypropylene[32]. These proteins bind to the Fc region of the IgG, causing the antibodies to bind to the column proteins and allowing all other antibody types or contaminating proteins to pass through the column, yielding only the IgG antibodies after elution[32]. A glycine buffer (pH~2) was used to break the strong bond between the G protein in the column, while dissociating the disulfide bonds of the antibodies. However, rapid buffer neutralization was achieved following elution in 500 mM Tris-HCl, which retain neutral pH and prevented any additional IgG denaturation by the glycine buffer[31].

A Bradford protein assay is one of the most commonly used protein quantification methods used in research labs[29]. While there are more accurate quantification assays, such as the Lowry method, the Bradford assay provides a rapid and more sensitive way to quantify protein

without the use of many reagents[29]. The assay uses the color change of Coomassie dye (Bradford Reagent) from brown to blue, signifying protein binding to the dye, to approximate the concentration of protein in the solution, which is assumed to be directly proportional to the spectroscopy readings[29]. The anionic blue form of the dye has a maximum absorbance of 590, but most assays are measured at 595 due to a recorded absorbance shift once the protein has bound to the dye[29]. IgGs are somewhat fragile in that they can easily denature in room temperature conditions, so the speed and specificity of the Bradford assay provided was the optimal choice compared to other quantification methods that take longer and require many more reagents, which may possible even result in the introduction of contaminants. One drawback of the Bradford assay is that it detects all proteins, not just antibodies, so while the concentration can be assumed by the assay, the purity of the sample cannot[29]. To address this issue, we performed an SDS-PAGE to check for any protein contamination.

To check the purity of the eluted IgG, SDS-PAGE was performed on the IgG sample and an IgG standard control. The reducing agent β-ME was used to reduce the IgG from a tertiary molecular structure (150 kD) to a primary structure that consists of two heavy chains (50 kD) combined with two light chains (25 kD). β-ME can break the disulfide bonds separating the chains, while separation of these chains are determined by SDS-PAGE, and compared to known IgG controls for identification. Therefore, additional banding patterns observed in the SDS-PAGE would serve as non-specific protein contamination. In our results, the IgG standard reduced to 50 kD and 25 kD sizes, while our HB46 IgG samples had a slightly higher band shifting around 28 kD. Upon further review, the light chains of monoclonal antibodies are known to have a range of molecular weights (20 kD to 30 kD) upon reducing treatment[7]. Knowing that the standard was pure and properly concentrated, the secondary banding was not contamination,

but in fact most likely a result of possible incomplete reduction by the β-ME. Excluding the secondary banding of the antibodies, there were no other bands in the gel, therefor there was no extra-protein contamination in the concentrated antibodies.

DENV-2 antibodies must successfully bind to the virus for SERS viral fingerprinting accuracy and selectivity. To ensure HB46 antibodies were capable of binding to DENV-2, a focus forming unit assay (FFU) was performed. To compare the FFU identification of HB46, this assay was first performed using a flavivirus-specific antibody (4G2) that can detect all 4 serotypes of DENV[23]. The results showed that HB46 IgG are capable of identifying DENV-2, as compared to 4G2 IgG, which suggest HB46 IgG are selective for DENV2. Additional studies using all serotypes of DENV (1-4) and HB46 in a FFU assay would further confirm the selectivity of HB46 to DENV2, compared to DENV1, 3, or 4, which is planned for the near future. Selective identification of different DENV serotypes in blood donations becomes useful during a SERS assay for diagnosis and the prevention of ADE occurring in patients who have already been infected with DENV. This selective DENV serotype testing could also allow health care professionals and epidemiologists to identify infection trends and prevent further infection in endemic areas.

Additionally, the immunostain results using 4G2 indicated differential sizes of FFU are produced with each serotype of DENV. Studies have shown that serotypical differences can be detected through FFU due to the reaction of the antibodies to each different antigen, with some be more active than others, with the more active antigens creating larger and the less active antigens creating smaller foci[9]. Further identification of HB46 sensitivity was performed using serial dilutions of DENV-2, which suggests the sensitivity of HB46 antibody to detect virus, using an immunostaining assay was $3.5x10^3$ PFU/ml. During SERS, the sensitivity of HB46

would be increased since multiple IgGs are conjugated to an AuNP, and it has been shown that AuNP-4G2 complexes can detect as low as 10 PFU/ml of sample[23]. In brief, HB46 IgG samples were capable of detecting 10 FFU/ml of DENV2, therefore are optimal to use for AuNP conjugation and subsequent SERS applications.

Further directions for this study is to perform a SERS assay using specific HB46 IgGs bound to AuNPs. For this, serial dilutions of DENV-2 will be performed in blood followed by the addition of AuNP-HB46 complexes, and following an incubation period these samples would be subjected to SERS. To confirm HB46 specificity, we will also dilute other flaviviruses, such as WNV or Zika virus, in blood and add our AuNP-HB46 complexes to be subjected to SERS, which would determine if SERS can provide a viral fingerprinting database for public use. Previous studies have used 4G2 antibodies conjugated to AuNP complexes, but these antibodies are only specific for flaviviruses in general and does not detect specific DENV serotypes which could be useful for diagnostic purposes[23]. By using a SERS assay that is antigen specific, the diagnostic aspect could be introduced, whereby a DENV-infected patient could know which serotype of DENV they have, along with how much viremia is in their body (indicating the stage of infection), and aid in their doctor's course of treatment. It will also give blood banks more cost effective, rapid and selective tools to screen for the DENV in endemic areas. This is especially useful in these areas where cheaper equipment is feasible to use, while it is also less labor intensive than RT-PCR. This allows for blood banks to test large amounts of samples in very little time, making it an ideal tool for endemic areas.

In summary, since different viral spectra are generated for each virus type in a SERS assay, this study provides a successful and reproducible protocol to initiate the development of an alternative method of blood screening that is sensitive, rapid and cost-efficient. Therefore,

generation of DENV-2 specific antibodies can be reproduced using this established protocol with other DENV serotypes, which can be integrated into future AuNP conjugation designs and SERS analyses.

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