University of Alberta

Ultrasensitive Point-of-Care Dengue Diagnostics and Vaccine Applications

by

Advaita Ganguly

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Pharmaceutical Sciences

Pharmacy and Pharmaceutical Sciences

Fall 2013
Edmonton, Alberta

ABSTRACT

Dengue virus infections can result in a range of clinical manifestations from asymptomatic infection to dengue fever and the severe disease dengue haemorrhagic fever/dengue shock syndrome. The disease is now endemic in more than 100 countries in Africa, the Americas, the eastern Mediterranean, Southeast Asia, and the Western Pacific, threatening more than 2.5 billion people. The World Health Organization estimates that there may be 50 million to 100 million cases of dengue virus infections worldwide every year, which result in 250,000 to 500,000 cases of Dengue Hemorrhagic fever and 24,000 deaths each year. The dengue virus non-structural NS1 protein is a 46–50 kDa glycoprotein when expressed in infected mammalian cells. A high circulating level of NS1 was demonstrated in the acute phase of dengue infection by antigen capture ELISAs. The precise function of dengue NS1 protein remains unclear. However, antigen detection of non-structural dengue antigens may be of benefit for an early stage rapid diagnosis of infection due to its long half-life in the blood. Five high affinity monoclonal antibodies were developed and characterized against the recombinant dengue NS1 protein using hybridoma technology. Anti-NS1 and anti-HRPO hybridomas were fused and sorted to develop a series of bi-specific antibodies. The recombinant NS1 protein was also used to immunize chickens for the development of Anti-NS1 chicken IgY polyclonal antibody. The different combinations of anti-NS1 mAbs, bi-specific antibodies and chicken IgY were used in the development of simple, rapid, inexpensive, highly sensitive, specific and easy to perform assays for the detection of dengue virus infection.

The dengue envelope protein was also expressed using recombinant techniques for the evaluation of its role as a vaccine candidate. The envelope protein is known to induce neutralizing antibodies against dengue virus. The recombinant dengue envelope protein was used to immunize mice as a vaccine candidate. Serum antibody analysis confirmed virus neutralization characteristics. We also raised monoclonal antibodies against the envelope protein to better understand dengue pathogenesis as well as potential reagents in dengue diagnostic development.

PREFACE

Conference Presentations

Advaita Ganguly, Raimar Loebenberg, Hoon H Sunwoo, 2012. **Rapid Point of Care Diagnostic for PRRS detection in Swine**, 33rd International Society for Animal Genetics Conference, Cairns, Australia

Advaita Ganguly, Saikiran Sharma, Mavanur R Suresh and Hoon H Sunwoo, 2011 **Development of Low Dose DC Targeted Dengue DNA Vaccine**, Research and Development Day, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (November 2011)

Advaita Ganguly, Dipankar Das, Xinli Tang, Pravin Bhatnagar, Mavanur R Suresh, 2010 **Development of Immunoswab Assay for Detection of Dengue Virus NS1 Antigen,** 2nd Pan American Dengue Research Network Meeting (November 2010), Cancun, Mexico

Advaita Ganguly, Dipankar Das, Xinli Tang, Pravin Bhatnagar, Mavanur R Suresh, 2010 **Monoclonal Antibody Immunoassay Based Detection of Dengue Virus NS1 Antigen,** Research and Development Day, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (November 2010)

Advaita Ganguly, Dipankar Das, Xinli Tang, Pravin Bhatnagar, Mavanur R Suresh, 2010 **Monoclonal Antibody Immunoassay Based Detection of Dengue Virus NS1 Antigen**, 28th Annual meeting of the European Society for Pediatric Infectious Diseases (May 2010), Nice, France

Advaita Ganguly, Pravin Bhatnagar, Xinli Tang, Dipankar Das, Mavanur R Suresh, 2009 **Development of Monospecific and Bispecific Antibodies against Dengue Virus NS1 protein**, World Congress of the World Society for Pediatric Infectious Diseases (November 2009), Buenos Aires, Argentina

Advaita Ganguly, Sunundha Mongkolaungkoon, Dipankar Das and Mavanur R Suresh, 2009, **Monoclonal antibodies against Dengue virus serotype 1 envelope protein - Development of a prospective vaccine**, Medical Biodefense Conference (October 2009), Munich, Germany

Advaita Ganguly, Sunundha Mongkolaungkoon, Dipankar Das and Mavanur R Suresh, 2009, **Expression of Dengue Virus Envelope and NS1 Proteins for Prospective Therapeutic and Diagnostic Applications**, International Meeting on Emerging Diseases and Surveillance (February 2009), Vienna, Austria

Advaita Ganguly, Pravin K Bhatnagar, Dipankar Das, Mavanur R Suresh, 2008, **Monoclonal Antibodies against Dengue Virus serotype 1 envelope Protein – Development of a Prospective Vaccine Research and Development Day**, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (October 2008)

Advaita Ganguly, Dipankar Das, Mavanur R Suresh, 2007, **Expression of the Dengue Virus Envelope protein in *E. coli* – A Potential Candidate for Vaccine Development**, Research and Development Day, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (October 2007)

Journal publications

Advaita Ganguly, Pravin Bhatnagar, Xinli Tang, Hoon H Sunwoo, Mavanur R Suresh, 2013, **A Rapid Point of care Immunoswab Assay for Dengue Virus NS1 Antigen Detection**, Diag Microbiol Infect Dis (*Accepted)*

Advaita Ganguly, Dipankar Das, Ravindra B Malabadi, Hoon H Sunwoo, Mavanur R Suresh, 2013, **Enhanced Prokaryotic Expression of the Dengue Virus Envelope protein**, J Pharm Pharmaceut Sc *(In Press)*

Advaita Ganguly, Ravindra B Malabadi, Raimar Loebenberg, Mavanur R Suresh and Hoon H Sunwoo, 2013, **Development of an ultrasensitive hetero-sandwich ELISA assay based on bispecific monoclonal antibody for the detection of dengue NS1 protein**, J Pharm Res, 7(5): 374-380

Advaita Ganguly, Ravindra B Malabadi, Raimer Loebenberg, Mavanur R Suresh and Hoon H Sunwoo, 2013, **Dengue Diagnostics: Current Scenario**, Research in Biotechnology, 4(2): 19-25

Advaita Ganguly, Ravindra B Malabadi, Raimer Loebenberg, Mavanur R Suresh and Hoon H Sunwoo, 2013, **A Mini-review of Dengue Vaccine Development**, Research in Pharmacy 3(2): 18-25

Hoon H. Sunwoo, Arivazhagan Palaniyappan, **Advaita Ganguly**, Pravin K. Bhatnagar, Ayman O.S. El-Kadi and Mavanur R. Suresh, 2012 **Quantitative and sensitive detection of SARS-CoV spike protein using bispecific monoclonal based enzyme-linked immunoassay**, J Virol Methods. 2012 Sep 17. pii: S0166-0934(12)00317-5. doi: 10.1016/j.jviromet.2012.09.006

Ravindra B. Malabadi, **Advaita Ganguly**, Hoon H Sunwoo, Mavanur R Suresh. 2012, **Role of bi-specific monoclonal antibodies in immunodiagnostic assay**, Research in Pharmacy 2(3): 08-14

Saifur Rahman Khan, **Advaita Ganguly**, Ravindra B. Malabadi, Hoon H. Sunwoo, Mavanur R. Suresh. 2012, **Gene Delivery System: A Developing Arena of Study for the New Era of Medicine**, Recent Patents on DNA & Gene Sequences 6(1)

Ravindra B. Malabadi, **Advaita Ganguly**, Archana Parashar, Jaime A. Teixeira da Silva, Mavanur R. Suresh, Hoon Sunwoo. 2011, **Overview of Plant Derived Vaccine Antigens: Dengue Virus**, J Pharm Pharm Sci, 14 (3): 400-413

Saifur Rahman Khan, **Advaita Ganguly**, Ravindra B. Malabadi, Hoon H. Sunwoo, Archana Parashar, Jaime A. Teixeira da Silva, Mavanur R. Suresh. 2011, **Targeting strategies and nanocarriers in vaccines and therapeutics**, Research in Biotechnology, 2(6): 08-20

Book chapter

Archana Parashar, Susmita Sarkar, **Advaita Ganguly**, Saikiran Sharma, Mavanur R. Suresh, 2011 **Bispecific Antibodies for Diagnostic Applications**, Ed. Dr. Roland Kontermann (U of Stuttgart), Springer, Heidelberg

Manuscripts submitted

Advaita Ganguly, Pravin Bhatnagar, Hoon H Sunwoo, Mavanur R Suresh, 2013, **Production and Characterization of Monoclonal Antibodies against Dengue Virus Envelope Protein** *(submitted)*

Advaita Ganguly, Ravindra B Malabadi, Raimer Loebenberg, Mavanur R Suresh and Hoon H Sunwoo, 2013 **Diagnostics of Dengue type 1 NS1 protein using IgY** *(submitted)*

Advaita Ganguly, Pravin Bhatnagar, Xinli Tang, Hoon H Sunwoo, Mavanur R Suresh, 2013 **Production and Characterization of Monospecific and Bispecific Antibodies against Dengue Virus NS1 protein** *(submitted)*

Advaita Ganguly, Saikiran Sharma, Ravindra B Malabadi, Raimer Loebenberg, Mavanur R Suresh and Hoon H Sunwoo, 2013, **Dendritic Cell targeted formulations for immunization against Dengue** (*under prep*)

ACKNOWLEGEMENT

I wish to express my sincere gratitude to my supervisor Dr. Mavanur R Suresh for his support and enthusiasm. His encouragement and guidance helped me accomplish my goals. I am very grateful to Dr. Raimer Loebenberg and Dr. Hoon H Sunwoo for guiding me in finishing my graduate program in the unfortunate absence of Dr. Suresh. I am obliged to them for their constant support, guidance, and constructive advice. The completion of this thesis would have not been possible without their assistance.

I would like to extend special thanks to my supervisory and candidacy committee members, Dr. Michael Doschak, Dr. Steve McQuarrie and Dr. John Mercer for continuous guidance, encouragement, support and suggestions during my PhD program. I also extend my sincere thanks to Dr. Arno Siraki for his excellent insights.

I would also like to acknowledge Dr. Dipankar Das, Dr. Pravin Bhatnagar, Dr. Welson Wang, Dr. Ravindra B Malabadi, Dr. Vivek Mishra, Dr. Palaniyappan and the entire Dr. Suresh research group for their kind support and suggestions.

I am also very grateful to Dr. Aravinda deSilva, University of North Carolina, Chapel Hill, USA, for his kind help in the characterization studies of the monoclonal antibodies and giving me the opportunity to train in his laboratory. I specially thank The Natural Science and Engineering and Research Council of Canada [NSERC] for my funding and the Faculty of Pharmacy and Pharmaceutical Sciences for all support. I would like to specially thank everyone in RFNS, Alberta Health Services and Comprehensive Tissue Center, Alberta Health Services for their support.

Finally, I wish to thank all my friends and family for their love, support and encouragement throughout.

CONTENTS

List of Tables

List of Figures

List of Symbols and Abbreviations

µg	microgram
µg/ml	microgram/milliliter
Ab	Antibody
ADE	Antibody dependent enhancement
Ag	Antigen
AP	Alkaline phosphatase
β –ME	β-mercaptoethanol
BSA	Bovine serum albumin
bsMAb	Bispecific monoclonal antibody
CAb	Capture antibody
cell/ml	cell per millilitre
CO_2	Carbon dioxide
DAb	Detection antibody
DBSA	Dialyzed bovine serum albumin
DC	Dendritic Cell
DENV	Dengue Virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue Shock Syndrome
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylene diamine tetraacetic acid

ELISA	Enzyme linked immunosorbent assay
Env	Envelope
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GAM-HRPO	Goat anti-mouse IgG conjugated to horseradish peroxidase
h	hour
HAT	Hypoxanthine-aminopterine-thymidine
HT	Hypoxanthine-aminopterin
HGPRT	Hypoxanthine-guanine-phosphoribosyl transferase
HRPO	Horseradish peroxidase
IgG	Immunoglobulin class G
IgY	Immunoglobulin class Y [chicken]
IMAC	Immobilized Metal affinity chromatography
LOD	Limit of detection
MAb	Monoclonal antibody
m-APBA	m-Amino phenyl boronic acid agarose
mg	milligram
min	minute
ml	millilitre
ng	nanogram
ng/ml	nanogram/milliliter

Ni-NTA	Nickel nitrilotriacetic acid
NS1	Non-structural protein 1
OD	Optical density
PBS	Phosphate saline buffer
PBST	Phosphate saline buffer containing 0.05% tween-20
PEG	Polyethylene glycol
POC	Point of care
PSG	Penicillin-streptomycin-glutamine
RPM	Revolution per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
St-HRPO	Streptavidin tagged horseradish peroxidase
TMB	3,3’,5,5’- Tetramethylbenzidine
TRITC	Tetramethylrhodamine isothiocyanate
WHO	World Health Organisation

1

INTRODUCTION

1.1 Dengue Diagnostics: Current Scenario

1.1.1 BACKGROUND

Dengue virus is currently recognized as one of the most infectious mosquito borne viruses, affecting people in more than 100 countries [1,2]. The disease is endemic, in mostly tropical and sub-tropical areas, and recently, an evolving concern in industrialized countries, particularly, in Europe and the USA [3, 4, 5]. Current assessment in developing countries reveals that dengue fever is one of the most significant emerging diseases of modern times. It is estimated that with more than 3 billion people currently at risk, huge fatalities are to be expected. Annually about 100 million cases of dengue fever and dengue hemorrhagic fever [DHF] are reported [6, 7, 8]. The potential global threat of dengue disease spreading stems from increased travel to and from endemic areas. Of particular concern is the mutational development of new dengue virus strains being more and more exposed to susceptible populations, particularly, in children. Globally, due to the quick spread of both virus and hosts, dengue disease has become an epidemic and an urgent public health concern [9, 10]. Phenotypically, dengue viruses are single stranded RNA viruses that belong to the family *Flaviviridae*. There are four serotypes, i.e. DEN-1, DEN-2, DEN-3 and DEN-4. Primarily the Aedes aegypti mosquitoes transmit these four serotypes to humans. Dengue virus is a positive-stranded encapsulated RNA virus wherein the genomic RNA is about 11 kb in size and comprises of three structural proteins [nucleocapsid protein [C], a membrane associated protein [M], and an envelope protein [E], and seven nonstructural [NS] proteins. Infection with any of the four serotypes induces a life-long protective immunity to that particular serotype but confers only partial immunity against later, secondary infections by the other three serotypes [11,

12]. Secondary infection with various dengue virus serotypes is a major risk factor for the more severe and often fatal DHF and the Dengue Shock Syndrome [DSS] due to the phenomenon of antibody-dependent enhancement [ADE] [13, 14].

There is no available anti-dengue vaccine or therapy for the treatment of dengue secondary infections [15]. Development of an effective dengue vaccine is extremely challenging primarily because it must be tetravalent so that it confers protection against all four serotypes but it's efficacy must also, overcome ADE. Furthermore, there is no proper animal model for dengue vaccine trials. Currently, some candidate vaccines are in various stages of clinical trials but an efficient, safe, low-cost vaccine remains to be developed. Dengue infection can range from an asymptomatic infection to a more severe form of the disease. The more severe forms of infection include the possibility of developing additional complications such as hepatitis and/or other side effects [16, 17]. The diagnostic tests developed must be able to determine the severity of the infection at every level while simultaneously, identify the different serotypes. Vector control efforts, another form of palliative intervention, have also been extensively adopted in dengue endemic countries but results are far from satisfactory [18]. Currently, a number of different diagnostic tests are being administered including virus isolation [17], hemagglutination inhibiton [HI] [23], plaque reduction neutralization test [PRNT] [43], reverse transcription polymerase chain reaction [RT-PCR] [45] and immunoassay for antibody detection [19, 20, 21]. Other novel technologies are also being aggressively pursued with objectives of reducing costs through improving viral test sensitivity, specificity, rapidity and simplicity of application. The current scenario surrounding tests currently being used for detection of dengue infection along with future objectives for promising technologies are discussed in the following section.

1.1.2 CURRENT DIAGNOSTICS FOR DENGUE VIRUS INFECTION

Simplified detection tests are very important for timely and accurate diagnosis of dengue for imparting proper care and treatment to the patients. As dengue is endemic in mostly developing countries it is very important to develop a diagnostic test that can be carried out with minimal training and above all the test must be fast and can be performed at any setting [22]. The most commonly used methods for detection of dengue virus infection are antibody detection assays, viral isolation and RT-PCR based diagnostics. Virus isolation is considered as the standard protocol in identifying dengue infection. Virus isolation is typically carried out from clinical samples using mosquito cell lines [e.g. AP-61, C6/36] [23]. Associating immunofluorescence techniques with dengue specific monoclonal antibodies identifies the virus. Inoculating mosquitoes is one of the better methods for virus isolation inoculation [24, 25] owing to its higher sensitivity. Reports confirm that virus isolation from whole blood is more sensitive compared to serum [26]. But the necessity of proper infrastructure is a major deterrent for the routine use of this method. Another method of detection of dengue infection is the detection of dengue viral antigens. As high concentrations of one of these antigens [NS1] has been reported in the blood of patients with primary as well as secondary dengue infections up to 10 days post disease onset [27, 28], detection of this antigen by an ELISA based assays is commonly employed to evaluate the severity of the infection. An assay, composed of polyclonal and monoclonal antibodies as capture and detection antibodies, respectively, along with purified NS1 from dengue infected cells as a standard, has already been developed. Analysis of clinical samples with this assay correlated highly with others therefore, additional assays were developed along the same lines employing these basic principles.

Subsequently, many of the kits developed became commercially available [29, 30]. Detecting viral antigens by indirect immunofluorescence assays have also been studied wherein monoclonal antibodies and fluorescein conjugated anti mouse or anti human antibodies are used. A visual end point [color change] constitutes a positive or a negative result. The MAC-ELISA is a commonly used dengue serological testing procedure. The assay contains all the four dengue serotype antigens to detect IgM antibodies in clinical samples. One of the major problems of this assay is its limited specificity and cross-reactivity with other flavivirus antigens. It has been reported that the ratio between IgM and IgG could be a parameter to differentiate between primary and secondary dengue infection. This has been commercially exploited in the PanBio detection kit. Falconar and colleagues have improved upon this and established better standards to define the ratios for 100% classification of serologically classical dengue infections [53, 54]. Microsphere-based immunoassays or MIA are gaining importance as an alternative for the diagnosis of many infectious diseases [56]. MIA is based on the covalent bonding of antigen or antibody to microspheres or beads and detection is by flow cytometry. MIA is faster than a MAC-ELISA and has potential to identify antibody responses to multiple pathogens simultaneously [53].

Multiple methods have been pursued for the serological detection of dengue specific antibodies that include the HI test [19], PRNT. The IgM /IgG ELISA based on a sandwich format and the HI test are the most commonly used serological assays. In the past, the HI test was used to differentiate between primary and secondary dengue virus infections but recently, antibody capture assays have become more popular. The HI test needs pretreatment to get rid of haemagglutination inhibitors and also the test is prone to flavivirus cross reactivity as well as serotype cross reactivity. Consequently, there was

the complement fixation [CF] test which, when compared to other tests has a higher sensitivity but the CF antibodies are found only after a week for a short period of time [34]. Of all the tests developed for identifying dengue infection, it appears that antibody detection ELISAs has become the most efficacious assay; the key advantages being its sensitivity and specificity. A number of kits are available in the market based on this principle [31, 32, 33]. The nucleic acid sequence based amplification or NASBA is an RNA amplification method based on chemiluminescence for detection of mRNA. This method has been successfully used to detect other pathogens such as malaria [35, 36]. This technology was then applied for the detection of dengue of all four serotypes and the results were comparable to a standard immunofluorescent-based virus isolation assay [37]. The PRNT is considered to be a standard assay to measure dengue-neutralizing antibodies. The assay analyses plaque reduction at a 50% end point. PRNT is capable of assaying dengue serotype specific neutralizing antibodies. The assay is efficient but the assay is prone to inter assay variations, which could come from virus stocks being used, cell lines to develop plaques or even the culture media being used. Standardization of the assay is currently being focused on [55].

Dengue RNA can also be detected in clinical samples from humans or in mosquitoes by reverse-transcription–polymerase chain reaction [RT-PCR] amplification technique [38, 39]. Another technique, known as dot blot immunoassay, is also available, however, this methodology is costly, and therefore routine use in developing nations is unlikely. Other sources utilized by researchers to detect serotype specific antibodies in clinical samples involved analysis of recombinant proteins domains of dengue virus serotypes, collected in strips, and then validated by PCR based diagnosis [40, 41]. Dengue virus antigens can also be visualized in samples with labelled monoclonal

antibodies, end point visualization done with fluorescent dyes and enzymes. Earlier dengue infections as well as current infections can be detected by Dengue Immunoglobulin G assays if timely serum collection can be done. Assays are generally done using dilutions of each serum sample and used to determine a primary or a secondary infection. IgG based assays are very useful but it also has a major limitation in that it is not serotype specific although the assay possess higher sensitivity than the HI assay. On the other hand IgM based dengue diagnosis**,** a similar assay, is commonly used in clinical settings. IgM appears within 5 days of onset of illness and has both high specificity and sensitivity. The antigen purity is a major parameter in IgM based assays. There are a lot of commercial kits that use antigens from all the four serotypes in the assay thus determining any dengue infection and not being serotype specific. The flaw in this assay is that IgM circulates till about 3 months post onset of illness therefore is not a proper validation of a current infection. Nonetheless, rapid assays based on this principle have been developed for point of care applications [42].

The continued and concerted effort in developing low cost and sensitive diagnostic applications for dengue is praiseworthy but more research is needed to overcome the many limitations of the existing technologies. Virus isolation is a time intensive process, requires skilled personnel and expensive infrastructure and cannot distinguish between a primary and secondary dengue virus infection. RNA amplification techniques are also costly requiring technically trained personnel. The dengue antigen detection assays are simple, rapid and inexpensive but lacks the sensitivity of a viral isolation or nucleic acid amplification technique. Another important parameter is the proper validation and effectiveness of the various commercial assays particularly in the developing countries [43]. The current focus should be to improve upon the existing

technologies by minimizing the drawbacks and the simultaneous persuasion for new technologies such as the use of biosensors. A biosensor based on liposomal amplification has already been developed to diagnose serotype specific synthetic dengue sequence [44]. Although promising outcomes have been reported, considerable research remains to be done to advance these technologies to the next level. Biosensor based detection has the potential to be considerably more sensitive than conventional assays. Dengue biosensors currently available are chip based having two specific monoclonal antibodies, immobilized into a piezoelectric transducer, for detection of envelope and NS1 protein from Dengue virus [56]. Multiple antibody coating facilitates the capture of different antigens, which increases the detection signal. This technology reported a 100-fold higher sensitivity than conventional enzyme immunoassays. Self-assembled monolayer based covalent immobilization of a bio-receptor conjugate of a dengue antigen and bovine serum albumin was recently performed on a gold chip [45, 59]. In this assay, indirect competitive inhibition based diagnoses of IgM antibodies are done. Bovine serum albumin was used as a blocking reagent to reduce non-specific binding thereby improving diagnostic selectivity. A surface plasmon resonance [SPR] was also used to analyze enhancement in the resonance angle of the surface-deposited sample in presence of Dengue virus. The SPR procedure has been beneficial to overcome signal resolution of optical fibers and other devices that are common issues. SPR facilitates real-time and label-free analysis but miniaturization of various components of SPR-based devices is a challenge that has to be addressed before the technology can be used for widespread diagnostic applications. [57,58]. However, these biosensor technologies need to overcome constraints such as interference issues between electrochemical substances and

the unpredictability of the biomolecule immobilization on the sensor surface, both conditions that may impact sensitivity and specificity [45].

1.1.3 FUTURE DIRECTION AND CONCLUSION

Recent developments in dengue diagnostics have been progressive along with the evolution of novel technologies that demonstrate enhanced viral detection. Evident improvements in new and/or existing methods for dengue infection have been made. Along with the progress made, however, research also indicates that there is still an enormous potential available towards the development of an ideal dengue virus detection system. This assay would be exceptionally simple to use, inexpensive, rapid, as well as highly specific. First and foremost, the development of immunoassays based on the NS1 antigen, has been very significant, and this has led to a number of commercial kits that are in huge demand. The use of bi-specific antibodies can significantly enhance the sensitivity of the existing assay due to their monovalency for an antigen as well as simultaneous binding to an enzyme. Bi-specific antibodies are engineered bi-functional molecules having dual binding specificities in a single entity. These antibodies, in particular, have been successfully exploited in the development of immunodiagnostic assays [46, 47, 48, 49]. In our laboratory we have developed a tetravalent diagnosis kit for dengue virus that is a sensitive, low cost immunoassay employing bi-specific antibodies. This assay has many positives such as specificity, cheap to produce, easy to use, along with immediate detection. Furthermore, it can be performed in any point of care setting by personnel with minimal technical expertise and, without the use of complex instruments [50, 51, 52]. This novel diagnostic application, in combination with

other existing technologies needs to be evolved to provide timely therapeutic intervention to tackle this devastating, often fatal disease.

1.1.4 REFERENCES

1. World Health Organization. Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control, 2nd edn. World Health Organization: Geneva, 1997

2. Castleberry JS, Mahon CR. Dengue fever in the Western Hemisphere. Clin Lab Sci 2003; 16: 34–38

3. Rodriguez-Tan RS, Weir MR. Dengue: a review. Tex Med 1998; 94: 53–59

4. Malison MD, Waterman SH. Dengue fever in the United States: a report of a cluster of imported cases and review of the clinical, epidemiologic, and public health aspects of the disease. JAMA 1983; 249: 496–500

5. Gascon J, Giner V, Vidal J, et al. Dengue: a re-emerging disease. A clinical and epidemiological study in 57 Spanish travelers. Med Clin [Barc] 1998; 111: 583–586

6. Monath TP. Dengue: the risk to developed and developing countries. Proc Natl Acad Sci USA 1994; 91: 2395–2400

7. Rigau-Perez JG, Clark GG, Gubler DJ, et al. Dengue and dengue haemorrhagic fever. Lancet 1998; 352: 971–977

8. Laue T, Emmerich P, Schmitz H. Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. J Clin Microbiol 1999; 37: 2543–2547

9. Guzman MG, Kouri G. Advances in dengue diagnosis. Clin Diagn Lab Immunol 1996; **3**: 621–627

10. Lupi O, Tyring SK. Tropical dermatology: viral tropical diseases. J Am Acad Dermatol 2003; 49: 979–1000.

11. Leyssen, P., E. D. Clercq, and J. Neyts, Perspectives for the treatment of infections with Flaviviridae. Clin. Microbiol. Rev 2000; 13:67–82

12. Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss, Nucleotide sequence of yellow fever virus: Implications for flavivirus expression and evolution. Science 1985; 229:726–733

13. Halstead, S. B., H. Shotwell, and J. Casals, Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. J. Infect. Dis. 1973; 128:15–22.

14. Halstead, S. B. Pathogenesis of dengue: challenge to molecular biology. Science 1988; 239:476–481

15. Sabchareon A, Lang J, Chanthavanich P, Yoksan S, Forrat R, Attanath P, Sirivichayakul C, Pengsaa K, Pojjaroen-Anant C, Chambonneau L, Saluzzo JF, Bhamarapravati N. Safety and immunogenicity of a three dose regimen of two tetravalent live-attenuated dengue vaccines in five- to twelve-year-old Thai children. Pediatr Infect Dis J. 2004 23[2]: 99–109

16. Mackenzie JS, Gubler DJ, Petersen LR. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med. 2004: 10[Suppl 12]: S98–109

17. Eltzov E, Atias D, Gheber L, Marks RS, Dengue Virus Diagnostics in Detection of bacteria, viruses, parasites and fungi [ed. Mariapia Viola-Magni], Springer Publishing, Berlin, NATO Science for Peace and Security Series A: Chemistry and Biology, 2010, DOI 10.1007/978-90-481-8544-3_12

18. AnandaRao R, Swaminathan S, Fernando S, Jana AM, Khanna N, A custom-designed recombinant multiepitope protein as a dengue diagnostic reagent. Protein Expr Purif. 2005 41[1]:136–147

19. Vaughn DW, Nisalak A, Kalayanarooj S, Solomon T, Dung NM, Cuzzubbo A, Devine PL, Evaluation of a rapid immunochromatographic test for diagnosis of dengue virus infection.J Clin Microbiol. 1998: 36[1]:234–238

20. Vaughn DW, Nisalak A, Solomon T, Kalayanarooj S, Nguyen MD, Kneen R, Cuzzubbo A, Devine PL, Rapid serologic diagnosis of dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections. Am J Trop Med Hyg 1999: 60[4]:693–698

21. Palmer CJ, King SD, Cuadrado RR, Perez E, Baum M, Ager AL, Evaluation of the MRL diagnostics dengue fever virus IgM capture ELISA and the PanBio Rapid Immunochromatographic Test for diagnosis of dengue fever in Jamaica. J Clin. Microbiol. 1999: 37[5]: 1600–1601

22. Blacksell SD, Commercial Dengue Rapid Diagnostic Tests for Point-of-Care Application: Recent Evaluations and Future Needs? J Biomed. Biotech. 2012: Article ID 151967, 12 pages, doi:10.1155/2012/151967

23. Guzman, M. G., and G. Kouri. Advances in dengue diagnosis. Clin. Diagn. Lab. Immunol. 1996: 3:621–627

24. Kuberski TT, Rosen L. A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. Am J Trop Med Hyg. 1977: 26:533-7

25. Kuberski TT, Rosen L. Identification of dengue viruses, using complement fixing antigen produced in mosquitoes. Am J Trop Med Hyg 1977: 26:538-43

26. Chonticha, K. et al. Dengue virus detection using whole blood for reverse transcriptase PCR and virus isolation. J. Clin. Microbiol. 2007: 45: 2480–2485

27. Dussart, P. et al. Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin. Vaccine Immunol. 2006: 13, 1185–1189

28. Das D, Mongkolaungkoon S, Suresh MR, Super induction of dengue virus NS1 protein in *E.coli*. Protein Expr. Purif. 2009: 66[1]: 66-72

29. Dussart P., et al. Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin. Vaccine Immunol. 2006: 13:1185–1189

30. Dussart P., et al. Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human serum. PLoS Negl. Trop. Dis. 2008: 2:e280

31. Innis, B. L., A. Nisalak, S. Nimmannitya, S. Kusalerdchariya, V. Chongswasdi, S. Suntayakorn, P. Puttisri, and C. H. Hoke. An enzymelinked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am. J. Trop. Med. Hyg. 1989: 40: 418–427

32. Bundo, K., and A. Igarashi. Antibody-capture ELISA for detection of immunoglobulin M antibodies in sera from Japanese encephalitis and dengue hemorrhagic fever patients. J. Virol. Methods. 1985: 11:15–22.

33. Burke, D. S., A. Nisalak, and M. A. Ussery. Antibody capture immunoassay detection of Japanese encephalitis virus immunoglobulin M and G antibodies in cerebrospinal **fl**uid. J. Clin. Microbiol. 1982: 15:1034–1042.

34. World Health Organization. Regional Guidelines on Dengue/DHF Prevention and Control. Regional Publication 29/1999. World Health Organization: Geneva

35. Schoone G. J., Oskam L., Kroon N. C., Schallig H. D., Omar S. A. Detection and quantification of Plasmodium falciparum in blood samples using quantitative nucleic acid sequence-based amplification. J. Clin. Microbiol. 2000: 38:4072–4075

36. Berndt C, Muller U, Bergmann F, Schmitt U, Kaiser R, Muller C, Comparison between a nucleic acid sequence-based amplification and branched DNA test for quantifying HIV RNA load in blood plasma. J Virol. Meth. 2000: 89[1-2]: 177-81

37. Teles FRR, Prazeres DMF, Lima-Filho JL, Trends in dengue diagnosis, Rev. Med. Virol. 2005: 15: 287–302

38. Miagostovich MP, dos Santos FB, de Araujo ES, et al. Diagnosis of dengue by using reverse transcriptase- polymerase chain reaction. Mem Inst Oswaldo Cruz 1997; 92: 595–599

39. Yenchitsomanus PT, Sricharoen P, Jaruthasana I, et al. Rapid detection and identification of dengue viruses by polymerase chain reaction [PCR]. Southeast Asian J Trop Med Public Health 1996; 27: 228–236

40. Maniatis T, Fritsch EF, Sambrook J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1982

41. Ludolfs D, Schilling S, Altenschmidt J, et al. Serological differentiation of infections with dengue virus serotypes 1 to 4 by using recombinant antigens. J Clin Microbiol 2002; 40: 4317–4320.

42. Hunsperger E. et al. Evaluation of commercially available anti-dengue virus immunoglobulin M tests. Emerg. Infect. Dis. 2009: 15, 436–440

43. Peeling RW, H. Artsob, J.L. Pelegrino, P. Buchy, M.J. Cardosa, S. Devi, D.A. Enria, J. Farrar, D.J. Gubler, M.G. Guzman, S.B. Halstead, E. Hunsperger, S. Kliks, H.S. Margolis, C.M. Nathanson, V.C. Nguyen, N. Rizzo, S. Vazquez, S. Yoksan, Evaluation of diagnostic tests: dengue, Nat. Rev. Microbiol, 2010: 12 [Suppl.], pp. S30–S38

44. Baeumner AJ, Schlesinger NA, Slutzki NS, et al. Biosensor for dengue virus detection: sensitive, rapid, and serotype-specific. Anal Chem 2002; 74: 1442–1448

45. Vasquez S, Lemos G, Pupo M, et al. Diagnosis of dengue virus infection by the visual and simple AuBioDOT immunoglobulin M capture system. Clin Diagn Lab Immunol 2003; 10: 1074–1077

46. Kreutz FT, Suresh MR, Novel bispecific immunoprobe for rapid and sensitive detection of prostate-specific antigen. Clin Chem. 1997: 43[4]: 649–656

47. Kammila S, Das D, Bhatnagar PK, Sunwoo HH, Zayas-Zamora G, King M, Suresh MR, A rapid point of care immunoswab assay for SARS-CoV detection. J Virol. Methods, 2008: 152[1–2]: 77–84

48. Sunwoo HH, Palaniyappan A, Ganguly A, Bhatnagar PK, El-Kadi AOS, Suresh MR, Quantitative and sensitive detection of the SARS-CoV spike protein using bispecific monoclonal antibody-based enzyme linked immunoassay, J Virol Methods, 2012: 187[1]: 72-8

49. Sarkar S, Tang XL, Das D, Spencer JS, Lowary TL, Suresh MR, A bispecific antibody based assay shows potential for detecting tuberculosis in resource constrained laboratory settings. PLoS ONE. 2012: **7**[2]: e32340. doi:10.1371/journal. pone.0032340.

50. Malabadi RB, Ganguly A, Teixeira da Silva JA, Parashar A, Suresh MR, Sunwoo HH, Overview of plant-derived vaccine antigens: Dengue virus. Journal of Pharm. & Pharmaceut. Sci. 2011: 14:400-413

51. Malabadi RB, Ganguly A, Sunwoo HH, Suresh MR, Role of bi-specific monoclonal antibodies in immunodiagnostic assay. Res. In Pharm. 2012: 2[3]: 08-14

52. Parashar A, Sarkar S, Ganguly A, Sharma SK, Suresh MR, Bispecific Antibodies for Diagnostic Applications, Ed. Dr. Roland Kontermann [U of Stuttgart], Springer, 2011 Heidelberg

53. Falconar AK, de Plata E, Romero-Vivas CM. Altered enzyme-linked immunosorbent assay immunoglobulin M [IgM]/IgG optical density ratios can correctly classify all primary or secondary dengue virus infections 1 day after the onset of symptoms, when all of the viruses can be isolated. Clin Vaccine Immunol, 2006: 13: 1044–1051

54. Buchy P, Yoksan S, Peeling RW, Hunsperger E, Laboratory Tests for the diagnosis of dengue virus infection. Scientific Working Group, Report on Dengue, 1-5 October 2006, Geneva, Switzerland

55. Endy TP, Nisalak A, Vaughn DW, Diagnosis of Dengue Virus Infections, Dengue, Tropical Medicine: Science and Practice, 2008: Vol 5 [Edited by Scott B Halstead]

56. Su CC, T.-Z. Wu, L.-K. Chen, H.-H. Yang, D.-F. Tai, Anal. Chim. Acta. 2003: 479 117–123

57. Kumbhat S, K. Sharma, R. Gehlot, A. Solanki, V. Joshi, J. Pharm. Biomed. Anal. 2010: 52, 255–259

58. Lee WG, Y.-G. Kim, B.G. Chung, U. Demirci, A. Khademhosseini, Adv. Drug Deliv. Rev. 2010: 62, 449–457

59. Teles FRR, Biosensors and rapid diagnostic tests on the frontier between analytical and clinical chemistry for biomolecular diagnosis of dengue disease: A review, Analytica Chimica Acta, 2011: 687, 28–42

1.2 A Mini-review of Dengue Vaccine Development

1.2.1 BACKGROUND

In the last 20 years, dengue virus has spread rapidly and is endemic in more than 100 countries. Dengue virus infection represents a major growing public health problem with an estimated 2.5 billion people at risk of infection in tropical and subtropical countries [1]. Early reports on dengue fever outbreaks date back to about two hundred years. Recent assessments reveal, that the major burden of this fatal disease is borne, in particular, by South East Asians and South and Central Americans [2,3]. Dengue Fever is a debilitating and often fatal *flaviviridae* infection transmitted by *Aedes* mosquitoes [4]. There are more than 100 million cases of dengue infections annually and about half a million reports of the more severe and life-threatening dengue haemorrhagic fever [DHF] and dengue shock syndrome [DSS] [5,6]. There are four serotypes of dengue virus [DV1, DV2, DV3 and DV4]. All the serotypes are competent in causing asymptomatic manifestations as well as the more severe and fatal DHF and DSS. Dengue virus possesses a single-stranded, positive-sense RNA genome. Translation of the viral RNA results in a single polypeptide that is processed by proteases, generating three structural proteins and seven non-structural [NS] proteins [7,8]. The structural proteins consist of the capsid, the membrane and the envelope whereas the non-structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. There is also a membrane precursor protein [prM], which assists in the folding of the envelope protein. The envelope protein plays an important role in the infection process and is also reported to be the target molecule for the development of neutralizing antibodies [9,10].

Dengue infection has become a huge burden; both in terms of lives lost, particularly in children, but also to the economic setback induced by the costly efforts to contain the disease. A primary infection, with any of the serotypes, would induce lifetime immunity against that particular serotype; but, a subsequent infection, with a different serotype, can increase the severity of the disease. This common, secondary infection is known as antibody dependent enhancement [ADE]. Hence, the development of an effective vaccine, catering to all the four serotypes, which would be safe and cost effective, is of great significance. Children and young adults who are the most susceptible to infections [11] would benefit appreciably from a multi-focal vaccination. Currently there are no licensed vaccines available for dengue infection, although there are vaccines available for a number of closely related viruses. Although the World Health Organization has prioritized the development of a dengue vaccine for a long time, effective progress has been extremely slow to develop and has also been very costly to sustain [12, 13, 14]. Due to ADE, the foremost issue associated with dengue vaccine development is the need for concurrent protective immunity against all four serotypes [15, 16, 17]. This short article summarizes various approaches undertaken towards a successful dengue vaccine and the challenges associated with it.

1.2.2 VACCINES: EFFORTS AND CHALLENGES

With the ever-increasing severity of the dengue virus and its crucial impact on loss of life, economics of the endemic region etc., the need of an effective vaccine is becoming more and more significant [30]. The increase in dengue incidence in the past decade and the simultaneous presence of four different serotypes has posed a greater threat to the communities and has also witnessed higher volumes of the more severe forms of dengue

infection. Although focused and concerted efforts have been made over the past 70 years for the development of an effective dengue vaccine, successful application has not been realized. Following the end of World War II, Sabin and colleagues reported on the efficacy of one of the first formulations of a dengue vaccine. Their work was based on a mouse model which involved live-attenuated virus passage multiple times [18, 19] before efficacy. After about 15 passages in the mouse brain, the virus became attenuated enough for trial in humans [4, 11]. Much later, Halstead and coworkers made efforts by attenuating dengue virus in dog kidney cells [20]. Other applications were focused on inactivated and subunit virus candidates [2]. The major obstacle faced by researchers in the development of an effective vaccine is the phenomenon of ADE. The presence of non-neutralizing antibodies developed during a primary infection of dengue was found to greatly increase the severity of a second dengue infection with a different serotype. The severity of the second dengue infection is enhanced by formation of immune complexes as well as by facilitating viral access to Fc**γ**-Receptor possessing cells [21,22]. Therefore an ideal vaccine candidate should be able to circumvent the ADE phenomenon and be tetravalent in nature or a combination of four monovalent vaccines delivered as a single vaccine. Potential candidates could well be a vaccine or vaccines that would be able to confer immunity against all the four different serotypes. Also, the need to understand the underlying molecular principles that govern pathogenesis of dengue is very important [29]. Currently, with the advent of recombinant DNA technology over the past decade, new, and more aggressive applications are being pursued and tested for efficacy. Furthermore, the absence of a promising animal model to test the vaccine candidates has also been a deterrent in dengue vaccine research. Since emerging economies of the world bear the greatest burden of dengue fatality, the development of an affordable vaccine is

essential for future developments. These developments might well involve targeting envelope glycoproteins as part of the virus structure and recently considered as the major target for vaccine development. Glyco-protein functionality appears to include virus attachment and subsequent cell entry. Domain III of the envelope protein is considered most important owing to its role in cell receptor binding [28]. Other proteins such as the membrane and NS1 are also thought to be protective [26]. Monoclonal antibodies against envelope and pre membrane proteins have also been found to be efficient in protecting against severe infection [23, 24]. There is also evidence that protective anti dengue antibodies are effective in children with maternal inheritance [25]. Cell mediated immune responses have also been shown to play a role in active viral clearance [27].

Live attenuated-vaccine developmental efforts are considered to be one of the most effective approaches. A wild type dengue virus can be attenuated by serial passages in tissue culture and alternatively, a chimeric vaccine candidate can be generated by modification of a pre-existing licensed yellow fever virus vaccine [31]. Another important feature of a live attenuated-vaccine is its ability to induce sustained immune responses; very closely mimicking a response in case of a natural infection. Sanofi Pasteur has developed a chimeric vaccine based on the yellow fever vaccine by incorporating dengue premembrane and envelope genes of dengue [31, 32]. Recently, an efficacy trial of the vaccine in Thailand demonstrated an impressive safety profile along with the ability to protect against three of the four-dengue serotypes. Further clinical trials are underway to validate vaccine efficacy and safety [33]. Inactivated virus vaccines are also in preclinical stages of development and one of the dengue serotype 2 vaccines manufactured by the Walter Reed Army Institute for Research is scheduled to begin clinical trials shortly [34]. Inactivated vaccines have certain advantages over the

attenuated ones**,** including inability to become pathogenic, and also being able to induce an immune response in all four dengue serotypes due to immunogenic equality [2]. Recombinant subunit vaccine candidates**,** based on the envelope protein, have also been developed. There has also been a study completed by researchers at the Pedro Kouri Tropical Medicine Institute where they used domain III of the envelope protein and fused it with a carrier protein to develop monovalent constructs for all the four serotypes. Each of the constructs developed was able to induce neutralizing immune responses and also demonstrated protection against viral challenge [35]. Another bivalent effort is based on a domain III and STF2D fusion protein conjugate. There is also a tetravalent conjugate of Domain III and Ag473 fusion protein being evaluated in animals [39, 40]. A lot of Dengue vaccine efforts based on virus like particles [VLP] are in preclinical phases. Virus-like particles lack replication material but are able to mimic antigen presentation like a natural viral infection [36]. A tetravalent vaccine candidate has been developed by Cytos Biotechnology by chemically attaching envelope domain III to an *E.coli* expressed VLP. Another monovalent candidate has been in development in ICGEB, India where the basis for the vaccine is the envelope domain III and *P.pastoris* expressed VLP [37, 38]. The Carolina Vaccine Institute [CVI] has been a pioneer in the development of an alpha virus replicon vector expressing envelope protein. This strategy was demonstrated by immunizing monkeys resulting in the generation of neutralizing antibodies and protection from viral challenge as well. GenPhar Inc., developed bivalent vaccine candidates based on an adenovirus construct. Viral challenge studies resulted in absolute protection against dengue type 1 and 3 whereas a significant reduction in viremia was observed against type 2 and 4 [41]. Another strategy, adopted by the Pasteur Institute, is centered on a tetravalent dengue antigen from a live attenuated measles vaccine vector. The construct

expresses domain IIIs of all the four serotypes along with the membrane protein ectodomain. Analysis in murine models showed induction of neutralizing antibodies against all the serotypes. Clinical trials of the construct have been planned in the near future [38]. With genetic engineering and recombinant technology rapidly making progress, the concept of DNA vaccines has been gaining considerable momentum. Studies have been conducted with monovalent constructs of dengue in macaques that resulted in production of neutralizing antibodies. It also conferred protection to the vaccinated macaques when challenged with a wild type dengue virus strain [42]. A follow up study was done by the same research group, which involved techniques to enhance the neutralizing efficacy of the earlier DNA vaccine construct. Results from these later studies revealed that neutralization efficacy could be improved significantly from that demonstrated from earlier constructs and was stable for at least 6 months post vaccination. All of the monkeys vaccinated, barring one, were completely protected from a virus challenge [43]. These important findings led to the transition of DNA based dengue vaccines towards implementation of clinical trials [44]. Advantages of DNA based strategies involve its ability to induce both humoral and cell mediated immune responses, lack of complicity like other vaccine approaches, and technically, found to be simpler to develop. However, certain drawbacks were evident, low levels of immunogenicity have been observed in data collected from the clinical trials. Another limitation is that of insufficient cell uptake [29] leading to reduced protein expression. Nonetheless, promising DNA based strategies are being explored further in other ongoing preclinical and clinical phases. Further studies on DNA based vaccinations including design, adjuvant selections, and alternative delivery systems are also being evaluated. DNA based vaccines require additional studies as they appear to have the potential of

being an effective strategy to counter dengue and other infectious agents. These vaccines are definitely more cost effective, technically easier to manufacture, have enhanced stability and are able to deliver multiple agents in one single construct.

1.2.3 TARGETING STRATEGIES FOR DENGUE: A VIABLE ALTERNATIVE

Dendritic cells [DCs] are a unique class of antigen presenting cells that have an important role in both innate and cell mediated immunity. DCs have a significant role to play in the activation of T- and B-cell immune responses and are deemed to be more efficient than other antigen presenting cells. Targeting dengue vaccines to specific surface receptors of DCs may be an important, alternative strategy for enhancing vaccine efficacy. Indeed, this strategy has been found to enhance antigen delivery to the DCs more effectively than traditional vaccination methods; wherein enzymes and other cells might express adverse events before being taken up by the DCs [45, 46]. DCs internalize antigens using its many cell surface receptors including Gb3, CD40, Fc receptors, C-type lectin receptors etc and present them to both MHC I and II pathways [47, 48]. A lot of studies have been conducted on DC cell receptors and antigen targeting.

Dendritic Cell Receptor [DEC-205] is part of the C-type lectin receptor family that has been extensively studied. It is present on both mature and immature dendritic cells and targeting DEC-205 results in improved cross-presentation of antigens compared to other receptors [49, 50, 51]. Reports illustrate that DEC-205 targeting resulted in an improvement in antigen presentation to activation of T-Cells [53]. We have recently developed an array of bispecific antibody based delivery vectors to target DCs with biotin labeled antigens that could be proteins, DNA, peptides, or gangliosides [45, 52]. In this

strategy, the antibody has dual specificity i.e. one arm can bind to any biotinylated antigen and the other arm is specific to the DC receptor DEC-205.

Specific targeting of DEC-205 resulted in a dramatic reduction in antigen dosage by approximately 500 fold when compared with non-targeted antigens. Low dose animal studies confirmed DEC-205 targeted strategy along with co-stimulatory anti-CD40 monoclonal antibody application. Results revealed a significant increase in both humoral and adapted immune responses for SARS-CoV, Ebola GP1, and, MUC-1 peptide [45]. Another study, targeting mucosal DCs, in a murine model with SARS NP DNA, induced an evident strong immune response. In another study, when CD40 monoclonal antibody was used to stimulate DC maturation, the immune responses were also found to significantly increase [54]. Additional dengue virus specific DC targeted vaccine candidates are currently being investigated [unpublished data]. DC targeted systems involving bispecific antibodies could be, potentially, a viable alternative for the development of efficacious dengue vaccines. Results from the studies on SARS-CoV, Ebola and other viruses suggest that similar strategies should be employed and tested on the dengue virus as soon as possible. Hopefully, application of these strategies will drastically reduce dengue fatalities around the world. Potentially, millions of healthy, intelligent individuals could be saved.

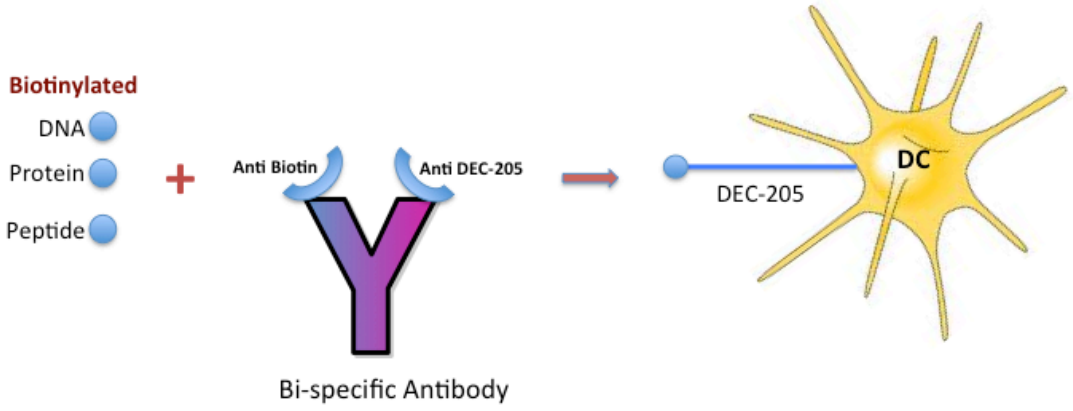


Fig 1.1: Schematic representation of DC Targeted Strategy

1.2.4 References

1. WHO. Strengthening implementation of the global strategy for dengue fever/dengue haemorrhagic fever prevention and control—report of the Informal Consultation. Geneva, Switzerland: World Health Organization, 1999. http://www.whoindia.org/LinkFiles/Dengue_GlobalStrategy.pdf [accessed July 24th, 2012].

2. Whitehead SS, Blaney JE, Durbin AP, Murphy BR. 2007. Prospects for a dengue virus vaccine. Nat. Rev. Microbiol. 5:518–28

3. Cardosa MJ, 1998, Dengue vaccine design: issues and challenges, British Medical Bulletin 54 [2], 395-405

4. Edelman R, 2007, Dengue Vaccines approach the finish line, Clin. Infect. Dis. 45 [suppl 1]: S56–60.

5. Gubler DJ. 2002 The global emergence/resurgence of arboviral diseases as public health problems. Arch Med Res 33: 330–42

6. Gubler DJ. 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 10: 100–03

7. Lindenbach, B. D. & Rice, C. M. in Fields Virology [edsKnipe, D. M. & Howley, P. M.] 1043–1125 [LippincottWilliams and Wilkins, Philadelphia, 2001].

8. Clyde, K. & Harris, E. 2006. RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required forviral replication. J. Virol. 80, 2170–2182

9. Mukhopadhyay, S., Kuhn, R. J. & Rossmann, M. G. 2005. A structural perspective of the flavivirus life cycle. Nature Rev. Microbiol. **3**, 13–22.

10. Kuhn, R. J. et al, 2002. Structure of dengue virus. Implications for flavivirus organization, maturation,and fusion. Cell 108, 717–725

11. Jin X, Block OT, Rose R, Schlesinger J, 2009. Dengue vaccine development and dengue viral neutralization and enhancement assays. Antiviral Therapy 14, 739-749

12. Almond J, Clemens J, Engers H, Halstead S, Khiem HB, Pablos-Mendez A, et al. 2002. Accelerating the development and introduction of a dengue vaccine for poor children, 5–8 December 2001, Ho Chi Minh City, VietNam. Vaccine 20, 3043–6.

13. Brandt WE, 1990. From the World Health Organization: development of dengue and Japanese encephalitis vaccines. J Infect Dis 162, 577–83.

14. Stephenson JR, 2006. Developing vaccines against flavivirus diseases: past success, present hopes and future challenges. Novartis Found Symp 277, 193–201.

15. Guzman, M. G., M. Alvarez, R. Rodriguez-Roche, L. Bernardo, T. Montes, S. Vazquez, L. Morier, A. Alvarez, E. A. Gould, G. Kouri, and S. B. Halstead. 2007. Neutralizing antibodies after infection with dengue 1 virus. Emerg. Infect. Dis. 13:282–286

16. Kliks, S. C., A. Nisalak, W. E. Brandt, L. Wahl, and D. S. Burke. 1989. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am. J. Trop. Med. Hyg. 40, 444–451.

17. Morens, D. M., and S. B. Halstead. 1990. Measurement of antibody-dependent infection enhancement of four dengue virus serotypes by monoclonal and polyclonal antibodies. J. Gen. Virol. 71, 2909–2914

18. Sabin AB, Schlesinger RW, 1945. Production of Immunity to dengue with virus modified by propagation in mice. Science. 101:640-642

19. Sabin AB, 1952, Research on Dengue during World War II. Am J Trop Med Hyg. 1:30-50.

20. Thomas SJ, Endy TP, 2011. Vaccines for the prevention of Dengue: Development update. Human Vaccines 7:6, 674-684

21. Calvert, AE, Huang, CY, Kinney, RM, Roehrig, JT, 2006. Non-structural proteins of dengue 2 virus offer limited protection to interferon-deficient mice after dengue 2 virus challenge. J. Gen. Virol. 87, 339–346

22. Halstead, SB, 2003. Neutralization and antibody dependent enhancement of dengue viruses. Adv. Virus Res. 60, 421–467

23. Kaufman, BM. et al. 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am. J. Trop. Med. Hyg. 41, 576–580

24. Crill, WD, Roehrig, JT, 2001. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J. Virol. 75, 7769–7773

25. Pengsaa, K et al, 2006. Dengue virus infections in the first 2 years of life and the kinetics of transplacentally transferred dengue neutralizing antibodies in thai children. J. Infect. Dis. 194, 1570–1576

26. Schlesinger, JJ, Brandriss, MW, Cropp, CB, Monath, TP, 1986. Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. J. Virol. 60, 1153–1155

27. Samuel, MA, Diamond, MS, 2006. Pathogenesis of West Nile Virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. J. Virol. 80, 9349–9360

28. Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-

Petty S, et al, 2008. Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nat. Struct. Mol. Biol. 15[3], 312–7

29. Murrrel, S, Wu, SC, Butler, M, 2011. Review of dengue virus and the development of a vaccine. Biotechnology Advances 29, 239–247

30. Malabadi, RB, Ganguly, A, daSilva, JAT, Parashar, A, Suresh, MR, Sunwoo, HH, 2011. Overview of Plant-Derived Vaccine Antigens: Dengue Virus, J. Pharm Pharmaceut. Sci. 14[3], 400-413

31. Guy, B, Almond, JW, 2007. Towards a dengue vaccine: Progress to date and remaining challenges, Comp. Immun. Microbiol. Infect. Dis. 31, 239-252

32. Murphy, BR, Whitehead, SS, 2011. Immune response to dengue virus and prospects for a vaccine, Annu. Rev. Immunol, 29, 587-619

33. http://www.sanofipasteur.com/articles/1118-sanofi-pasteur's-dengue-vaccine-demonstrates-proof-of-efficacy.html [accessed as on Sept 01, 2012]

34. Putnak, R. et al, 2005. An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. Vaccine 23, 4442–4452

35. Hermida L, Bernardo L, Martin J, Alvarez M, Prado I, Lopez C, et al, 2006. A recombinant fusion protein containing the domain III of the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. Vaccine 24, 3165–71

36. Jennings GT, Bachmann MF. 2008. The coming of age of virus-like particle vaccines. Biol Chem 389, 521–36

37. Spohn G, Jennings GT, Martina BE, Keller I, Beck M, Pumpens P, et al. 2010. A VLP based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. Virol J 7, 146

38. Schmitz, J, Roehrig, J, Barrett, A, Hombach, J, 2011. Next generation dengue vaccines: a review of candidates in preclinical development. Vaccine 29, 7276-7284

39. Chen HW, Liu SJ, Liu HH, Kwok Y, Lin CL, Lin LH, et al. 2009. A novel technology for the production of a heterologous lipoprotein immunogen in high yield has implications for the field of vaccine design. Vaccine 27, 1400–9

40. Leng CH, Liu SJ, Tsai JP, Li YS, Chen MY, Liu HH, et al. 2009. A novel dengue vaccine candidate that induces cross-neutralizing antibodies and memory immunity. Microbes Infect 11, 288–95

41. Holman DH, Wang D, Raviprakash K, Raja NU, Luo M, Zhang J, et al. 2007. Two complex, adenovirus-based vaccines that together induce immune responses to all four dengue virus serotypes. Clin Vaccine Immunol 14, 182-9

42. Raviprakash K, Porter KR, Kochel TJ, Ewing D, Simmons M, Phillips I, et al. 2000. Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques. J Gen. Virol. 81, 1659–67

43. Raviprakash K, Ewing D, Simmons M, Porter KR, Jones TR, Hayes CG, et al. 2003. Needle-free Biojector injection of a dengue virus type 1 DNA vaccine with human immunostimulatory sequences and the GM-CSF gene increases immunogenicity and protection from virus challenge in Aotus monkeys. Virology 315, 345–52

44. Danko JR, Beckett CG, Porter KR, 2011. Development of dengue DNA vaccines. Vaccine 29, 7261-7266

45. Wang WW, Das D, Suresh MR, 2009. A versatile Bifunctional Dendritic Cell Targeting Vaccine Vector, Mol. Pharmaceutics 6[1], 158-172

46. Tacken, PJ, Torensma R, Figdor, CG, 2006. Targeting antigens to dendritic cells in vivo, Immunobiology 211[6−8] 599-608

47. Figdor, CG, van Kooyk Y, Adema, GJ, 2002. C-type lectin receptors on dendritic cells and Langerhans cells, Nat. Rev. Immunol. 2[2] 77-84

48. Tacken, PJ, de Vries, IJ, Torensma, R, Figdor, CG, 2007. Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting, Nat. Rev. Immunol. 7[10] 790-802

49. Bozzacco, L, Trumpfheller, C, Siegal, FP, Mehandru, S, Markowitz, M, Carrington, M, Nussenzweig, MC, Piperno, AG, Steinman, RM, 2007. DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes, Proc. Natl. Acad. Sci. U.S.A. 104[4], 1289-94

50. Jiang W, Swiggard WJ, Heufler C, Peng M, Mizra A, Steinman RM, Nussenzwig MC, 1995. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 375: 151

51. Banchereau J, Steinman RM, 1998. Dendritic cells and the control of immunity, Nature, 392: 245

52. Wang WW, Das D, Tang XL, Budzynski W, Suresh MR, 2005. Antigen targeting to dendritic cells with bispecific antibodies. J Immunol Meth 306, 80-92

53. Bonifaz, LC, Bonnyay, DP, Charalambous, A, Darguste, DI, Fujii, SI, Soares, H, Brimnes, MK, Moltedo, B, Moran, TM, Steinman, RM, 2004. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination, J. Exp. Med. 199 [6] 815– 824

54. Raghuwanshi, D, Mishra, V, Das, D, Kaur, K, Suresh, MR, 2012. Dendritic Cell targeted chitosan nanoparticles for Nasal DNA immunization gainst SARS CoV Nucleocapsid protein, Mol Pharm, 9[4]: 946-956

1.3 Bispecific antibodies for Diagnostic Applications

1.3.1 BACKGROUND

Bi-specific monoclonal antibodies are artificially engineered immunoglobulins with two distinct binding specificities [1, 2, 3]. On the other hand, monospecific antibodies are the naturally occurring major IgG class having two identical antigen binding paratopes with two identical heavy and light chains [3]. Bispecific monoclonal antibodies [bsMAbs] can be produced mainly by three methods: 1) chemically linking two antibody molecules, 2) fusion of two different hybridomas and 3) using recombinant DNA approaches. Bispecific mononclonal antibodies have been extensively exploited in both diagnostic and therapeutic areas. Bispecific antibodies were first generated 37 years ago by chemical methods [4, 3]. Hybridoma technology opened a wide range of applications in fundamental and applied immunology [5, 1, 2]. In 1975, a major breakthrough was made by Kohler and Milstein [1975] in generating continuous *invitro* cultures of fused lymphocytes secreting antibodies with predetermined specificity. Fusion of a normal B cell [plasma cell] with myeloma cell [cancerous plasma cell] resulted in the generation of a hybrid cell known as hybridoma [5, 1, 2]. This hybridoma not only possessed the immortal-growth properties of the myeloma cell but also secreted an antibody product of the B cells. Kohler and Milstein were awarded a Nobel prize in 1984 for this work [5, 1-3]. This landmark discovery has revolutionized the use of bispecific antibodies [bsMAbs] because of the specificity of antigen binding abilities. Therefore, bsMAbs have many applications in the area of immunodetection, immunohistochemistry, adioimmunotherapy, adioimmunodiagnosis, and immunotherapy to targeted drug delivery, vaccine development and gene therapy [1-3, 5-7]. Furthermore, bsMAbs with

intrinsic binding sites to any two antigens has the capability to form uniform, homogeneous and reproducible immunoconjugates with one or two entities in a predetermined order [1-3]. This chapter highlights recent diagnostic applications of bispecific antibodies in immunoswab assay which might play an important role in controlling few of the following human infectious diseases.

1.3.2 SARS [severe acute respiratory syndrome].

Severe acute respiratory syndrome [SARS] is a severe form of pneumonia due to Corona virus [SARS-CoV] infection, and has adapted to human to human transmission [8-10]. The development of specific diagnostic tests against SARS-CoV is an essential step for effective treatment of infected patients and could prevent future SARS outbreaks [9]. There are three major diagnostic methods are currently available: 1) viral RNA detection using [RT]-PCR [11, 12], 2) antibody detection [13, 14] and 3) antigen detection [14, 15]. The most predominant SARSCoV virus derived protein throughout the infection is the nucleocapsid protein [NP] [16]. In our laboratory, five monoclonal antibodies [bsMAbs] against recombinant nucleocapsid protein [NP] of severe acute respiratory syndrome [SARS] causing coronovirus [CoV] were developed by hybridoma technology [9]. A highly sensitive, rapid and simple bsMAb based immunoswab assay was also developed in our laboratory for early detection of SARS-CoV [9, 17, 18, 20]. In the immunoswab assay, easy-touse swabs were first coated with anti-SARS CoV monoclonal antibody to capture NP in the test sample followed by detection with bsMAb [9, 17]. The immunoswab assay showed NP detection limits of 10 pg/mL [1 pg/swab] in saline, 20–

200 pg/mL [1–10 pg/swab] in pig nasopharyngeal aspirate and 500 pg/mL [25 pg/swab] in rabbit serum. This assay was completed within a period of approximately 45 minutes, which make it a rapid test for SARS diagnosis [17]. In another development, an ultrasensitive ELISA using mouse monoclonal IgG combined with chicken polyclonal IgY antibody for SARS virus infection was also validated. The immunoassay method of detecting SARSCoV NP antigen could be used as a cost effective diagnostic kit for checking the SARS suspected individuals.

1.3.3 Dengue

Dengue is one of the viral diseases found throughout the world. The mosquito which is responsible for transmission of dengue virus between humans and monkeys is known as Aedes aegypti [20-25]. Dengue virus is a member of the genus Flavivirus [family Flaviviridae] [20, 26, 27]. In many cases of infected individuals dengue fever [DF] presents as the dengue hemorrhagic fever [DHF] or dengue shock syndrome [DSS] [20, 27]. Dengue virus is endemic and significantly causing the death of many children [20, 22, 28-31]. Till today, dengue disease is not under control and effective immunization may provide protection for controlling dengue infections [20-22, 24]. Potential vaccines for dengue has been challenging, and there are many problems associated with the development of dengue vaccine [20, 24, 32, 33]. Long duration immunity to only one serotype is observed in many infected individuals. On the other hand dengue infected individuals showed short duration immunity to the other serotypes [21, 24, 34]. One of the major problems is that short duration immunity is lost after 3-4 months, and the individual has no immunity to the other three dengue serotypes [20, 22]. Currently there

is no diagnostic assay available for the detection of dengue virus infection at the acute or early stages. This thesis focuses on the development of a novel, highly sensitive immunodiagnostic assay for the detection of dengue virus using bsMAb. The assay is highly specific and easy to perform since it does not require any technical expertise or sophisticated instruments. The general format of the immunodiagnostic assay involves coating of dengue virus antigen-specific monoclonal antibody as the capture antibody on calcium alginate tipped swabs with aluminum/or plastic shafts as per previously published protocol [35].

1.3.4 Chikungunya

Chikungunya is another viral disease, which was reported for the first time in Tanzania [36, 37]. The epidemic cycle of chikungunya is similar to those of dengue and urban yellow fever. Chikungunya virus [CHIKV] is a mosquito-borne alpha virus belongs to the family of Togaviridae [38, 39, 40]. Geographically, chikungunya virus [CHIKV] is distributed in Africa, India and South East Asia [39, 41]. The symptoms of the diseases involves fever, headache, rash, nausea, vomiting, myalgia, and arthralgia/arthritis [40, 42, 43]. The severity of the disease is characterized by the neurological manifestations such as acute limb weakness, and joint pain with a fatal haemorrhagic condition [37, 44]. Chikungunya was reported as a major epidemic disease during 2004-2007 in Indian Ocean islands and India [39, 44, 45, 46]. No licensed vaccine or effective drug is available in the market throughout the world for chikungunya disease. However, the expression level of the CHIKV protein is very low in mammalian or bacterial or insect cells. Furthermore, the expressed protein is easily degraded. Therefore, there is an urgent need for the development of diagnostic assay for the early detection of the disease in the

infected individuals. However, current methods of early detection of chikungunya antigen in the infected individuals using PCR amplification are very expensive [44]. The PCR methods involve use of technically trained manpower, costly chemicals, and sophisticated instruments for the assay which the developing countries cannot afford. Till today, there are no reports of immunoswab assays as the diagnostic approach for chikungunya. Therefore, a sensitive, in expensive diagnostic test using immunoswab assay development for the detection of early stages of the chikungunya viral infection is being pursued. This immunoswab assay is simple, reliable, fast, and less expensive.

1.3.5 Tuberculosis [TB]

The gram positive bacterium which is a causative agent for tuberculosis is known as *Mycobacterium tuberculosis* [47-49]. Tuberculosis [TB] is still a major health problem in many countries throughout the world. *M. bovis* Bacillus Calmette-Guerin [BCG] is the only live bacterial vaccine in use that has shown no major side-effects. However, BCG vaccination is ineffective against adult pulmonary TB, and therefore, an urgent need for potential vaccines is a high priority. Furthermore, HIV patients and multi-drug resistant mycobacterium strains have contributed to the re-emergence of TB [50]. The major problem is that there is no specific diagnostic approach to find out the early stages of TB. Unfortunately, the false negative results of current TB diagnostic test such as sputum smear microscopy [SSM] are not reliable [47-50]. In addition, clinical symptoms and results of chest x-rays are nonspecific [50]. PCR based diagnosis is very expensive, which needs costly sophisticated instrumentation and time consuming too. Another simple method is the culture method for the detection of TB. The major drawback of this method is a time consuming since it takes 2-7 weeks to obtain results. Therefore, early

diagnosis is very important since TB is a highly contagious disease at the early stages of development. Therefore, our laboratory has developed a novel, and highly sensitive immunodiagnostic assay for the detection of *M. tuberculosis* using bsMAb [50]. TB diagnostic test was developed as per [50]. The assay is highly specific, sensitive, and less expensive. Therefore, the use of bsMAb in the immunodiagnostic assays plays an important role in the modern medicine. This method of diagnostic approach is very simple, rapid and cost effective. Early diagnosis always helps the patients in controlling the spread of disease at the right time. It will also help the physicians for recommending the proper medication for controlling the disease in the infected individual.

1.3.6 *Escherichia coli* O157:H7

E.coli O157:H7 belongs to the group of Verocytotoxin or Shiga toxin producing *E.coli* that has surfaced as an important food- and water-borne pathogen worldwide. It causes non-bloody or bloody diarrhea [hemorrhagic colitis] and hemolytic uremic syndrome characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. *E.coli* O157: H7 is therefore classified as enterohemorrhagic *E.coli* [EHEC]; it is reported to cause about 73,000 cases of infections per year in the United States alone. Several outbreaks of *E.coli* O157:H7 infection have been associated with recreational and municipal drinking water in North America, United Kingdom, and Japan. Current methods to detect *E.coli* O157:H7 in water and food are traditional enrichment and plating on Sorbitol MacConkey agar and Rainbow agar selective media and other immunological methods. The main drawbacks of these methods include time requirement [24–48 h for culture], difficulty in isolating DNA from sample [for PCR] and low sensitivity. It is important to note that low colony forming units [CFU] of *E.coli* O157:H7

present in contaminated food and water is generally sufficient to cause a severe infection, therefore having a sensitive method for detection during investigations of outbreaks is vital. A highly sensitive bsMAb-based immunodetection assay was developed by our laboratory for the detection of *E.coli* O157:H7 in water samples. *E.coli* O157:H7 whole bacteria and *E.coli* O157:H7 lipopolysaccharide [LPS] were used to first generate a monoclonal antibody, the hybridoma of which, was fused with anti-HRPO secreting monoclonal antibody hybridoma to generate a quadroma producing bsMAb specific for both *E.coli* O157 and HRPO. The bsMAb was subsequently purified using benzhydroxamic acid agarose column and used in sandwich ELISA immunoassay. The anti-*E.coli* O157:H7 monoclonal antibody was used as the capture antibody to bind the bacteria from samples and the bsMAb was used as the detection antibody. The detection limits of the assay were found to be 100 and 750 CFU/ml of sample for tap water and lake water, respectively. The immunoassay was further adapted to an immunofilter assay format to suit public health applications such as in water testing laboratories. As low as 50 CFU of *E.coli* O157:H7/100 ml water were detected indicating that the assay is highly sensitive. Moreover the assay is highly specific since it did not detect a 500-fold excess of other bacterial strains such as *Salmonella, Pseudomonas*, and as well non-O157:H7 and non-pathogenic *E.coli*. A higher signal to noise ratio and a clean background was achieved in the assay since bsMAb served as a high specific activity probe. The bsMAb-based immunodetection assay therefore functions as a robust, ultrasensitive, and quick method for the detection of *E.coli* O157:H7. It has distinct advantages as it eliminates the amplification step required by other current assays and could easily be developed as a routine screening assay for detection of *E.coli* O157:H7 in water bodies especially in developing countries [51].

The use of bsMAb in immunodiagnostic assays has resulted in the development of next generation immunoassays that are highly sensitive, rapid, simple, and cost-effective. The specificity of the assays approaches the theoretical limit of immunodetection making them ultrasensitive. Importantly, these assays are invaluable as a point of care diagnostic tool. Such assays are of immense value especially in some resource-constrained countries to combat the spread of diseases. Rapid detection of communicable diseases has huge impact on the current global health scenario where an infectious disease can make quick transition from an epidemic to a pandemic like the recent outbreak of swine flu. Most of the current diagnostic assays measure specific antibodies to bacterial or viral antigens and therefore may report a previous infection since antibodies circulate in the blood 6 months post infection. Such assays rarely detect infection at initial stages making early intervention almost impossible. In contrast, bsMAb-based diagnostics measure specific bacterial or viral antigens rather than the antibodies and therefore accurately reflect active diseased state and make early-stage detection possible. In the coming years, bsMAb will prove valuable for use in diagnosis of different types of cancer. Since bsMAb-based cancer diagnosis has many advantages and is highly sensitive it will be useful not only for early detection of cancer but also convenient for monitoring the progression of the diseased state and assessing response to treatment. Given the immense potential of bsMAb as excellent immunoprobes, there are efforts to engineer newer forms of these antibodies with significantly improved binding specificity and avidity. bsMAb await a plethora of novel applications in the field of diagnostics.

1.3.7 REFERENCES

1. Suresh MR, Cuello AC, Milstein C [1986a] Advantages of bispecific hybridomas in one step immunocytochemistry and immunoassays. PNAS. 83: 7989-7993.
2. Suresh MR, Cuello AC, Milstein C [1986b] Bispecific monoclonal antibodies from hybrid hybridomas. Meth. Enzym. 121: 210-215.
3. Cao Y, Suresh MR [1998] Bispecific antibodies as novel bioconjugates. Bioconjugate Chemistry. 9[6]: 635-644.
4. Nisonoff A, Rivers MM [1961] Recombination of a mixture of univalent antibody fragments of different specificity. Arc. Biochem. Biophys. 93:460.
5. Kohler G, Milstein C [1975] Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256:495.
6. Khan SR, Ganguly A, Malabadi RB, Sunwoo HH, Parashar A, Teixeira da Silva JA, Suresh MR [2011] Targeting strategies and nanocarriers in vaccines and therapeutics. Res. Biotech. 2[6]:08-20.
7. Khan SR, Ganguly A, Malabadi RB, Sunwoo HH, Suresh MR [2012] Gene delivery system: A developing arena of study for new era of medicine. DNAG. 6[1]: 2-9 [8].
8. Nicholls J, Dong XP, Jiang G, Peiris M [2003] SARS: clinical virology and pathogenesis. Respirology 8 Supplement. S6-8.
9. Das D, Kammila S, Suresh MR [2010] Development, characterization and application of monoclonal antibodies against severe acute respiratory syndrome coronovirus nucleocapsid protein. Clin. Vacc. Imm. 17[12]: 2033-2036.
10. Du L, He Y, Zhou Y, Liu S, Zheng BJ, Jiang S [2009] The spike protein of SARS-CoV, a target for vaccine and therapeutic development. Nat. Rev.

Microbiol. 7:226-236.

11. Jiang SS, Chen TC, Yang JY, Hsiung CA, Su IJ, Liu YL, Chen PC, Juang JL [2004]. Sensitive and quantitative detection of severe acute respiratory syndrome coronavirus infection by real-time nested polymerase chain reaction. Clin. Infect. Dis. 38:293-296.
12. Poon LL, Chan KH, Wong OK, Cheung TK, Ng I, Zheng B, Seto WH, Yuen KY, Guan Y, Peiris JS [2004] Detection of SARS coronovirus in patients with severe acute respiratory syndrome by conventional and real-time quantitative reverse transcription-PCR assays. Clin. Chem. 50: 67-72.
13. Chan PK, Ng KC, Chan RC, Lam RK, Chow VC, Hui M, Wu A, Lee N, Yap FH, Cheng FW, Sung JJ, Tam JS [2004] Immunofluorescence assay for serologic diagnosis of SARS. Emer. Infect. Dis. 10: 530-532.
14. Li YH, Li J, Liu XE, Wang L, Li T, Zhou YH, Zhuang H [2005] Detection of the nucleocapsid protein of severe acute respiratory syndrome coronvirus in serum: comparision with results of other viral markers. J. Virol. Meth. 130: 45-50.
15. Che XY, Qiu LW, Pan YX, Wen K, Hao W, Zhang LY, Wang YD, Liao ZY, Hua X, Cheng VC, Yuen KY [2004] Sensitive and specific monoclonal antibodybased capture enzyme immunoassay for detection of nucleocapsid antigen in sera from patients with severe acute respiratory syndrome. J. Clin. Microbiol. 42:2629-2635.
16. Hiscox JA, Cavanagh D, Britton P [1995] Quantification of individual subgenomic mRNA species during replication of the coronavirus transmissible gastroenteritis virus. Vir. Res. 36:119-130.
17. Kammila S, Das D, Bhatnagar PK, Sunwoo HH, Zayas-Zamora G, King M,

Suresh MR [2008] A rapid point of care immunoswab assay for SARS-CoV detection. J. Virol. Meth. 152[1-2]: 77-84

18. Suresh MR, Bhatnagar PK, Das D [2008] Molecular targets for diagnostics and therapeutics of severe acute respiratory syndrome [SARS-CoV]. J. Pharm. Pharmaceut. Sci. 11[2]:1s-13s.
19. Bhatnagar PK, Das D, Suresh MR [2008] Sequential affinity purification of peroxidase tagged bispecific anti-SARSCoV antibodies on phenylboronic acid agarose. J Chromat. Anal. Tech. Biomed. Life Sci. 863[2]: 235-241.
20. Murrell S, Wu S-C, Butler M [2011] Review of dengue virus and the development of a vaccine. Biotech. Adv. 29:239- 247.
21. Schieffelin JS, Costin JM, Nicholson CO, Orgeron NM, Fontaine KA, Isern S, Michael SF, Robinson JE [2010] Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived from a naturally infected patient. Virol. J. doi: 10.1186/1743-422X-7-28.
22. Shrivastava A, Dash PK, Tripathi NK, Sahni AK, Gopalan N, Lakshmana Rao PV [2011] Evaluation of a commercial Dengue NS1 enzyme-linked immunosorbent assay for early diagnosis of dengue infection. Ind. J. Med. Mirobiol. 29[1]: 51-55
23. Malabadi RB, Parashar A, Ganguly A, Suresh MR [2010] Expression of dengue virus envelope protein in a different plant system. Faculty Research and Development day, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada, 19th November 2010, page no-31.
24. Malabadi RB, Ganguly A, Teixeira da Silva JA, Parashar A, Suresh MR, Sunwoo HH [2011] Overview of plant-derived vaccine antigens: Dengue virus. J. Pharm. Pharmaceut. Sci. 14:400-413.

25. Monath TP [1994] Dengue: the risk to developed and developing countries. PNAS. 85:7627-7631.
26. Halstead SB [1988] Pathogenesis of dengue: challenges to molecular biology. Science. 239 [4839]: 476-481.
27. Halstead SB [1989] Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenic cascade. Rev. Infect. Dis.. 11[4]: S830-9.
28. World Health Organization [2009] Vector borne viral infections: dengue fever [online]. http.//www.who.int/vaccine_research/ diseases/vector/en/index1.html.
29. World Health Organiziation. W.P. R [2010] Dengue in the Western Pacific Region [online]. http.//www.wpro.who.int/health_topic s/dengue/overview.htm
30. Simasathien S, Watanaveeradej V [2005] Dengue vaccine. J. Med. Assoc. Thai. 88[3]: S363-77.
31. Gubler DJ, Meltzer M [1999] Impact of dengue/dengue hemorrhagic fever onthe developing world. Adv. Vir. Res. 53: 35-70.
32. Holmes E, Twiddy SS [2003] The origin, emergence and evolutionary genetics of dengue virus. Inf Gen Evolution. 3:19-28
33. Rico-Hesse R [1990] Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology. 174[2]: 479-93.
34. Sabin AB [1989] Research on dengue during World War II. Amer. J. Trop. Med. Hyg. 1[1]: 30-50.
35. Tang XL, Peppler MS, Irvin RT, Suresh MR [2004] Use of bispecific antibodies in molecular velcro assays whose specificity approaches the theoretical limit of immunodetection for Bordetella pertussis. Clin. Diag. Lab. Imm. 11[4]: 752-757
36. Robinson MC [1955] An epidemic of virus disease in Southern Provinces,

Tanganyika Territory in 1952-53. I Clinical features. Trans Royal Soc Trop Med Hyg. 49:28-32.

37. Muthumani K, Lankaraman KM, Laddy DJ, Sundaram SG, Chung CW, Sako E, Wu L, Khan A, Sardesai N, Kim JJ, Vijayachari P, Weiner DB [2008] Immunogenicity of novel consensus-based DNA vaccines against Chikungunya virus. Vaccine. 26:5128-5134.
38. Strauss JH, Strauss EG [1994] The alphaviruses: gene expression, replication and evolution. Microbiol Rev. 58:491-562.
39. Tiwari M, Parida M, Santhosh SR, Khan M, Dash PK, Lakshmana Rao PV [2009] Assessment of immunogenic potential of Vero adapted formalin inactivated vaccine derived from novel ECSA genotype of Chikungunya virus. Vaccine. 27:2513-2522.
40. Akahata W, Yang ZY, Andersen H, Sun S, Holdaway HA, Kong WP, Lewis MG, Higgs S, Rossamann MG, Rao S, Nabel GJ [2010] A VLP vaccine for epidemic Chikungunya virus protects non-human primates against infection. Nat. Med. 16[3]: 334-338.
41. Mavalankar D, Shastri P, Raman P [2007] Chikungunya epidemic in India: A major public-health disaster. Lancet Infect. Dis. 7:306-307.
42. Simon F, Savini H, Parola P [2008] Chikungunya: a paradigm of emergence and globalization of vector-borne diseases. Medical Clin North America. 92:1323-1343.
43. Powers AM, Logue CH [2007] Changing patterns of chikungunya virus: reemergence of a zoonotic arbovirus. Journa of General Virology. 88:2363-2377.
44. Mohan A, Kiran DHN, Manohar IC, Prabath Kumar D [2010] Epidemiology,

clinical manifestations, and diagnosis of chikungunya fever: Lessons learned from the re-emerging epidemic. Ind. J. Dermatol. 55[1]: 54-63.

45. Paramasivan R, Phillip Samuel P, ThenmozhiV, Rajendra R, Victor Jerald LS, Dhananjeyan KJ [2009] Chikungunya virus isolated in Lakshadweep islands in the Indian Ocean: Evidence of the central/East African genotype. Japanese J. Infect. Dis. 62:67-69.
46. Gardner J, Anraku I, Le TT, Larcher T, Major L, Roques P, Schroder WA, Higgs S, Suhrbier A [2010] Chikungunya virus Arthritis in adult wild-type mice. J. Virol. 84[16]: 8021-8032.
47. Sarkar S, Suresh MR [2011] An overview of tuberculosis chemotherapy – a literature review. J. Pharm Pharmaceut. Sci.. 14: 148–161.
48. Chaudhary M, Gupta S, Khare S, Lal S [2010] Diagnosis of tuberculosis in an era of HIV pandemic: a review of current status and future prospects. Ind. J. Med. Microbiol. 28: 281–289.
49. Gennaro ML [2000] Immunologic diagnosis of tuberculosis. Clin. Infect. Dis. 30: S243–246.
50. Sarkar S, Tang XL, Das D, Spencer JS, Lowary TL, Suresh MR [2012] A bispecific antibody based assay shows potential for detecting tuberculosis in resource constrained laboratory settings. PLoS ONE. 7[2]: e32340. doi:10.1371/journal. pone.0032340.
51. Guttikonda S, Tang XL, Yang BM, Armstrong GD, Suresh MR, [2007] Monospecific and bispecific antibodies against E.coli O157 for diagnostics. J Imm Meth. 327[1-2]: 1-9

1.4 Objectives and Hypotheses

The foremost aim of this research is to develop minimally invasive inexpensive points of care diagnostics for dengue infection. We endeavored to design and develop a simple dipstick type lateral flow device or a swab based immunoassay for detection of Dengue virus infection. Such formats provide instant visual end points for the presence of markers specific to dengue virus infection. The diagnostic formats chosen are similar to the common pregnancy test with ease of use. We have exploited the combined potential of mAbs, bsMAbs and Chicken IgY in combination assays for detection of dengue. Another aim of this thesis was to develop an effective vaccine candidate for dengue that could successfully neutralize dengue virus infection. As an extension of the aforementioned aim, the development of monovalent and tetravalent dendritic cell targeted low dose DNA dengue vaccine formulations were also pursued.

The major proteins of dengue virus include the membrane glycoprotein, envelope glycoprotein protein and the core nucleocapsid protein. The envelope glycoprotein along with non-structural protein [NS1] of dengue virus is considered a good candidate as therapeutic targets for the development of therapeutic antibodies. The NS1 is also a good diagnostic target. We delved on a multi-pronged approach to ultimately generate the full length monoclonal and bi-specific antibodies for oligoclonal monoclonal antibody cocktail as therapeutics and bi-specific antibody as diagnostic reagents.

1.4.1 Hypotheses

We hypothesize that sandwich ELISA based on second generation self assembling bi-specific antibodies with binding specificities to dengue virus antigens NS1 in one paratope and an enzyme such as HRPO in the second paratope (BsMAb) in combinations with MAb and IgY can successfully detect antigen levels seen in patient body fluid samples for screening applications both in hospital settings as well as at point of care level.

1.4.2 Salient features

- The immunoassay could be an inexpensive economic alternative to PCR and quantitative PCR.
- The immunoassay shall have enhanced sensitivity in the bsMAb based format in comparison to the conventional chemically conjugated mAb [biotin labeled] based format.
- The designed immunoassay shall be able to detect Dengue specific antigen at the point of care level and also able to detect specific antigen from clinical samples in a hospital setting or a physician's clinic.
- Another immunoassay involving chicken IgY as a diagnostic probe with good sensitivity and less expensive would be an ideal supplementary diagnostic to detect Dengue infection

1.4.3 Continuing and future research goals

- Development of envelope antigen and monoclonal antibodies against the protein can also be used to further substantiate dengue-screening assays
- The Dengue envelope protein as a vaccine candidate would potentially be able to neutralize viral infection
- Anti envelope monoclonal antibodies can be effective in therapeutic strategies as well as in understanding dengue pathogenesis in combating dengue and also for fundamental understanding of the disease
- Dendritic cell targeted monovalent and tetravalent DNA vaccines can be a significant strategy in the quest for developing low dose dengue vaccines with high efficacy

To achieve the aforesaid aims and objectives the research plan undertaken is illustrated in the following flow diagram [Fig 1.2].

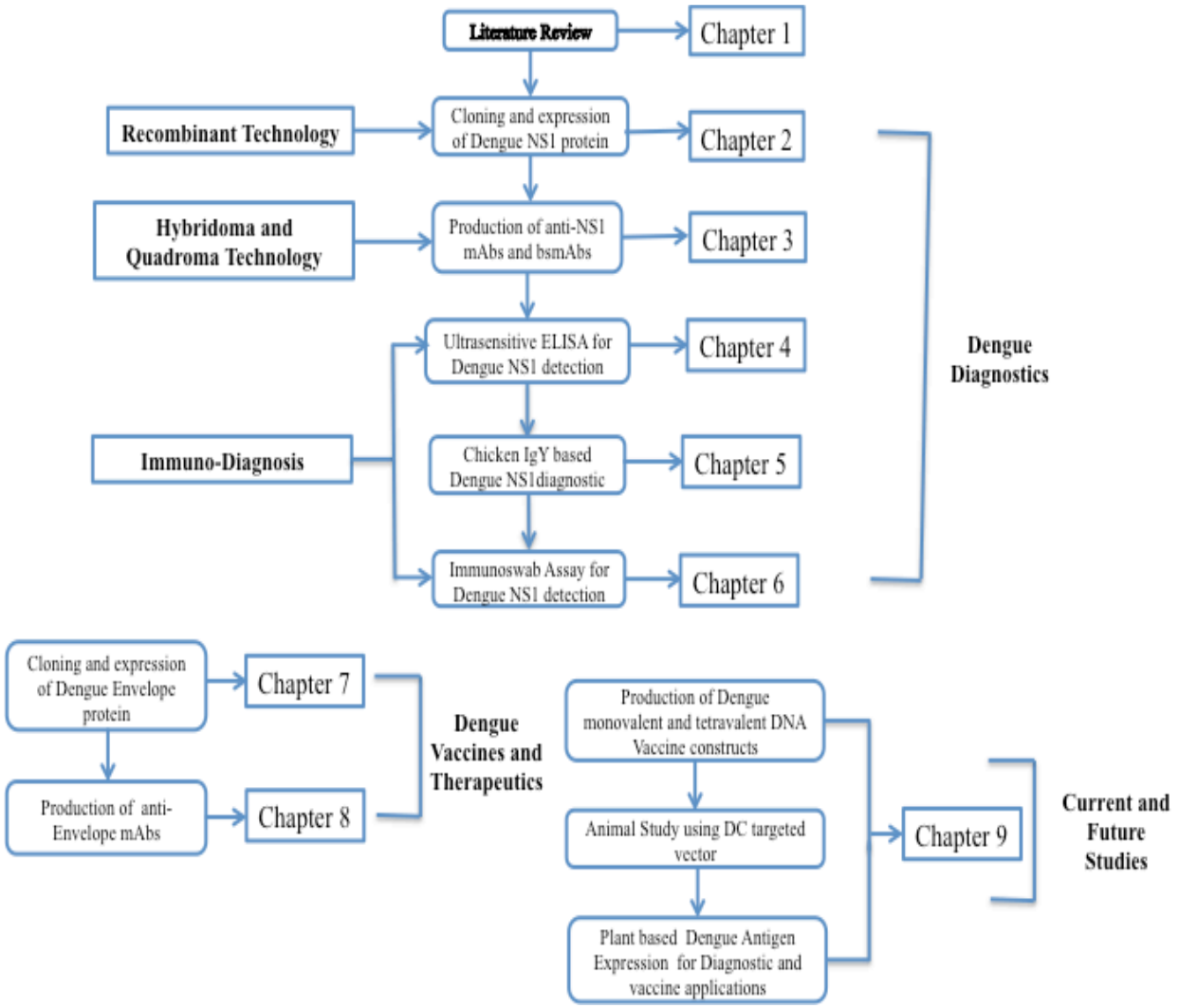


Figure 1.2 Schematic representation of research objectives

2

EXPRESSION OF DENGUE VIRUS NS1 PROTEIN IN *E.coli*

2.1 Introduction

Dengue fever is an important mosquito-borne viral disease of humans. This has been a recurrent phenomenon throughout the tropics in the past decade. During 2002, more than 30 Latin American countries reported over a million dengue fever [DF] cases with large number of dengue hemorrhagic fever [DHF]. Annually, there are an estimated 100-million dengue virus infections worldwide [1]. Increasingly cases of the more severe and potentially lethal DHF and dengue shock syndrome [DSS] are reported with children bearing much of the disease burden. Dengue virus is endemic in at least 100 countries worldwide and causes more human cases than any other mosquito-borne virus. The mortality rate of DHF in most countries is 5%, primarily among children and young adults. In several Asian countries, this virus is the leading cause of hospitalization and death in children. Hence, there is an urgent need for the development of diagnostic, prophylactic and therapeutic reagents to manage DHF. The dengue virus non-structural NS1 protein is a 46–50 kDa glycoprotein expressed in infected mammalian cells. All non-structural proteins are intracellular proteins with the exception of dengue NS1 protein, which exists as secreted as well as a membrane associated protein. Both forms are demonstrated to be immunogenic [2–4]. It was also reported that NS1 is one of 7 NS proteins produced during viral replication. It possesses not only group specific but also type specific determinants and has been recognized as an important antigen in dengue infection [2,4,5]. A high circulating level of NS1 was demonstrated in the acute phase of dengue by antigen capture ELISAs [2,6]. The precise function of dengue NS1 protein remains unclear. However, antigen detection of non-structural dengue antigens may be of benefit for an early stage rapid diagnosis of infection due to its long half-life in the blood.

The usefulness of this study was to clone and express of DEN-1 full-length NS1 gene in *Escherichia coli*, for future development of monoclonal antibodies exploiting hybridoma and quadroma technology for rapid point of care applications. In this study, we report the successful cloning and very high-level expression of the NS1 protein and purification from *E.coli* as inclusion bodies and subsequent refolding.

2.2 Materials and Methods

2.2.1 Vector and chemicals

Restriction endonucleases and modifying enzymes were purchased from New England Biolabs [Mississauga, Canada]. The anti-His6 MAb was purchased from Novagen Inc. [Madison, USA]. Prestained low range protein molecular weight markers, 40% acrylamide: bisacrylamide, glycine and protein assay reagents were purchased from Bio-Rad [Mississauga, Canada]. ECL nitrocellulose membrane, X-ray film and Western blotting reagent were purchased from Amersham Pharmacia Biotech [BaiedUrfe, Quebec, Canada]. Glutathione [reduced and oxidized], sodium deoxycholate, L-arginine, GAM-HRPO, urea and other general molecular biology grade reagents were purchased from Sigma [Oakville, Canada]. Ni–NTA agarose, plasmid DNA isolation and gel extraction kits were obtained from Qiagen [Mississauga, Canada].

2.2.2 Construction of plasmid **[pDS21NS1]**

The NS1 full-length nucleotide sequence of dengue [DEN-1] was codon optimized for *E.coli* expression and chemically synthesized by GENEART Inc., Germany. The codon

optimized NS1 gene, containing plasmid obtained from GENEART Inc. and the expression vector pBM802 [7] were digested with NdeI and EcoRI, gel purified and ligated. The ligation mixtures were transformed in *E.coli* top 10 cells and bacterial colonies were analyzed by plasmid DNA isolation and restriction digestion fragment mapping [8].

2.2.3 Analysis of recombinant clones

Single bacterial colonies were cultured in 2 ml TB medium [8] containing 5 μg/ml of tetracycline [Tet5] and were incubated overnight at 37 ºC with shaking [250 rpm]. The overnight culture was diluted to 1/100th volume in 10 ml fresh TB/Tet5 medium and grown at 37 ºC. The bacterial culture was induced when the optical density [OD600nm] reached approximately 0.5–0.6 with arabinose [0.2% [w/v]] overnight [~16 h] at 37 ºC, where as in control sample arabinose was not added. The bacterial culture of test and control samples were harvested by centrifugation at 5000g for 10 min at 4 ºC and the total cell lysate was prepared [8]. Total cell protein [TCP] was analyzed by SDS–PAGE using 10% polyacrylamide gel [9] with a Mini Protean III apparatus [Bio-Rad]. The protein gel was stained with 0.25% [w/v] Coomassie Brilliant Blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 30% methanol.

2.2.4 Expression optimization [Temp, Time and Inducer]

The expression of the NS1 protein was optimized for three different temperatures, time durations and inducer [arabinose] concentrations. Bacterial growth condition was similar to that described above. For arabinose dose optimization, the bacterial culture was induced with different concentrations of arabinose [2%, 0.2%, 0.02%, 0.002% and

0.0002% [w/v]] and allowed to grow overnight [16 h] at 30 ºC. For temperature optimization, the bacterial culture was induced with arabinose [0.2% [w/v]] and allowed to grow overnight [16 h] at three different temperatures [37, 30 and 24 ºC]. For time optimization, the bacterial culture was induced with arabinose [0.2% [w/v]] and allowed to grow for 0 h, 2 h, 4 h, 6 h and overnight [~16 h] at 30 ºC. Total cell proteins from each optimization experiment were analyzed by SDS–PAGE and Western blot to select the ideal condition for optimum protein expression.

2.2.5 Medium scale expression and purification

A single bacterial colony was inoculated in 10 ml TB/Tet5 medium and allowed to grow overnight at 37 ºC shaker. The overnight culture was diluted [1:100] in fresh 4 X 1 L TB/Tet5 medium and grown at 37 ºC until an OD600nm of 0.5–0.6 was reached. Expression was done by optimized conditions as described in the previous section. Induction was initiated by adding 0.2% [w/v] arabinose and bacterial culture was incubated for 16 h with vigorous shaking at 30 ºC. Bacterial culture was harvested by centrifugation at 5000g for 20 min at 4 ºC and total cell protein [TCP] from induced and uninduced culture was analyzed by SDS–PAGE and Western blot probed with anti-His6 MAb.

2.2.6 Purification of inclusion bodies

The purification of inclusion bodies was done according to previously published method [10]. Briefly, 19.6 g of bacterial wet pellet from 4 L bacterial culture was suspended in 196 ml PBS [10 ml PBS per g of pellet] and completely lysed by passing through a French Press [20,000 psi]. The total cell lysate was clarified by centrifugation at 27,000g for 30 min at 4 ºC and supernatant was collected as total soluble protein. The pellet was

resuspended in lysis buffer [Table 2.1] and then 2% sodium deoxycholate was added. The mixture was incubated at room temperature for 30 min with gentle shaking and centrifuged at 27,000g for 30 min at 4 ºC. The pellet was resuspended in lysis buffer and washed thrice at 27,000g for 20 min at 4 ºC to completely remove sodium deoxycholate.

2.2.7 Immobilized Metal Affinity Chromatography (IMAC) purification

Inclusion bodies were solubilized in denaturing buffer B [Table 2.1] for 1 h at room temperature with gentle shaking. Solubilized denatured rNS1 proteins from insoluble materials were separated by centrifugation at 27,000g for 30 min at 4 ºC. Final yield of solubilized denatured protein was determined by protein assay using BSA as the standard protein [11]. A Ni–NTA column was prepared by loading the Ni–NTA agarose on a plastic column [Bio-Rad] and equilibrated with 10 bed volumes of buffer B. Twenty milligrams of solubilized denatured rNS1 protein was loaded on the column and the column was washed with 5–10 bed volumes with buffer C. After complete wash, bound protein was eluted with buffer D and buffer E. All the eluted fractions were analyzed by SDS–PAGE prior to refolding.

Table 2.1 Buffer used for purification of inclusion bodies and refolding

Buffer	Composition
Lysis Buffer	50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA
Buffer B	8M Urea, 100 mM NaH_2PO_4, 10 mM Tris- Cl, pH 8.0
Buffer C	8M Urea, 100 mM NaH_2PO_4, 10 mM Tris- Cl, pH 6.3
Buffer D	8M Urea, 100 mM NaH_2PO_4, 10 mM Tris- Cl, pH 5.9
Buffer E	8M Urea, 100 mM NaH_2PO_4, 10 mM Tris- Cl, pH 4.5
TA Buffer	50 mM Tris, pH 8.0, 0.4 M L- arginine

2.2.8 Refolding

Protein assay was done to quantitate the amount of protein eluted from the Ni–NTA column and it was estimated about 0.4– 0.6 mg/ml with a total amount of around 12–14 mg. Refolding was done in three different concentrations to evaluate the best refolding condition. The eluted protein was adjusted to 100, 75 and 50 μg/ml with refolding TA buffer and refolding was done by dialysis in TA buffer in the presence of 1.0 mM GSH [glutathione, reduced], 0.1 mM GSSG [glutathione, oxidized] for 3 days with two changes at 4 ºC. Final dialysis was done in PBS pH 7.4 at 4 ºC.

2.2.9 Western blot analysis

TCP, inclusion bodies, IMAC eluted fractions or refolded rNS1 protein were electrophoresed on SDS–PAGE using 10% polyacrylamide gel and then electroblotted onto Hybond ECL nitrocellulose membranes [12]. The nitrocellulose membrane was blocked with 5% skim milk in PBST [0.1% Tween 20 in 1X PBS, pH 7.3] for overnight at 4 ºC. The membrane was washed four times with PBST and incubated with anti-His6 MAb for 1 h. After washing, the membrane was incubated with HRPO labeled goat anti-mouse IgG [GAM-HRPO] for 1 h. Finally, the membrane was washed with PBS and enhanced chemiluminescence based detection was performed to visualize the binding.

2.2.10 In gel digestion

Protein identification was performed at the Institute for Biomolecular Design, University of Alberta, Edmonton, Alberta, Canada. Briefly, stained bands [spots] were excised and an automated in-gel tryptic digestion was performed on a Mass Prep Station [Micromass, UK]. The gel pieces were destained, reduced with DTT, alkylated with iodoacetamide, then digested with trypsin [Promega Sequencing Grade Modified] and the resulting

peptides were extracted from the gel and analyzed via LC/MS. LC/MS was performed on a CapLC HPLC [Waters, USA] coupled with a Q-ToF-2 mass spectrometer [Micromass, UK]. Tryptic peptides were separated using a linear water/acetonitrile gradient [0.2% formic acid] on a Picofrit reversed-phase capillary column, [5 micron BioBasic C18, 300 Angstrom pore size, 75 micron ID x 10 cm, 15 micron tip] [New Objectives, MA, USA], with an in-line PepMap column [C18, 300 micron ID x 5 mm] [LC Packings, CA, USA] used as a loading/desalting column [13].

2.3 Results

2.3.1 NS1 gene cloning and small-scale expression

The full-length codon optimized NS1 gene was cloned in the correct reading frame with the His6 tag at the C-terminal and designated as pDS21NS1 for high-level expression of proteins as inclusion bodies in *E.coli* [7]. The correct size recombinant clones were selected for protein expression. The plasmid containing full-length NS1 gene was isolated for expression. Expression results showed that all the NS1 clones selected were expressing the target protein of approximately 46 kDa at different levels when analyzed by SDS– PAGE where as in control sample [without arabinose] there was no expression of the target protein [Fig. 2.1]. The expression of rNS1 protein was confirmed by Western blot probed with anti-His6 MAb. The best NS1 clone was chosen for the expression optimization and further studies.

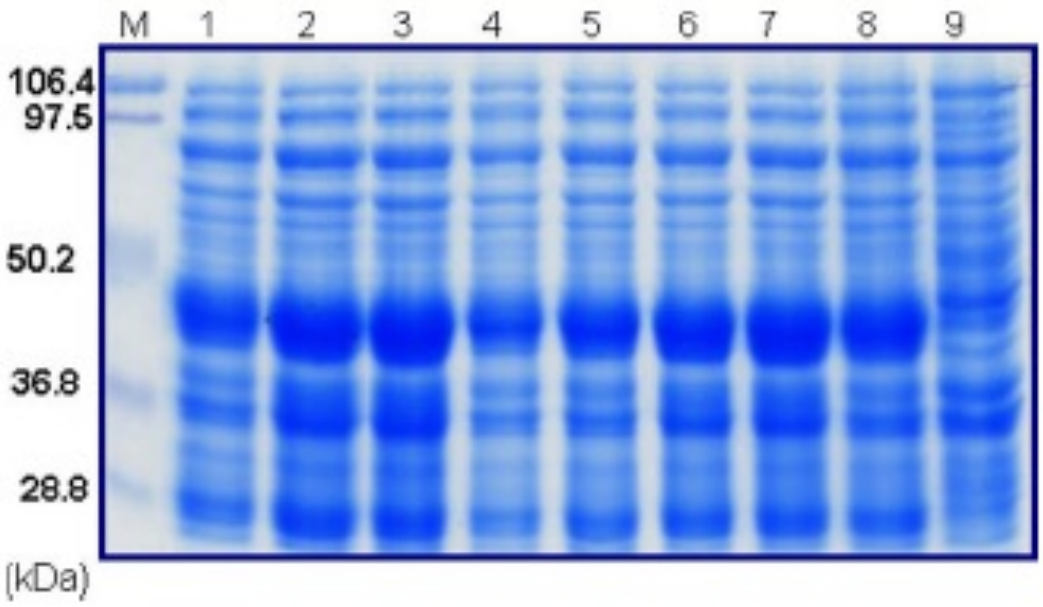
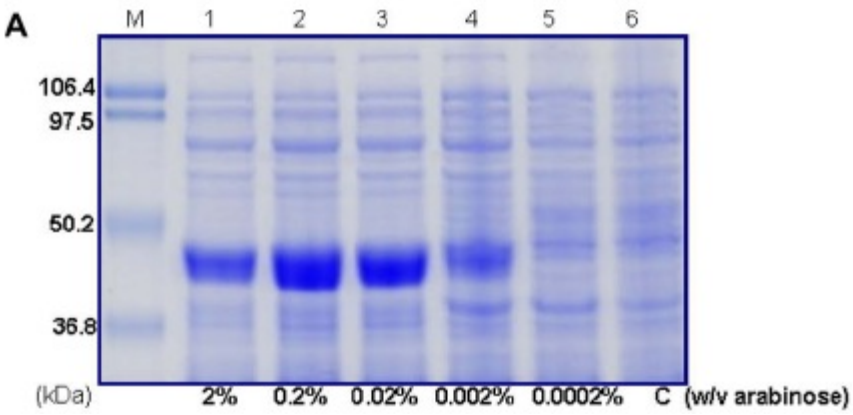
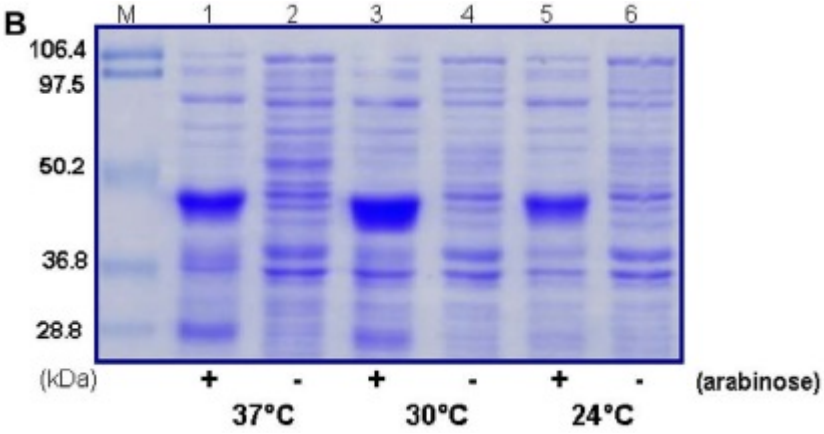


Fig 2.1: SDS–PAGE analysis of different NS1 clones expression in *E.coli*. Lane M: standard protein molecular weight markers, lanes 1–8: clone# 1, 3, 4, 5, 7, 9, 11, and 12, respectively, lane 9: control.

2.3.2 Expression optimization [Inducer, Temp and, Time]]

The NS1 protein was successfully expressed as inclusion bodies in *E.coli*. The optimal conditions for rNS1 protein expression were 0.2% [w/v] arabinose concentrations [Fig. 2.2A], 30ºC temperature [Fig. 2.2B] and 16 h induction time [Fig. 2.2C].


A
M
1
2
3
4
5
6
106.4
97.5
50.2
36.8
(kDa)
2%
0.2%
0.02%
0.002%
0.0002%
C
(w/v arabinose)


B
M
1
2
3
4
5
6
106.4
97.5
50.2
36.8
28.8
(kDa)
+
-
+
-
+
-
(arabinose)
37°C
30°C
24°C

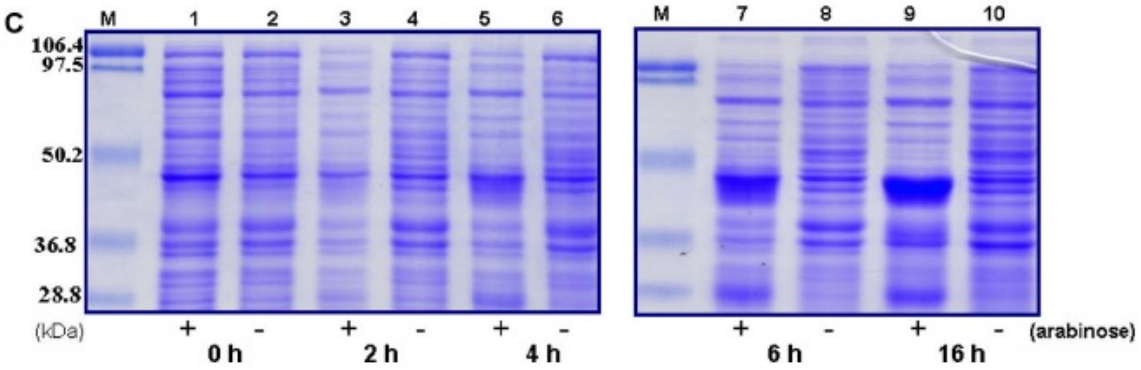


Fig 2.2: Expression optimization. [A] SDS–PAGE analysis of arabinose concentration dependent rNS1 protein expression. Bacterial cultures were induced with different concentrations of arabinose and grown at 30 ºC for overnight. Lane M: standard protein molecular weight markers, lanes 1–5: bacterial cultures were induced with 2%, 0.2%, 0.02%, 0.002%, 0.0002% arabinose [w/v], respectively, lane 6: control. [B] SDS–PAGE analysis of temperature dependent rNS1 protein expression. Bacterial cultures were induced with 0.2% arabinose [w/v] and grown at different temperatures [37, 30 and 24 ºC] for overnight. Lane M: standard protein molecular weight markers, lanes 1 and 2: 37 ºC, lanes 3 and 4: 30 ºC, lanes 5 and 6: 24 ºC. ‘‘+” indicates arabinose was added, ‘‘-” indicates arabinose was not added [control]. [C] SDS–PAGE analysis of time dependent rNS1 protein expression. Bacterial cultures were induced with 0.2% arabinose [w/v] and grown at 30 ºC for different time period. Lane M: standard protein molecular weight markers, lanes 1 and 2: 0 h, lanes 3 and 4: 2 h, lanes 5 and 6: 4 h, lanes 7 and 8: 6 h, lanes 9 and 10: overnight [16 h]. ‘‘+” indicates arabinose was added, ‘‘-” indicates arabinose was not added [control].

2.3.3 Medium scale of NS1 expression

The bacterial expression vector pBM802 was designed for high level expression of recombinant protein in *E.coli* as inclusion bodies. The medium scale expression of the rNS1 was performed and there was high-level expression in *E.coli* when analyzed by SDS–PAGE [Fig. 2.3]. Inclusion bodies were prepared from the bacterial pellet by a French Press. Following complete bacterial cell lysis, the insoluble inclusion bodies were separated from total soluble protein by centrifugation. The pellet was washed with sodium deoxycholate and subsequently washed with lysis buffer to remove any sodium deoxycholate. The final yield of denatured soluble inclusion bodies was estimated to be approximately 230–250 mg/L of initial bacterial culture. The purity of the inclusion bodies along with different washes was analyzed by SDS–PAGE.

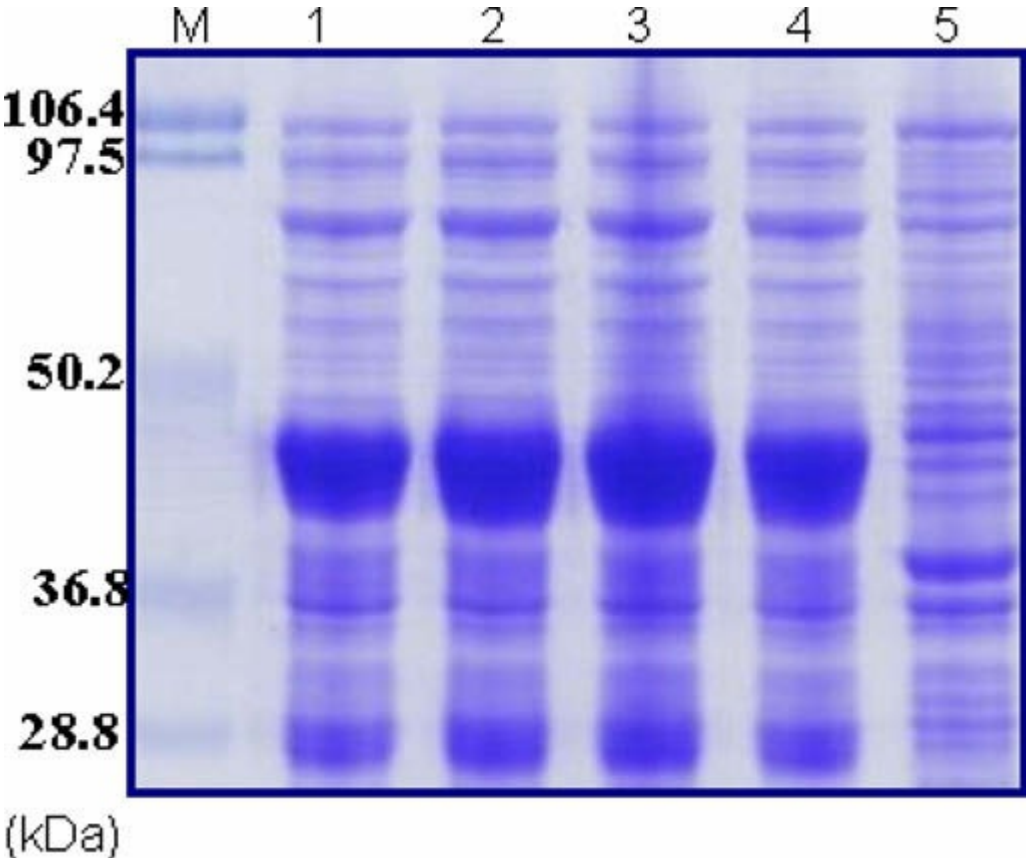


Fig 2.3: SDS–PAGE analysis of medium scale production of rNS1 protein. Lane M: standard protein molecular weight markers, lanes 1–4: bacterial culture flask 1–4 [f1–f4], respectively, lane 5: control.

2.3.4 Purification and refolding

The rNS1 protein was predominantly expressed in *E.coli* as inclusion bodies. The rNS1 protein was isolated from inclusion bodies from bacterial shake flask culture and purified by IMAC chromatography under denaturing conditions [Fig. 2.4A]. The purity of the eluted rNS1 protein was analyzed by SDS–PAGE prior to refolding. Renaturing conditions, protein concentrations in the refolding buffer as well as suitable buffer compositions are important to simulate correct folding, formation of the proper disulfide bond and proper association of different domains. It has been reported that the presence of arginine in refolding buffer played an important role in solubilization, inhibiting the aggregation of refolding intermediates and thus increases the yield of the refolded protein [14]. It has been previously demonstrated in literature that addition of GSH/GSSG into the refolding buffer facilitates disulfide bond formation and thus enhances renaturation of the protein [15]. The refolding step was done for 3 days by dialysis and any aggregate formed during refolding was removed by centrifugation. The supernatant was collected as refolded rNS1 for further use. Thus the in vitro refolding proved to be successful in recovering soluble protein expressed in *E.coli* as inclusion bodies. The purification steps and yield of rNS1 protein from 1 L of *E.coli* culture are summarized in Table 2. The purity of the refolded rNS1 protein was evident from the SDS–PAGE [Fig. 2.4B] and Western blot [Fig. 2.4C] with a single band of approximate molecular weight of 46 kDa. Its purity was estimated to be greater than 90%.

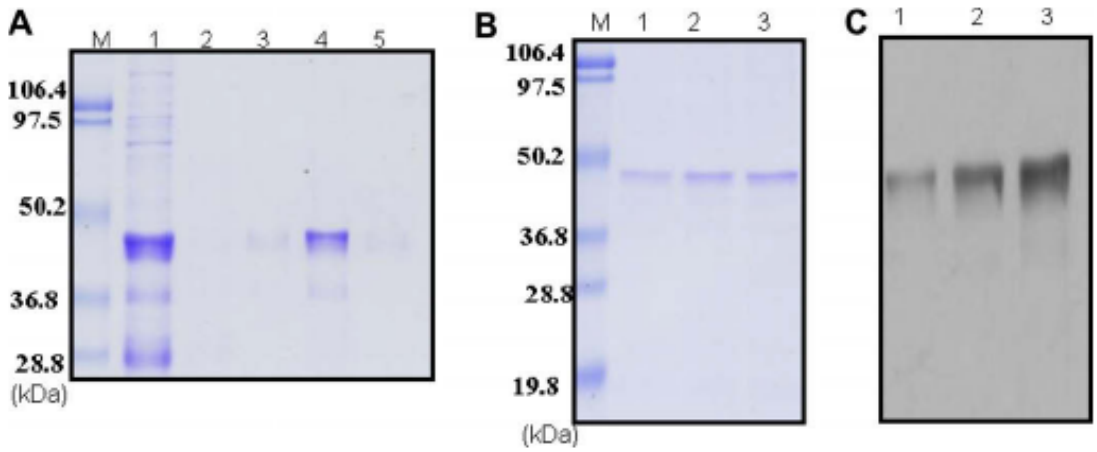


Fig 2.4: IMAC purification and refolding. [A] SDS–PAGE analysis of IMAC purified rNS1 protein. Lane M: standard protein molecular weight markers, lane 1: unbound protein, lanes 2 and 3: washes, lanes 4 and 5: elutions. [B] SDS–PAGE analysis of refolded rNS1 protein. Lane M: standard protein molecular weight markers, lanes 1–3: recovered rNS1 protein refolded at 50, 75, 100 µg/ml, respectively. [C] Western blot analysis of refolded rNS1 protein probed with anti-His_6 MAb. Lanes 1–3: recovered rNS1 protein refolded at 50, 75, 100 µg/ mL respectively.

2.3.5 In Gel Digestion

Protein identification from the generated LC/MS data was done by searching the NCBI [National Center for Biotechnology Information] non-redundant database [Database: NCBInr 20071130 [5678482 sequences; 1961803296 residues]] using Mascot Daemon search methodology [http://www.matrixscience.com]. Mascot search results showed significant hits for the DEN-1 polyprotein which includes NS1 [gi|20135604 polyprotein (dengue virus type 1)].

2.4 Discussion

Rapid diagnostics of any infectious disease can lead to early therapeutic intervention of probable cases as well as suspected cases. In many viral diseases, virus shedding is greatest during the early symptomatic phase, i.e. around and immediately following the onset of symptoms. It has been reported that the NS1 antigen was found circulating from the first day after the onset of symptoms up to day 9. The NS1 levels ranged from 0.04 to 2 μg/ml in acute phase serum samples [from day 0 to 7], and the level for a convalescent phase serum [day 8 and later] was 0.04 μg/ml. In secondary phase infection, NS1 levels ranged from 0.01 to 2 μg/ml and were not detectable in convalescent phase sera. An antigen capture ELISA has been developed for detection of serum NS1 early in primary and secondary dengue infection [6]. According to these studies the presence of NS1 in human sera can be confirmed between days 0 and 9 [16,17] and with a peak at days 6–10 [18]. Dengue NS1 antigen testing is suggested as a helpful tool for the early diagnosis of dengue infection after the onset of fever [19,20]. Commercially available dengue NS1 antigen capture ELISA has been evaluated for the detection of NS1 from patient's

samples in different stages [17, 20–22]. It is therefore an important antigen for rapid viral diagnosis. It has been reported in the literature that different expression systems have been exploited for NS1 expression and with a typical yield of 10–30 mg/L in bacteria [23], 25 mg/L of multi-epitope dengue protein in bacteria [24] and 70 mg/L in yeast [25]. Successful expression of NS1 protein has also been reported in both baculovirus and mammalian expression systems [26–29]. Here, we have also exploited the bacterial expression system for the production of rNS1 protein. Bacterial expression is perhaps the most commonly employed expression system for the production of non-glycosylated recombinant proteins. The organism is relatively simple to manipulate and the small scale analysis of many different parameters can be optimized in a short period of time. This allows the rapid identification and optimization of several growth and induction conditions for medium scale production. Many eukaryotic genes cannot be expressed efficiently in the *E.coli* host due to the difference in codon preference as well as toxicity of foreign protein and mRNA instability for the expression of protein encoded by the gene. It is also a very well known fact that heterologously expressed eukaryotic proteins are not post-translationally modified when it is expressed in *E.coli*. It is also difficult to express as soluble protein or facilitate the secretion of expressed protein into the culture medium. In addition, proteins expressed in large amounts tend to precipitate, forming inclusion bodies [10, 30, 31] and present an advantage with respect to higher yield and especially the purification of expressed protein. It has been demonstrated in the literature that genes can be codon optimized to the host translational system with the significantly higher expression level than native genes [7]. Based on this knowledge, we have obtained the codon optimized NS1 gene from GENEART Inc. for expression in *E.coli*. In the present study, we have cloned and purified the dengue NS1 protein in *E.coli* for the

development of monoclonal antibody and subsequently bispecific antibodies for early diagnostic applications. NS1 gene was cloned under the control of the pBAD promoter for high-level expression of recombinant protein as inclusion bodies in the bacterial cytoplasm. The final yield of purified inclusion bodies was estimated by Bradford protein assay [11] to be approximately 230– 250 mg/L of initial bacterial culture which is 10- to 25-fold higher compared to previous reports using the native NS1 gene sequence. We have expressed several viral antigens in *E.coli* exploiting codon optimized genes and the NS1 expression is the most robust yield we have achieved [Table 2.3]. The bacterial cell lysis by French Press and washing steps with detergent were used successfully to purify inclusion bodies from soluble protein. SDS–PAGE analysis clearly demonstrated that lysis by French Press and several washings with lysis buffer increased the purity of the inclusion bodies since the bulk of the *E.coli* soluble proteins were separated. The purity of inclusion bodies was also judged by SDS–PAGE and Western blot. The purification method exploits the immobilized metal affinity chromatography [IMAC] under denaturing conditions. The effectively adsorbed His-tagged protein could be purified to homogeneity [10]. IMAC purification under denaturing conditions yielded significant amount of pure rNS1 protein with a single band as judged by SDS–PAGE and with a typical yield of 60– 80% of starting denatured inclusion bodies [Table 2.2]. Most recombinant proteins are expressed in *E.coli* as inclusion bodies and different refolding methods have been reported to renature proteins from inclusion bodies [10,32]. The IMAC eluted rNS1 protein was refolded in TA buffer in the presence of a redox pair [GSH/GSSG] with three different protein concentrations [Table 2.4].

Table 2.2. Purification of dengue rNS1 protein from *E.coli* in 1 L culture.

Purification Steps	Total protein [mg]	rNS1 [mg]	Recovery [%]
Inclusion bodies	250	232	92.8
IMAC elutions[a]	20	12	60
Refolding	12	12	100

[a] In each batch of IMAC purification, 20 mg of protein was used

Table 2.3. Expression yields of various codon optimized genes

Protein	Yield [mg/L]	Reference
SARS- CoV NP	70	[7]
Dengue NS1	230-250	This report
H5N1 HA1	15-20	Unpublished

Table 2.4. Different refolding conditions and yield.

Protein	GSH: GSSG [mM]	Refolding TA Buffer	Protein Conc. In Refolding buffer [µg/ml]	Yield [%]
rNS1	1: 0.1	50 mM Tris, pH 8.0, 0.4 M L- arginine	100	No aggregation [100]
rNS1	1: 0.1	50 mM Tris, pH 8.0, 0.4 M L- arginine	75	No aggregation [100]
rNS1	1: 0.1	50 mM Tris, pH 8.0, 0.4 M L- arginine	50	No aggregation [100]

There are 12 cysteines conserved among NS1 proteins of flaviviruses that form disulfide bond [S–S] which suggests an important role in structure and function of the protein. The crystal structure of NS1 protein is yet not known but Wallis et al. has predicted 6 disulfide bonds present in the dengue NS1 protein [33]. Therefore the addition of GSH/GSSG into the refolding buffer which maintained the oxidizing environment enhances S–S bond formation and hence increases the solubility of the recombinant protein [15, 34]. The protein concentration plays a crucial role in refolding conditions and it was observed that when the concentration was above 100 μg/ mL, aggregation was evident. The purity of the refolded protein was judged by SDS–PAGE [Fig. 2.4B] and Western blot [Fig. 2.4C] data indicated that anti-His6 MAb reacted with a single band of around 46 kDa, suggesting that we had successfully purified rNS1. The identity of purified rNS1 protein was confirmed by in vitro gel digestion, mass spectrometry, and NCBI non-redundant database searching. The refolded rNS1 antigen is being used to immunize mice to develop monoclonal antibodies. The polyclonal antibodies from mouse serum were strongly reacting with recombinant antigen [Fig. 2.5A] in Western blot [Fig. 2.5B]. The rNS1 protein will be useful to select a pair of monoclonal antibodies with non-overlapping different specificities for antigen capture ELISA and point of care rapid assays [35]. In addition, the rNS1 antigen based ELISA could be the basis of developing a sensitive serum diagnostic to monitor dengue outbreaks by detecting the early human anti-dengue antibodies.

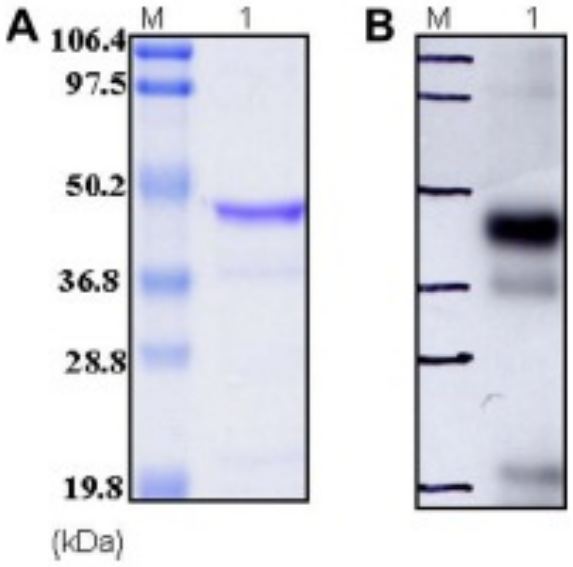


Fig 2.5: SDS–PAGE analysis of refolded rNS1 protein. [A] Lane M: standard protein molecular weight markers, lane 1: refolded rNS1 protein. [B] Western blot analysis of refolded rNS1 protein probed with mouse anti-NS1 polyclonal antibodies. Lane 1: refolded rNS1 protein.

2.5 References

1. D.J. Gubler, M. Meltzer, Impact of dengue/dengue hemorrhagic fever on the developing world, Adv. Virus Res. 53 [1999] 35–70.

2. P.R. Young, P.A. Hilditch, C. Bletchly, W. Halloran, An antigen capture enzymelinked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients, J. Clin. Microbiol. 38 [2000] 1053–1057.

3. M. Flamand, F. Megret, M. Mathieu, J. Lepault, F.A. Rey, V. Deubel, Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion, J. Virol. 73 [1999]
6104–6110.

4. A.K. Falconar, P.R. Young, Immunoaffinity purification of native dimer forms of the flavivirus non-structural glycoprotein, NS1, J. Virol. Methods 30 [1990] 323–332. [5] E.A. Henchal, L.S. Henchal, B.K. Thaisomboonsuk, Topological mapping of unique epitopes on the dengue-2 virus NS1 protein using monoclonal
antibodies, J. Gen. Virol. 68 [Pt. 3] [1987] 845–851.

6. S. Alcon, A. Talarmin, M. Debruyne, A. Falconar, V. Deubel, M. Flamand, Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections, J. Clin. Microbiol. 40 [2002] 376–381.

7. D. Das, M.R. Suresh, Copious production of SARS-CoV nucleocapsid protein employing codon optimized synthetic gene, J. Virol. Methods 137 [2006] 343–346.

8. J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

9. U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 [1970] 680–685.

10. D. Das, F. Jacobs, H. Feldmann, S.M. Jones, M.R. Suresh, Differential expression of the Ebola virus GP[1,2] protein and its fragments in E.coli, Protein Expr. Purif. 54 [2007] 117–125.

11. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 [1976] 248–254.

12. H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. USA 76 [1979] 4350–4354.

13. D. Das, T.M. Allen, M.R. Suresh, Comparative evaluation of two purification methods of anti-CD19-c-myc-His6-Cys scFv, Protein Expr. Purif. 39 [2005] 199–208.

14. K. Tsumoto, M. Umetsu, I. Kumagai, D. Ejima, J.S. Philo, T. Arakawa, Role of arginine in protein refolding, solubilization, and purification, Biotechnol. Prog. 20 [2004] 1301–1308.

15. C. Wei, B. Tang, Y. Zhang, K. Yang, Oxidative refolding of recombinant prochymosin, Biochem. J. 340 [Pt. 1] [1999] 345–351.

16. P.Y. Shu, L.K. Chen, S.F. Chang, Y.Y. Yueh, L. Chow, L.J. Chien, C. Chin, H.H. Yang, T.H. Lin, J.H. Huang, Potential application of nonstructural protein NS1 serotype-specific immunoglobulin G enzyme-linked immunosorbent assay in
the seroepidemiologic study of dengue virus infection: correlation of results with those of the plaque reduction neutralization test, J. Clin. Microbiol. 40 [2002] 1840–1844.

17. P. Dussart, B. Labeau, G. Lagathu, P. Louis, M.R. Nunes, S.G. Rodrigues, C. Storck-Herrmann, R. Cesaire, J. Morvan, M. Flamand, L. Baril, Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum, Clin. Vaccine Immunol. 13 [2006] 1185–1189.

18. H. Xu, B. Di, Y.X. Pan, L.W. Qiu, Y.D. Wang, W. Hao, L.J. He, K.Y. Yuen, X.Y. Che, Serotype 1-specific monoclonal antibody-based antigen capture immunoassay for detection of circulating nonstructural protein NS1: implications for early diagnosis and serotyping of dengue virus infections, J. Clin. Microbiol. 44 [2006] 2872–2878.

19. A. Chuansumrit, W. Chaiyaratana, V. Pongthanapisith, K. Tangnararatchakit, S. Lertwongrath, S. Yoksan, The use of dengue nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection, Pediatr. Infect. Dis. J. 27 [2008] 43–48.

20. K. Lapphra, A. Sangcharaswichai, K. Chokephaibulkit, S. Tiengrim, W. Piriyakarnsakul, T. Chakorn, S. Yoksan, L. Wattanamongkolsil, V. Thamlikitkul, Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness, Diagn. Microbiol. Infect. Dis. 60 [2008] 387–391.

21. V. Kumarasamy, S.K. Chua, Z. Hassan, A.H. Wahab, Y.K. Chem, M. Mohamad, K.B. Chua, Evaluating the sensitivity of a commercial dengue NS1 antigencapture ELISA for early diagnosis of acute dengue virus infection, Singapore Med. J. 48 [2007] 669–673.

22. V. Kumarasamy, A.H. Wahab, S.K. Chua, Z. Hassan, Y.K. Chem, M. Mohamad, K.B. Chua, Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection, J. Virol. Methods 140 [2007] 75–79.

23. J.L. Huang, J.H. Huang, R.H. Shyu, C.W. Teng, Y.L. Lin, M.D. Kuo, C.W. Yao, M.F. Shaio, High-level expression of recombinant dengue viral NS-1 protein and its potential use as a diagnostic antigen, J. Med. Virol. 65 [2001] 553–560.

24. R. AnandaRao, S. Swaminathan, S. Fernando, A.M. Jana, N. Khanna, A customdesigned recombinant multiepitope protein as a dengue diagnostic reagent, Protein Expr. Purif. 41 [2005] 136–147.

25. J.M. Zhou, Y.X. Tang, D.Y. Fang, J.J. Zhou, Y. Liang, H.Y. Guo, L.F. Jiang, Secreted expression and purification of dengue 2 virus full-length nonstructural glycoprotein NS1 in Pichia pastoris, Virus Genes 33 [2006] 27–32. [26] C.R. Parrish, W.S. Woo, P.J. Wright, Expression of the NS1 gene of dengue virus type 2 using vaccinia virus. Dimerisation of the NS1 glycoprotein, Arch. Virol. 117 [1991] 279–286.

27. X. Qu, W. Chen, T. Maguire, F. Austin, Immunoreactivity and protective effects in mice of a recombinant dengue 2 Tonga virus NS1 protein produced in a baculovirus expression system, J. Gen. Virol. 74 [Pt. 1] [1993] 89–97.

28. M.J. Pryor, R.C. Gualano, B. Lin, A.D. Davidson, P.J. Wright, Growth restriction of dengue virus type 2 by site-specific mutagenesis of virus-encoded glycoproteins, J. Gen. Virol. 79 [Pt. 11] [1998] 2631–2639.

29. H. Leblois, P.R. Young, Maturation of the dengue-2 virus NS1 protein in insect cells: effects of downstream NS2A sequences on baculovirus-expressed gene constructs, J. Gen. Virol. 76 [Pt. 4] [1995] 979–984.

30. D. Das, S.L. Gares, L.P. Nagata, M.R. Suresh, Evaluation of a western equine encephalitis recombinant E1 protein for protective immunity and diagnostics, Antiviral Res. 64 [2004] 85–92.

31. I. Kurucz, J.A. Titus, C.R. Jost, D.M. Segal, Correct disulfide pairing and efficient refolding of detergent-solubilized single-chain Fv proteins from bacterial inclusion bodies, Mol. Immunol. 32 [1995] 1443–1452.

32. D. Das, J. Kriangkum, L.P. Nagata, R.E. Fulton, M.R. Suresh, Development of a biotin mimic tagged ScFv antibody against western equine encephalitis virus: bacterial expression and refolding, J. Virol. Methods 117 [2004] 169–177.

[33] T.P. Wallis, C.Y. Huang, S.B. Nimkar, P.R. Young, J.J. Gorman, Determination of the disulfide bond arrangement of dengue virus NS1 protein, J. Biol. Chem. 279 [2004] 20729–20741.

34. J.Q. Guo, S.Y. You, L. Li, Y.Z. Zhang, J.N. Huang, C.Y. Zhang, Construction and high-level expression of a single-chain Fv antibody fragment specific for acidic isoferritin in Escherichia coli, J. Biotechnol. 102 [2003] 177–189.

35. S. Kammila, D. Das, P.K. Bhatnagar, H.H. Sunwoo, G. Zayas-Zamora, M. King, M.R. Suresh, A rapid point of care immunoswab assay for SARS-CoV detection, J. Virol. Methods 152 [2008] 77–84.

3

PRODUCTION AND CHARACTERIZATION OF MONOSPECIFIC AND BISPECIFIC ANTIBODIES AGAINST DENGUE VIRUS NS1 PROTEIN

3.1 Introduction

Dengue is one of the leading vector borne diseases worldwide**,** and more than 100 million people are infected every year [1,2,3]. Dengue fever is an important mosquito-borne viral disease of humans. This has been a recurrent phenomenon throughout the tropics in the past decade. During 2002, more than 30 Latin American countries reported over a million dengue fever [DF] cases with large number of dengue hemorrhagic fever [DHF]. Annually, there are an estimated 100-million dengue virus infections worldwide [4]. Increasingly cases of the more severe and potentially lethal DHF and dengue shock syndrome [DSS] are reported with children bearing much of the disease burden. Dengue virus is endemic in at least 100 countries worldwide and causes more human cases than any other mosquito-borne virus. The mortality rate of DHF in most countries is 5%, primarily among children and young adults. In several Asian countries, this virus is the leading cause of hospitalization and death in children. There have been a large number of dengue epidemics that resulted in enormous economic and human loss in parts of Asia and South America [5]. Hence, there is an urgent need for diagnostic, prophylactic and therapeutic reagents to manage DHF. Dengue virus has four serotypes, which are antigenically distinct [6]. Among those, the dengue virus non-structural [NS] 1 protein is a 46–50 kDa glycoprotein expressed in infected mammalian cells. All 7 NS proteins are intracellular proteins with the exception of dengue NS1 protein, which exists as secreted as well as a membrane-associated protein. Both forms are demonstrated to be immunogenic [7-9]. It was also reported that NS1 is one of 7 NS proteins produced during viral replication. It possesses not only group specific but also type specific determinants and has been recognized as an important antigen in dengue infection

[7,9,10]. A high circulating level of NS1 was demonstrated in the acute phase of dengue by antigen capture ELISAs [7,11]. The precise function of dengue NS1 protein remains unclear. However, antigen detection of non-structural dengue antigens may be of benefit for an early stage rapid diagnosis of infection due to its long half-life in the blood. At present there are no vaccines or drugs available to counter this deadly disease and diagnosis of infections particularly in endemic regions are clinical [12]. Dengue causes a range of syndromes ranging from asymptomatic infection to a severely debilitating and life threatening hemorrhagic condition [13]. Detection of non-structural dengue proteins will be of great importance for an early stage rapid diagnosis of infection as a result of its long half-life in the blood [1]. Morbidity and mortality incidence can be significantly reduced with early and accurate detection [14]. Dengue NS1 antigen screening is one of the important tools for early detection of dengue infection after the onset of fever [2,3]**.**

BsMAbs have many applications in the area of immunodetection and have been exploited previously for the targeted drug delivery, vaccine development and gene therapy [3,15,16]. In this study, we have produced monoclonal antibodies, and bispecific antibodies against dengue NS1 protein. The monoclonal antibodies were characterized by dengue NS1 recombinant antigen based ELISA, SDS-PAGE and Western blot. The isotypes of the antibodies were also determined**,** and subsequently used to develop preliminary a specific and sensitive antigen capture ELISA for the detection of dengue NS1. The sensitivity and specificity of a preliminary antigen capture ELISA was also assessed.

3.2 Materials and Methods

3.2.1 Materials

Fetal bovine serum [FBS] was obtained from PAA cell culture company [Ontario, Canada] and streptomycin–penicillin–glutamine was obtained from Gibco [NY, USA]. Polyethylene glycol [PEG] 1300–1600, HAT and HT supplement, goat anti-mouse IgG conjugated with horseradish peroxidase [GAM–HRPO], bovine serum albumin [BSA], fluorescein isothiocyanate [FITC], tetramethylrhodamine isothiocyanate [TRITC], horseradish peroxidase [Type VI], protein G-agarose were purchased from Sigma [St. Louis, MO, USA]. Cell strainers for collection of spleen and streptavidin–HRPO [St–HRPO] were obtained from BD Biosciences Pharmingen [MA, USA]. Hyperflasks were purchased from Corning [NY, USA]. Tetramethylbenzidine [TMB] and hydrogen peroxide [H_2O_2] detecting reagent was obtained from KPL laboratories [Gaithersburg, MD, USA]. Slide-A-LyzerR for dialysis was obtained from Pierce [Rockford, IL, USA]. TMB stabilized substrate for HRP was obtained from Promega Corporation [Madison, USA]. Non-sterile flat bottom 96-well ELISA plates were obtained from Nunc International Maxisorp [Rochester, NY, USA]. Sterile flat bottom 96-well cell culture plates for hybridoma clone production, cell culture flat bottom plates and cell culture flasks respectively, were obtained from Corning Incorporated [NY, USA].

3.2.2 Preparation of Dengue non-structural protein [NS1]

A full-length codon optimized NS1 gene was cloned in a bacterial expression vector and the expressed protein was purified from *E.coli* cultures [1]. The gene was cloned in correct reading frame with His_6 tag at the C-terminal end for high-level expression within inclusion bodies of *E.coli.* Restriction digestion mapping and SDS-PAGE showed that the right size clones [48kDa] were selected. Immobilized metal-affinity chromatography [IMAC] was used under denaturing conditions to adsorb the His6-tagged protein to finally elute the pure NS1. The glycosylated NS1 was used to generate anti-NS1 MAbs, and for screening bsMAbs,

A single bacterial colony was inoculated in 10 ml Terrific Broth/Tet5 medium and grown overnight at 37ºC shaker. The overnight culture was diluted [1:100] in fresh 1 L TB/Tet5 medium and grown at 37 ºC until an OD_{600nm} of 0.5–0.6 was reached. NS1 induction was done by adding 0.2% [w/v] arabinose according to optimized parameters and bacterial culture was further incubated for 16 h with vigorous shaking at 30 ºC. Culture was harvested by centrifugation at 5000g for 20 min at 4 ºC and the pellet was subject to determine total cell protein [TCP] from induced and uninduced culture. The NS1 protein was further analyzed by SDS–PAGE and also by Western blot probed with anti-His_6 MAb.

The pellet [5 g of bacterial wet pellet] from 1 L bacterial culture was suspended in 50 ml Phosphate Buffered Saline [PBS] and completely lysed by passing through a French Press [20,000 psi]. The total cell lysate was clarified by centrifugation at 27,000g for 30 min at 4 ºC and supernatant was collected as total soluble protein. The pellet was resuspended in lysis buffer and then 2% sodium deoxycholate was added. The mixture

was incubated at room temperature for 30 min with gentle shaking and centrifuged at 27,000g for 30 min at 4 ºC. The pellet was resuspended in lysis buffer and washed three times at 27,000g for 20 min at 4ºC to completely remove sodium deoxycholate.

3.2.3 IB solubilization and immobilized metal affinity chromatography [IMAC] purification

The washed pellets, inclusion bodies, were solubilized in denaturing buffer [8M Urea, 100mM NaH_2PO_4, 10 mM Tris-Cl, pH 8.0] for 1 h at room temperature [RT] with gentle shaking. Solubilized denatured recombinant NS1 proteins from insoluble materials were separated by centrifugation at 27,000 X g for 30 min at 4 ºC. A Ni–NTA column was prepared by loading the Ni–NTA agarose on a plastic column [Bio-Rad] and equilibrated with 10 bed volumes of the denaturing buffer. Twenty milligrams of solubilized denatured His_6 tagged rNS1 protein were loaded on the column and the column was washed with 5–10 bed volumes with washing buffer [8M Urea, 100mM NaH_2PO_4, 10 mM Tris-Cl, pH 6.3]. After complete wash, bound protein was eluted with elution buffer [8M Urea, 100mM NaH_2PO_4, 10 mM Tris-Cl, pH 5.9] and subsequently with the same elution buffer at pH 4.5. The eluted fractions were analyzed by SDS–PAGE prior to refolding.

3.2.4 Refolding

Protein assay was done to quantitate the amount of protein eluted from the Ni–NTA column with a total amount of ~4 mg. Refolding was done in three different concentrations to evaluate the best refolding condition. The eluted protein was adjusted to

100, 75 and 50 µg/ml with refolding Tris-Arginine buffer. The refolding was done by dialysis in Tris-Arginine buffer in the presence of 1.0 mM GSH [glutathione, reduced], 0.1 mM GSSG [glutathione, oxidized] for 3 days with two changes at 4 ºC. Final dialysis was done in PBS pH 7.4 at 4 ºC.

3.2.5 Western blot analysis

Total cell protein, inclusion bodies, IMAC eluted fractions or refolded dengue NS1 protein were electrophoresed on SDS–PAGE and then electroblotted onto Hybond ECL nitrocellulose membranes. The nitrocellulose membrane was blocked with 5% skim milk in PBST [0.1% Tween 20 in PBS, pH 7.3] for overnight at 4 ºC. The membrane was washed four times with PBST and incubated with anti-His6 MAb for 1 h. After washing, the membrane was incubated with HRPO labeled goat anti-mouse IgG [GAM-HRPO] for 1 h. Finally, the membrane was washed with PBS and enhanced chemiluminescence based detection was performed to determine the binding.

3.2.6 Preparation of anti-dengue NS1 mouse monoclonal hybridomas

6–8 week old female BALB/c mice were immunized 3 times intraperitoneally with 25 ng of NS1 antigen on day 0, and 14 using complete and incomplete Freund's adjuvant, respectively, and finally with 10 µg of antigen on day 28 using PBS [pH 7.3] [Table 3.1]. The immune response to the antigen was assessed by measuring the serum titer in mouse using indirect ELISA [Figure 3.1]. The mice with highest titer were ethically sacrificed and their spleens collected on day 3 after the last antigen injection. The splenocytes were fused with Sp2/0 murine myeloma cell line [ATCC #CRL 1581,

negative HGPRT] at a ratio of 5:1 using 50% [w/v] polyethylene glycol [PEG] according to the previously established technique [17]. The anti-dengue NS1 hybridomas obtained from five mice were then harvested and characterized by ELISA. These MAbs were used to generate quadromas.

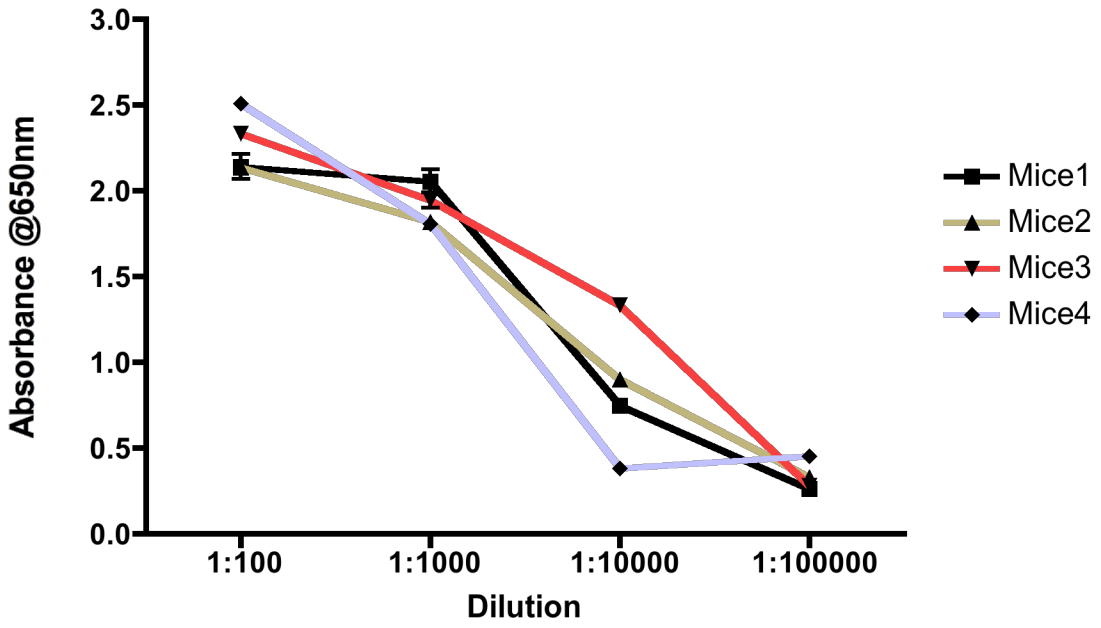


Fig. 3.1 Titer of ELISA indicating serum titer in mice immunized with recombinant dengue recombinant NS1 antigen. Values are the means of triplicate samples. Vertical bars indicate the Standard Deviation.

Time [days]	NS1 antigen	Adjuvant	Injection volume	Route
	Check pre-immune titer from mice via tail bleed			
Day 0	**25 µg**	**CFA**	**0.2 ml**	**IP**
Day 14	**25 µg**	**IFA**	**0.2 ml**	**IP**
Day 21	**10 µg**	**PBS**	**0.2 ml**	**IP**
Day 24	**Check titer from mice followed by hybridoma fusion**			

Table 3.1 Immunization protocol of balb/c mice with recombinant NS1 antigen for MAb production

3.2.7 Screening of MAbs by indirect ELISA

Antibody secretion by each of the five hybridoma cells was detected by indirect ELISA. 100 µL of purified dengue NS1 protein [10 µg/ mL] was used per for coating 96 well plates [Nunc-Immuno Maxisorb^{TM} plates, Nunc] overnight at 4ºC. To avoid nonspecific binding, the wells were blocked with 2% BSA, for 2 h at 37ºC. After washing, the wells were incubated with 100 µL supernatant from each hybridoma clone for 2 h at 37 ºC. The wells were washed three times with PBS and the bound antibodies were detected using goat anti mouse IgG conjugated with horseradish peroxidase [GAM-HRPO] [Sigma] as secondary antibody at a 1:10, 000 dilution for 1 h at 37 ºC. 100 µL of TMB substrate was finally added to the wells. After 15 min of color development, optical density was measured at 650 nm at V_{max} kinetic ELISA plate reader. Clones having ELISA values three times higher than in the blank were considered positive. Serum of non-immunized mouse was used as negative control. Positive sera from hyper immunized mice and RPMI media were used as positive control and blank, respectively.

3.2.8 Cloning and recloning of anti dengue NS1 hybridomas

Best clones of each hybridoma were selected for further cloning and recloning. Hybridoma containing high titer of MAb were grown in culture media [RPMI with 1% PSG and 5% FBS] and subsequently by limiting dilution, 1 cell/well of each hybridoma was added in 96-well plates and incubated in a CO_2 incubator at 37ºC. In about 2 weeks, cell clusters appear and the MAbs are screened for dengue NS1 by indirect ELISA. Re-clonings were repeated until 100% cloning efficiency was attained.

3.2.9 Production of MAb supernatant

The five anti dengue NS1 MAbs were cultured in large scale by two methods. In the first method cells were grown in small 75 mm^3 flasks and then expanded in large 175 mm^3 flasks. The supernatant were then harvested and stored at -20^0C. The cells in the flasks were then resuspended again with fresh media. In the second method, HYPERflask^{TM} [bioreactor] was used to produce large volumes of MAb supernatant. Cells were cultured in 25 mm^3 flasks and then seeded in to the HYPERflask^{TM}. The supernatant was collected and stored when the cells undergo cell death. Fresh cell cultures were then used to seed the HYPERflask^{TM} for continuous cell culture. RPMI-1640 with, 5% v/v FBS and supplemented with 2 mM L-glutamine, 50 U/mL penicillin and streptomycin was used and production was in an CO_2 incubator at 37^0C.

3.2.10 Relative affinity analysis of anti dengue NS1 antibodies

All the MAbs were analysed for affinity against the dengue NS1 antigen by indirect ELISA with modifications. 96-well plates were immobilized with different dilutions of NS1 overnight at 4ºC and were subsequently washed and blocked. Following this, the MAbs were added in triplicates at a concentration of 10 µg/ mL. The bound antigen was detected by GAM-HRPO followed by addition of TMB substrate as mentioned earlier. Results were comparable to values obtained while performing titer check prior to hybridoma development.

3.2.11 Crossreactivity of anti dengue NS1 antibodies

The MAbs generated were analysed by ELISA and Western blot against other viral proteins available in our laboratory which included SARS nucleoprotein, SARS S1, Dengue envelope, Western equine encephalitis E1 and Ebola GP1.

3.2.12 Cell lines for quadroma fusion

Mouse anti-dengue NS1 hybridoma and rat anti-HRPO YP4 hybridoma were chosen for developing quadromas. The anti-HRPO YP4 is a well-characterized rat hybridoma. It was selected for drug resistance to 8-azaguanine, making it sensitive to aminopterine in HAT medium. YP4 hybridoma secretes monospecific anti-horseradish peroxidase [HRPO] antibodies [IgG2a] and was gifted by the late Dr. C. Milstein, Medical Research Council for Molecular Biology, Cambridge, United Kingdom.

3.2.13 Development of anti-dengue NS1/anti-HRPO quadromas

The production of anti-dengue NS1/anti-HRPO quadromas involved maintaining the two hybridoma cell lines in logarithmic growth phase containing RPMI medium with 5% FBS at 37ºC supplemented with 5% CO_2. A stock solution of tetramethyl rhodamine isothiocyanate [TRITC, 0.5 mg/mL] or fluorescein isothiocyanate [FITC, 0.5 mg/mL] was diluted in 1:5 ratios to be used as the working solution. The following steps as reported earlier were then followed for successful completion of a quadroma fusion [18,19]. Briefly, 2×10^7 cells/ mL of anti-dengue NS1 hybridomas and YP4 hybridomas were separately resuspended in RPMI pH 7.4 and 6.8, respectively. Anti-dengue NS1 hybridomas were then labeled with TRITC [red fluorescence] and YP4 cells were labeled

with FITC [green fluorescence]. Following 30 min incubation at 37 ºC in a 5% CO_2 incubator, the cell suspensions were washed and mixed in a 50 mL tube and centrifuged at 459 × *g* for 7 min. Two mL of PEG was added to the cell pellet slowly over a period of 2 min, with gentle mixing. Following addition of PEG, the cell suspension was incubated at 37ºC for 3 min. Twenty mL of serum free RPMI medium was added to dilute the toxic effects of PEG. Flow cytometry [FACSAria, Becton Dickinson] with an argon ion 488 nm air cooled laser [Dept of Medical Microbiology and Immunology, University of Alberta] was used to sort cells with dual fluorescence and seeded at 1 cell/well in 96-well sterile culture plates with RPMI containing 20% FBS. The plates were incubated at 37 ºC with 5% CO_2. The clones were screened twice by bridge ELISA. The best clones were subject to recloning to select strong positive and highly specific quadroma secreting bsMAbs against dengue NS1.

3.2.14 Screening method to detect quadromas secreting bsMAb [bridge ELISA]

The 96-well plate was coated with 5 µg/mL purified dengue NS1 protein overnight at 4ºC. Non specific sites were blocked with 2% bovine serum lbumin [BSA] at 37ºC for 2 h. The plate was then washed three times with Phosphate Buffered Saline – 0.05% Tween 20 [PBS-T]. 100 µL of various quadroma cell culture supernatants was serially diluted with PBS-BSA and added followed by incubation for 1 h at 37 ºC. 100 µL of 10 µg/mL [diluted in 2% DBSA] HRPO was added to the microtiter plate followed by washing with PBS-T three times. 100 ìL of TMB substrate was finally added to the wells. Positive quadromas secreting bsMAb were selected after 15 min of color development, measured at 650 nm at V_{max} ELISA plate reader. PBS was used as blank and negative controls were wells not having dengue NS1 antigen.

3.2.15 Purification of monospecific MAbs

Cell culture supernatant comprising MAbs were centrifuged at 4 °C at 7900 rpm for 30 min to remove the cells. The crude supernatant was loaded on to a Protein-G sepharose column equilibrated with PBS at pH 7.4. The bound immunoglobulins were eluted by 0.1 M glycine, pH 2.8 and the fractions were neutralized with 1 M Tris pH 9. The pooled elutes were finally dialyzed with three changes of PBS at pH 7.2 [Figure 3.2].

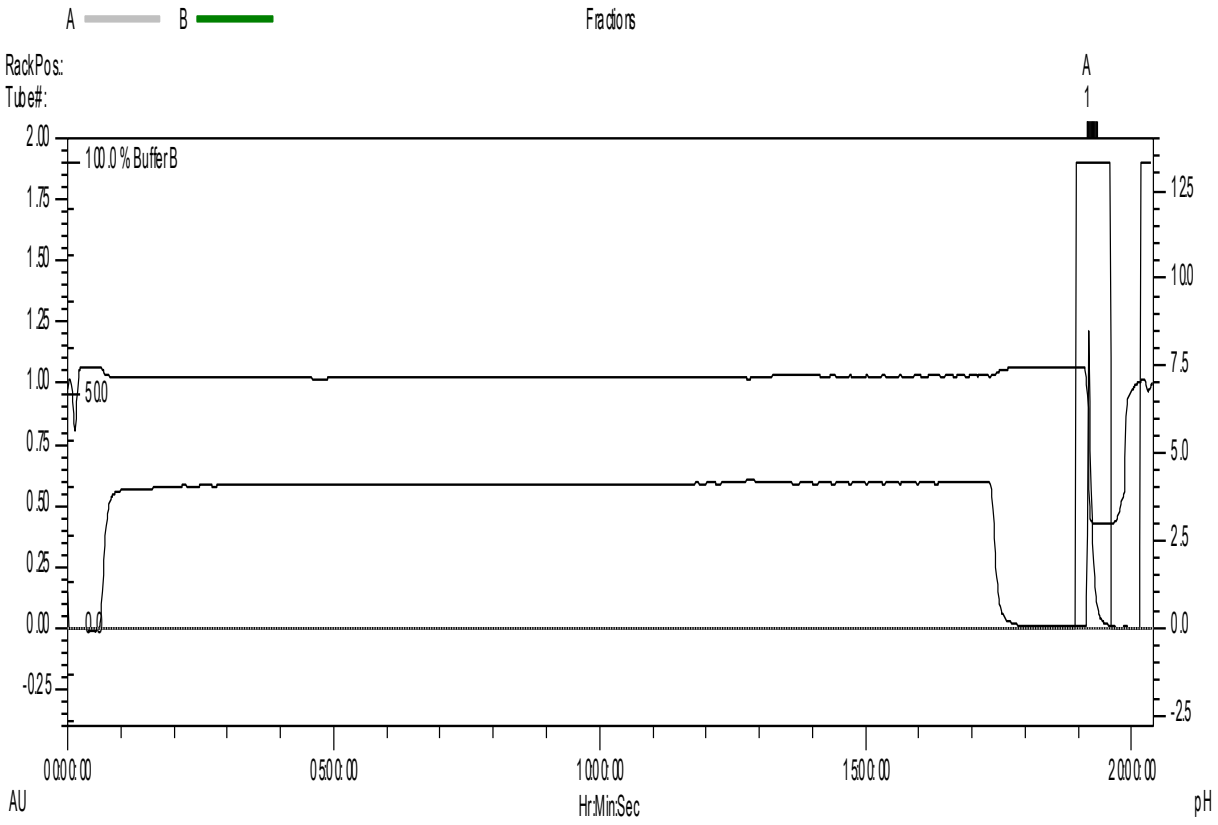


Fig. 3.2 Purification Profile of anti NS1 MAb using affinity chromatography

3.2.16 Subclass determination of MAbs

The immunoglobulin subclass was determined using the Isostrip mouse monoclonal antibody isotyping kit according to instructions from the manufacturer [Roche, USA].

3.2.17 Affinity purification of HRPO labeled bispecific antibodies [bsMAbs]

The purification of the quadromas was carried out by a novel method using *m*-aminophenylboronic acid agarose column [APBA] established in our laboratory [20]. The APBA column was briefly saturated with HRPO to capture the bsMAb along with the monoclonal anti-HRPO MAb, leading to eliminating the monoclonal anti-dengue NS1 species. Following a thorough washing with potassium phosphate buffer, the bound antibodies were eluted with potassium phosphate buffer containing 0.1M sorbitol. The pooled elutes were finally dialyzed with PBS at pH 7.2. Protein estimation was done by Bradford assay [21].

3.3 Results

3.3.1 Production of Anti–Dengue NS1 MAbs

Fusion of spleen cells from all the Balb/C mice (5 in total) immunized with recombinant dengue NS1 and SP2/0 murine myeloma cells produced multi-hybridoma clones secreting MAbs against dengue NS1 protein. Five positive hybridoma clones [P148.1, P148.7, P148.9, P148.L1 and P148.L2] were selected by indirect ELISA for production of MAbs against dengue NS1 protein.

3.3.2 Purification of MAbs

Each of the five hybridomas was grown in large quantities in bioreactors and the supernatant was purified through a protein G column. P148.L1 and P148.L2 showed high yields of MAbs at 0.812 mg/mL and 0.790 mg/mL (mean concentration obtained with 5 batches of 1500 mL each MAb supernatant purified), respectively [Table 3.2].

Anti dengue NS1 MAb	Concentration [mg/mL]	Total amount of protein recovered [mg]
P148.1	0.757	11.35
P148.7	0.634	9.51
P148.9	0.688	10.32
P148.L1	0.812	12.18
P148.L2	0.790	11.85

Table 3.2 Anti dengue NS1 MAb yield after affinity purification (Mean of 5 batches of MAb supernatant purified. Each batch volume of 1500 mL)

3.3.3 Isotype determination of MAbs

Isotyping of the five MAbs was done by using a commercially available isotyping kit [Roche]. Results confirmed that the heavy chain of three of the anti dengue NS1 MAbs [P148.1, P148.7, and P148.9] was of the IgG_1 type whereas that of P148.L1 and P148.L2 was of the IgG_2 type. The light chain was found to be kappa for all of the MAbs.

3.3.4 Cross reactivity analysis

Cross reactivity analysis with the aforementioned proteins by Western blot and ELISA confirmed the specificity of the MAbs for the dengue NS1 protein, indicating that the MAbs could not react to Ebola protein SARS S1, SARS NP, Ebola GP1, Western Equine Encephalitis E1 both in ELISA and Western blot [Table 3.3].

Type of Antigen	Reactivity
Dengue type 1 NS1	+
SARS S1	-
SARS NP	-
Ebola GP1	-
Western Equine Encephalitis E1	-
PBS [Negative Control]	-

Table 3.3 Cross reactivity of anti dengue NS1 MAbs by Western blot and ELISA

3.3.5 Specificity of the anti dengue NS1 hybridomas

Specificity of the antibodies was evaluated by SDS-PAGE and subsequent Western blot. Analysis confirmed the specificity of purified MAbs against the dengue NS1 antigen [data not shown].

3.3.6 Development of Anti–Dengue NS1 and HRPO Quadromas

The development of anti-NS1/anti-HRPO quadromas involved maintaining the two hybridoma cell lines [anti-NS1 and anti-HRPO] in logarithmic growth phase containing RPMI medium with 10% FBS at 37 °C supplemented with 5% CO_2. Trypan blue staining of over 90% was observed before the cells were used for fusion. A stock solution of tetramethyl rhodamine isothiocyanate [TRITC, 0.5 mg/mL] and fluorescein isothiocyanate [FITC, 0.5 mg/mL] was diluted in 1:5 ratios to be used as the working solution. The following steps as reported earlier were then followed for successful completion of a quadroma fusion [19]. Briefly, 2×10^7 cells/mL of anti-NS1 hybridomas [P148.L1, P148.L2] and YP4 hybridomas were separately resuspended in RPMI pH 7.4 and 6.8, respectively. Anti-NS1 hybridomas were then labelled with TRITC [red fluorescence] and YP4 cells were labelled with FITC [green fluorescence]. Following 30 min incubation at 37 °C in a CO_2 incubator the hybridoma cell suspensions were washed and mixed in a 50 mL tube and centrifuged at 459 × g for 7 min. To the cell pellet, 2 mL of PEG was added drop by drop over a period of 2 min, with gentle mixing. Upon the addition of PEG, the cell suspension was then placed at 37 °C in a CO_2 incubator for 3 min, followed by addition of 20 mL of FBS free RPMI medium to dilute the toxic effects of PEG. Flow cytometry was then used to sort cells with dual

fluorescence and were seeded at 1 cell/well in 96 well sterile tissue culture plates with RPMI containing 20% FBS. The plates were incubated at 37 °C with 5% CO_2. The clones were then screened twice using a bridge ELISA [22,23]. The cloning of quadromas was performed based on the limiting dilution culture method. The two best clones of each fusion P156.1 and P156.2 were then chosen to undergo further recloning [3–4 times] to select positive and highly specific quadromas secreting bsMAb against Dengue NS1. The yield of fused quadroma cells showed 0.8% [Figure 3.3]. Successive recloning ensured strong and stable bsMAb production. After culture, bsMAbs from two clones were purified by APBA column. The yield of bsMAbs from each quadroma was 4.15 mg /L of culture supernatant.

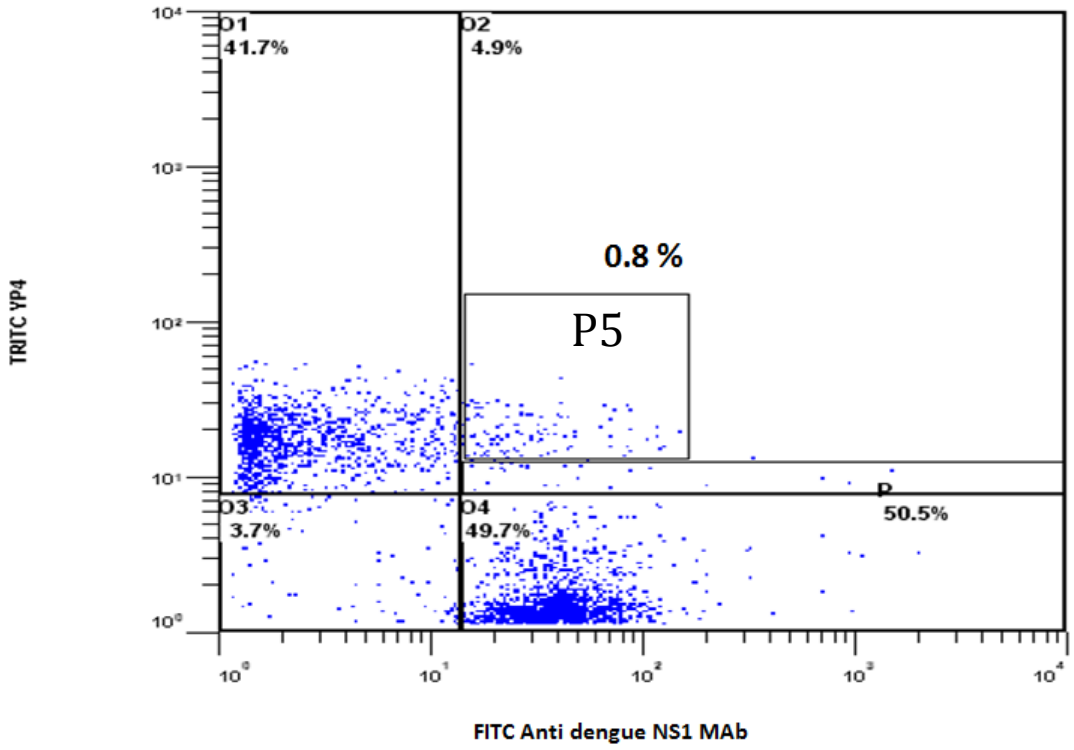


Fig. 3.3 FACS analysis after PEG fusion of the two fluorescent hybridomas.

3.3.7 Bridge ELISA

A bridge ELISA was done to screen the purified bsMAbs [Figure 3.4] from two quadromas by coating the recombinant dengue NS1 antigen and by adding P156.1 or P156.2 as a detecting probe.

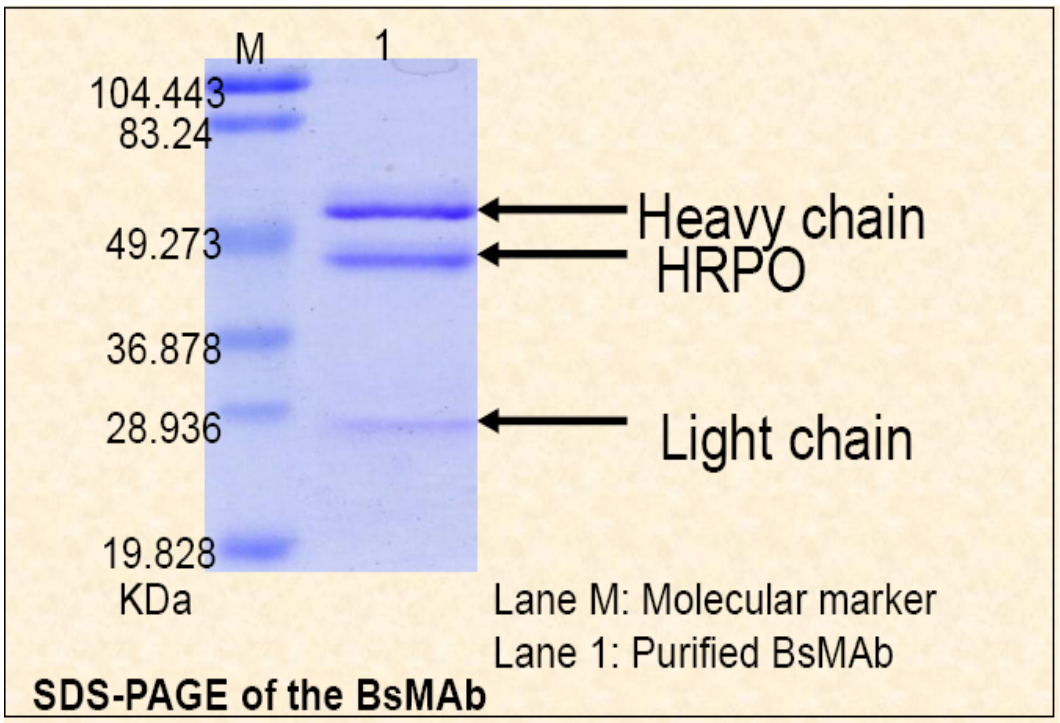


Fig. 3.4 SDS-PAGE of purified bsMAb

Two bsMAbs showed absorbance at 0.5 and 0.55, respectively, indicating the high specificity of bsMAb [Table 3.4].

Primary Quadroma Clones	**Dilution of bsMAb supernatant for screening of positive clones**					
	Neat	**1:10**	**1:100**	**1:1000**	**1:10000**	**Blank**
P156.1	0.351	0.578	0.255	0.137	0.070	0.031
P156.2	0.313	0.539	0.209	0.115	0.045	

Clones after 4th recloning

P156.1	0.869	1.187	0.456	0.161	0.079
P156.2	0.836	1.240	0.413	0.144	0.064

Table 3.4 Screening of quadroma cell lines secreting bsMAb [anti-NS1/anti-HRPO] at different dilutions

3.3.8 Direct detection of recombinant NS1 using bsMAb

Assays were done to detect NS1 protein in 96-well plate with bsMAB-HRPO complex as the detecting antibody. Different concentrations of the purified protein were immobilized on the plate. Detecting antibody concentration was 1 μg/mL and we achieved very low limit of detection [Figure 3.5].

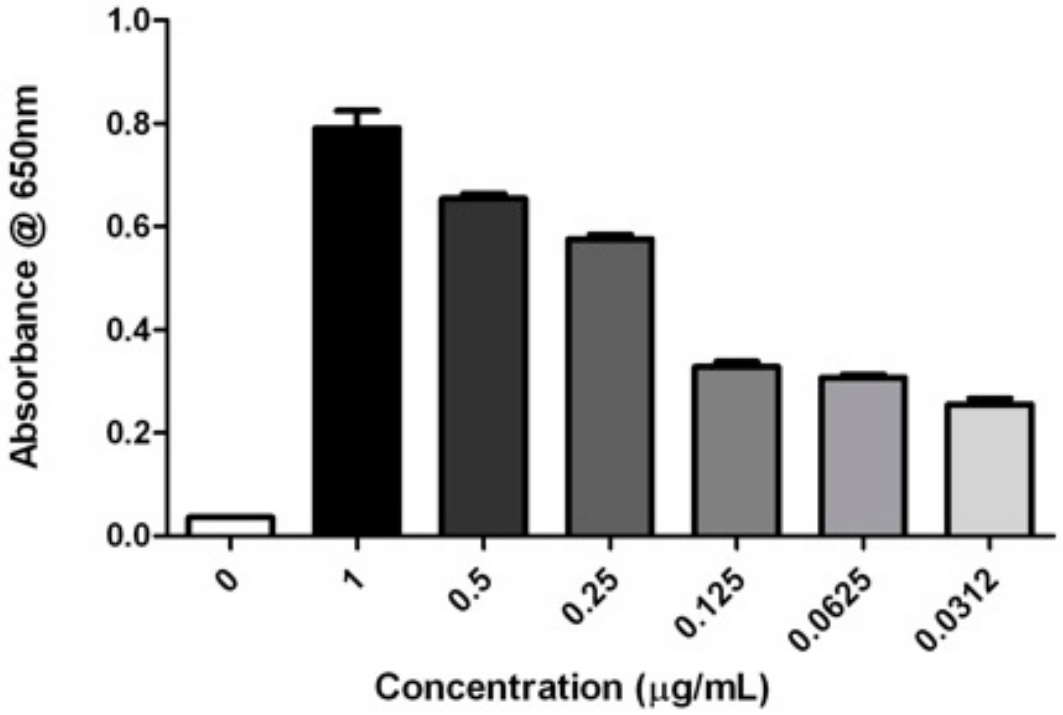


Fig. 3.5 ELISA for direct detection of Dengue NS1 antigen using bsMAb. Vertical bars indicate the standard deviation. (X-axis: NS1 antigen concentration; Y-axis: Absorbance)

3.3.9 Hetero-Sandwich ELISA to detect recombinant NS1

Preliminary sandwich ELISA formats were performed to detect recombinant NS1 protein in a 96-well microtiter plate using P148.L2 or P1148.L1 MAb as the capture antibody [CAb] and the bsMAb–HRPO [P156.1] [Figure 3.6] complex as the detecting antibody [DAb] [24,25]. Evaluation of the ELISA formats for detection of NS1 showed that bsMAb was sensitive and capable of detecting nanogram levels of antigen [data not shown]. Good sensitivity coupled with minimum background was observed during detection of dengue NS1 when CAb concentration was 10 μg /mL and DAb concentration was 1 μg /mL. Further optimization of these assays would enable development of a rapid screening procedure for dengue suspected individuals.

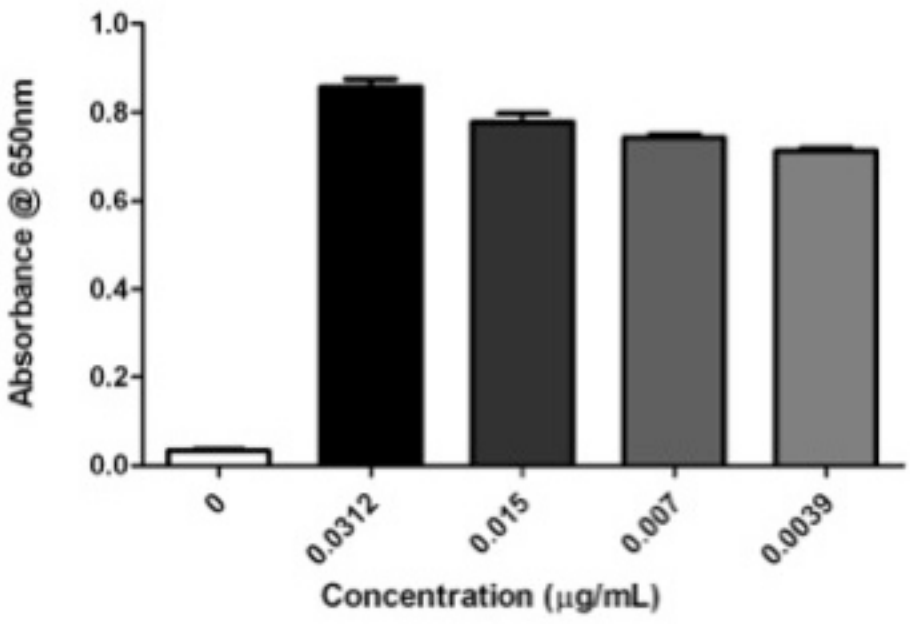


Fig. 3.6 Sandwich ELISA for detection of Dengue NS1 antigen using bsMAb. Vertical bars indicate the Standard Deviation. (X-axis: NS1 antigen concentration; Y-axis: Absorbance)

3.4 Discussion

The main objective of this study was to develop a panel of MAb [Figure 3.7] and bsMAb for dengue NS1. The P148 series of MAbs developed showed high specificity for recombinant dengue NS1 antigen. These panels of MAbs were analyzed against recombinant dengue envelope protein and other viral proteins and were found to be non cross-reactive.

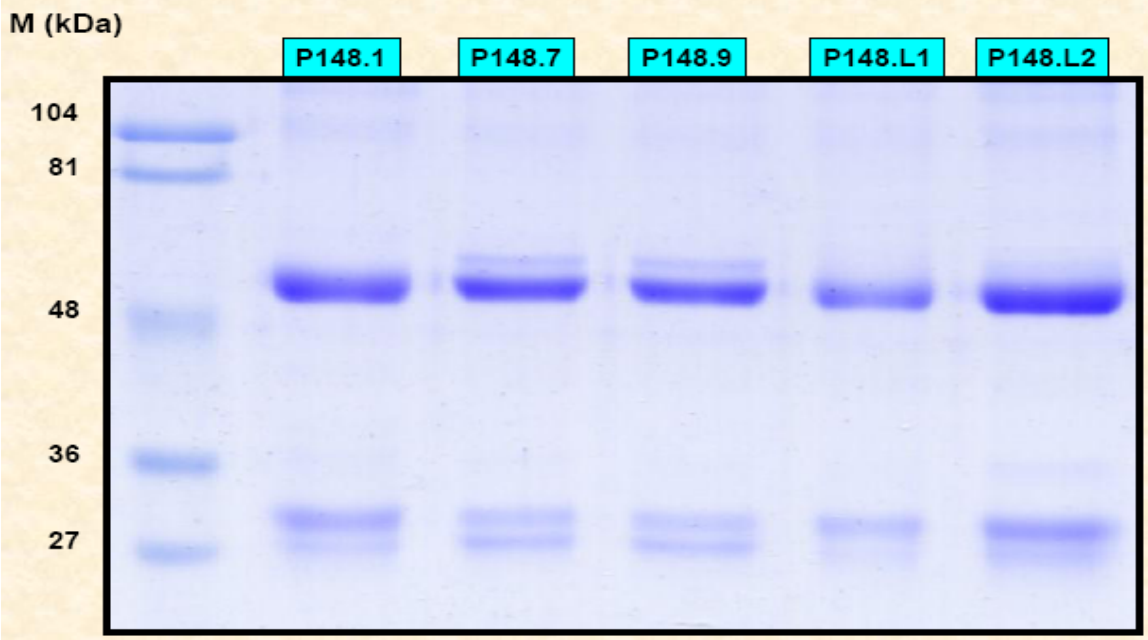


Fig. 3.7 SDS-PAGE of purified anti dengue NS1 MAbs

The P156 series bsMAb which is functionally monovalent also shows the same pattern of strain reactivity [26]. This is expected since the paratope would be the same in both the antibodies except that the bsMAb is monovalent for each paratope. We chose the hybrid–hybridoma approach to generate the P156.1 and P156.2 bsMAbs from the P148 monoclonal antibody owing to its simple method [23,24,27]. The bridge ELISA for screening of bsMAbs provides a specific labeling of enzyme to antibody, leaving the second paratope for dengue NS1 epitope binding [26,28]. More over the APBA agarose matrix employed for the affinity purification provide good yields of quadromas associated with HRPO in two steps [20,24,26]. The use of bsMAb overcomes the drawbacks of chemical coupling that results in inactivation of the enzyme and antibody component. The bsMAb approach develops a highly specific activity moiety with every molecule bound to HRPO. The use of bsMAb avoids the limitations of chemical coupling that could lead to inactivation of both enzyme and antibody components. The bsMAb approach generates a high specific activity tracer with every molecule bound to HRPO. The bsMAb has molecular uniformity unlike chemical conjugation of enzyme and antibody widely used. The bsMAb–HRPO conjugate provides a clean signal to background noise ratio and theoretically has the highest specific activity of the antibody tracer contributing to the higher sensitivity and lower backgrounds [19]. Our bsMAb–HRPO along with the panel of MAbs would be used to develop an ultrasensitive and rapid dengue detection assay to be used in point of care settings and sudden outbreaks. The purity of the anti-dengue NS1 MAbs was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE], and Western blot analysis showed the specificity of purified MAbs to dengue NS1 antigen [data not shown].

The codon-optimized recombinant NS1 gene was used to determine the specificity of the MAbs by Western blotting. When the protein G-purified antibodies were used all the MAbs were found to react specifically to full-length dengue NS1 antigen. There was no cross-reactivity between the MAbs when reacted with other viral antigens [Table 3.1]. The specificity of the newly developed MAbs for dengue NS1 was evaluated by Western blotting. Sandwich assays with all five MAbs exhibited strong binding only with **d**engue NS1 and no cross-reactivity with other proteins was observed. Previous articles stated that the NS1 antigen was found circulating from day 1 after the onset of fever up to day 9 and NS1 levels were between 0.04 and 2 μg/mL in acute-phase serum samples [from day 0 to 7], and for a convalescent phase serum [day 8 and later] was around 0.04 μg/mL. In secondary infection, NS1 concentrations were between 0.01 and 2 μg/mL and absolutely not detectable in convalescent-phase sera [11,29]. Dengue NS1 antigen analysis is reported to be an important tool for the early detection of dengue infection after the onset of fever [30,31]. Commercially available dengue NS1 antigen capture ELISA has been evaluated for the diagnosis of NS1 from clinical samples at various stages [29,31,32,33, 14,36]. It is therefore, an important antigen for rapid viral diagnosis. Our preliminary assays to screen NS1 antigen was able to detect nanogram levels. We designed two different formats to detect recombinant NS1 antigen. Direct detection method involved coating of plates with different concentrations of recombinant antigen and detecting with bsMAb. In this case the limit of detection was found to be 0.0312 μg/mL whereas in the sandwich format the cut-off increased to 0.0039 μg/mL. Thus, our new MAbs and bsMAbs could be useful reagents for diagnostics, as well as functional analysis of NS1 and basic research of dengue infection.

Antibody capture ELISAs along with the hemagglutination inhibition [HI] test are routinely used techniques for the detection of dengue virus infections. The HI test was used to distinguish between primary and secondary dengue infections. Subsequently antibody capture ELISA started to be routinely used. There are several drawbacks of the HI test among which the most significant is its inability to detect isotype-specific antibody response, and furthermore high cross-reactivity of flavivirus-specific IgG antibodies, which make accurate diagnosis extremely complex [34, 35]. NS1 antigen circulates in serum from the day of infection; therefore, development of a sensitive antigen capture diagnostic would be very beneficial for accurate and fast detection of dengue for early therapeutic intervention.

3.5 References

1. Das, D., Mongkolaungkoon, S., Suresh, M.R., 2009. Super induction of dengue virus NS1 protein in E.coli. Protein Expr. Purif. 66, 66-72.

2. Malabadi, R.B., Ganguly, A., Teixeira da Silva, J.A., Parashar, A., Suresh, M.R., Sunwoo, H.H., 2011. Overview of plant-derived vaccine antigens: Dengue virus. J. Pharm. Pharmaceut. Sci. 14,400-413

3. Malabadi, R.B., Ganguly, A., Hoon, H.H., Suresh, M.R., 2012. Role of bispecific monoclonal antibodies in immunodiagnostic assays. Res. Pharm. 2[3], 08-14.

4. Gubler, D.J., Meltzer, M., 1999. Impact of dengue/dengue hemorrhagic fever on the developing world, Adv. Virus Res. 53, 35–70.

5. Whitehorn, J., Farrar, J., 2010. Dengue. Br. Med. Bull. 95[1], 161-173

6. Calisher, C. H., Karabatsos, N., Dalrymple, J. M., Shope, R. E., Porterfield, J. S., Westaway, E. G., Brandt, W. E., 1989. Antigenic relationships between ¯aviviruses as determined by cross-neutralization tests with polyclonal antisera. J. Gen. Virol. 70, 37-43

7. Young, P.R., Hilditch, P.A., Bletchly, C., Halloran, W., 2000. An antigen capture enzyme linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients, J. Clin. Microbiol. 38, 1053–1057.

8. Flamand, M., Megret, F., Mathieu, M., Lepault, J., Rey, F.A., Deubel, V., 1999. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion, J. Virol. 73, 6104–6110.

9. Falconar, A.K., Young P.R., 1990. Immunoaffinity purification of native dimer forms of the flavivirus non-structural glycoprotein, NS1, J. Virol. Methods 30, 323–332.

10. Henchal, E.A., Henchal, L.S., Thaisomboonsuk, B.K., 1987. Topological mapping of unique epitopes on the dengue-2 virus NS1 protein using monoclonal antibodies, J. Gen. Virol. 68, 845–851.

11. Alcon, S., Talarmin, A., Debruyne, M., Falconar, A., Deubel, V., Flamand, M., 2002. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections, J. Clin. Microbiol. 40, 376–381.

12. Farrar, J., Focks, D., Gubler, D., Barrera, R., Guzman, M. G., Simmons, C., Kalayanarooj, S., Lum, L., McCall, P. J., Lloyd, L., Horstick, O., Dayal-Drager, R., Nathan, M. B., Kroeger, A., 2007. Towards a global dengue research agenda. Tropical Medicine & International Health. 12[6], 695–699

13. Kalayanarooj, S., Vaughn, D.W., Nimmannitya, S., 1997. Early clinical and laboratory indicators of acute dengue illness. J. Infect. Dis. 176, 313–21.

14. Xu, H., Di, B., Pan, Y., Qiu, L., Wang,. Y, Hao, W., He, L., Yuen, K., Che, X., 2006. Serotype 1-Specific Monoclonal Antibody-Based Antigen Capture Immunoassay for Detection of Circulating Nonstructural Protein NS1: Implications for Early Diagnosis and Serotyping of Dengue Virus Infections. J. Clin. Micro. 44[8], 2872–2878

15. Khan, S.R., Ganguly, A., Malabadi, R.B., Sunwoo, H.H., Parashar, A., Teixeira da Silva, J.A., Suresh, M.R., 2011. Targeting strategies and nanocarriers in vaccines and therapeutics. Res. Biotech.. 2[6], 08-20.

16. Khan, S.R., Ganguly, A., Malabadi, R.B., Sunwoo, H.H., Suresh, M.R., 2012. Gene delivery system: A developing arena of study for new era of medicine. *Recent Patents on DNA & Gene Sequences.* 6[1], 2-9 [8].

17. Kohler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497.

18. Das, D., Suresh, M.R., 2005 Producing bispecific and bifunctional antibodies. Methods Mol Med 109, 329–346

19. Tang, X.L., Peppler, M.S., Irvin, R.T., Suresh, M.R., 2004. Use of bispecific antibodies in molecular velcro assays whose specificity approaches the theoretical limit of immunodetection for *Bordetella pertussis*. Clin. Diagn. Lab. Immunol. 11, 752–757.

20. Bhatnagar, P.K., Das, D., Suresh, M.R., 2008. Sequential affinity purification of peroxidase tagged bispecific anti-SARS-CoV antibodies on phenylboronic acid agarose. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 863, 235–241.

21. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254

22. Suresh, M.R., Cuello, A.C., Milstein, C., 1986a. Advantages of bispecific hybridomas in one-step immunocytochemistry and immunoassays. Proc. Natl. Acad. Sci. U.S.A. 83, 7989–7993.

23. Suresh, M.R., Cuello, A.C., Milstein, C., 1986b. Bispecific monoclonal antibodies from hybrid hybridomas. Methods Enzymol. 121, 210–228.

24. Kammila, S., Das, D., Bhatnagar, P.K., Sunwoo, H.H., Zayas-Zamora, G., King, M., Suresh, M.R., 2008. A rapid point of care immunoswab assay for SARS-CoV detection, J. Virol. Methods 152, 77–84.

25. Guttikonda, S., Tang, X.L., Yang, B.M., Armstrong, G.D., Suresh, M.R., 2007. Monospecific and bispecific antibodies against E.coli O157 for diagnostics. J Immunol Methods 327[1–2], 1–9

26. Sarkar, S., Tang, X.L., Das, D., Spencer, J.S., Lowary, T.L., ,Suresh, M.R., 2012. A Bispecific Antibody Based Assay Shows Potential for Detecting Tuberculosis in Resource Constrained Laboratory Settings. PLoS ONE 7[2]: e32340. doi:10.1371/journal.pone.0032340

27. Kreutz, F.T., Xu, D.Z., Suresh, M.R., 1998. A new method to generate quadromas by electrofusion and FACS sorting. Hybridoma 17[3], 267–273

28. Gupta, S., and M. R. Suresh. 2002. Affinity chromatography and co-chromatography of bispecific monoclonal antibody immunoconjugates. J. Biochem. Biophys. 51, 203-216

29. Dussart, P., Labeau, B., Lagathu, G., Louis, P., Nunes, M.R., Rodrigues, S.G., Storck-Herrmann, C., Cesaire, R., Morvan, J., Flamand, M., Baril, L., 2006. Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum Clin. Vaccine. Immunol., 13, 1185–1189

30. Chuansumrit, A., Chaiyaratana, W., Pongthanapisith, V., Tangnararatchakit, K., Lertwongrath, S., Yoksan S., 2008. The use of dengue nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection Pediatr. Infect. Dis. J., 27, 43–48

31. Lapphra, K., Sangcharaswichai, A., Chokephaibulkit, K., Tiengrim, S., Piriyakarnsakul, W., Chakorn, T., Yoksan, S., Wattanamongkolsil, L., Thamlikitkul, V., 2008, Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness. Diagn. Microbiol. Infect. Dis. 60[4], 387-391

32. Kumarasamy, V., Chua, S.K. Hassan, Z., Wahab, A.H., Chem, Y.K., Mohamad, M., Chua K.B., 2007. Evaluating the sensitivity of a commercial dengue NS1 antigen-capture ELISA for early diagnosis of acute dengue virus infection Singapore Med. J. 48, 669–673

33. Kumarasamy, V., Wahab, A.H., Chua, S.K., Hassan, Z., Chem, Y.K., Mohamad, M., Chua, K.B., 2007. Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection J. Virol. Methods, 140, 75–79

34. Shu, P. Y., Chen, L. K., Chang, S. F., Yueh, Y. Y., Chow, L., Chien, L. J., Chin,C., Lin, T. H., and J. H. Huang. 2003. Comparison of capture immunoglobulin M [IgM] and IgG enzyme-linked immunosorbent assay [ELISA] and nonstructural protein NS1 serotype-specific IgG ELISA for differentiation of primary and secondary dengue virus infections. Clin. Diagn. Lab. Immunol. 10, 622–630

35. Pawitan, J.A., 2011. Dengue Virus Infection: predictors for severe dengue Acta Med Indones, 43[2], 129-135

36. Thamlikitkul V., 2008. Evaluation of an NS1 antigen detection for diagnosis of acute dengue infection in patients with acute febrile illness Diagn. Microbiol. Infect. Dis. 60, 387–391

4

DEVELOPMENT OF AN ULTRASENSITIVE HETERO-SANDWICH ELISA ASSAY BASED ON BISPECIFIC MONOCLONAL ANTIBODY FOR THE DETECTION OF DENGUE NS1 PROTEIN

4.1 Introduction

Dengue fever [DF] is an acute febrile illness caused by a mosquito-borne flavivirus. The more severe form of DF is known as dengue hemorrhagic fever [DHF], and dengue shock syndrome [DSS], which can be fatal, especially among young children. This accounts for the majority of the 5% annual case-fatality rate in countries where DF is endemic [1,2,3]. Global infestation of all four dengue virus serotypes throughout the tropical areas has led to more severe outbreaks. Efficacy in diagnostics as well as costs, are important concerns for disease management. The dengue viruses are enveloped and contain a single, positive-sense RNA genome of about 11 kb that is composed of three structural protein genes that encode the nucleocapsid protein, a membrane-associated protein, an envelope protein, and seven nonstructural [NS] proteins [4,5]. NS proteins do not form a part of the virion structure, but are expressed on the surface of infected cells [6]. The function of NS1 is yet to be fully defined but reports mention a viral RNA role in replication process [7]. The nonstructural protein, NS1 is a 46- to 50-kilodalton [kDa] glycoprotein that is expressed in both membrane-associated and secreted forms [5,8]. One of the problems associated with patient management during dengue infection relates to quick and accurate diagnosis. Initial symptoms are often similar to other diseases such as malaria, which is often prevalent in areas where infection is endemic. Thus, being able to accurately identify the different virus strains with a rapid, cheap, and sensitive diagnoses, is essential for proper patient care. Common methodologies used for detection of dengue infection are virus isolation, RNA and specific IgM/IgG antibodies diagnosis in patients' sera. In general, combinations of these methods are mostly used [9]. A significant limitation of these techniques, however, is time; usually, it takes from 3 to 5 days after the onset of the symptoms to detect anti-dengue IgM and from 1 to 14 days for

anti-dengue IgG to become detectable [11]. Also, viral isolation is expensive and time consuming and requires proper cell culture infrastructure in laboratories to be confirmed. Cell culture propagation is inherently time consuming and thus costly. The PCR based methods, although sensitive, are also expensive and time consuming. Clinical access to this data is also limited [9]. Commercial anti-dengue antibody diagnosis is available however; results cannot be confirmed until at least 4-5 days after onset of suspected dengue infection [10]. During the acute phase of dengue infection, found in patients with primary and secondary symptoms, enhanced NS1 protein levels have been found [11]. Hence, immediate detection of the NS1 protein after the onset of suspected Dengue infection may prove to be a viable alternative to the other methods currently employed.

The objective of the present study, therefore, is to develop a highly sensitive ELISA assay for the detection of dengue NS1 antigen using high affinity monoclonal antibody [mAb] and bispecific antibody [bsMAb] detection. In comparison to traditional methods employed, our diagnosis for NS1 protein is more sensitive, takes less time to complete, thus less money spent, while leading to, potentially, a more efficacious treatment.

4.2 Materials and Methods

4.2.1 Cell line

An anti-horse radish peroxidase [HRPO] rat hybridoma cell line [YP4], and an anti-dengue NS1 murine hybridoma cell line [P148], was used in this study. YP4 was a kind gift from the late Dr. C. Milstein [Medical Research Council for Molecular Biology, Cambridge, United Kingdom] while the P148 producing anti-NS1 mAb was developed and characterized in our laboratory. The quadroma cell line [P156] was also developed in our laboratory fusing P148 and YP4.

4.2.2 Other materials and reagents

Cell culture media RPMI 1640 and penicillin-streptomycin-glutamine [PSG] were purchased from Gibco [Grand Island, New York, USA]. Fetal bovine serum [FBS] was purchased from PAA laboratories [Pasching, Austria]. Goat anti-mouse IgG conjugated to horseradish peroxidase [GAM-HRPO], bovine serum albumin [BSA], polyethylene glycol [PEG] 1300–1600, fluorescein isothiocyanate [FITC], tetramethylrhodamine isothiocyanate [TRITC], HRPO Type IV, protein G-agarose, *m*-amino phenyl boronic acid [*m*-APBA] agarose, and long chain sulfosuccinimidyl NHS biotin were purchased from Sigma Chemicals [St. Louis, Missouri, USA]. Streptavidin tagged HRPO [St-HRPO] was purchased from BD Biosciences [San Jose, California, USA]. Tetramethylbenzidine [TMB] was purchased from BioFx Laboratory [Burlington, North Carolina, USA]. For Western blots, hybond-ECL nitrocellulose membranes were procured from Amersham Biosciences, [Freiburg, Germany] and the Western blot detection system was procured from GE Healthcare [Waukesha, Wisconsin, USA]. Nylon

fibre swabs were bought from Micro Rheologics [Brescia, Italy]. Non-sterile flat bottom NUNC maxisorp 96-well ELISA plates were purchased from VWR [Ontario, Canada]. Fluorescence activated cell sorter, FACS Aria [BD Biosciences, USA], was accessed from the Department of Medical, Microbiology and Immunology, University of Alberta. For protein purification, we used a Biologic Duoflow system [Bio-Rad, USA] while the ELISA absorbance was read using **a** Versa max microplate reader [Molecular Devices, USA]. Rabbit serum was obtained from the Health Sciences Laboratory Animal Services [HSLAS], University of Alberta.

4.2.3 Protein preparation

The full length dengue virus type 1 NS1 nucleotide sequence was codon optimized for prokaryotic expression and synthesized from GENEART [Burlington, Ontario, Canada]. The optimized NS1 gene was PCR amplified and cloned in the correct reading frame in pBM802 vector along with the His_6 tag at the C-terminal for enhanced expression of proteins in inclusion bodies of *E.coli*. The recombinant clones were analyzed by restriction digestion fragment mapping and the correct clones were subsequently selected for protein expression. Protein purification was done by IMAC chromatography from inclusion bodies according to a previous protocol [12]. The NS1 protein was used to develop anti-NS1 mAb and bsMAb for the development of this ultrasensitive immunoassay.

4.2.4 Preparation of anti dengue NS1 hybridomas

Immunizations were performed in accordance with the guidelines of the Institutional Animal care and use committee. Six to eight week old female BALB/c mice (n=5) were immunized intraperitoneally 3 times with 25 µg of dengue envelope antigen

on day 0, and 14 using complete and incomplete Freund's adjuvant, and once with 10 μg of antigen on day 28 using phosphate buffered saline [PBS] pH 7.3. The humoral immune response to the antigen was assessed by measuring the titer of polyclonal antibody in mouse serum using indirect ELISA. The mice with highest titer were splenectomized on day 3 after the last antigen injection. The splenocytes were fused with SP2/0 myeloma cells at a ratio of 5:1 using 50% [w/v] polyethylene glycol [PEG] according to the technique established by Kohler and Milstein [1975] [13]. Using this methodology, five anti-NS1 mAbs [P148.1, P148.7, P148.9, P148.L1, P148.L2] were developed and characterized.

4.2.5 Purification of mAb

The production, purification and characterization of the anti dengue NS1 mAb' were performed by affinity chromatography according to the published protocol [14,15]. This purified mAb antibody was subsequently used in the ELISA assay, as the capture antibody.

4.2.6 Production of bispecific monoclonal antibody from quadroma

The bsMAb was developed by fusing two different hybridoma cell lines, P148.L1 anti-NS1 mAb and YP4 anti-HRPO mAb each hybridoma at 2×10^7 cells was separately isolated from the two cell lines in their logarithmic growth phase. The anti-HRPO YP4 is a well-characterized rat hybridoma that was previously selected for drug resistance to 8-azaguanine, making it sensitive to aminopterine in HAT medium. The P148.L1 [re-suspended in RPMI media, pH 7.4] was labeled with the red dye TRITC. The YP4 [re-suspended in RPMI media, pH 6.8] was labeled with the green dye FITC. Both

hybridomas were incubated for 30 min in a 5% CO_2 chamber [37°C]. Excess dye was removed by repeated washes [x3] with RPMI serum free media. The cells were thoroughly mixed and then centrifuged at 459× *g* for 7 minutes. The pellet was collected and suspended in RPMI. The supernatant was removed and the fusion of the two cell lines was done by drop-wise addition of 2 ml of polyethylene glycol to the cell pellet with continuous stirring for 2 minutes at 37°C. The toxic effect of PEG was immediately addressed by diluting the mixture with 20 ml of serum free RPMI media. This mixture was then centrifuged at 114× *g* for 5 minutes and the cell pellet was again suspended in RPMI media containing 10% FBS. The fused cells were sorted by fluorescence-activated cell sorting [FACS] and the dual positive cells were seeded in a 96-well sterile tissue culture plate at a concentration of 1 cell/well. The cells were cultured in 20% FBS media at 37°C with 5% CO_2 and their growth was regularly monitored. Based on cell growth, after approximately two weeks of culture, the cells were screened for their activity using the bridge ELISA technique.

4.2.7 *Purification of bsMAb*

The stable, cloned bsMAb secreting cells were seeded in a hyper flask for large-scale expansion. 7–10 days later the supernatant was harvested and centrifuged at 5000 rpm for 30 minutes. The collected supernatant was passed through a 0.22 µm filter to remove cell debris and the clarified supernatant was further processed to obtain pure bsMAb antibody. The purified bsMAb was then used as the detection antibody in the bsMAb ELISA immunoassay. An earlier published two-stage purification procedure, developed in our lab, was adopted for bsMAb purification [16].

4.2.8 Optimization of the capture antibody concentration

Purified P148.L2 mAb was subsequently selected as the capture antibody [Cab] in the formation of the ELISA assay. A microtitre plate was coated overnight at 4°C with 100 µl of various concentrations of P148.9 mAb ranging from 0 to16 µg/ml in triplicate. The plates were then blocked with 200 µl of 3% dialysed BSA [DBSA] in PBS at 37°C for 3 hours. 100 µl of 5 ng/ml dengue NS1 recombinant antigen was then added and incubated for 2 hours, and subsequently 4 µg/ml of P156 bsMAb [DAb] was added and incubated for 1 hour. The plate was washed [x3] with PBST after each of the steps mentioned above. Lastly, TMB was added for color development and read at 650 nm using a microplate reader.

4.2.9 Optimization of the detection antibody concentration

P156 bsMAB was used as the detection antibody. A fixed concentration of capture antibody [10 µg/ml] was used to coat a microtitre plate and different dilutions of detection antibody ranging from 0 to 16 µg/ml were used. The assay protocol and the concentration of the other parameters were identical as capture antibody optimization and the results were also similarly analyzed.

4.2.10 Optimization of the conjugate Streptavidin-HRPO

Serial two-fold dilutions of the conjugate St-HRPO [in PBS with 1% BSA] ranging from 1:4,000 to 1:48,000 were used in the assay. The previously optimized concentrations of the other components such as CAb [4 µg/ml], DAb [2 µg/ml] and dengue NS1 antigen [5

ng/ml] were kept constant. The assay was performed as described in section 2.10 and the data was similarly analyzed.

4.2.11 Biotinylation of anti dengue NS1 mAbs

Anti-NS1 mAbs were biotinylated by using long arm biotinaimdo hexanoic acid-3-sulfo-N-hydroxysuccinimide ester. 1µg each of protein-G purified [five anti-spike mAbs] in PBS, pH 7.4 was added to 20 µl of long chain biotin [30 µg/ml] and incubated at room temperature [RT] for 1 h. 10 µl of glycine [100 µg/µl] was then added and the solution kept on a shaker for 10 min [17]. The solution was then dialyzed in a slide-A-lyzer against PBS, pH 7.4 overnight at 4ºC.

4.2.12 ELISA formats

4.2.12.1 Indirect ELISA

Hybridoma culture supernatants were assayed for binding to Dengue NS1 coated 96 well plates. Plates were coated with 100 µl of purified Dengue NS1 [5µg/ml] in PBS and incubated overnight [4ºC] and then blocked with 3% BSA for 2 h at 37ºC. The ELISA plates were then washed three times with PBS containing 0.05% Tween 20 [PBS-T]. 100 µl of conjugated goat anti-mouse IgG HRPO, diluted [1:2,000] in 1% BSA in PBS was then added to the wells and incubated for 1 h at 37ºC. The plate was again washed 3 times with PBST. TMB substrate was added to the plate and incubated 10 minutes, then read at [650 nm] for antibody detection using a V_{max} ELISA plate reader. Mouse immune and preimmune sera were diluted 1:1,000 with 1% BSA in PBS for use as positive and negative controls respectively.

4.2.12.2. ELISA format for detection of bsMAb in quadroma culture supernatants

The fused quadroma cells generally secrete three stable antibodies, the two parent mAbs [P148 and YP4] and the newly fused bsMAb antibody. A bridge ELISA technique was adopted [18] to screen for clones that secrete bsMAb, The 96-well plates were immobilized with 100 µl of recombinant dengue NS1 antigen [10µg/ml] and incubated at 4°C for overnight. The plates were washed [x3] with PBST [PBS with 0.05% Tween-20] and the non-binding sites were blocked with 2% bovine serum albumin [BSA] at 37°C for 2 hrs. After washing [x3**],** 100 µl of diluted [neat, 1:10, 1:100] cell supernatant was added and incubated for 1 hour at room temperature [RT]. The plates were again washed [x3] with PBST and 100 µl of HRPO [10 µg/ml] was added and incubated for 30 minutes at RT. The plates were washed [x6] to remove excess unbound HRPO and finally, 100 µl of TMB substrate was added and color development was read at 650 nm using a microplate reader. The control was RPMI media only. The clones with maximum bsMAb secretion capacity were identified and re-cloned by the standard limiting dilution method. Briefly, the cells were placed in a tissue culture plate at a concentration of 1 cell/well. They were then cultured as before, and positive clones were screened using bridge ELISA. The above cloning and screening steps were repeated until a stable clone was obtained.

4.2.12.3 Sandwich ELISA using biotinylated mAb

All incubations were done at 37 °C. Washing [4-5x] was done with PBST after each step. The assay was performed with Dengue anti-NS1 mAb [P148.L2 or P148.L1] as capture antibody and the biotinylated P148.L2 mAb as detection antibody. The anti-

NS1 mAb P148.L2 was biotinylated with NHS-LC-Biotin [Sigma, USA] as per manufacturers' instruction. A microtiter plate [NUNC, Denmark] was coated with 100 µL of purified NS1 mAb P148.L2 in 0.05 M carbonate buffer at 4 °C overnight. Nonspecific binding sites were blocked with 200 µL of 2% BSA for 2 hr. Different concentrations of the dengue NS1 antigen ranging from 20 ng/ mL to 0 [20, 10, 5…0] were used, then the plate was incubated at 37 °C for 1 h. Thorough washing [3-5x] was completed and 100 µL of the biotin labeled P148.L2 mAb [2 µg/mL] was added to each well and incubated at 37 °C for 1 h. After incubation, the plate was washed [3-5x] and streptavidin-HRPO [Sigma, USA] was added and incubated at 37 °C for 30 min. Subsequently, TMB substrate [Kirkegaard & Perry Laboratory, USA] [32] was added. OD_{650} was measured after 15 min using an ELISA V_{max} kinetic microplate reader [Molecular Devices Corp., USA].

4.2.12.4 Sandwich ELISA using bsMAb

Except as otherwise indicated, all incubation steps were performed at 37 °C for 1 h. Washing five times was conducted by PBS-T between each step. Plates were coated with 100 µl of purified anti-NS1 mAb [P148.9L2 or P148.L1] in 50 mM carbonate buffer [pH 9.6]. The remaining sites on the well surface were blocked with 200 µl of blocking buffer [3% [w/v] BSA in PBS-T] at 37 ºC for 1 h. A volume of 100 µl of Dengue NS1 [serial dilution in 1% [w/v] BSA in PBS-T] was added to the wells, which was then followed by an additional 100 µl of bsMAb-HRPO complex [P156]. Plates were washed [3-5x] and TMB substrate was added for colour development and subsequently read at 650 nm after 5 min incubation using an ELISA plate reader.

4.2.13 Assay quality control

Intra and inter-plate precision profiles were generated using, six replicate standard curves on one plate, and six replicate plates, each with triplicate standard curves [19].

4.2.14 Statistical analyses

The student's *t*-test [one-tailed *t*-test] was used to analyze the significant difference [$p < 0.05$] between the control [zero antigen] and samples.

4.3 Results

The NS1 nucleotide sequence of Dengue virus was codon optimized for prokaryotic expression and synthesized from GENEART [Burlington, Ontario, Canada]. The optimized NS1 gene was PCR amplified and cloned in the proper reading frame in pBM802 vector along with the His_6 tag at the C-terminal for higher expression of proteins in inclusion bodies of *E.coli*. The recombinant clones were analyzed by restriction digestion fragment mapping and the correct clones were selected for protein expression. Purification of protein from inclusion bodies was done as per established protocol [12]. Mice were immunized with recombinant dengue NS1 antigen and the polyclonal titer estimated by indirect ELISA indicating a robust immune response [FIG 4.1].

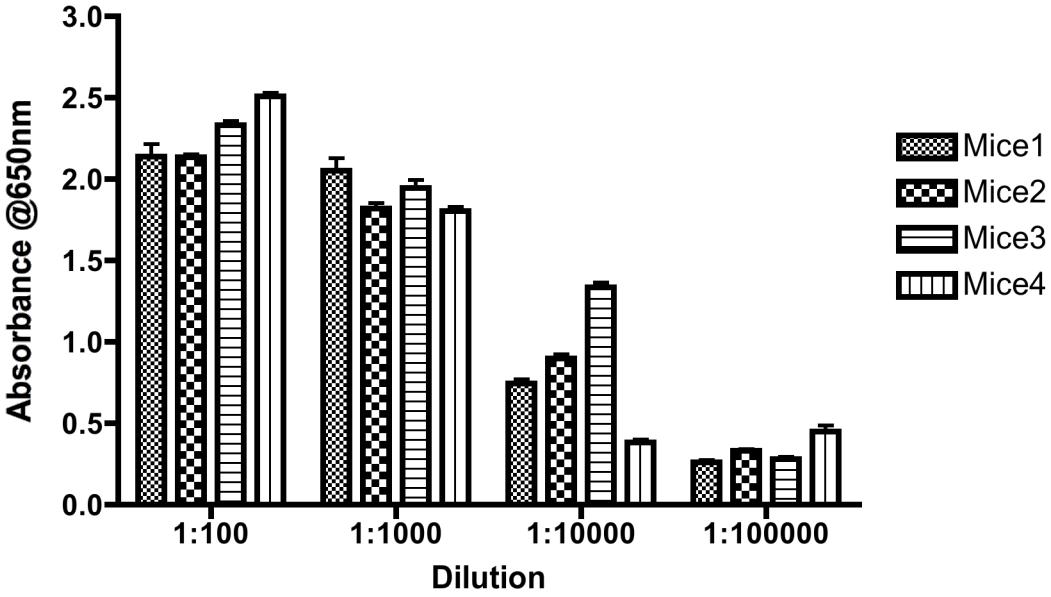


Fig 4.1 Titer of ELISA indicating robust immune response in mice immunized with recombinant dengue NS1 antigen. Values are the means of triplicate samples. Vertical bars indicate the standard deviation.

The mAbs were purified by affinity chromatography as mentioned earlier. The dual step bsMAb purification was done according to the established procedure [16]. The purified entity from the first stage comprised of a mixture of three antibodies, two parent mAbs [anti NS1 mAb and YP4] and bsMAb, all being of the IgG isotype. The second step of purification involved using *m*-amino phenyl boronic acid [*m*-APBA] agarose to remove the anti NS1 mAb as its presence could influence the sensitivity of the assay. The bsMAb, obtained from *m*-APBA column, was attached to the reporter molecule HRPO via the HRPO-binding paratope. After two steps of purification an enhanced bsMAb activity was observed in the ELISA assay. The purified hybridomas and quadromas were analyzed by SDS-PAGE under reduced conditions [data not shown], which confirmed the high purity of the antibodies. Cross reactivity studies with other viral recombinant antigens like SARS, WEE and Ebola yielded negative results.

The concentration of bsMAb chosen for this study was 2 µg/ ml as the detecting antibody [FIG 4.2]. An optimization of P148.L2 mAb as the capture antibody was 4 µg/ml [FIG 4.3]. The optimal dilution for streptavidin-HRPO was found to be 1:8000 [FIG 4.4]. These values were selected based on their statisctical significance. These different optimization assays were independently repeated twice and performed in triplicate.

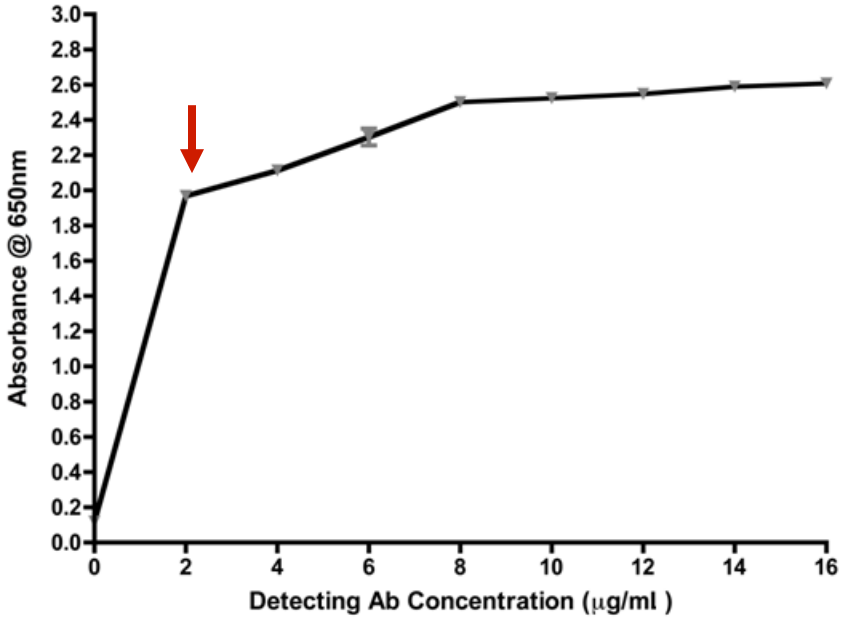


Fig 4.2 Optimization of detection antibody [DAb] against NS1 in sandwich ELISA. Arrow indicates optimized concentration.

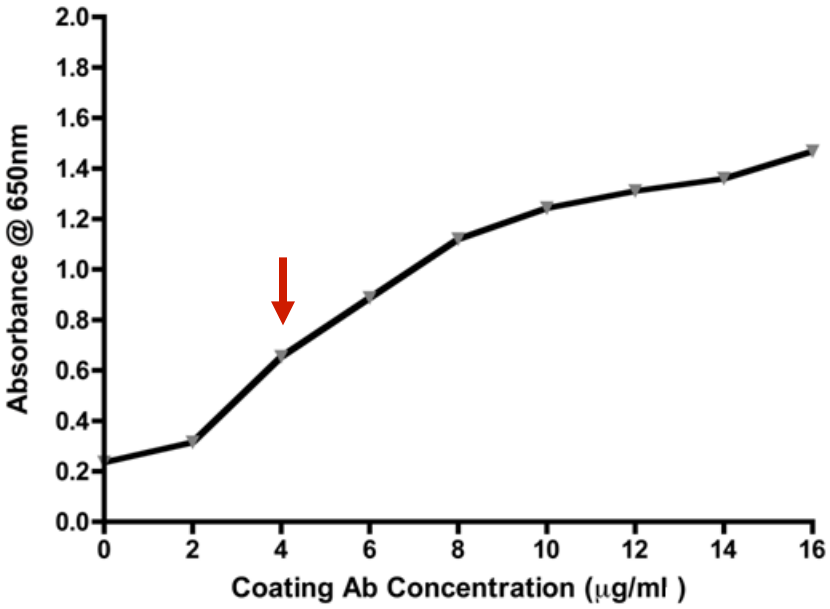


Fig 4.3 Optimization of the capture antibody [CAb] against NS1 in sandwich ELISA. Arrow indicates optimized concentration.

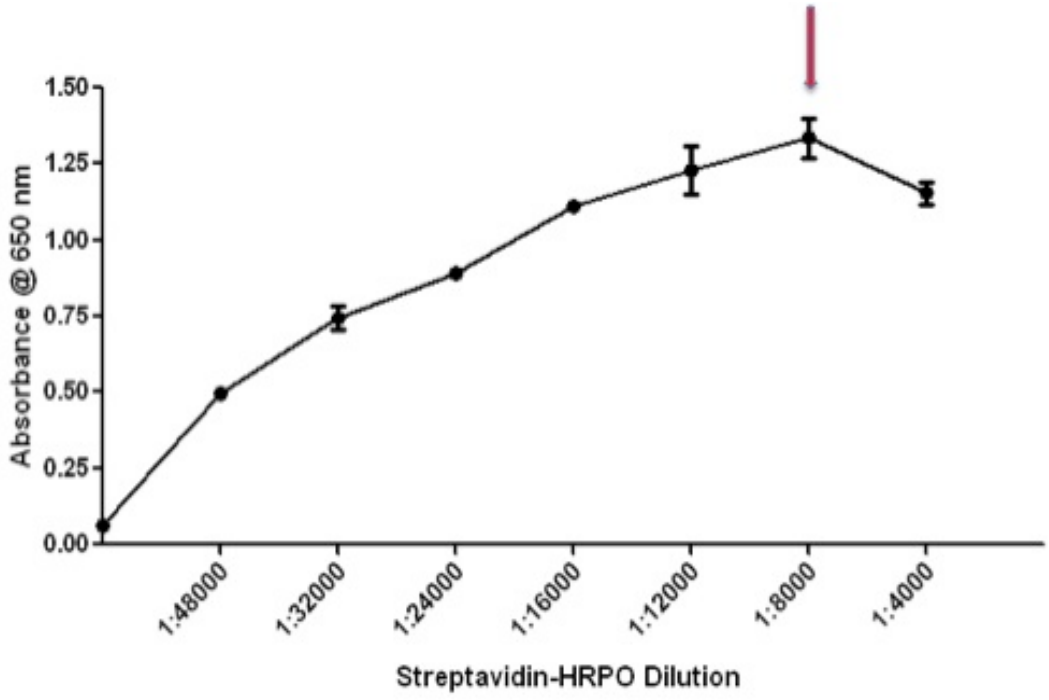
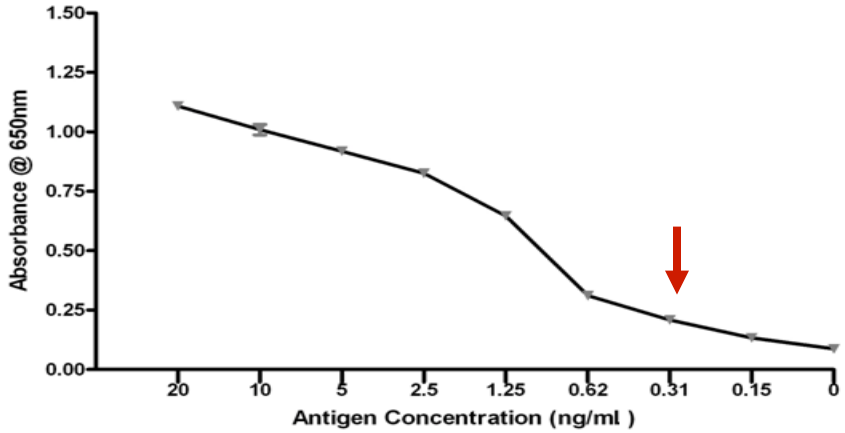


Fig 4.4 Optimization of streptavidin-HRPO conjugate. Arrow indicates optimized concentration.

These optimal levels of antibodies were used to develop sandwich assay having nanogram level cut-offs with recombinant dengue NS1 antigen [dilutions from 20 ng/ml to 0.156 ng/ ml; n = 3]. Figure 4.5A and 4.5B illustrates that the detection limit of the bsMAb based sandwich ELISA assay was found to be 0.3125 ng/ml or 31.25 pg/ml [$p<0.02$] of dengue NS1 antigen [$P < 0.05$].

4.5A



4.5B

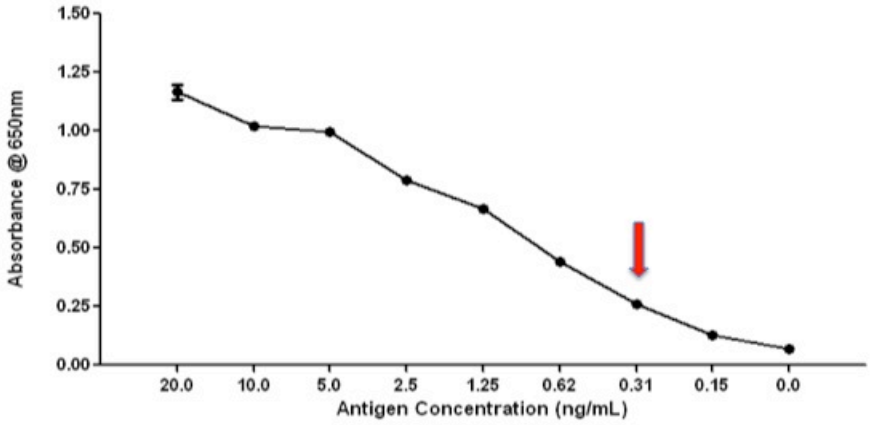


Fig 4.5 ELISA for the detection of NS1 antigen using bsMAb. **[A]** Sandwich ELISA with anti-Dengue-NS1 mAb [P148.L2] as a coating antibody and bsMAb-HRPO complex [P156] as a detection antibody. **[B]** Sandwich ELISA with anti-Dengue-NS1 mAb [P148.L1] as a coating antibody and bsMAb-HRPO complex [P156] as a detection antibody. The color was developed using TMB substrate. The results are expressed as absorbance reading at 650 nm wavelengths. Arrows indicate the cut-off values or limits of detection.

We also prepared a modified sandwich ELISA assay using a biotin-conjugated mAb as the detection antibody and the same mAb as the capture antibody. Biotin conjugated detection antibody provided high cut-offs because of the non-reversible binding nature of biotin to streptavidin. However, comparative analysis with quadromas based immunoassay sensitivity was found to be higher. FIG 4.6A and 4.6B illustrates that the assay sensitivity was found to be about 0.625 ng/ml or 62.5 pg /ml [$p<0.02$] which is double that of the bispecific immunoassay. To increase the sensitivity of the sandwich assay, we had to increase the concentration of the biotin labeled DAb [data not shown].

4.6A

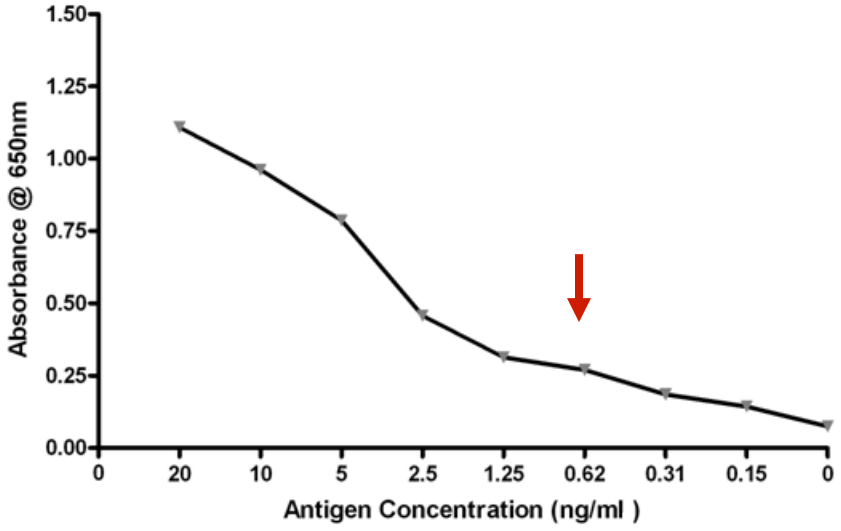


Fig 4.6A ELISA for detection of Dengue NS1 antigen using biotinylated mAb. Sandwich ELISA with anti-Dengue-NS1 mAb [P148.L2] as a coating antibody and same mAb labeled with biotin as a detection antibody. The biotinylated mAb [P148.L2] was detected using Streptavidin-HRPO.

4.6B

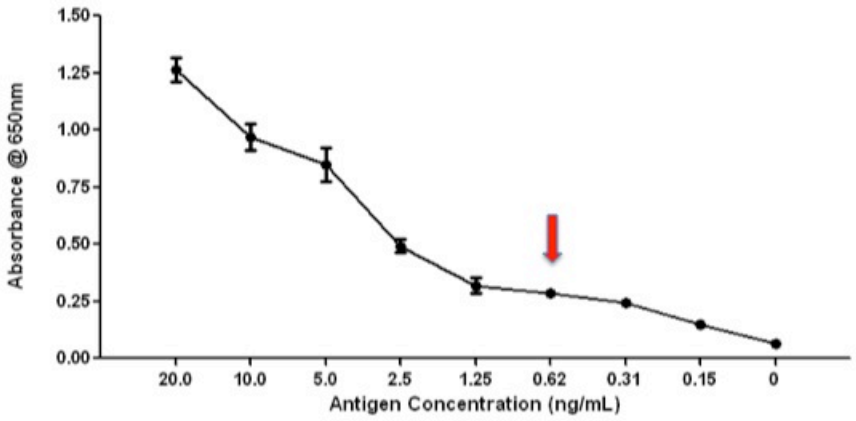


Fig 4.6B ELISA for detection of Dengue NS1 antigen using biotinylated mAb. Sandwich ELISA with anti-Dengue-NS1 mAb [P148.L1] as a coating antibody and another mAb labeled with biotin [P148.L2]. The biotinylated mAb was detected using Streptavidin–HRPO. The results are expressed as absorbance reading at 650 nm wavelengths. Arrows indicate the limit of detection.

These results indicate that by using the bsMAb as the capture antibody instead of the DAb antibody, detection limit was improved. The sandwich assay developed using different mAbs generated against dengue NS1 antigen as the capture antibody and the dual specific quadroma as the detecting antibody, was clearly illustrated, that all the monoclonal antibodies reacted well for the sandwich assay when used as capture antibodies with NS1 as the antigen and bsMAb as the detecting antibody, however, with different relative affinity [FIG 4.7].

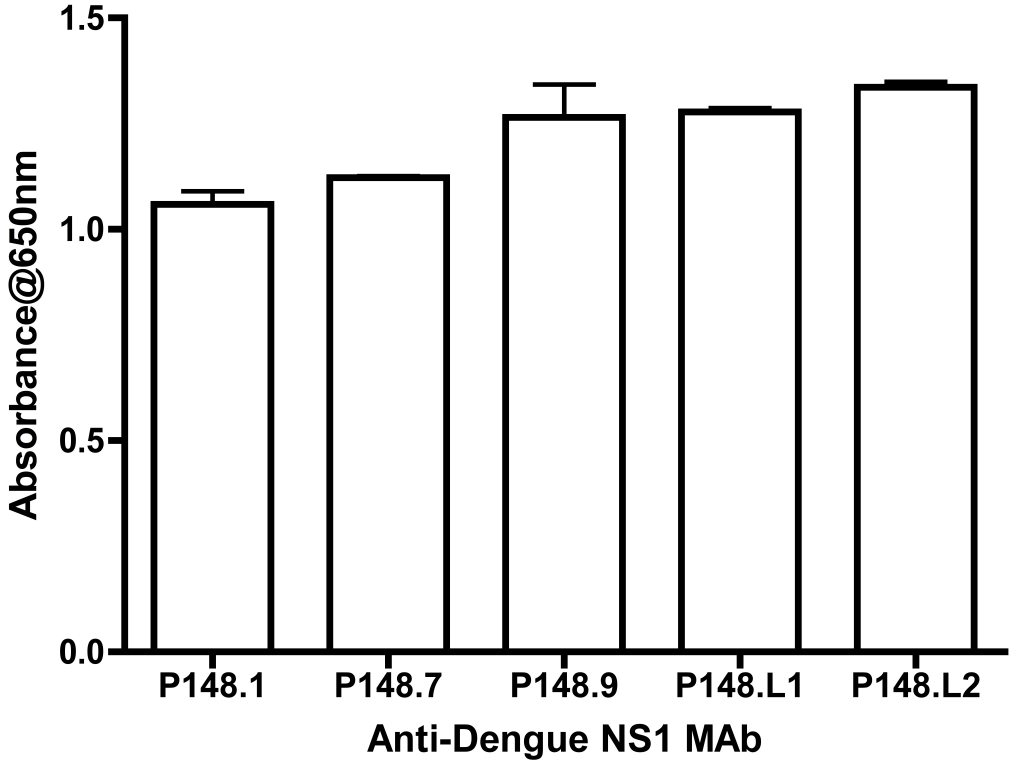


Fig 4.7 Titer of Sandwich ELISA with 5 dengue anti-NS1 monoclonal antibodies: anti-dengue mAbs were coated in the ELISA plate. Ten micrograms per milliliter of NS1 antigen was added and detected by bsMAb. Values are the means of triplicate samples. Vertical bars indicate the standard deviation. OD is optical density

The best sandwich pair found, was when P148.L2 and bsMAb were used as capture antibodies and detecting antibodies respectively. Since we found no significant difference in affinities between the different sandwich combinations we identified the best pair and subsequently used these for the development of the ultrasensitive immunoassay. A range of different anti dengue NS1 mAbs and bsMAb concentrations [n = 6] were used to determine the most efficacious diagnostic pair. The biotinylated mAb based assay might be prone to a slightly higher background at times due to the random conjugation process involved in labeling resulting in skewed ratio between the mAb and biotin. The random conjugation process could also result in partial blocking of the antigen-binding site as the biotin conjugation site is variable [Sarkar et al., 2012]. On the contrary, the bsMAb with dual paratopes specific for different antigens would always maintain a balance leading to low background.

4.4 Discussion

Rapid and accurate detection of dengue infections in a laboratory setting or, more importantly on site, along with the ability to differentiate between multiple infections during the acute phase of illness, is an absolute necessity for timely clinical intervention and epidemiological control in dengue endemic areas [20]. Furthermore, dengue detection kits must be easy to use and inexpensive particularly in resource-constrained countries. An ideal assay would be something that is convenient, sensitive, specific, and above all affordable and which would be able to quickly and accurately detect viral infections [21]. For enhancing early public health interventions for control of dengue outbreaks, it is absolutely essential to detect acute infections during the initial period of clinical symptoms. Early diagnosis of infection remains a challenge. Currently, a number

of techniques are routinely used for the diagnosis of dengue virus infection. These applications include viral isolation and identification, RT-PCR based identification of viral genome or antibody capture assays. Virus isolation is still considered foremost for diagnosis of acute dengue infection, but it is very expensive and time intensive [9]. Reverse transcriptase-polymerase chain reaction [RT-PCR] is not readily available at all clinical laboratories primarily because of its high cost. Measuring levels of anti-dengue antibodies is dependent on the patients' immune system to produce IgM or IgG antibodies against dengue virus antigens. Therefore, all these techniques have limitations in providing a rapid diagnosis of infection at a low cost. Detectable antibodies especially IgM can only be measured after 5 days post infection [4,22,23]. To address some of these problems and limitations we have developed an ultrasensitive quadroma based diagnostic tool for the detection of dengue NS1. Quadromas have been shown to play important roles in modern medicine, and have been extensively used in therapeutic applications including vaccine development, and gene therapy [24,25,26]. Bispecific monoclonal antibodies [bsMAbs] have recently been used in the development of immunoassays for diagnosis of bacterial and viral infectious diseases [27,28]. In this study, by using bsMAb as the detecting antibody, we increased the sensitivity of the assay considerably to 31.25 pg /ml which is substantially lower than current dengue detection assays. Furthermore, with the use of second-generation quadromas, we were able to significantly lower the antigen detection limit thereby enabling us to diagnose dengue infection at its earliest phase.

To our knowledge, the development of bsMAb secreting quadroma as a bifunctional immunoconjugate possessing two paratopes as a diagnostic reagent is the first of its kind against dengue virus NS1. The presence of intrinsic enzyme binding

activity within the bsMAb makes it an ultra-sensitive tool in the development of specific diagnostics against dengue infections particularly for those with negligible NS1 background. The sensitivity of the assay developed, in detecting low levels of NS1 antigen, suggests its usefulness and subsequent potential for detecting dengue virus particles as well. Our assay could effectively be adopted in a sandwich format as well as an indirect ELISA format for detection of dengue virus or NS1 antigen. This assay is particularly user friendly and reduces the number of steps required for diagnosis. Based on our current strategies, we are developing an immunoswab assay for the detection of dengue virus NS1 as a point of care diagnostic tool. Hence, we are in the process of developing a highly sensitive and inexpensive detection system for all the diagnostic markers associated with dengue virus infection. In 2002, Alcon and co-workers [20], by using their NS1 antigen-capture ELISA, reported that dengue virus NS1 antigen could be detected up to 9 days after onset of fever. Hence, selection of our ultrasensitive dengue NS1 protein-capture ELISA immunoassay involving hybridomas and quadromas would be a worthy choice for a fast, accurate and inexpensive alternative to the presently employed methods. Future studies include optimization of the assay using clinical samples as well as transfer of the technology to other platforms for ease of use and to different settings with minimal facilities.

This rapid ultrasensitive sandwich ELISA could also be extended to help control other infectious pathogens. Literature cites a number of studies wherein mAbs in combination with polyclonal antibodies have been employed for developments of NS1 capture ELISA with good specificities [27]. Our endeavor elucidates the use of bsMAb secreting quadroma, which was developed using one of the anti dengue NS1 mAbs as the detecting antibody. With respect to polyclonal antibodies, the quadromas offer some

evident advantages. bsMAbs can be developed in perpetuity with stable batch reproducibility [21,29,30]. This is very significant in the context of assay robustness over time. Traditional diagnostic assays involving monoclonal antibodies and polyclonal antibodies need an extra step in the context of the addition of a secondary antibody chemically tagged to a certain enzyme [31]. Enzyme-antibody tagging by chemical methods is difficult to perform repeatedly while also maintaining similar efficacy [21]. In contrast, our second-generation bsMAb secreting quadroma is already conjugated with HRPO during purification [16], thereby reducing the additional steps of secondary antibody addition, and thereafter the multiple washing steps. This modification, alone, significantly minimizes the time to carry out the assay. Our assay is able to detect the dengue NS1 antigen suggesting that this assay could be useful in detecting the whole virus as soon as the infection sets in, rather than later, when the antigen gets secreted in body fluids.

4.5 Conclusions

We have developed a sensitive dengue virus NS1 diagnostic tool by optimizing a sandwich ELISA immunoassay for the detection of the NS1 antigen. We evaluated the efficacy of a panel of monoclonal antibodies [mAbs] with high affinity and specificity for the NS1 dengue 1 antigen along with a combination of different bi-specific monoclonal antibodies [bsMAb] for antigen detection. By using recombinant NS1 protein from dengue virus, we established a detection sensitivity of 31.25 pg/ ml. For the future, the sandwich ELISA developed could be translated to other infectious diseases and perhaps be viewed as a possible replacement for other diagnostic techniques that are more

expensive, time consuming and labor intensive. Implementation of this “time saving” diagnostic tool could assist in preventing serious viral outbreaks by allowing earlier therapeutic interventions.

4.5 References

1. Gubler, D. J., Clark, G. G. 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerging Infectious Diseases 1, 55–57

2. Malabadi, R. B., Ganguly, A., Teixeira da Silva, J. A., Parashar, A., Suresh, M. R., Sunwoo, H. H. 2011. Overview of plant-derived vaccine antigens: Dengue virus, Journal of Pharmacy and Pharmaceutical Sciences 14[3], 400-413

3. Malabadi, R. B., Ganguly, A., Sunwoo, H. H., Suresh, M. R. 2012. Role of bi-specific monoclonal antibodies in immunodiagnostic assay. Research in Pharmacy 2[3], 08-14

4. Kumarasamy, V., Wahab, A.H.A., Chua, S.K., Hassan, Z., Chem, Y.K., Mohamad, M., Chua, K.B. 2007. Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. Journal of Virological Methods. 140, 75–79

5. Falconar, A., Young, K. I., Young, P. R. 1990. Immunoaffinity purification of the native dimer forms of the flavivirus non-structural glycoprotein, NS1. Journal of Virological Methods 30, 323-332

6. Schlesinger, J.J., Brandriss, M. W., Putnak, J. R., Walsh, E. E. 1990. Cell surface expression of yellow fever virus nonstructural glycoprotein NS1: consequences of interaction with antibody Journal of General Virology 71, 593–599

7. Lindenbach, B. D., Rice, C. M. 1997. Trans-complementation of yellow fever virus NS1 reveals a role in early RNA replication. Journal of Virology 71, 9608-9617

8. Falconar, A., Young, K. I., Young, P. R. 1991. Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 non-structural glycoprotein NS1. Journal of General Virology 72, 961–965

9. Osorio, L., Ramirez, M., Bonelo, A., Villar, L. A., Parra, B. 2010. Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection. Virology Journal. 361

10. Halstead, S.B., 2008. Dengue Virus–Mosquito Interactions. Annu. Rev. Entomol. 2008. 53:273–91

11. Lima, M. dR, Q., Nogueira, R. M. R., Schatzmayr, H. G., Santos, F. Bd., 2010. Comparison of Three Commercially Available Dengue NS1 Antigen Capture Assays for Acute Diagnosis of Dengue in Brazil. PLoS Neglected Tropical Diseases 4[7]: e738. doi:10.1371/journal.pntd.0000738

12. Das, D, Mongkolaungkoon, S., Suresh, M. R. 2009. Super induction of dengue virus NS1 protein in E.coli. Protein Expression and Purification 66, 66-72

13. Kohler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497

14. Shahhosseini, S., Das, D., Qiu, X., Feldmann, H., Jones, S. M., Suresh, M. R. 2007. Production and characterization of monoclonal antibodies against different epitopes of Ebola virus antigens. Journal of Virological Methods 143, 29-37

15. Das, D., Suresh, M. R. 2005. Producing bispecific and bifunctional antibodies. Methods In Molecular Medicine 109, 329–346

16. Bhatnagar, P.K., Das, D., Suresh, M.R. 2008. Sequential affinity purification of peroxidase tagged bispecific anti-SARS-CoV antibodies on phenylboronic acid agarose. Journal of Chromatography B: Analytical Technologies in the Biomedical Life Sciences 863, 235–241

18. Guttikonda, S., Tang, X. L., Yang, B. M., Armstrong, G. D., Suresh, M. R. 2007. Monospecific and bispecific antibodies against E.coli O157 for diagnostics. Journal of Immunological Methods. 327[1–2], 1–9

19. Sunwoo, H. H., Wang, W., Sim, J. S. 2006. Enzyme Linked-Immunoassay for the Quantitation of E.coli O157:H7. Immunology Letters 106, 191-193

20. Alcon, S., Talarmin, A., Debruyne, M., Falconar, A., Deubel, V., Flamand, M. 2002.

Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. Journal of Clinical Microbiology 40, 376-381

21. Sarkar, S, Tang, X. L., Das, D., Spencer, J. S., Lowary, T. L., Suresh, M. R. 2012. A Bispecific Antibody Based Assay Shows Potential for Detecting Tuberculosis in Resource Constrained Laboratory Settings. PLoS ONE 7[2]: e32340. doi:10.1371/journal.pone.0032340

22. Zainah, S, Abdul Wahab, A.H., Mariam, M., Fauziah, M. K., Khairul, A. H., Roslina, I., Sairulakhma, A., Kadimon, S. S., Mat Jais, M. S., Chua, K. B. 2009. Performance of a commercial rapid dengue NS1 antigen immunochromatography test with reference to dengue NS1 antigen-capture ELISA. Journal of Virological Methods 155, 157–160

23. Qiu, L.W., Di, B., Wen, K., Wang, X. S., Liang, W. H., Wang, Y. D., Pan, Y. X., Wang, Ding, Y. Q., Che, X. Y. 2009. Development of an antigen capture immunoassay based on monoclonal antibodies specific for dengue virus serotype 2 nonstructural protein 1 for early and rapid identification of dengue virus serotype 2 infections. Clinical and Vaccine Immunology 16[1], 88–95

24. Khan S. R., Ganguly, A., Malabadi, R. B., Sunwoo, H. H., Parashar, A., Teixeira da Silva, J. A., Suresh, M. R. 2011. Targeting strategies and nanocarriers in vaccines and therapeutics. Research in Biotechnology 2[6], 08-20

25. Khan, S. R., Ganguly, A., Malabadi, R. B., Sunwoo, H. H., Suresh, M. R. 2012. Gene delivery system: A developing arena of study for new era of medicine. Recent Patents on DNA & Gene Sequences 6[1], 2-9

26. Wang, W., Das, D., Tang, X. L., Budzynski, W., Suresh, M. R. 2005. Antigen targeting to dendritic cells with bispecific antibodies. Journal of Immunological Methods 306[1-2], 80-92

27. Parashar, A., Sarkar, S., Ganguly, A., Sharma, S., Suresh, M. R. 2011. Bispecific antibodies for diagnostic applications; Bispecific Antibodies. Ed Dr. Kontermann RE. ISBN-978-3-642-20909-3, Springer Heidelberg Dordrecht London New York: 349-358

28. Das, D, Kammila, S., Suresh, M. R. 2010. Development, characterization and application of monoclonal antibodies against severe acute respiratory syndrome coronovirus nucleocapsid protein. Clinical and Vaccine Immunology. 17[12], 2033-2036

29. Hoon, H. H., Palaniyappan, A., Ganguly, A., Bhatnagar, P. K., El-Kadi, A. O. S., Suresh, M. R. 2012. Quantitative and sensitive detection of SARS-CoV spike protein using bispecific monoclonal antibody-based enzyme-linked immunoassay. Journal of Virological Methods. [*In press*]

30. Ritter, M.A. 2000. Polyclonal and monoclonal antibodies. Methods in Molecular Medicine 40, 23–34.

31. Cao Y, Suresh, M.R. 1998. Bispecific antibodies as novel bioconjugates. Bioconjugate Chemistry 9, 635–44

32. Blake, D, Jones, R. M., Blake II, R., Pavlov, A., Darwish, I., Yu, H. 2001. Antibody-based sensors for heavy metal ions. Biosensor and Bioelectronics 16, 799-809

5

DIAGNOSTIC OF DENGUE TYPE 1 NS1 PROTEIN USING CHICKEN IgY

5.1 Introduction

Dengue is one of the foremost vector borne diseases globally**,** and more than 100 million people are infected every year [1,2]. Dengue fever is an important mosquito-borne viral disease and has been a fatal issue in the tropical regions. The severe Dengue Hemorrhagic Fever [DHF] and dengue shock syndrome [DSS] are mostly associated with children. There have been huge dengue epidemics causing economic damage and loss of human life in Asia and South America [3]. There is immediate requirement for efficient and sensitive diagnostic, prophylactic and therapeutic agents for effective management of Dengue fever. Dengue virus has four serotypes, which are antigenically distinct [Das 2009]. The dengue virus non-structural 1 [NS1] protein is a 46–50 kDa glycoprotein expressed in infected mammalian cells. All the NS proteins are intracellular proteins with the exception of dengue NS1 protein, which exists as secreted as well as a membrane-associated protein. Both forms have been known to be immunogenic [4-6]. It was also reported that NS1 is produced during viral replication. It possesses group specific as well as type specific determinants and is considered an important antigen in dengue infection [5-7]. High circulating levels of NS1 were demonstrated in the acute phase of dengue by antigen capture ELISAs [6,8]. The exact function of dengue NS1 still remains unknown but antigen detection of non-structural dengue antigens may be of significant usefulness for an early stage rapid detection of infection owing to its long half-life in blood. Currently, there are no vaccines or drugs available to combat this challenging disease and diagnoses of infections particularly in endemic regions are generally clinical [3,9]. Dengue induces asymptomatic infection to a severely weakening and life threatening hemorrhagic version [10]. Morbidity and mortality incidence can be considerably

lowered with early and accurate detection [13]. Dengue NS1 antigen screening is one of the coomonly utilized tools for early detection of dengue infection after the onset of fever [11,12].

Therefore requirement for the development of sensitive and highly specific diagnostic kits is essential. Polyclonal antibody [pAb] and mAb are used for different clinical diagnostic applications. Pathogenic strains of bacteria and viruses can be detected by exploiting pAb for its enhanced sensitivity due to its ability of binding to multiple epitopes.

Avian pAb against different antigens produces chicken IgY in humongous amounts when compared to mammalian and rodent species. Chicken IgY can also be obtained by non-invasive methods by collection of antibodies in the egg yolks of hens hyper immunized with the specific antigen. Utilizing hens to produce polyclonal antibodies is a viable alternative to the use of other animal models mainly due to lower cost of productions and also being more ethical [14].

Chicken IgY is similar to mammalian IgG structurally and in affinity, but its molecular weight [~180 kDa] is higher than IgG [~150 kDa] as it comprises of extra heavy chain constant domain and carbohydrate chain. Chicken egg yolk antibodies [IgY] have been successfully employed for diagnostic, prophylactic and therapeutic purposes [15]. It has been established in applications includes quantitation of tumor biomarkers [16, 30], as immunotherapeutic agents against *Candida albicans* [31], detection of *E.coli* O157 [17], *Salmonella* [18,19] and SARS [15,20].

This study involved the development an ultrasensitive heterosandwich ELISA diagnostic method using mouse mAb and chicken egg yolk IgY directed against dengue virus NS1 antigen.

5.2 Materials and Methods

5.2.1 Materials

Acrylamide: bisacrylamide [40%], prestained low range protein molecular weight markers and protein assay reagent were purchased from Bio-Rad Laboratories Ltd. [Ontario, Canada]. Western blotting reagents were purchased from Amersham Pharmacia Biotech [BaiedUrfe, Quebec, Canada]. Bovine serum albumin [BSA], rabbit anti-chicken IgY–horseradish peroxidase [HRP], 2-2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] and other general reagents were purchased from Sigma [St. Louis, MO, USA]. Microtiter 96-wells plates were purchased from Costar Inc [Cambridge, MA, USA] and 3,3′,5,5′-Tetramethylbenzidine [TMB] substrate from KPL Inc [Frederick, MD, USA]. The ELISA V_{max} kinetic microplate reader was obtained from Molecular Devices Corp [Sunnyvale, CA, USA].

5.2.2 Protein preparation

Full-length Dengue type 1 NS1 protein was expressed in *Escherichia coli* and purified according to established method [21]. Full-length NS1 was used for chicken immunization as well as an antigen for the development of the heterosandwich ELISA.

5.2.3 Generation of mAbs

Five 6-8 weeks old Balb/C mice were injected intraperitoneally with 25 μg of NS1 antigen emulsified with an equal volume of Freund's complete adjuvant [FCA] and subsequently boosters with Freund's incomplete adjuvant [FIA] at two week intervals. A final booster injection with 10 μg of NS1 in phosphate buffered saline [PBS] was given 2

days prior to hybridoma fusion. Mice were sacrificed and spleens were harvested for hybridoma fusion according to our published methods [22]. We have developed and characterized 5 mAbs directed against Dengue NS1 protein.

5.2.4 Chicken Immunization

Immunization of hens was carried out as previously established [23]. Dengue NS1 [100 μg of protein/ml] was suspended in PBS [pH 7.3] and emulsified with an equal volume of FIA. Two 23-weeks-old Single Comb White Leghorn chickens were intramuscularly injected with the emulsified antigen [0.25 ml] at four different sites in the breast muscles. A booster immunization was given after two weeks. Eggs were collected everyday and preserved at 4°C until further purification and characterization of the antibodies.

5.2.5 Purification of anti-Dengue NS1 IgY polyclonal antibody

Water-soluble fraction [WSF] comprising of specific IgY against Dengue NS1 antigen was prepared from egg yolk by modified water dilution method [19]. 8 volumes of cold distilled water [acidified with 0.1 M HCl] was mixed with the egg yolk was separated from egg white. Following this cold acidified distilled water [pH 2.0] was added slowly to make the final dilution of 1:10 and the mixture was then adjusted to a pH of 5.0-5.2 and incubated at 4°C for 12 h. The WSF was then separated by centrifugation [3,125 x g at 4°C] for 20 min. Subsequently the IgY-rich WSF was mixed with 60% saturation of ammonium sulfate to precipitate the IgY and purified by Sephacryl S-300 gel chromatography. Finally the purified IgY was titrated by indirect ELISA. Non-specific IgY obtained from non-immunized eggs was also prepared to serve as a control.

5.2.6 SDS-PAGE and Western blot analysis

Purified IgY was electrophoresed on SDS-PAGE using 10% polyacrylamide gel to determine the purity of the antibody [24]. Recombinant dengue NS1 was electroblotted onto Hybond ECL nitrocellulose membranes [25] and blocked with 5% skim milk in PBS-T for 1 h at RT. The membrane was subsequently washed 4 times with PBS-T and incubated for 1 h at RT with anti-Dengue NS1 IgY [1 μg/mL]. After washing 4 times with PBS-T, the nitrocellulose membrane was incubated with rabbit anti-chicken IgY-HRP for 1 h at RT. Finally, the membrane was washed with PBS 4 times and chemiluminescent detection was performed to observe the specific binding.

5.2.7 Determination of titer of Dengue NS1 specific IgY by ELISA

Anti Dengue NS1 IgY was analyzed by ELISA according to a previously published method [26]. 96-well plates were coated with 100 μl [10 μg/ml] of Dengue NS1 in carbonate-bicarbonate buffer [0.05 M, pH 9.6] and incubated at 37°C for 90 min. Following incubation the plates were washed four times with PBS containing 0.05% Tween 20 [PBS-T]. Then 200 μl of 2% bovine serum albumin [BSA] solution [w/v] in PBS-T was added to each well for blocking non specific binding and the plates were again incubated at 37°C for 45 min. Following another washing step, dengue specific IgY or non-specific IgY as a control at a dilution of 1:5,000 in PBS-T was added to each well [100 μl/ well] and the plates were again incubated at 37°C for 1 h. The plates were again washed 3-4 times with PBS-T and 100 μl of rabbit anti-chicken IgY-horseradish peroxidase [HRP, diluted 1:5,000 in PBS-T] was added to each well and incubated at 37°C for 90 min. The plates were then washed four times with PBS-T, followed by the

addition of 100 µl of freshly prepared 2-2'-azino-bis substrate solution in 0.05 M phosphate citrate buffer [pH 5.0] containing 30% hydrogen peroxide. Absorbance was measured at 405 nm after 30 min using an ELISA V_{max} kinetic microplate reader. The ELISA value of specific IgY activity was determined by subtracting the value of the control IgY from that of specific IgY.

5.2.8 Heterosandwich ELISA

96-well plates were coated with 100 µl [10 µg/ml] of all five dengue anti-NS1 mAbs [P148.1, P148.7, P148.9, P148.L1 and P148.L2] and incubated at 4^{o}C overnight. After washing four times with PBS-T, the plate was blocked with 2% BSA for 2 h at RT. Following washing, 100 µl of recombinant dengue NS1 [10 µg/ml in 1% BSA] antigen was added and incubated for 1 h at RT. The plate was then washed four times with PBS-T and incubated with 100 µl anti dengue NS1-IgY [5 µg/ml diluted in 1% BSA] for 30 min at 37^{o}C. The plate was again washed with PBS-T and incubated with 100 µl of rabbit anti-chicken IgY-HRP [1:5000 diluted in 1% BSA] for 45 min at 37^{o}C. After another washing, TMB substrate and hydrogen peroxide [1:1] was added and absorbance was measured at 650 nm using an ELISA V_{max} kinetic microplate reader after 5 min. The wells containing capture mAbs without dengue NS1 antigen were used to determine a nonspecific adsorption.

5.2.9 Ultrasensitive ELISA

We selected anti-NS1 mAb P148.L2 as capture antibody and the chicken IgY as detecting antibody in the development of an ultrasensitive ELISA for Dengue NS1

detection. NS1 antigen optimization was done by coating microtitre plates with 100 µl of 8 µg/ml of P148.L2 mAb in PBS [pH 7.3] at 4^{o}C overnight. The plates were then washed five times with PBS-T and blocked with 2% BSA for 2 h at RT. After washing four times with PBS-T, 100 µl of serially diluted concentrations of recombinant NS1 antigen diluted in 1% BSA was added and incubated for 1 h at RT. The plate was then washed four times with PBS-T and incubated with 100 µl IgY [5 µg/ml diluted in 1% BSA] for 1 h at 37^{o}C. Detecting antibody concentration optimization was done similarly. Following blocking 100 µl of 1 µg/ml of NS1 antigen diluted in 1% BSA was added and incubated for 1 h at RT. After washing, the plate was incubated with 100 µl of different concentrations of IgY diluted in 1% BSA for 1 h at 37^{o}C. Following incubation, the wells were washed and incubated with rabbit anti-chicken IgY-HRP [100 µl of 1:5,000 diluted in 1% BSA] for 45 min at 37^{o}C. The colour was developed with TMB substrate and hydrogen peroxide [1:1] for 5 min at RT. The well containing capture mAb without antigen was used as negative control. The absorbance was measured at 650 nm using an ELISA V_{max} kinetic microplate reader. A student "*t*" test was used to analyze the significant differences [$p < 0.05$] between the samples and control.

5.3 Results

Intramuscular immunization of hens was carried out with Dengue NS1 antigen and eggs collected everyday. Activities of anti-Dengue NS1 IgY from chickens immunized with the recombinant antigen were analysed by indirect immunoassay at specified time point during immunization [Figure 5.1].

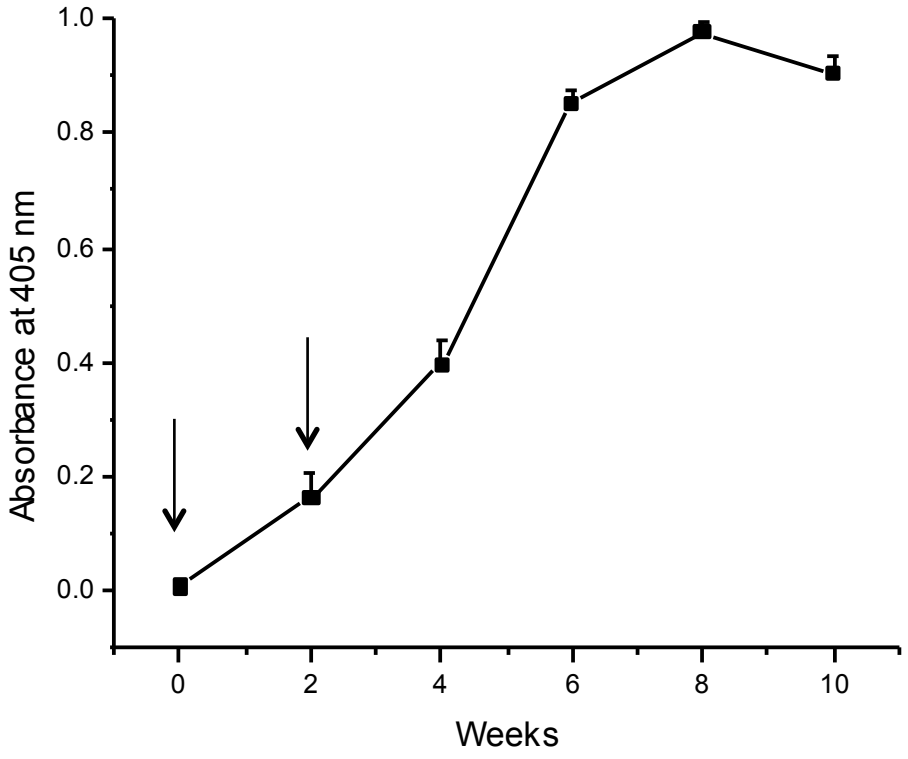


Figure 5.1. The change of specific activity of IgY in the egg yolk of chickens immunized with Dengue NS1 antigen. The level of IgY activity in a 3,000-fold dilution of IgY was measured by the ELISA using Dengue NS1 as an antigen and expressed as OD value 405 nm. Values are the mean of quadruple samples. Vertical bars indicate the standard deviation. Arrows indicate the week of immunization.

The IgY titer measured by indirect ELISA demonstrated an initial response coupled with activity enhancement. After a subsequent booster dose a rapid secondary response was observed with higher Dengue NS1 specific IgY activity at a peak of 8 weeks. This shows that high affinity anti-Dengue NS IgY could be obtained from egg yolks collected within the immunization period of 6 to 10 weeks. WSF of egg yolk was purified and analyzed for purity and specificity to NS1 antigen. The crude egg yolk IgY WSF showed only about 38-40% purity, indicating presence of contaminants such as alpha and gamma livetins, lipoproteins, fatty acid molecules, etc. Crude IgY WSF from immunized hens were again purified by ammonium sulfate precipitation and finally by Sephacryl S-300 gel chromatography. The purified IgY was then analyzed by SDS-PAGE under reducing conditions, which confirmed that the purity of IgY was greater than 95% [Figure 5.2]. The specificity of the anti-Dengue IgY was also analyzed by Western blot, strong binding between IgY and recombinant dengue NS1 protein was observed. Cross reactivity studies with recombinant Dengue envelope protein and SARS NP proteins were negative.

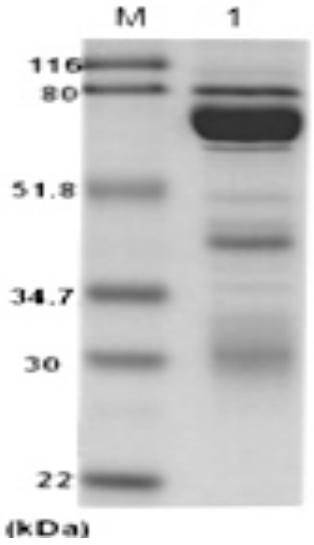


Figure 5.2. SDS-PAGE of purified IgY [Lane M: standard protein molecular weight markers, lane 1: IgY antibody]

Heterosandwich immunoassayassay was designed employing different anti-Dengue NS1 mAbs as capture antibody and IgY as detecting antibody. All the five mAbs made good heterosandwich when used as capture antibodies, with NS1 as antigen and IgY as detecting antibody with different affinities [Figure 5.3]. The best heterosandwich pairs were when P148.L1 and P148.L2 mAbs were used as capture antibodies respectively with IgY as the detecting antibody. There were no significant differences in affinities between the pairs therefore we selected P148.L2 as capture antibody and IgY as detecting antibody for the development of the ultrasensitive ELISA.

Different anti-Dengue NS1 IgY concentrations [n=4] were used for optimization of detecting antibody concentration that would be used in the ultrasenstive ELISA and also enhance assay sensitivity [limit of detection]. The optimized concentration of Dengue NS1 IgY was 2 µg/ml [Figure 5.4].

The optimized anti-Dengue NS1 IgY concentration was used to develop the ultrasensitive immunosorbent assy with NS1 antigen [in serial dilutions from 4 µg/ml to 0.12 ng/ml; n=4] with P148.L2 as capture antibody [8 µg/ml]. When 2 µg/ml of anti-Dengue NS1 IgY [as detecting antibody] was used in the assay with P148.L2 mAb as capture antibody, 0.96 ng/ml of NS1 antigen could be detected which was found to be statistically significant [$p < 0.05$] [Figure 5.5].

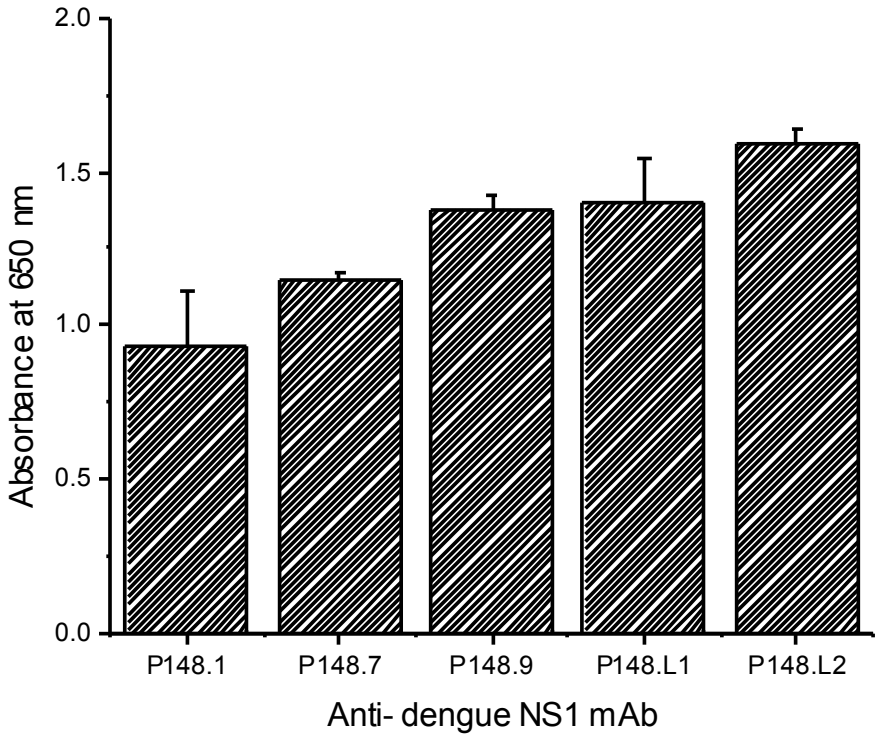


Figure 5.3 Heterosandwich ELISA with five anti-Dengue NS1 mAbs: Anti-Dengue NS1 antibodies were coated in ELISA plate. 10 μg/ml NS1 antigen was added and detected by chicken IgY. Values are the mean of quadruplet samples. Vertical bars indicate the standard deviation.

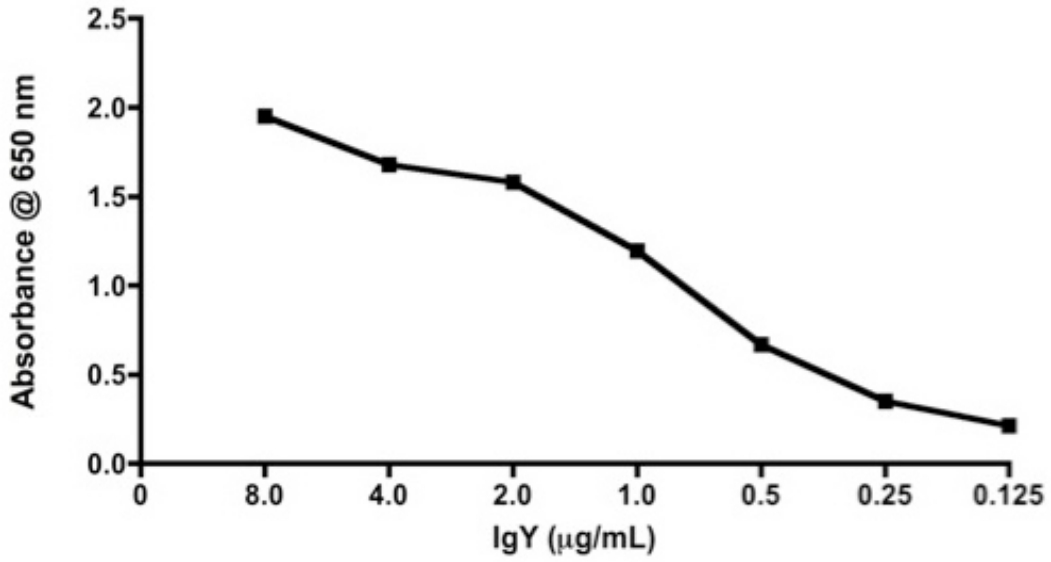


Figure 5.4 IgY optimization assay: P148.L2 mAb was coated in ELISA plate. 1 μg/ml of NS1 antigen was added and detected by different concentrations of chicken IgY. Values are the mean of quadruplet samples. Vertical bars indicate the standard deviation.

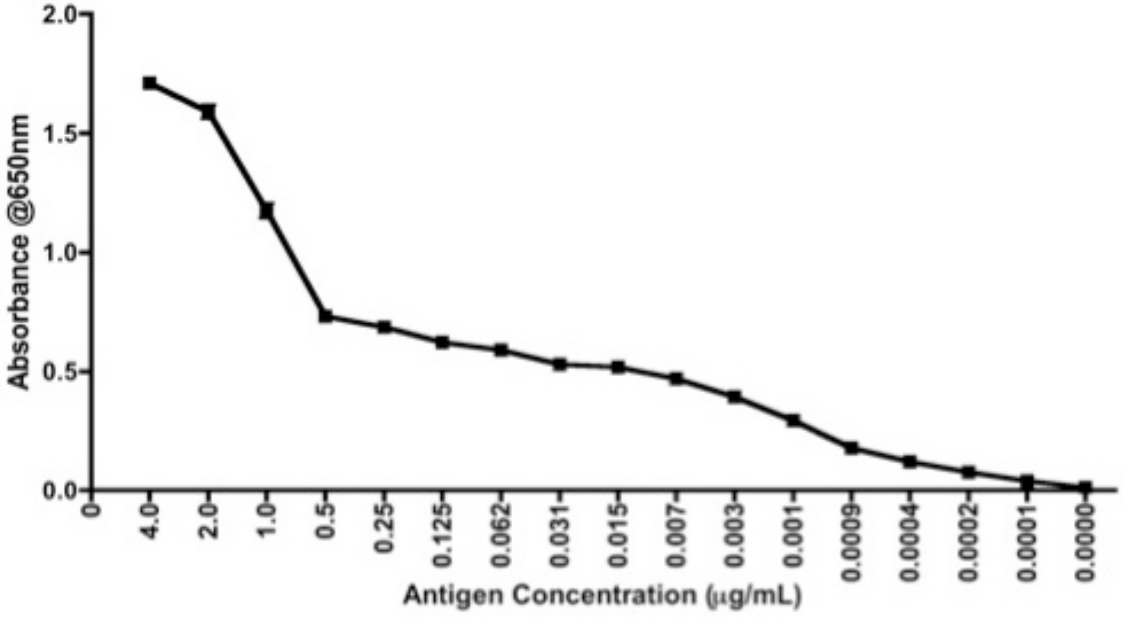


Figure 5.5 Ultrasensitive ELISA: P148.L2 mAb was coated in ELISA plate. Each serially diluted NS1 antigen [from 4 µg/ml to 0.12 ng/ml] was added and detected by chicken IgY. Values are the mean of quadruplet samples. Vertical bars indicate the standard deviation.

5.4 Discussion

In this study we have developed an ultrasensitive heterosandwich ELISA to detect dengue NS1 using both murine monoclonal antibodies and chicken IgY for the rapid and early diagnosis of dengue infection. Serological techniques are widely used for dengue detection but not sensitive enough before 5 days after infection [27]. Virus isolation procedures and PCR based detection systems are also established methods but both are extremely time intensive and expensive. It has been reported that NS1 antigen is in circulation up to day 9 after onset of fever at a concentration range of 0.04-2 μg/ml. Another report suggested that dengue type 1 specific NS1 protein could be detected even up to day 18 [28]. Therefore a low cost sensitive diagnostic for screening of dengue infection is very important. Chicken were intramuscularly immunized to generate the anti-Dengue NS1 IgY antibody as reports suggest that avian maternal antibodies are transferred from serum to egg yolk to confer passive immunity to their offspring in the form of IgY antibody. Reports confirm that intramuscular immunization in chicken results in enhanced antibody production characterized by higher specificity [29]. Chickens are also known to be more tolerant towards immunological adjuvants [14]. This technology has been widely used for development of antibodies against a variety of antigens both viral [32] and bacterial [15,26]. IgY antibodies developed are of high quality due to the phylogenetic gap between mammals and birds. IgY antibodies also have characteristic high affinity and avidity [33]. Another advantage is IgY production is more ethical as it involves daily collection of eggs and not repetitive bleeding of animals [34]. There is also significantly higher yield of chicken IgY in relatively less time is

beneficial antigen specific generation of IgY antibodies [35]. It has also been demonstrated that IgY is stable at various conditions such as heat, pressure, alkalinity, acidity [> pH 3.5] as well as in presence of proteolytic enzymes like trypsin and chymotrpsin [36]. Therefore the ability to produce high amounts of antigen specific IgY with reasonably high stability makes production of IgY against various viral antigens a viable option to be used in diagnostics [37], antibiotic-alternative therapy [38] and therapeutics [39]. Anti-Dengue NS1 IgY generated increased over time and Dengue NS1 specific IgY titer also increased peaking around the eighth week. SDS-PAGE analysis confirmed the purity of the IgY. The structure of IgY is similar to mammalian IgG consisting of two heavy and two light chain but IgY has a mass of of 180 KDa that is higher than the IgG molecule [150 KDa]. The higher mass of IgY is due to a higher number of heavy chain constant domains and carbohydrate chains [15]. The presence of IgY was also analyzed by Western blot [data not shown]. The ultrasensitive IgY based sandwich immunoassay was able to detect upto 48 pg /well or 0.48 ng/ml of dengue NS1 antigen. This sensitivity is higher than comparable assays routinely used for the diagnosis of dengue infection but the advantage lies in its low cost and abundant supply of diagnostic IgY with high specificity. The combination of mAbs and IgY could potentially be an essential diagnostic tool for the better management of dengue fever globally.

5.5 Conclusion

In this study we have developed Dengue diagnostic tool by heterosandwich ELISA for detection of NS1 antigen using high affinity mAb and IgY [chicken egg yolk polyclonal antibody] for antigen detection with a limit of detection of 0.48 ng/ml or 48 pg /well of recombinant dengue NS1 antigen. This sensitive immunoassay being much more economical can potentially be replicated for the diagnoses of other infectious diseases. IgY based detection of Dengue NS1 antigen developed in the laboratory could be an effective alternative for screening purposes during a Dengue outbreak.

5.6 References

1. Ganguly A, Malabadi RB, Loebenberg R, Suresh MR, Sunwoo HH. Dengue diagnostics: current scenario. Res Biotech.2013a;4[2]:19**e**25.

2. Gubler, D. J., Clark, G. G. 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerging Infectious Diseases 1, 55–57

3. Ganguly A, Malabadi RB, Loebenberg R, Suresh MR, Sunwoo HH. A mini-review of dengue vaccine development. Res Pharm. 2013b;3[2]:18**e**25.

4. Falconar, A., Young, K. I., Young, P. R. 1991. Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 non- structural glycoprotein NS1. Journal of General Virology 72, 961–965

5. Falconar, A., Young, K. I., Young, P. R. 1990. Immunoaffinity purification of the native dimer forms of the flavivirus non-structural glycoprotein, NS1. Journal of Virological Methods 30, 323-332

6. Young PR, Hilditch PA, Bletchly C, Halloran W. 2000. An antigen capture enzyme linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol. 38:1053-1057

7. Ramirez AH, Moros Z, Comach G, Zambrano J, Bravo L, Pinto B, Vielma S, Cardier J, Liprandi F. 2009. Evaluation of dengue NS1 antigen detection tests

with acute sera from patients infected with dengue virus in Venezuela. Diag. Microbiol. Infect. Dis. 65:247–253

8. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. 2002. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencingprimary or secondary infections. J Clin Microbiol. 40: 376-381

9. Osorio L, Ramirez M, Bonelo A, Villar LA, Parra B. Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection. Virol J. 2010; 7:361.

10. Zainah S, Wahab AH, Mariam M, Fauziah MK, Khairul AH, Roslina I, Sairulakhma A, Kadimon SS, Jais MS, Chua KB. 2009. Performance of a commercial rapid dengue NS1 antigen immunochromatography test with reference to dengue NS1 antigen-capture ELISA. J Virol Meth. 155: 157-160

11. Malabadi, R. B., Ganguly, A., Sunwoo, H. H., Suresh, M. R. 2012. Role of bi-specific monoclonal antibodies in immunodiagnostic assay. Res. Pharm. 2[3]: 08-14

12. Malabadi RB, Ganguly A, Teixeira da Silva JA, Parashar A, Suresh MR, Sunwoo HH. 2011 Overview of plant-derived vaccine antigens: Dengue virus. J Pharm and Pharmaceut Sc. 14[3]:400-413

13. Shu PY, Chen LK, Chang SF, Su CL, Chien, Chin C, Lin TH, Huang JH. 2004. Dengue Virus serotyping based on envelope and membrane and nonstructural protein NS1 serotype specific capture immunoglobulin M enzyme linked immunosorbent assays. J. Clin. Microbiol. 42[6]: 2489-2494

14. Sudjarwo SA, Sudjarwo KE, Koerniasari, 2012. Purification and characterization protein of anti- dengue specific immunoglobulin Y for diagnostic kit of dengue. J Appl Pharm Sci. 2[12]: 007-012

15. Palaniyappan A, Das D, Kamilla S, Suresh M. R., Sunwoo H. H. 2012. Diagnostics of Severe acute respiratory syndrome-associated coronavirus [SARS-CoV] nucleocapsid antigen using chicken Immunoglobulin Y. Poultry Sci, 91 : 636–642

16. Xiao, Y., X. Gao, G. Gannot, M. R. Emmert-Buck, S. Srivastava, P. D. Wagner, M. D. Amos, and P. E. Barker. 2008. Quantitation of HER2 and telomerase biomarkers in solid tumors with IgY antibodies and nanocrystal detection. Int. J. cancer 122: 2178-2186.

17. Sunwoo, H. H., W. W. Wang, and J. S. Sim. 2006. Detection of *Escherichia coli* O157:H7 using chicken immunoglobulin Y. Immunol. Lett. 106:191-193.

18. Terzolo, H. R., V. E. Sandoval, M. I. Caffer, R. Terragno, and A. Alcain. 1998. Agglutination of hen egg-yolk immunoglobulins [IgY] against *Salmonella enterica*, serovar enteritidis. Rev. Argent Microbiol. 30:84-92.

19. Lee, E. N., Sunwoo, H. H., Menninen, K. and Sim, J. S. 2002. In vitro studies of chicken egg yolk antibody [IgY] against Salmonella enteritidis and Salmonella typhimurium. Poult. Sci. 81:632–641.

20. Kammila S, Das D, Bhatnagar PK, Sunwoo HH, Zamora GZ, King M, Suresh MR. 2008. A rapid point of care immunoswab assay for SARS-CoV detection. J Virol Meth. 152: 77-84

21. Das D, Mongkolaungkoon S, Suresh MR. 2009. Super induction of dengue virus NS1 in *E.coli*. Prot Exp Purif. 66: 66-72

22. Shahhosseini, S., Das, D., Qiu, X., Feldmann, H., Jones, S. M., Suresh, M. R. 2007. Production and characterization of monoclonal antibodies against different epitopes of Ebola virus antigens. Journal of Virological Methods 143, 29-37

23. Sunwoo, H. H., T. Nakano, W. T. Dixon, and J. S. Sim. 1996. Immune responses in 453 chickens against lipopolysaccharide of Escherichia coli and *Salmonella typhimurium*. Poult. Sci. 75: 342-345.

24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

25. Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350–4354

26. Sunwoo, H. H., Lee, E. N., Menninen, K., Suresh, M. R. and Sim, J. S. 2002. Growth inhibitory effect of chicken egg yolk antibody [IgY] on Escherichia coli O157:H7. J. Food Sci. 67:1486–1494.

27. Dussart P, Labeau B, Lagathu G, Louis P, Nunes MR, et al. [2006] Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. Clin Vaccine Immunol 13: 1185–1189

28. Xu, H., B. Di, Y. X. Pan, L. W. Qiu, Y. D. Wang, W. Hao, L. J. He, K. Y. Yuen, and X. Y. Che. 2006. Serotype 1-specific monoclonal antibody-based antigen capture immunoassay for detection of circulating nonstructural protein NS1: implications for early diagnosis and serotyping of dengue virus infections. J. Clin. Microbiol. 44:2872-2878.

29. Hirai K, Arimitsu H, Umeda K, Yokota K, Shen L, Ayada K. 2010. Passive oral immunization by egg yolk immunoglobulin [IgY] to Vibrio cholera effectively prevents cholera. Acta Med Okayama. 64:163-170

30. Liou JF, Chang CW, Tailiu JJ, Yu CK, Lei HY, Chen LR. 2010. Passive protection effect of chicken egg yolk immunoglobulins on enterovirus 7 infected mice. VaCCINE. 28: 8189-8196

31. Ibrahim el, S. M., A. K. Rahman, R. Isoda, K. Umeda, N. Van Sa, and Y. Kodama. 2008.*In vitro* and *in vivo* effectiveness of egg yolk antibody against *Candida albicans* [anti-CA IgY]. Vaccine 26: 2073-2080

32. Piela, T. H., C. M. Gulka, V. J. Yates, and P. W. Chang. 1984. Use of egg yolk in serological test [ELISA and HI] to detect antibody to Newcastle disease, infectious bronchitis, and Mycoplasma gallisepticum. Avian Dis. 28:877-883.

33. Krief A, Leteson JJ, Billen D. 2002. Comaprison between IgY technology from chickens and IgG technology from mice for production of tailor-made antibodies. Tetrahedron Lett. 43: 1843-1846

34. Tini M, Jewell UR, Camenish G, Chilov D, Gassmann M. 2002. Generation and Application of chicken egg-yolk antibodies. Comp Biochem Physiol A Mol Integr Physiol. 131: 569-74

35. Schade, R., W. Burger, T. Schoneberg, A. Schniering, C. Schwarzkopf, A. Hlinak, and H. Kobilke. 1994. Avian egg yolk antibodies. The egg laying capacity

of hens following immunisation with antigens of different kind and origin and the efficiency of egg yolk antibodies in comparison to mammalian antibodies. Altex 11:75-84.

36. Shimizu, M., H. Nagashima, K. Sano, K. Hashimoto, M. Ozeki. K. Tsuda, and H. Hatta. 1992. Molecular stability of chicken and rabbit immunoglobulin G. Biosci. Biotechnol. Biochem. 56:270-274.

37. Erhard, M. H., P. Schmidt, P. Zinsmeister, A. Hofmann, U. Munster, B. Kaspers, K. H. B. Wiesmuller, W. G. Bessler, and M. Stangassinger. 2000. Adjuvant effects of various lipopeptides and interferon-gamma on the humoral immune response of chickens. Poult. Sci. 79:1264-1270.

38. Carlander, D., H. Kollberg, P. E. Wejaker, and A. Larsson. 2000. Peroral immunotherapy with yolk antibodies for the prevention and treatment of enteric infections. Immunol. Res. 21:1-6.

39. Schade, R., X. Zhang, and H. Terzolo. 2007. In Use of IgY antibodies in human and veterinary medicine. Pages 213-222 in Bioactive Egg Compounds. Ed. R. Houopalahti, R. Lopez-Fandino, M. Anton, and R. Schade. Springer-Verlag: Berlin Heidelberg

6

HETEROSANDWICH IMMUNOSWAB ASSAY FOR DENGUE VIRUS NS1 ANTIGEN DETECTION

6.1 Introduction

Dengue is a mosquito borne disease caused by any of the four known serotypes of the dengue virus [DENV1-4]. According to the World Health Organization [WHO], around 100 million cases of dengue occur each year, and among them about half a million result in the severe Dengue haemorrhagic fever [DHF] [1-4]. Dengue fever and its significantly fatal variants, DHF and Dengue Shock Syndrome [DSS] are becoming very important public health concerns, mostly in the emerging economies thereby threatening development [2-6]. Dengue fever, DHF, and DSS represent a spectrum of disease resulting from infection with the dengue virus, which is transmitted primarily by the mosquito, *Aedes aegypti*. The spectrum of clinical illness is various from mild symptoms such as fever to death [9]. Dengue virus infection is clinically similar to many other acute febrile illnesses, in which serological testing plays an important role in early diagnosis and management [4, 7]. DHF and DSS, which are the more severe clinical manifestations of the disease, occur more commonly in individuals with a second or subsequent infection [8], and thus early laboratory diagnosis may be of prognostic value. Routine serological diagnosis relies on ELISA or hemagglutination inhibition [HI] based on the detection of dengue-specific IgM antibodies during the acute phase of infection [9]. However, anti dengue antibodies of the IgM class develop rather slowly. So during a primary dengue infection, antibodies detected by any of the prevalent methods like HI or neutralization assays are sluggish and also have low titers [22]. A rapid and sensitive immunoassay needs to detect Dengue NS1 which is nonstructural protein that localizes on the surface of Dengue virus infected cells and is released into the circulatory system. NS1 antigen half-life in blood has been found to be much higher than that of the virus

perse and has been detected in sera till about 18 days after symptoms occur [23]. Another major feature is serotype specific anti NS1 antibodies does not react with other dengue serotypes or even other flavivirus NS1 proteins [24].

Recently, bsMAbs that play an important role in modern medicine have been exploited in therapeutic applications including vaccine development, and gene therapy [10, 11]. BsMAbs were used in the development of immunoassays for diagnosis of bacterial and viral infectious diseases [12, 13]. The development of quadromas as bifunctional entities possessing two binding sites for dengue NS1 and an enzyme, horseradish peroxidase would help reduce diagnosis time significantly. The immunoswab assay based on bsMAbs, the first of its kind against dengue NS1, makes it a useful tool in the development of specific diagnostics against dengue NS1 with clean backgrounds along with sensitivity and rapidity. This articles reports bsMab based immunoswab assay in two different formats, one being a direct detection of NS1 antigen using a bsMAb and second is the heterosandwich assay for NS1 diagnosis. Both the formats are easy to use, sensitive, rapid and inexpensive.

6.2 Materials and Methods

6.2.1 Cell lines

Mouse hybridoma cell line P148.L1 was used to produce monoclonal antibody for the NS1 protein of dengue virus. The YP4 was a generous gift of the late Dr. C. Milstein, Laboratory of Molecular Biology, Medical Research Council, Cambridge, United Kingdom. The quadroma P156, a fuse with P148.L1 hybridoma producing anti-NS1 monoclonal antibody and a rat hybridoma YP4 producing an anti-HRPO mAb.

6.2.2 Materials

Fetal bovine serum [FBS] was obtained from PAA cell culture company [Ontario, Canada], streptomycin–penicillin–glutamine [PSG] and cell culture media RPMI was obtained from Gibco [NY, USA]. Bovine serum albumin [BSA], Goat anti-mouse IgG HRPO, horseradish peroxidase [Type VI], Protein G-agarose, m-amino phenyl boronic acid [m-APBA] agarose, was purchased from Sigma [St. Louis, MO, USA]. Streptavidin tagged HRPO was obtained from BD Biosciences [California, USA] Hyperflask^{TM} cell culture vessel was obtained from Corning Inc [NY, USA]. Tetramethylbenzidine [TMB] was obtained from BioFx Laboratory [North Carolina, USA]. Sterile swab with tip flocked with nylon fiber was obtained from micro Rheologics [Brescia, Italy]. Slide-A-LyzerR for dialysis was obtained from Pierce [Rockford, IL, USA]. Pooled rabbit serum was procured from Health Sciences Laboratory Animal Services [HSLAS], University of Alberta.

6.2.3 Protein preparation

Full-length dengue NS1 was expressed in *E.coli* and purified as illustrated previously [14]. The purified NS1 was used for antibody generation as well as an antigen for the development of the immunoswab assays.

6.2.4 Hybridoma culture and purification

P148.L1 cell bank was taken from liquid nitrogen storage, and thawed with RPMI media comprising of 5% FBS and 1% PSG. Subsequently the cells were grown in the above media at 37ºC and 5% CO_2 for anti-NS1 antibody production. On achievement of steady growth rate and confirming activity with ELISA, cells were expanded in a Hyperflask^{TM}. After 7 days, the supernatant was harvested at 7900 rpm for 20 mins at 4ºC. Following centrifugation, the supernatant was passed through a protein G column to obtain purified antibody using established procedure [15]. The eluted antibody was dialysed in PBS overnight at 4ºC. The integrity and activity of the purified hybridoma was determined by SDS-PAGE and Western Blot.

6.2.5 Development of anti-dengue NS1/anti-HRPO quadromas

The production of anti-dengue NS1/anti-HRPO quadromas involved maintaining the two hybridoma cell lines in logarithmic growth phase containing RPMI medium with 5% FBS at 37ºC supplemented with 5% CO_2. A stock solution of tetramethyl rhodamine isothiocyanate [TRITC, 0.5 mg/mL] or fluorescein isothiocyanate [FITC, 0.5 mg/mL] was diluted in 1:5 ratios to be used as the working solution. The following steps as reported earlier were then followed for successful completion of a quadroma fusion [Das and Suresh, 2005; Tang et al., 2004]. Briefly, 2×10^7 cells/ mL of anti-dengue NS1

hybridomas and YP4 hybridomas were separately resuspended in RPMI pH 7.4 and 6.8, respectively. Anti-dengue NS1 hybridomas were then labelled with TRITC [red fluorescence] and YP4 cells were labeled with FITC [green fluorescence]. Following 30 min incubation at 37 ºC in a 5% CO_2 incubator, the cell suspensions were washed and mixed in a 50 mL tube and centrifuged at 459 × *g* for 7 min. Two mL of PEG was added to the cell pellet slowly over a period of 2 min, with gentle mixing. Following addition of PEG, the cell suspension was incubated at 37ºC for 3 min. Twenty mL of serum free RPMI medium was added to dilute the toxic effects of PEG. Flow cytometry [FACSAria, Becton Dickinson] with an argon ion 488 nm air cooled laser [Dept of Medical Microbiology and Immunology, University of Alberta] was used to sort cells [fig 6.1] with dual fluorescence and seeded at 1 cell/well in 96-well sterile culture plates with RPMI containing 20% FBS. The plates were incubated at 37 ºC with 5% CO_2. The clones were screened twice by bridge ELISA. The best clones were subject to recloning to select strong positive and highly specific quadroma secreting bsMAbs against dengue NS1.

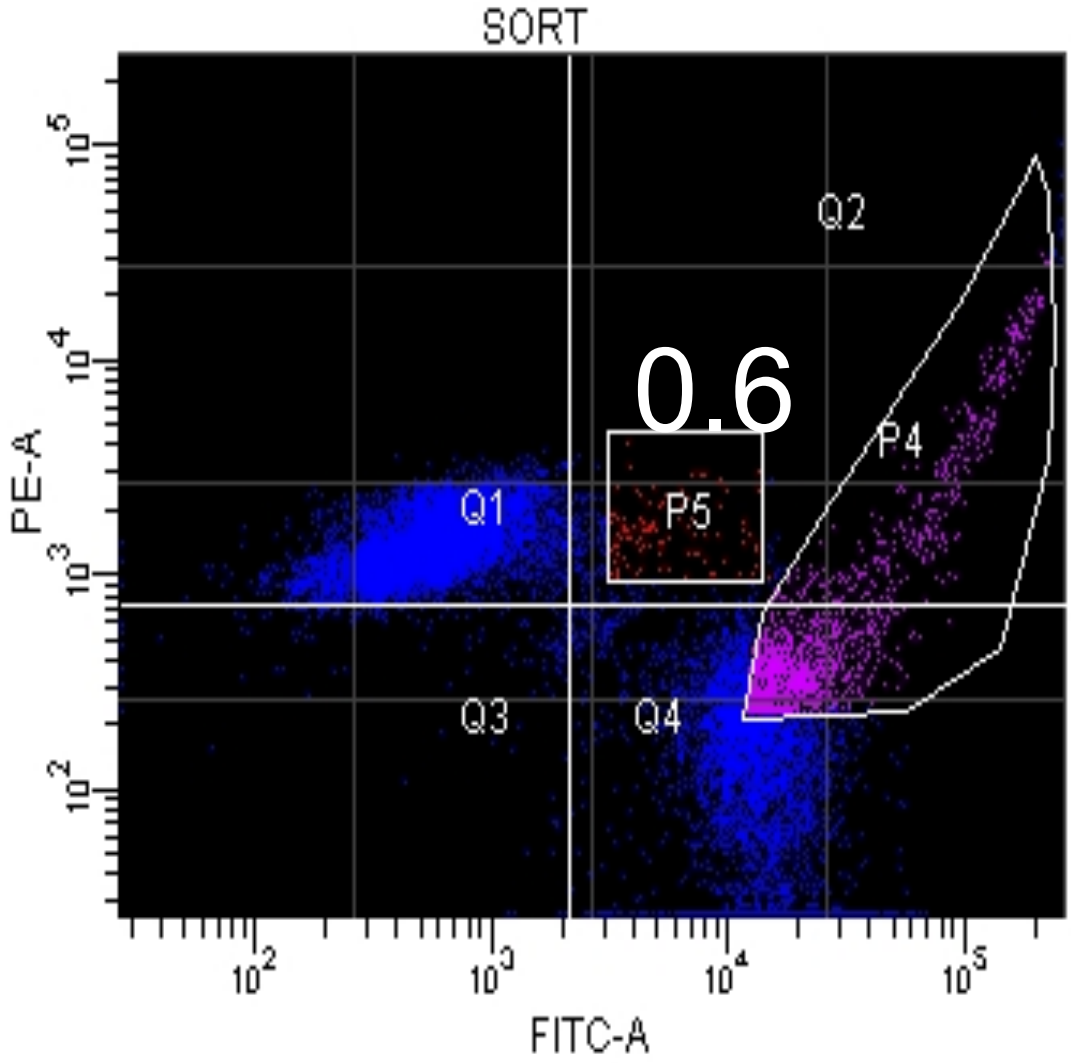


Fig 6.1 FACS analyses after PEG fusion of the two fluorescent labelled hybridomas. The dot plot analysis performed after PEG fusion shows the number of double-fluorescent cells [Area P5]. The double-positive cells from area P5 were sorted at one cell per well.

6.2.6 Screening method to detect quadromas secreting bsMAb [bridge ELISA]

The 96-well plate was coated with 5 µg/mL purified dengue NS1 protein overnight at 4ºC. Non specific sites were blocked with 2% Bovine Serum Albumin [BSA] at 37ºC for 2 h. The plate was then washed three times with Phosphate Buffered Saline – 0.05% Tween 20 [PBS-T]. 100 ìl of various quadroma cell culture supernatants was serially diluted with PBS-BSA and added followed by incubation for 1 h at 37 ºC. 100 µL of 10 ìg/mL [diluted in 2% DBSA] HRPO was added to the microtiter plate followed by washing with PBS-T three times. 100 ìL of TMB substrate was finally added to the wells. Positive quadromas secreting bsMAb were selected after 15 min of color development, measured at 650 nm at V_{max} ELISA plate reader. PBS was used as blank and negative controls were wells not having dengue NS1 antigen.

6.2.7 Quadroma culture and purification

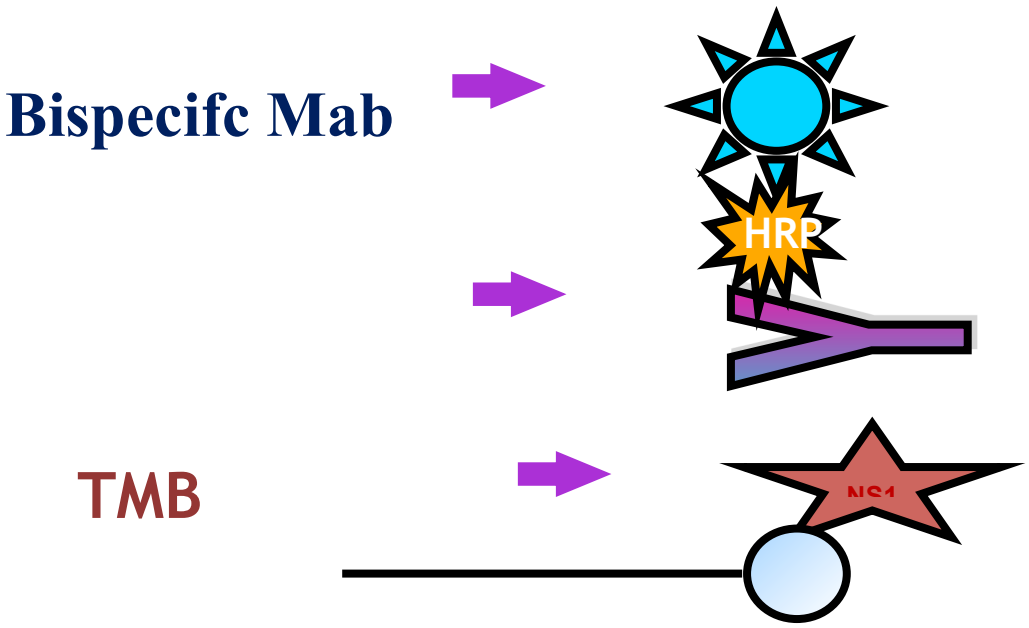
P156 cell line was removed from liquid nitrogen tank and quickly thawed employing aforementioned protocol. Cell line was subject to Hyperflask™ expansion for large quantities. After around 7-8 days, the supernatant was collected and centrifuged at 7900 rpm for 20 min at 4ºC. The supernatant was further filtered to remove any unwanted junk. Following this a new two stage purification procedure established in our laboratory was adopted [16]. After passing the supernatant through the protein G column, it was subject to another passage through a HRPO saturated APBA column which would bind to the quadroma as well as the anti-HRPO monoclonal antibody. Final elution of the bsMAb was done with 0.1M sorbitol containing phosphate buffer after several steps of washing.

The purified antibody was dialysed in PBS and analyzed by ELISA and SDS-PAGE for determination of functionality and integrity.

6.2.8 Immunoswab assay design and antigen spiking in different matrices

Immunoswab assay involved the use of nylon fiber tipped swabs along with a plastic robust shaft as previously established in our laboratory [15, 17]. The sharp end of the swab was used to coat the capture antibody or the antigen depending on the format [Figure 6.2] and subsequently used in antigen detection as discussed in the following sections. Dengue NS1 antigen was spiked in two different matrices, normal saline [pH 7.3–7.4] and naïve rabbit serum.

[A]



[B]

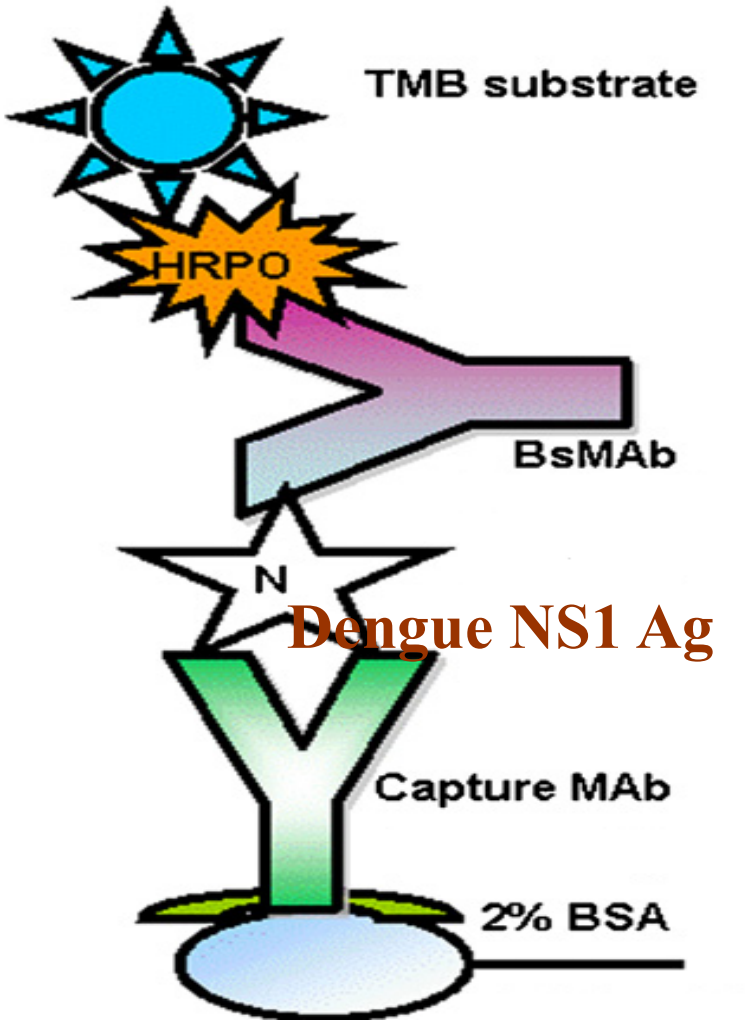


Fig 6.2 bsMAb based immunoswab assay format, [A] bsMAb based direct detection of dengue NS1, [B] bsMAb based heterosandwich assay for dengue NS1 detection

6.2.9 Direct detection of dengue NS1 using bsMAb

The swabs were spiked with different concentrations of NS1 antigen in saline and rabbit serum [50 µL]. The immunoswabs were then incubated at room temperature for 20 min, fixed with 50 µL of 95% ethanol for 5 min and dried. The swabs were finally blocked with 5% dialyzed BSA [DBSA] for 30 min. Control swabs without antigen spiking was also processed. The swabs were finally washed extensively with PBS-tween multiple times by simple fill-and-aspiration steps [15]. Subsequently, the swabs were incubated with bsMAb [5 µg / mL] for 30 mins. Following this, the swabs were again washed as mentioned above and incubated with TMB [50 µL] for color development.

6.2.10 bsMAb based heterosandwich assay for dengue NS1 detection

The nylon tipped swabs were coated with MAb [50 µL], and incubated at room temperature for 20 min. Then it was dried for 5 mins and fixed with 95% ethanol. Following fixation, the swabs were blocked with 5% DBSA for 30 min. The swabs were then washed with PBS-tween, and incubated for 20 min at room temperature with different concentrations of NS1 antigen spiked in different matrices. Next, the swabs were washed again as mentioned before and incubated with the detecting bsMAB for 30 min. After this the swabs were washed again and incubated with TMB for visual end point.

6.2.11 Stability of immunoswab

Stability studies with the immunoswabs were also carried out at three different temperatures [RT, 4ºC and - 20º C] and at 1, 2 and 4-week time points.

6.3 Results and Discussion

There is an urgent requirement for a rapid, inexpensive, and simple test for the detection of dengue virus, which result in huge human and economical suffering. Current immunosassay is based on IgM sera detection for dengue viral infection, however, in acute primary dengue virus infection, production of dengue IgM antibodies delays about 5-7 days after the dengue viral infection [18]. Therefore, insufficient levels of the antibody against dengue NS1 antigen during the early period of infection may result in false negative diagnosis. Since dengue NS1 is known to circulate in the system during acute phase of infection, it can be measured by antigen capture assays [18, 19]. In the present study, recombinant dengue NS1 antigen was chosen for developing an immunoswab assay to diagnose dengue virus infection.

The immunoswabs developed in two different formats consist of [1] being direct detection of antigen and [2] a heterosandwich assay. The direct detection of NS1 involved coating of the swabs with various concentrations of antigen and detecting was done with the bsMAb. In the direct immunoswab assay the swabs were incubated with various concentration of NS1 followed by detection with the bsMAb [P156]. The diagnostic time is considerably less in this format as the number of steps involved is greatly reduced from a traditional monoclonal based sandwich assay The limit of detection [LOD] was found to be different in saline and serum. The sensitivity of direct immunoswab assay was higher in saline [7.5 pg/swab or 150 pg/mL] [fig 6.3B] compared to 12.5 pg/swab or 250 pg/mL [fig 6.3A] when antigen was spiked in serum. Yet another factor is, when used in clinical settings or ports of entry, nonavailability of capture

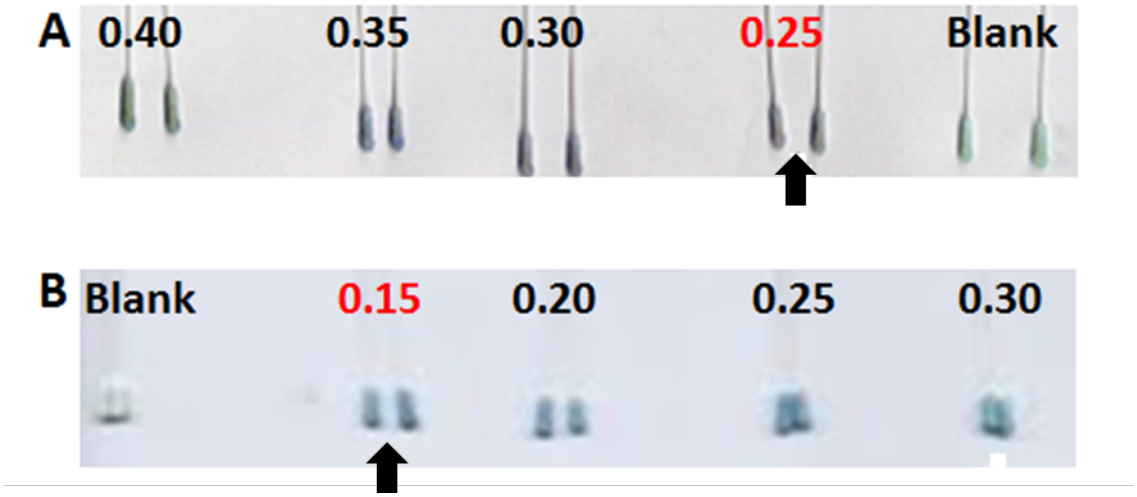


Fig 6.3 Anti-dengue NS1/anti-HRPO BsMAb immunoswab assay for detection of Dengue NS1, [A] in rabbit serum, [B] in saline. [↑] Limit of detection

antibody coated swabs would not be a hindrance in conducting assays particularly in urgent situations.

In the heterosandwich based immunoswab assay, the sensitivity was even higher. The LOD in saline was 15 pg/mL or 0.75 picogram per swab [fig 6.4B]. In serum, the sensitivity was 1 picogram per swab or 20 pg/mL [fig 6.4A]. BsMAbs generally have higher sensitivity owing to their greater molecular uniformity [20]. The assays defined above were done in duplicates and repeated three times and results were in accordance each time.

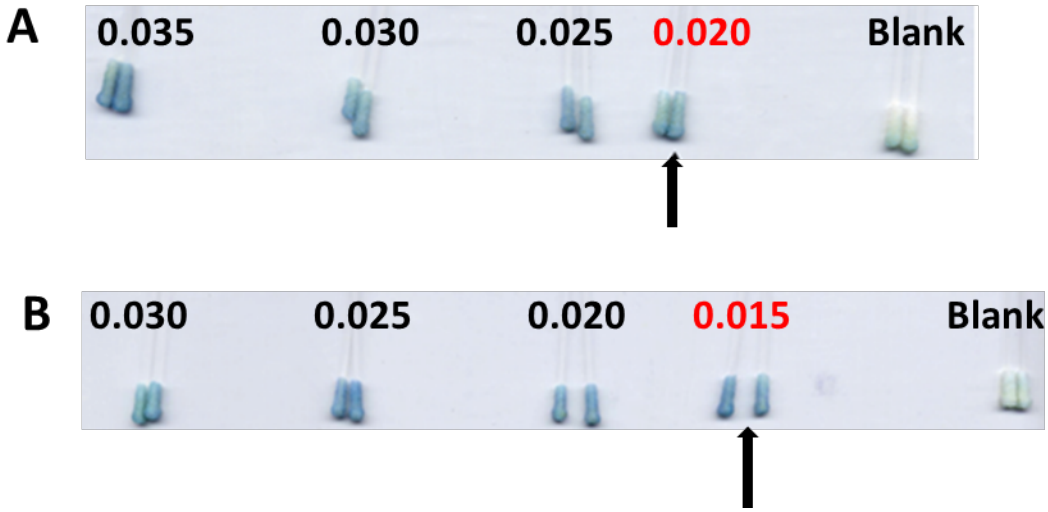


Fig 6.4 Anti-dengue NS1/anti-HRPO BsMAb heterosandwich immunoswab assay for detection of Dengue NS1, [A] in rabbit serum, [B] in saline. [↑] Limit of detection

The swabs were further analyzed for their stability and shelf life. 1, 2 and 4 week time periods were selected to understand the shelf-life under different temperature conditions. We selected the 1 pg/ swab as a reference to analyze the stability, which is also LOD for the heterosandwich immunoswab assay. The swabs remained constant and sensitive at all the different time points and temperature parameters. We suggest the swabs can be stored for further periods with good sensitivity, if it can be kept at sub zero conditions [15]. In certain dengue endemic countries where access to electricity is an issue maintaining sub zero temperatures for storage is challenging. Our immunoswabs have maintained good sensitivity when stored in room temperature upto 4 weeks. Storing the immunoswabs in iceboxes can be an alternative for prolonging sensitivity in developing countries.

The limit of detection for recombinant NS1 antigen using bsMAB as the detecting antibody was in picogram levels. The immunoswab assay involved rapid diagnosis of the NS1 antigen spiked in two matrices, saline and serum. This form of infection analysis of NS1 antigen was performed to essentially mimic actual screening of suspected individuals where minimally trained persons can perform the assay during a future dengue epidemic. The estimated time to perform the assay with the both the bsMAb based direct detection and the double antibody based heterosandwich assay format was much less than an hour. Another important factor is the level of labeling of bsMAb antibodies with HRPO during the purification step is potentially consistently one to one, which lessens batch variations and enhances overall assay reliability [17].

The first of its kind dengue immunoswab assay illustrated in this chapter is simple, rapid and easy to perform. Diagnostic end points can be obtained very fast in

about an hour's time. The assay can be performed by minimally trained personnel which is an advantage. The diagnostic end point can be analysed visually and expensive machinery is not essential. This particular assay can be the basis of an ideal detection system for a fatal infection like dengue and has all the attributes essential primarily for the developing world [21].

Therefore, the non structural protein NS1 as a biomarker is a very suitable diagnostic marker, which can be efficiently measured during the initial phase of the disease. The immunoswab assay is significant in measuring picogram levels of NS1 spiked in serum, indicating the potential use of the assay in public health. The use of highly specific bsMAb with negligible batch variations has the ability to do away with problems such as false negatives in case of prevalent antibody detection assays. Also UV based assays, PCR and virus isolation techniques are labor intensive and extremely costly. Antibodies with enzyme tags are used extensively in biochemical and immunochemical applications. The enzyme is generally labelled to a monoclonal antibody or a polyclonal antibody by a covalent linker [20]. This chemical cross-linking method can be a problem owing to variability and partial inactivation of the enzyme or the antibody; competition from unreacted entities which could lead to low specific activity; less shelf life; and intra batch variations in the sizes and properties of the conjugate [17]. Cross-linked large protein aggregates may induce nonspecific binding, resulting in noise enhancement in the assay. bsMAbs are bifunctionally engineered antibodies having two different antigen-binding sites in a single antibody molecule. Hence, use of our quadroma has resulted in the development of a novel first of its kind designer bsMAb nanoprobe that has one antigen-binding site capable of binding to

dengue NS1 and the other capable of binding to an enzyme [HRPO]. As the assay is fast the use of immunoswab-based dengue NS1 diagnosis during an outbreak or an epidemic will not cause unnecessary inconvenience to people. This kind of assay can significantly lower people's paranoia, anxiety and worry during dengue pandemics. The immunoassay can also be used in areas where sufficient health care is unavailable. Another significant advantage of this novel diagnostic is its extremely low cost/low tech approach. Although the assay demands a couple of washing (fill and aspirate) and incubation steps, we believe that can be easily done by the personnel with minimal technical training. Furthermore, the cost of the reagents required to perform the assay is less than 1.00 CAD per swab (cost analysis not shown) The use of immunoswab does not need sophisticated technology or highly skilled persons to carry out the test. Any person with minimal training can conduct this test in a primary healthcare or rural setting. It will not be a economic burden to people in developing countries who have to invest in expensive diagnostic procedures in case of pandemics and outbreaks. This assay is very beneficial in parts of the globe where unavailability of sophisticated and expensive technology can be a deterrent in diagnosis of Dengue and several other viral infections.

Future effort would include more optimization of the assay, use of clinical samples from dengue infected patients for analyses. More optimized development of the assay could allow dengue screening to be done in a physician's office, hospitals and healthcare facilities as well as ports of entry to monitor outbreaks of dengue as well as early diagnosis for therapeutic intervention. We would also develop similar immunoswab assays for other dengue serotypes.

6.4 References

1. Ramirez AH, Moros Z, Comach G, Zambrano J, Bravo L, Pinto B, Vielma S, Cardier J, Liprandi F. 2009. Evaluation of dengue NS1 antigen detection tests with acute sera from patients infected with dengue virus in Venezuela. Diag Micro and Infect Dis. 65:247–253

2. Gubler DJ, Meltzer M. 1999. Impact of dengue/dengue haemorrhagic fever on the developing world. Adv Virus Res. 53: 35–70

3. Malabadi RB, Parashar A, Ganguly A, Suresh MR. 2010. Expression of Dengue virus envelope protein in a different plant system, Faculty Research and Development day, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada: Pg 31.

4. Malabadi RB, Ganguly A, Teixeira da Silva JA, Parashar A, Suresh MR, Sunwoo HH. 2011 Overview of plant-derived vaccine antigens: Dengue virus. J Pharm and Pharmaceut Sc. 14[3]:400-413

5. Gibbons RV, Vaughn DW. 2002. Dengue: an escalating problem. Br Med. J324:1563-1566

6. Zainah S, Wahab AH, Mariam M, Fauziah MK, Khairul AH, Roslina I, Sairulakhma A, Kadimon SS, Jais MS, Chua KB. 2009. Performance of a commercial rapid dengue NS1 antigen immunochromatography test with reference to dengue NS1 antigen-capture ELISA. J Virol Meth. 155: 157-160

7. Gubler DJ. 1998. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 11[3]: 480-496

8. Halstead SB. 2002. Dengue. Curr Opin Infect Dis. 15: 471-476

9. Shu PY, Huang JH. 2004. Current advances in dengue diagnosis. Clin Diag Lab Immun. 11[4]: 642-650

10. Khan SR, Ganguly A, Malabadi RB, Sunwoo HH Archana Parashar, Teixeira da Silva JA, Suresh MR. 2011. Targeting strategies and nanocarriers in vaccines and therapeutics. Res Biotech. 2[6]:08-20

11. Khan SR, Ganguly A, Malabadi RB, Sunwoo HH, Suresh MR. 2012. Gene delivery system: A developing arena of study for new era of medicine. DNAG 6[1]: 2-9

12. Das D, Kammila S, Suresh MR. 2010. Development, characterization and application of monoclonal antibodies against severe acute respiratory syndrome coronovirus nucleocapsid protein. Clin Vac Immun. 17[12]: 2033-2036

13. Parashar A, Sarkar S, Ganguly A, Sharma S, Suresh MR, Bispecific antibodies for diagnostic applications. 2011. Bispecific Antibodies. Ed Dr. Kontermann RE. ISBN-978-3-642-20909-3, Springer Heidelberg Dordrecht London New York: 349-358.

14. Das D, Mongkolaungkoon S, Suresh MR. 2009. Super induction of dengue virus NS1 in *E.coli*. Prot Exp Purif. 66: 66-72

15. Kammila S, Das D, Bhatnagar PK, Sunwoo HH, Zamora GZ, King M, Suresh MR. 2008. A rapid point of care immunoswab assay for SARS-CoV detection. J Virol Meth. 152: 77-84

16. Bhatnagar PK, Das D, Suresh MR. 2008. Sequential affinity purification of peroxidase tagged bispecific anti-SARS-CoV antibodies on phenylboronic acid agarose. J Chromatogr B: Anal, Technol, Biomed, Life Sci. 863: 235-241

17. Tang XL, Peppler MS, Irvin RT, Suresh MR. 2004. Use of bispecific antibodies in molecular Velcro assays whose specificity approaches the theoretical limit of immunodetection for *Bordetella pertussis*. Clin Vac Immun. 11[4]: 752-757

18. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. 2002. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencingprimary or secondary infections. J Clin Microbiol. 40: 376-381

19. Young PR, Hilditch PA, Bletchly C, Halloran W. 2000. An antigen capture enzyme linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J Clin Microbiol. 38:1053-1057

20. Sarkar S, Tang XL, Das D, Spencer JS, Lowary TL, Suresh MR. 2012. A bispecific antibody based assay shows potential for detecting tuberculosis in resource constrained laboratory settings. *PLoS ONE.* 7[2]: e32340. doi:10.1371/journal.pone.0032340.

21. Mabey D, Peeling RW, Ustianowski A, Perkins MD. 2004. Diagnostics for the developing world. Nat Rev Microbiol. 2:231-240

22. Russell PK, Nisalak A, Sukhavachana P, Vivona S. 1967. A plaque reduction test for dengue virus neutralization antibodies. J Immunol. 99:285-290

23. Xu H, Di B, Pan YX, Qiu LW, Wang YD, Hao W, He LJ, Yuen KY, Che XY. 2006. Serotype 1-specific monoclonal antibody-based antigen capture immunoassay for detection of circulating nonstructural protein NS1: implications for early diagnosis and serotyping of dengue virus infections. J Clin Microbiol. 44[8]:2872-2878

24. Shu PY, Chen LK, Chang SF, Su CL, Chien, Chin C, Lin TH, Huang JH. 2004. Dengue Virus serotyping based on envelope and membrane and nonstructural protein NS1 serotype specific capture immunoglobulin M enzyme linked immunosorbent assays. J Clin Microbiol. 42[6]: 2489-2494

7

ENHANCED PROKARYOTIC EXPRESSION OF DENGUE VIRUS ENVELOPE PROTEIN

7.1 Introduction

Dengue virus is the most important arthropod-borne human pathogen. The incidence of dengue fever epidemics has increased significantly over the last few decades, and it is estimated that up to 100 million cases occur annually. In addition, a severe form of the disease, dengue hemorrhagic fever [DHF], has emerged in the same period causing 500,000 cases worldwide each year [1]. There are four different but antigenically closely related serotypes of dengue virus [DEN-1, 2, 3 and 4], and it is believed that DHF may result from secondary infection by different virus serotypes, in a process known as antibody mediated disease enhancement [2]. This feature has made vaccine development efforts against the dengue virus a difficult issue. Nevertheless, researchers around the world are exploring different approaches towards dengue vaccine development based on recombinant viral proteins expressed in different systems, inactivated viruses, conventional live attenuated viruses, antigen encoding plasmids and viral vectors encoding antigen genes [3].

The envelope [Env] protein of the dengue virus is the most studied antigen. Several studies confirm that the Env protein can be an effective vaccine candidate. Multiple strategies including subunit Env vaccines, DNA vaccines and attenuated viruses are being undertaken to develop a suitable vaccine for controlling the viral infection [4-7].

The dengue virus Env protein is around 50-55 kDa in size, with the N terminal corresponding to the ectodomain, which is exposed on the virion surface. The C terminal constitutes the trans-membrane hydrophobic domain and helps anchor the molecule on the lipid bilayer. The envelope is a multifunctional protein with important roles in host cell surface receptor binding [8]. The Env protein is also very important from the

viewpoint of humoral immunity against the virus [9]. Hence, efforts are made to express the Env protein in heterologous systems in order to develop it as a vaccine candidate.

The goal of this study was efficient cloning and expression of DEN-1 full length Env gene in *E.coli* that could be used to develop monoclonal antibodies [MAbs] by hybridoma technology or as a viable antigen for diagnostic and vaccine purposes. In this study, we report the successful cloning and high-level expression of the Env gene, purification from *E.coli* as inclusion bodies and its subsequent refolding. After purification with an affinity column and subsequent refolding, the protein was tested for its capability to induce robust humoral immune response in mice and virus blocking capability. The functionality of the refolded protein was further determined with heparan sulfate binding assay. Dengue virus infectivity has been reported to be dependent on the Env protein binding to heparan sulfate in the target cell [10]. The recombinant antigen has been evaluated as a diagnostic reagent. This article illustrates enhanced expression and functionality of the recombinant protein.

7.2 Materials and Methods

7.2.1 Vector and Chemicals

Restriction enzymes were procured from New England Biolabs [Mississauga, Canada]. The anti-His_6 was purchased from Novagen Inc [Madison, USA]. 40% acrylamide: bisacrylamide, prestained low range protein molecular weight markers and protein assay reagents were obtained from Bio-Rad [Mississuaga, Canada]. Hybond ECL nitrocellulose membrane, X-ray film and the ECL Western blotting reagents were obtained from Amersham Pharmacia Biotech [Baied Urfe, Quebec, Canada]. Glutathione [GSH] and glutathione disulphide [GSSG] were purchased from Boehringer Mannheim. Baby Hamster Kidney [BHK 21] cells, C6/36 mosquito cells were obtained from American Type Culture Collection [Virginia, USA], Dengue-1 virus [Hawaii strain] was maintained in the C6/36 cells. Briefly, monolayers of C6/36 were incubated with virus at a multiplicity of infection of 0.01 and incubated at 26°C in 5% CO_2 for 5 days, heparan sulfate, sodium deoxycholate, arginine, goat anti mouse-HRPO [GAM-HRPO], urea and other general reagents were obtained from Sigma [Oakville, Canada]. Ni-NTA agarose, plasmid DNA isolation kit and gel extraction kit were obtained from Qiagen [Mississauga, Canada].

7.2.2 Construction of Plasmid [pDS20Env]

Codon optimized Env nucleotide sequence for *E.coli* expression was chemically synthesized from GENEART, Germany. The codon optimized Env gene comprising of the plasmid and the expression vector pBM802 were NdeI and EcoRI digested followed by gel purification and ligation. The ligation mixtures were subsequently transformed in

E.coli Rosetta [ATCC 87064, *E.coli* DE3] by a heat shock method for recombinant protein expression. Screening of the resultant transformants was done by plasmid DNA isolation and restriction digestion [11].

7.2.3 Analysis of recombinant clones

Individual bacterial colonies were grown in 2 mL Terrific Broth [TB] medium [1.2% tryptone, 2.4% yeast extract, 0.4% [v/v] glycerol and 25 mM Hepes pH 7.2] comprising tetracycline and chloramphenicol, 5 μ gmL^{-1} and 34 μ gmL^{-1}, respectively, followed by overnight incubation at 37°C with shaking at 250 rpm. The culture was diluted 100 fold the next day in fresh TB medium comprising 5 μ gmL^{-1} tetracycline and 34 μ gmL^{-1} chloramphenicol and grown at 37°C with brisk shaking at 250 rpm. The bacterial culture was induced with 0.2% [w/v] of arabinose on reaching optical density [OD_{600nm}] of approximately 0.5-0.6 and was thereafter allowed to grow overnight [~16 h] at 37°C with shaking at 250 rpm. The bacterial culture was harvested by centrifugation at 5,000 x g for 10 min at 4°C and the total cell protein lysate was prepared [12]. The total cell protein was analyzed by SDS-PAGE using 10% polyacrylamide gel, according to Laemmli's method [13] and stained.

7.2.4 Expression optimization [Temperature, Time and Inducer]

The Env protein expression was optimized for three different sets of temperatures [37°C, 30°C and 24°C], varied time durations and inducer [arabinose] concentrations. Bacterial growth conditions were identical as defined earlier. The bacterial culture was induced with 0.2% [w/v] of arabinose at optical density [$OD_{600\ nm}$] of 0.5-0.6 and allowed to grow overnight [~16 h] at three different temperatures. For time optimization, the

bacterial culture was induced with 0.2% [w/v] of arabinose after an absorbance of 0.5 was reached. The cultures were further allowed to grow for 0 h, 2 h, 4 h, 6 h and overnight [~16 h] at 37°C. The bacterial cultures were induced with different concentrations of arabinose [2%, 0.2%, 0.02%, 0.002% and 0.0002%] and allowed to grow over night [~16 h] at 37°C. The total cell proteins obtained from each experiment were analyzed by SDS- PAGE and Western blot.

.

7.2.5 Medium scale expression and purification of the viral antigen

A fresh single colony of *E.coli* transformants containing pDS20Env was inoculated in 10 mL TB medium containing 5 μ gmL^{-1} of tetracycline and 34 μ gmL^{-1} chloramphenicol and allowed to grow overnight at 37°C in an incubator shaker at 250 rpm. The overnight culture was diluted 100 times in 1 L TB medium containing 5 μ gmL^{-1} of tetracycline and 34 μ gmL^{-1} chloramphenicol and grown at 37°C with shaking at 250 RPM until an $OD_{600\ nm}$ of 0.5–0.6 was reached. Induction was done by arabinose addition to a final concentration of 0.2% [w/v] and bacterial culture was incubated for 16 h with vigorous shaking [250 rpm] at 37°C. Pellets were collected by centrifugation at 5,000 X g for 20 min at 4°C. Total cell proteins from both uninduced and induced culture were analyzed by SDS–PAGE and Western blot.

7.2.6 Processing of inclusion bodies

The bacterial pellet [3 g] was resuspended in 30 mL PBS and lysed by French Press [20,000 psi]. The cell lysate was clarified by centrifugation at 27,000 X *g* for 30 min at 4°C and the supernatant was discarded. The pellet was again resuspended in 50

mL lysis buffer [50 mM Tris, pH 8.0, 200 mM NaCl and 1 mM EDTA] and adjusted to 2% sodium deoxycholate, incubated at room temperature for 30 min and centrifuged at 27,000 X g for 30 min at 4°C. The pellet was resuspended in lysis buffer and washed three times at 27,000 X g for 20 min at 4°C.

7.2.7 Protein purification [IMAC] under denaturing conditions

Inclusion bodies from the above step were solubilized in denaturing buffer [8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0] for 1 h at room temperature with occasional shaking. Solubilized proteins were separated from insoluble material by centrifugation at 27,000 X g for 30 min at 4°C and the final yield of solubilized protein was determined by Bradford assay. An IMAC based separation system was prepared by loading 9 mL of Ni–NTA agarose on a column. The column was equilibrated with 5 bed volumes of denaturing buffer [8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8]. Denatured soluble protein was loaded on the column and the column was washed initially with 5 bed volumes of washing buffer [8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3]. Bound protein was eluted with elution buffer [8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 4.5]. The various eluted fractions were analyzed by SDS–PAGE to measure the purity.

7.2.8 Refolding

Protein assay was done by Bradford method to quantify the amount of eluted protein from the IMAC column. The eluted protein was diluted to 75 μ gmL^{-1} and 60 μ

gmL^{-1} with Tris Arginine [TA] dialysis buffer [50 mM Tris, pH 8.0, 0.4 M L-arginine] to identify the appropriate refolding condition. Refolding was done by dialysis in TA buffer in the presence of 1.0 mM GSH and 0.1 mM GSSG, and changed three times over three days at 4°C. Final dialysis was done in PBS at 4°C. Any aggregation was removed by centrifugation and the supernatant was collected as a soluble refolded protein.

7.2.9 Western blot analysis

Total cell proteins, inclusion bodies, IMAC eluted fractions or refolded Env proteins were separated on SDS–PAGE using 10% polyacrylamide gel and transferred onto hybond ECL nitrocellulose membranes [14] with a transblot apparatus [Bio-Rad] according to the manufacturer's instructions. The membrane was blocked with 5% skim milk in PBST [0.1% Tween 20 in PBS, pH 7.3] for 1 h. The membrane was washed four times with PBST and incubated for 1 h with anti-His_6 MAb. After washing four times with PBST, the membrane was incubated with GAM-HRPO for 1 h. Finally, the membrane was washed with PBST four times and ECL-based detection was performed according to manufacturer's instructions.

7.2.10 In gel digestion

Protein identification by LC-MS was performed at the Institute of Biomolecular design, University of Alberta, Edmonton, Alberta, Canada.

7.2.11 Heparan Sulfate binding assay

Refolded protein was analysed for recognition of heparan sulfate by ELISA. Briefly, 96–well Maxisorp plates were coated with 0.5 µ gmL^{-1} heparan sulfate and

blocked with 3% bovine serum albumin [BSA]. Heparan sulfate coated wells were incubated with refolded protein for 1 h at 37°C. Serial dilutions of refolded Env protein were analysed. Then, wells were washed four times with PBST and incubated with 100 µl of diluted [1:1,000 in 5% BSA] anti-His_6 MAb for 1 h at room temperature. The wells were washed again and incubated with anti-mouse IgG HRP [at 1:1,000 dilutions in 5% BSA] for 1 h at room temperature. The color reaction was developed with TMB. The optical density was measured at 650 nm. Two negative controls [all assay components minus the recombinant protein and the other minus heparan sulfate] and a blank [containing only the detection reagents] were also included in the assay. The assay was repeated 3 times.

7.2.12 Immunization of mice

Groups of five balb/c mice were intra-peritoneally injected with 60 µg of purified Env protein in PBS emulsified with an adjuvant [protocol number 074/09/09, approved by Health Science Animal Protocol Committee, University of Alberta]. The protein was administered to the mice on days 0, 14 and 28 using Freund's complete adjuvant for the first administration and Freund's incomplete adjuvant for the second administration. The final injection was with 10 µg of protein in PBS. Mice were bled one week after the last administration and serum samples were then collected for further analysis.

7.2.13 Preparation of Dengue virus type 1 stock

The culture supernatant, obtained from dengue 1 virus [Hawaii strain]-infected C6/36 *Aedes albopictus* cell culture after removal of cellular junk by centrifugation, was used as the source of virus. The stock was titrated on BHK cells grown in culture plates. The

virus dilution at which 50% of the infected wells resulted in cytopathic effect was done by an established method [39] and viral titers were formulated as the reciprocal of the dilution in terms of tissue culture infective doses [$TCID_{50}$] [31].

7.2.14 Virus binding blocking assay

The ability of the Env protein to block dengue virus type 1 binding to cells in tissue culture was performed [31]. BHK cells were immobilized in 96-well plates [2×10^3 cells/well]. At about 60% confluency, they were pre-incubated for 30 min at 37 °C with 200 µl of 1× PBS containing either the test [Env] or control proteins. We used BSA as a control. Protein concentration in each case was from 0 to 20 µg per well. Following pre-incubating with the proteins 50 µL dilute Dengue type 1 virus [equivalent to 250 $TCID_{50}$] was added to each well and incubated for 30 min at 37 °C. After this, the protein/virus mixture was aspirated out the cells were washed three times with 1× PBS and fresh medium [200 µL/well] was added followed by incubation for 3 days in a humidified 10% CO_2 incubator at 37 °C. After 3 days the wells were scored for the presence or absence of cytopathic effects [CPE] by microscopic examination.

7.2.15 Envelope Protein as a diagnostic reagent

Access to clinical samples being limited, we spiked different concentrations of anti-dengue monoclonal antibody 8A5 in rabbit serum and neat serum as blank. We also used serum spiked with SARS antibodies as negative control. We blinded the tubes and

labelled the samples 1-7 randomly. The purified full length Env protein was diluted to 10 μgmL^{-1} in 0.1M bicarbonate buffer [pH 9.6] and 96–well Maxisorp plates were coated overnight at 4°C. The coated plates were washed with PBS and blocked with 2% BSA for 2 h at 37°C. The plates were washed again with PBS. 100 µL of the samples were added to the wells and incubated for 2 h at 37°C. The plates were washed again with PBS and anti-mouse IgG conjugated to horseradish peroxidase was added [1:10000 dilution] for 30 min at 37°C. The plates were washed again with PBS and TMB substrate added and absorbance was read at 650 nm. The assay was done in triplicate.

7.3 Results

7.3.1 Env gene cloning and expression

The full length Env gene was successfully cloned with the C-terminal His_6 tag and denoted as pDS20Env for higher expression of proteins in *E.coli* in the form of inclusion bodies [15]. Recombinant clones pertaining to the right size were selected for protein expression. The full length Env gene comprising plasmid was isolated for expression. Expression results of various clones showed that all the Env full length clones selected expressed the protein of interest at approximately 54 kDa at varying levels as determined by SDS-PAGE analysis. This was confirmed by Western blot probed with anti-His_6 MAb [Figure 7.1]. In the control sample, no expression of Env protein was observed. The best Env clone was selected for expression optimization and further studies.

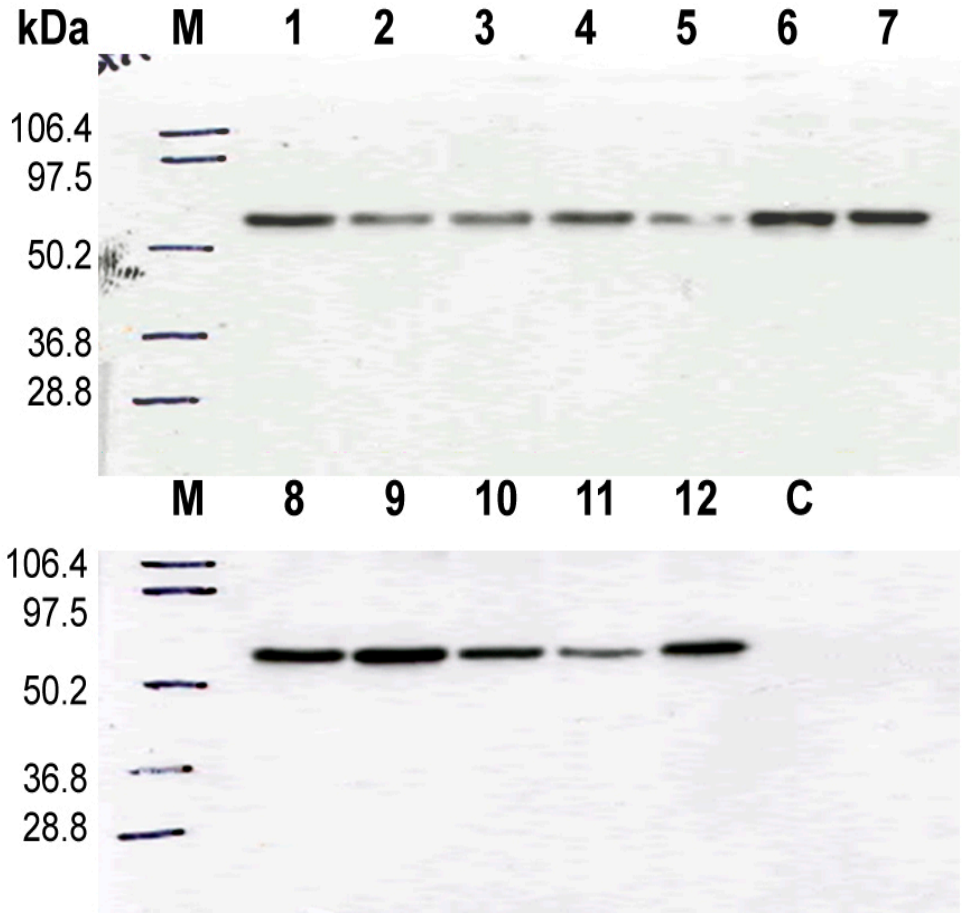


Fig 7.1 Envelope protein expression. Lane M: Marker, Lanes 1 – 12: Clone # 1 – 12, and Lane C is Control.

7.3.2 Expression optimization [Temperature, Time and Inducer]

The Env protein was successfully expressed as a recombinant protein in *E.coli*. The optimal conditions for Env protein expression were 0.2% [w/v] arabinose concentration [Figure 7.2a and 7.2b], 37°C temperature [Figure 7.3a and 7.3b] and 16 h induction time [Figure 7.4].

7.2a

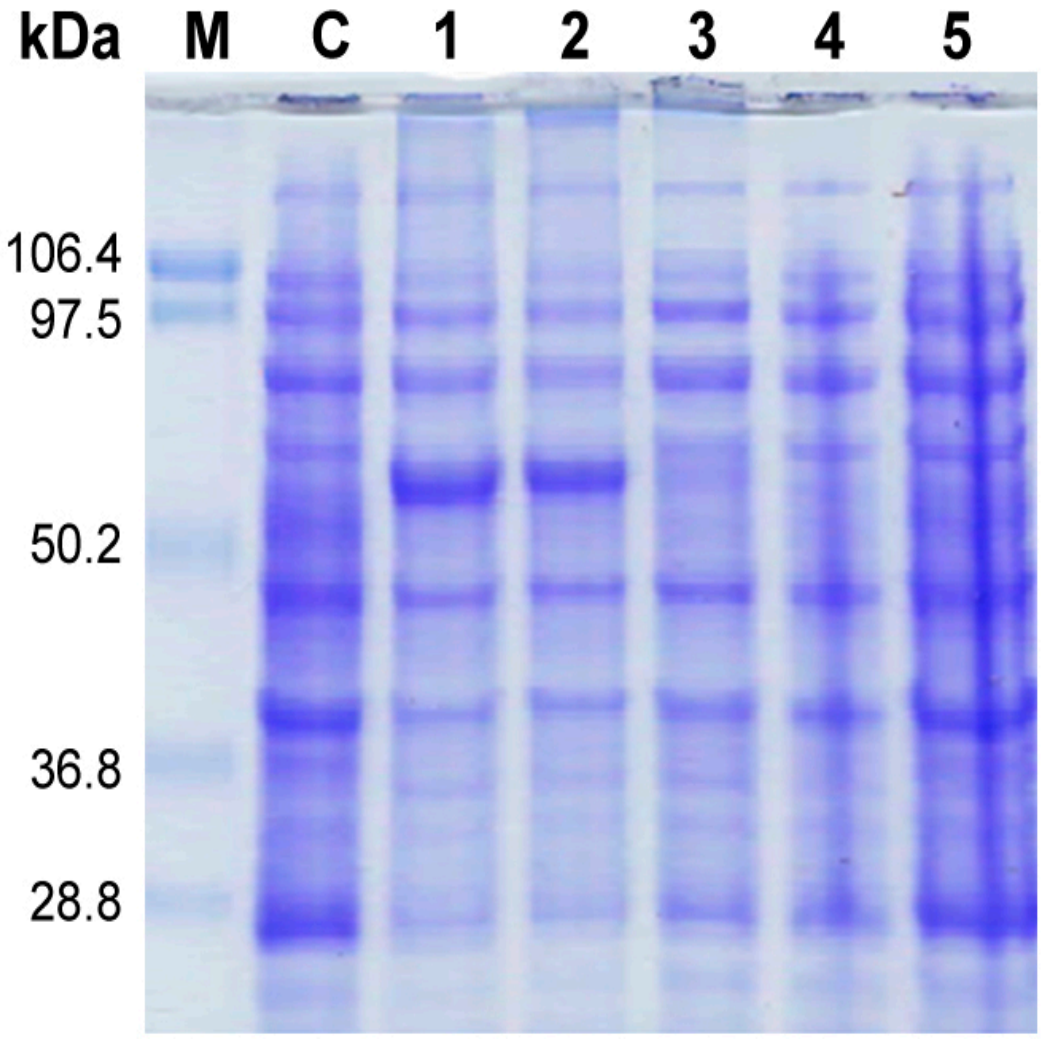


Fig 7.2A Arabinose dose optimization of envelope protein expression. SDS-PAGE. Lane M: prestained marker, Lane C: Control, Lane 1-5: 2%, 0.2%, 0.02%, 0.002%, 0.0002%.

7.2b

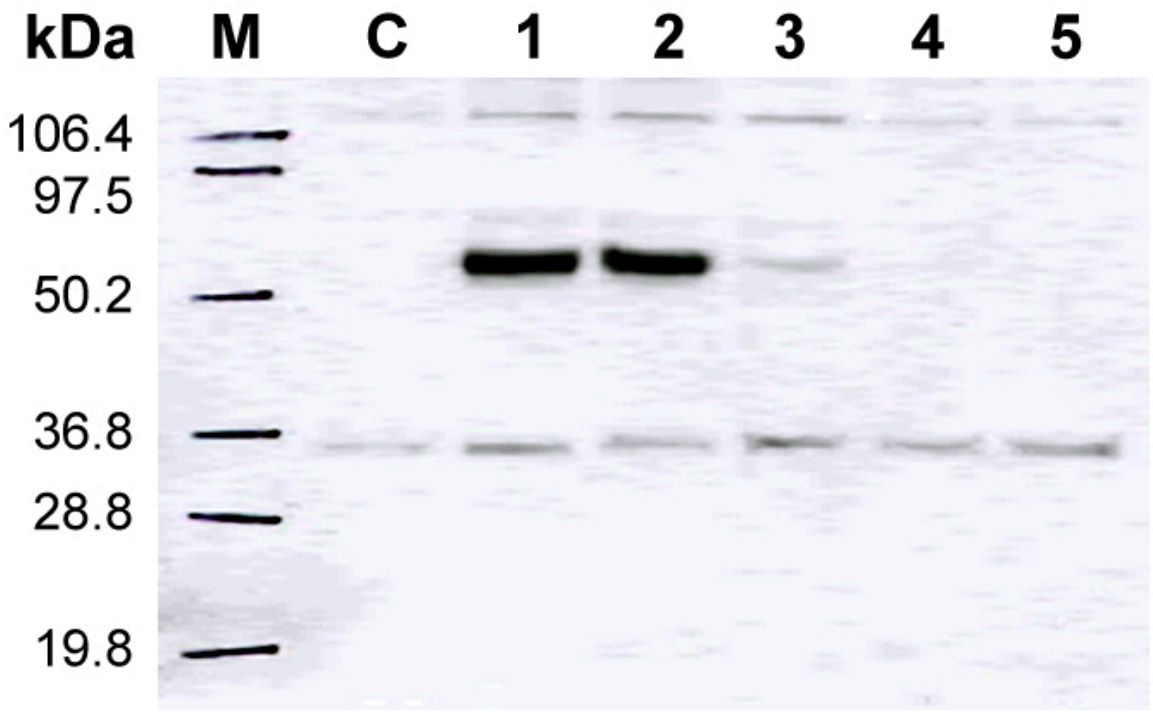
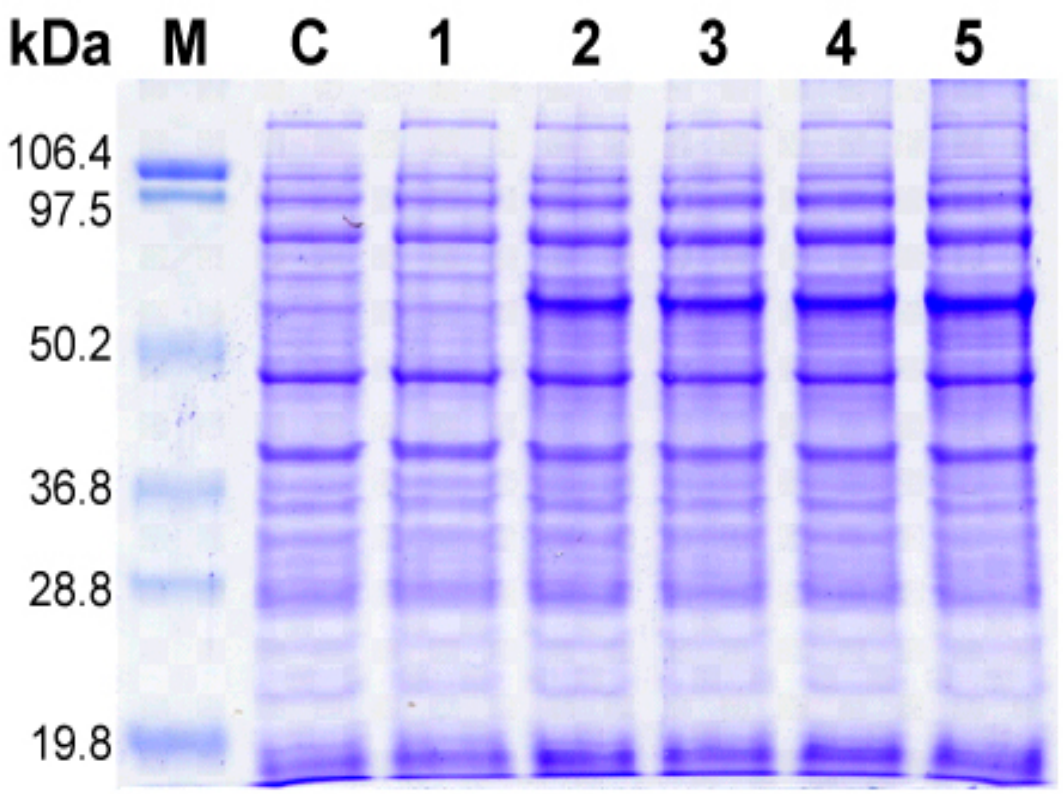


Fig 7.2B Arabinose dose optimization of envelope protein expression. Western blot. Lane M: prestained marker, Lane C: Control, Lane 1-5: 2%, 0.2%, 0.02%, 0.002%, 0.0002%.

7.3a



7.3b

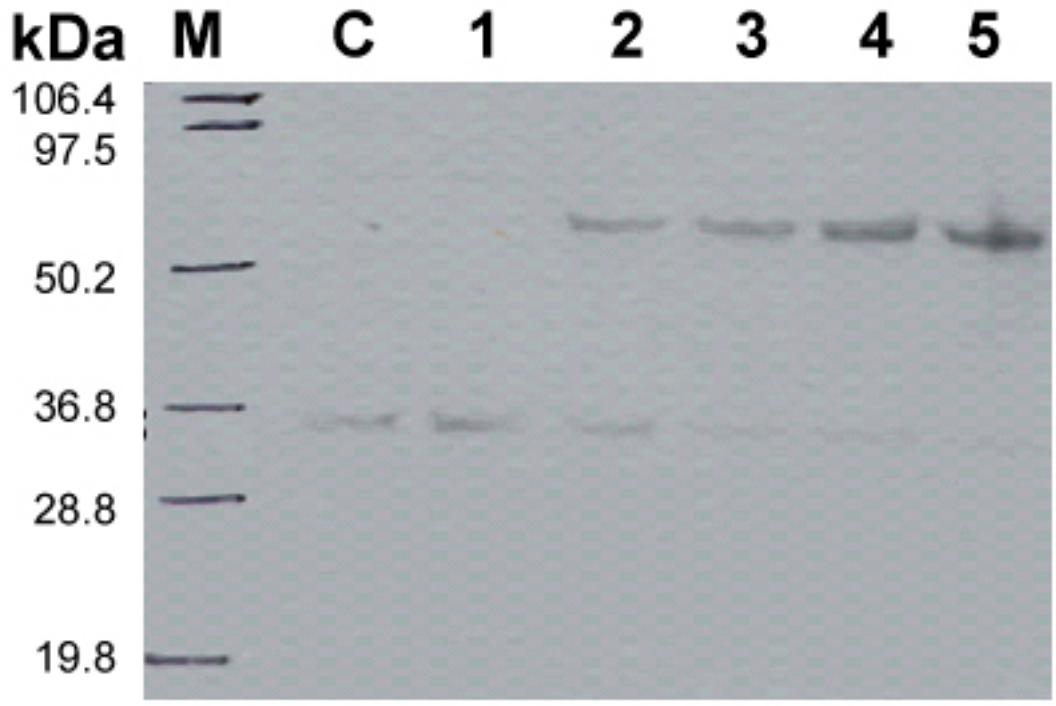


Fig 7.3 Time induction of envelope protein expression. **a** SDS PAGE. Lane M: prestained marker, Lane C: Control, Lane 1-5: 0 h, 2 h, 4 h, 6 h, overnight. **b** Western blot. Lane M: prestained marker, Lane C: Control, Lane 1-5: 0 h, 2 h, 4 h, 6 h and overnight.

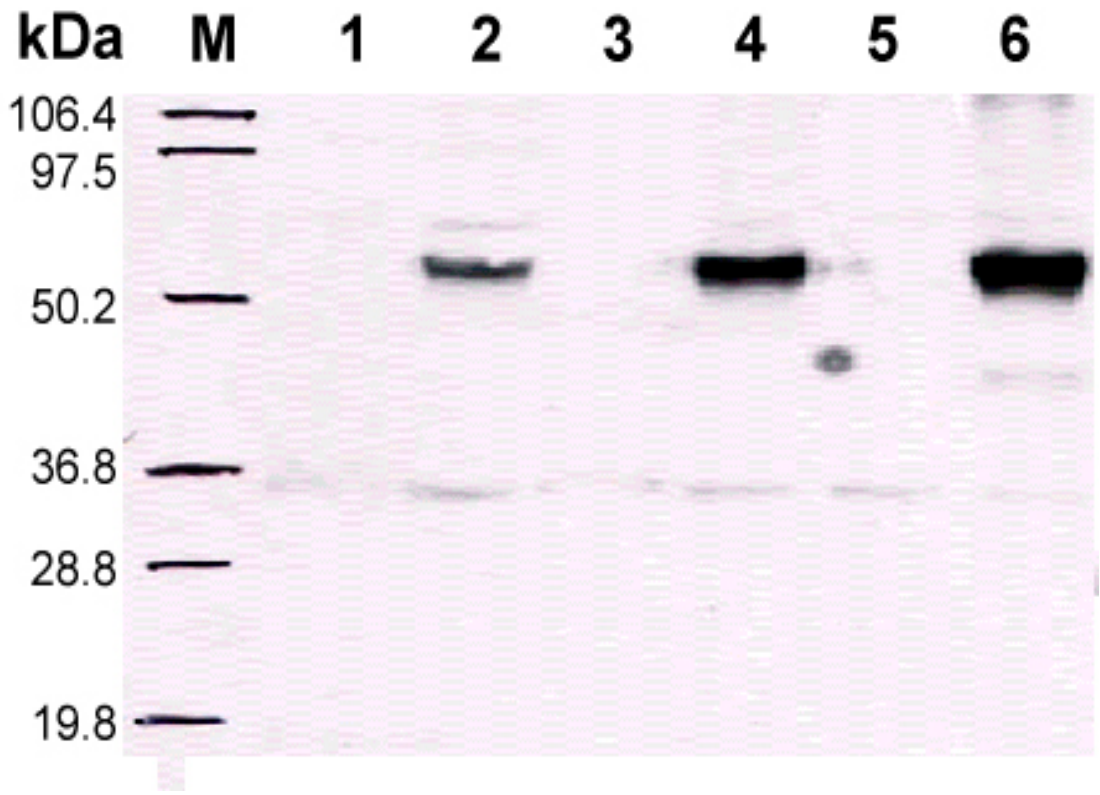


Fig 7.4 Temperature optimization of envelope protein expression by Western blot. Lane M: prestained marker, Lane 1: 24ºC Control, Lane 2: 24ºC Test, Lane 3: 30ºC Control, Lane 4: 30ºC Test, Lane 5: 37ºC Control, Lane 6: 37ºC Test.

7.3.3 Medium scale expression of Env protein

The recombinant Env was expressed and the bulk of the antigen was in the inclusion bodies [Figure 7.5]. Inclusion bodies were prepared from bacterial pellet by French press. After completion of cell lysis, the insoluble inclusion bodies were separated from the soluble bacterial protein by centrifugation and thereafter the pellets were washed with sodium deoxycholate. Any remaining sodium deoxycholate were removed from the inclusion bodies by subsequent washes with lysis buffer. The purity of the inclusion bodies along with the different washes was analyzed by SDS-PAGE. The final yield of purified soluble inclusion bodies was estimated by Bradford protein assay to be approximately 15-20 μ gmL^{-1} of bacterial culture.

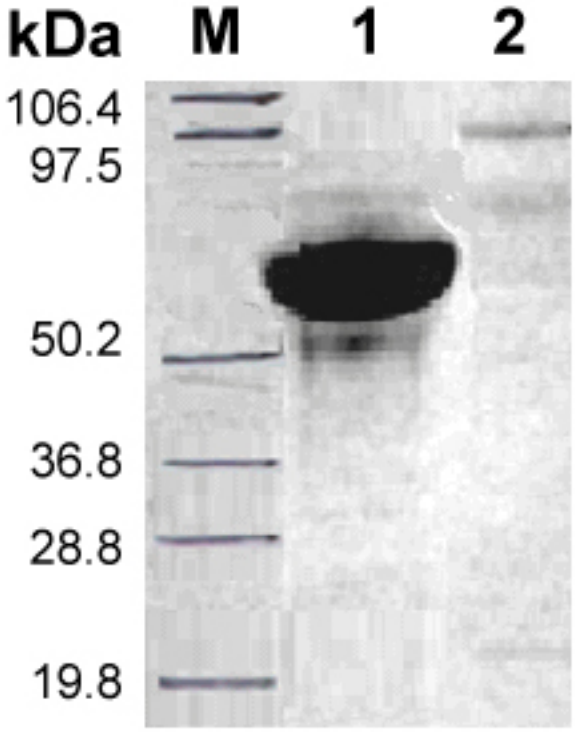


Fig 7.5 Accumulation of envelope protein in inclusion bodies [Lane 1] and soluble fractions [Lane 2] by Western blot. M: prestained marker.

7.3.4 Purification and Refolding

The insoluble protein was isolated from inclusion bodies with a final purified protein yield in the range of 15-20 mgL^{-1} of bacterial shake flask culture and purified by immobilized metal–affinity chromatography [IMAC] under denaturing conditions. The purification method involved IMAC for separating recombinant proteins from major bacterial contaminants. Employing this method, pure proteins were eluted out from the IMAC affinity column as determinated in the methods section. The eluted protein showed a single band of approximate molecular weight of 54 kDa, with purity greater than 90% [Figure 7.6]. Folding of proteins and disulphide bond formation and association of various domains require renaturing conditions and suitable buffers. The presence of arginine in the refolding buffer helped in solubilization, inhibiting aggregation of refolding intermediates and thereby increasing the yield [16]. Refolding was done over three days by dialysis. No aggregation was observed after refolding. The supernatant was collected as refolded Env protein for future use. Hence, the *in vitro* refolding was successful in recovering the soluble protein expressed in *E.coli* in form of inclusion bodies.

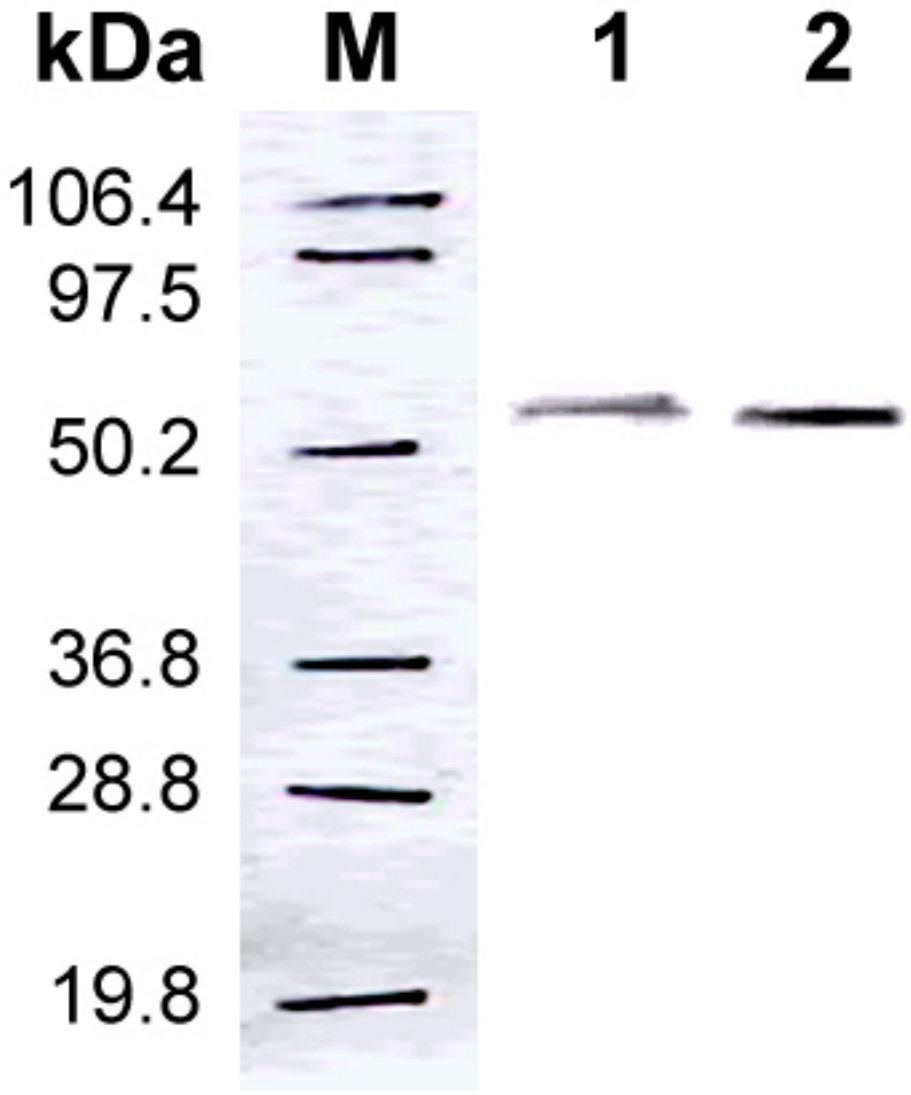


Fig 7.6 Refolded envelope protein by Western blot. Lane M: prestained marker, Lane 1: refolded envelope protein [75 μ gmL^{-1}], Lane 2: envelope protein [60 μ gmL^{-1}].

7.3.5 In Gel Digestion

Non-redundant National Center for Biotechnology Information [NCBI] database was searched for protein identification from the LC/MS data according to established protocol [11]. Significant hits were obtained from the search for the DEN- 1 polyprotein which included Env [gi]130423 polyprotein [Dengue virus type 1].

7.3.6 Immunogenicity of the purified Env protein

Mice were immunized intra peritoneally [60 μg at two week intervals] Sera from immunized mice were analyzed by ELISA [Figure 7.7] and Western blot [Figure 7.8]. The results indicate development of robust humoral immunity in mice. Immunized mice sera could also bind to the purified and refolded dengue Env protein on Western blot.

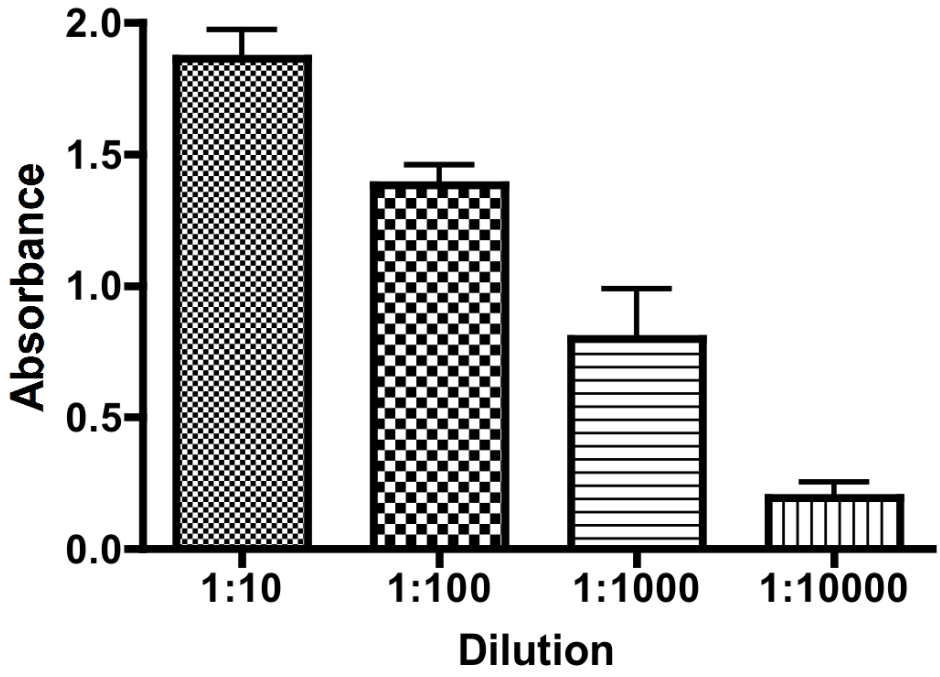


Fig 7.7 Indirect ELISA of mice sera immunized by Dengue envelope protein at absorbance of 650 nm. Analysis of humoral immune response to dengue envelope protein.

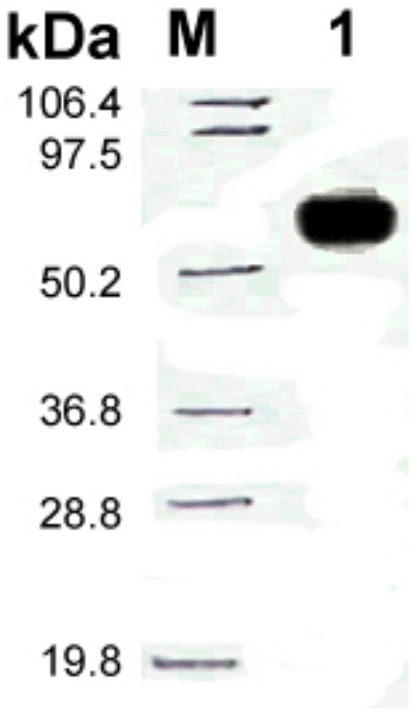


Fig 7.8 Western blot analysis of refolded envelope protein probed with mouse anti-Env polyclonal antibodies. Lane M: prestained marker, Lane 1: refolded envelope protein.

7.3.7 Refolded Env protein blocks Dengue virus – 1 infection

After refolding, the recombinant protein's biological functionality was assessed by virus blocking assay. Pre-incubated BHK cells with various concentrations of refolded Env protein were infected with Dengue type 1 virus. Total blocking was observed at concentrations 16 μg and higher [Figure 7.9]. This substantiates a successful refolding of the recombinant protein.

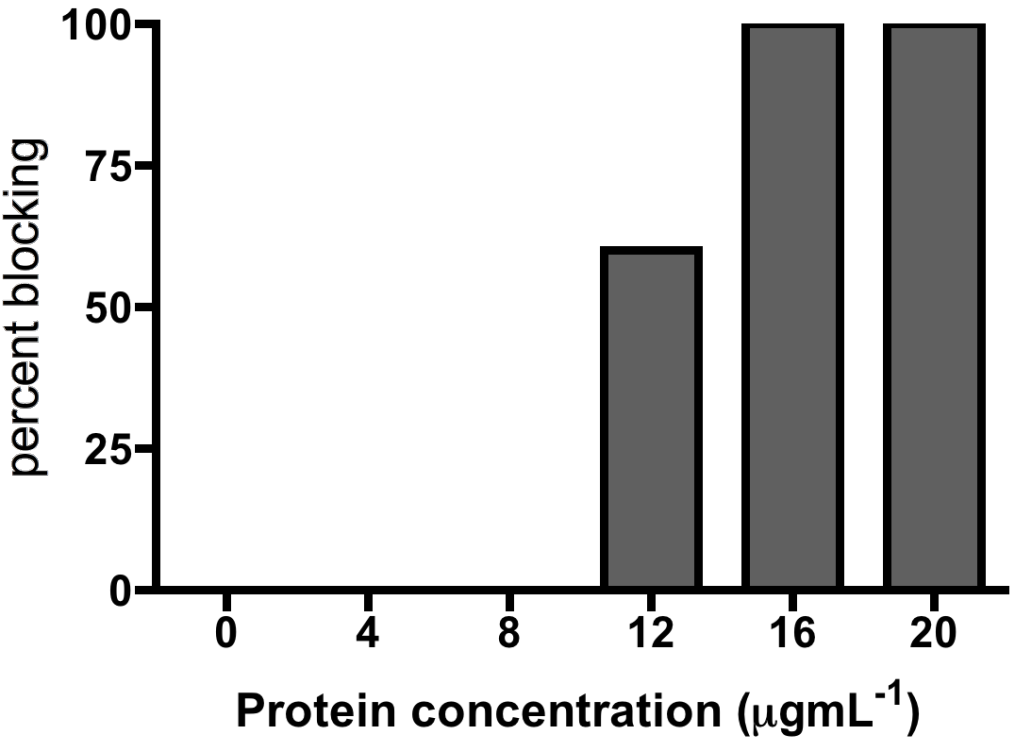


Fig 7.9 Biological function assessment of refolded Env protein. BHK cells were pre-incubated with various concentrations of Env protein and BSA [control] and then infected with dengue virus type 1 [250 $TCID_{50}$ per well]. After 3 days, the wells were examined under a microscope for cytopathic effects.

7.3.8 Binding Assay

The recombinant protein was found to be biologically functional based on the heparan sulfate binding assay [Figure 7.9]. Denatured protein was also analyzed in a parallel assay wherein the binding was not as significant as previously reported by pattnaik and co-workers [17].

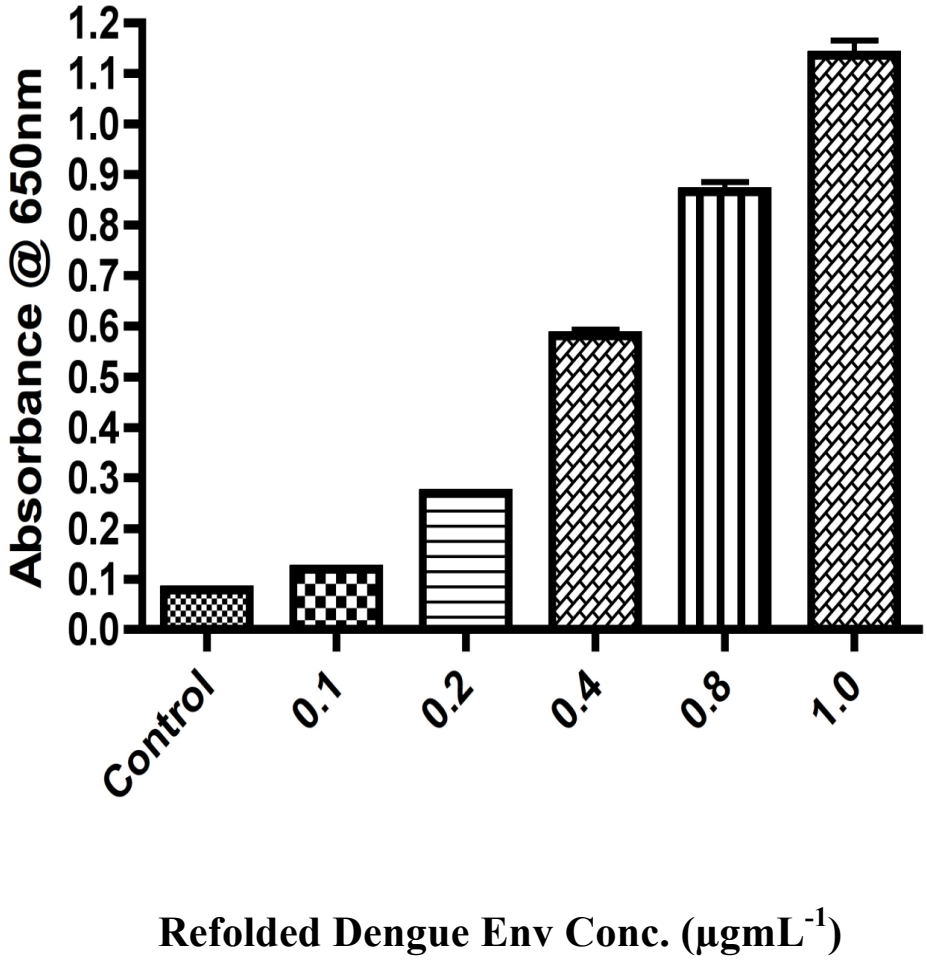


Fig 7.10 Receptor binding assay with purified and refolded Dengue env protein. ELISA plate wells were coated with 0.5 μgmL^{-1} cell free soluble heparan sulfate, except the control wells. Different concentrations of refolded protein were used. Refolded protein bound to heparan sulfate in a concentration dependant manner.

7.3.9 Diagnostic evaluation of anti-dengue antibodies using recombinant antigen

The recombinant env protein was used in indirect ELISA for detection anti dengue antibodies in the blinded spiked samples. We tested 5 samples with varying dengue antibody concentrations along with blank and negative control. The assay could positively detect the 5 samples that had spiked dengue antibodies and the remaining two samples yielded negative result. The specificity of the ELISA was found to be 100% [Table 7.1].

Range of OD value	Blinded Sample No	Original Sample
0.736 - 0.910	1	8 $ngmL^{-1}$ anti den MAb
0.588 - 0.692	2	6 $ngmL^{-1}$ anti den MAb
0.000 - 0.096	3	Neat Serum
0.211 - 0.289	4	2 $ngmL^{-1}$ anti den MAb
0.951 - 1.078	5	10 $ngmL^{-1}$ anti den MAb
0.000 - 0.127	6	10 $ngmL^{-1}$ SARS MAb
0.473 - 0.535	7	4 $ngmL^{-1}$ anti dengue MAb

Table 7.1 Range of OD_{650} values in blinded diagnostic assay

7.4 Discussion

The Env protein of Dengue viruses is widely recognized as a major subunit vaccine component. The Env protein is a multifunctional protein with proven roles in host cell surface receptor binding [18]. It is also one of the important targets to induce protective and prolonged immune response [19]. Earlier studies have used different expression systems involving yeast, insect cells and *E coli* for Env expression [20-22]. In most of the previous expression systems and purification methods the recombinant proteins were associated with fusion tags such as glutathione S-transferase [GST] and maltose binding proteins for expressing the protein in soluble form with subsequent affinity purification [23]. Most studies show that yields of the Dengue Env protein to be low associated with significantly high cost of production. Low expression levels and difficulty in purification has been a major hindrance in the development of Env based subunit vaccines [24]. Baculovirus based expression of the antigen has been reported to form aggregates [25]. The Env glycoprotein is also very important for vaccine and therapeutic aspects due to the presence of neutralizing epitopes [26-27].

In the present study we have used the *E coli* system for the production of Env protein which is one of the most frequently used method for recombinant protein expression. An advantage of the *E.coli* system is its convenient expression capability and low cost. It is known that *E.coli* expressed eukaryotic protein folding is difficult to obtain as a soluble protein. Large scale expression of proteins is conducible to precipitation thereby forming inclusion bodies [28-29]. Protein purification from inclusion bodies gives a better yield and is relatively simple to purify as previously demonstrated in our

laboratory. Earlier studies show that codon optimized genes boost expression level than the native gene [28].

In this study, we have obtained codon optimized Env gene chemically for expression in *E.coli*. Codon optimization substantially enhances gene expression that leads to higer level of protein expression. Codon optimization was carried out using optimizer software in Geneart. They follow a deterministic sliding window algorithm for multiparameter sequence optimization. The target sequences are determined with a quality attribute taking codon usage, GC content, mRNA structure and species-specific sequence motifs into consideration. The first codon of the best candidates' variation window is made absolute and the window is shifted by one codon position towards the 3' end [30]. We have cloned and purified the Dengue Env in *E.coli* for the development of MAbs for primarily translational applications. The Env protein could also be used for dengue detection and therapeutic and vaccine applications. The 6x His tag was inserted at the C-terminal end of the recombinant protein to augment easy purification. This tag has negligible immunogenicity and hence need not be removed from the recombinant protein [31]. We also analyzed a range of conditions to validate the optimal temperature, time and inducer concentration for highest protein expression [11].

The Env gene was cloned in presence of a promoter for high-level expression of recombinant protein as inclusion bodies. The pBM802 vector was used for cloning under the control of the pBAD promoter that is nothing but arabinose promoter hence arabinose was used as inducer [32]. This promotes high level expression of recombinant protein as inclusion bodies in the bacterial cytoplasm. The final yield of purified inclusion bodies was estimated by Bradford protein assay [33] and estimated to be approximately 15-20

mgL^{-1} [Table 7.2] of initial bacterial culture which is 5-10 fold higher compared to previous studies using the native Env gene sequence [19,22,31].

Dengue Envelope Type	**Affinity Tag**	**Host**	**Yield**	**Reference**
Dengue 1 Env	His_6	*E.coli*	15-20 mg L^{-1}	This article
Dengue 1 Env	GST	*E.coli*	2 mg L^{-1}	[22]
Dengue 1 Env	GST	*Pichia*	0.1 mg L^{-1}	[22]
Dengue 2 Env	HBsAg	*Pichia*	0.5 mg L^{-1}	[38]
Dengue 2 Env	MBP	Sf9	1 mg10^{-9} cells	[21]

Table 7.2 Comparative analysis of production of full length Env proteins

The purity of inclusion bodies was determined by SDS-PAGE and Western blot. French Press lysis of cells and subsequent washing steps with detergent were adopted to purify the inclusion bodies from soluble proteins. SDS-PAGE analysis demonstrated that lysis by French Press and further washing with PBS substantially enhanced the purity of the inclusion bodies as a large amount of the *E.coli* soluble proteins could be separated. The purification method comprising of IMAC under denaturing conditions adsorbed the His-tagged protein [23]. IMAC purification under denaturing conditions produced better amounts of pure Env protein with one band as determined by SDS-PAGE analysis. Bradford assay estimated a final concentration of around 80% of the initial amount. Recombinant proteins are expressed in *E.coli* as inclusion bodies and various refolding methods have been described to renature the proteins from inclusion bodies [23]. The IMAC purified Env protein was refolded using TA buffer consisting of a redox pair [GSH/GSSG] with two different protein concentrations [75 μ gmL^{-1} and 60 μ gmL^{-1}]. No aggregation was visible with both the concentrations during the process of refolding. SDS-PAGE and Western blot analyses were used to determine the purity of the refolded protein. Anti-His_6 MAb reacted with a single band of ~54 kDa, suggesting a successful purification of recombinant Env. The identity of purified protein was further confirmed by *in vitro* gel digestion, mass spectrometry, and NCBI non-redundant database search which proved that the purified protein band to be the dengue polyprotein of our interest. The protein was also probed with dengue Env MAbs thus confirming proper refolding. The Env protein was also successful in inducing a robust humoral immune response in balb/c mice as evidence from the high antibody titers and western blot analysis. The refolded Env protein was used as an antigen to immunize mice for development of MAbs. The polyclonal antibodies from mice sera were strongly binding with the

recombinant protein in Western blot [Figure 7.8]. The conformation of the Env protein plays an important role during viral infection [34]. We did not analyze the crystal structure of the recombinant Env protein but putting it in perspective to findings by various research groups we can predict our refolded protein to be having a postfusion conformation. It has been reported that exposure to detergent and acidic pH would lead to trimeric postfusion conformation [35]. As our procedure involves similar parametric conditions we can anticipate the structural conformation. In addition anti dengue MAbs 8A5 and 12A1 were sourced from our collaborators in the United States to probe our refolded Env protein in Western blot as well as standard ELISA and we found desired level of interaction. Further assessment of the functional integrity of the refolded protein was conducted by performing a dengue virus-blocking assay [Figure 7.10]. The assay demonstrated the ability of the recombinant Env protein to completely block dengue virus infectivity. The functionality of the recombinant protein was further analyzed with heparan sulfate based assay. The recombinant protein may also be used as a diagnostic antigen. Our mimic blinded optimized diagnostic assay resulted in 100% specificity and sensitivity. Further optimization would offer a rapid alternative to whole virus antigen based diagnostic assays. This expression system can be exploited for other recombinant proteins as well which would be biologically functional and also in developing a dengue vaccine owing to high yield protein production at a very reasonable cost. The recombinant Env protein will be used to develop several MAbs with different specificities from immunized mice. MAbs against dengue Env is known to protect mice against dengue infection [36].

In summary the Den-1 full length Envelope protein was efficiently expressed in the *E.*coli system. We hope to develop several MAbs in future to develop an oligoclonal

antibody cocktail-based therapeutic formulation against all the dengue virus serotypes. The full length Env protein could also be used for dengue diagnostic studies [37]. Development of drugs inhibiting the Env mediated dengue infection can be exploited with a functional recombinant protein. This would be highly inexpensive procedure for the development of antigens for large-scale use in terms of therapeutics and diagnostics.

7.5 References

1. Whitehead SS, Blaney JE, Durbin AP, Murphy BR: Prospects for a dengue virus vaccine. *Nat Rev Microbiol* 2007, 5:518-528.

2. Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E: Lethal Antibody Enhancement of Dengue Disease in Mice Is Prevented by Fc Modification. *PLoS Pathog* 2010, 6[2]:e1000790. doi:10.1371/journal.ppat.1000790.

3. Malabadi RB, Ganguly A, Silva JA, Parashar A, Suresh MR, Sunwoo H: Overview of plant-derived vaccine antigens: Dengue virus. *J Pharmac Pharm Sci* 2011, 14:400-413.

4. Men R, Wyatt L, Tokimatsu I, Arakaki S, Shameem G, Elkins R, Chanock R, Moss B, Lai CJ: Immunization of rhesus monkeys with a recombinant of modified vaccinia virus Ankara expressing a truncated envelope glycoprotein of dengue type 2 virus induced resistance to dengue type 2 virus challenge. *Vaccine* 2000, 18:3113-3122.

5. Mota J, Acosta M, Argotte R, Figueroa R, Mendez A, Ramos C: Induction of protective antibodies against dengue virus by tetravalent DNA immunization of mice with domain III of the envelope protein. *Vaccine* 2005, 23:3469-3476.

6. Putnak R, Barvir DA, Burrous JM, Dubois DR, D'Andrea VM, Hoke CH, Sadoff JC, Eckels KH: Development of a purified, inactivated, dengue-2 virus vaccine prototype in Vero cells: immunogenicity and protection in mice and rhesus monkeys. *J Infect Dis* 1996, 174:1176-1184.

7. Wu SF, Liao CL, Lin YL, Yeh CT, Chen LK, Huang YF, Chou HY, Huang JL, Shaio MF, Sytwu HK: Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. *Vaccine* 2003, 21:3919-3929.

8. Chen Y, Maguire T, Marks RM: Demonstration of binding of dengue virus envelope protein to target cells. *J Virol* 1996, 70:8765-8772.

9. Megret F, Hugnot JP, Falconar A, Gentry MK, Morens DM, Murray JM, Schlesinger JJ, Wright PJ, Young P, Van Regenmortel MH: Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue virus envelope glycoprotein. *Virol* 1992, 187:480-491.

10. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM: [1997] Dengue Virus Infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 1997, 3:866-871.

11. Das D, Mongkolaungkoon S, Suresh MR: Super induction of dengue virus NS1 protein in E.coli. *Protein Expr Purif* 2009, 66:66-72.

12. Sambrook J, Fritsch EF, Maniatis, T: Molecular Cloning: A Laboratory Manual, vol. I. 2nd edition. *Cold Spring Harbor Laboratory Press,* 1989. ISBN 0-87969-309-6

13. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227:680-685.

14. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979, 76:4350-4354.

15. Das D, Suresh MR: Copious production of SARS-CoV nucleocapsid protein employing codon optimized synthetic gene. *J Virol Methods* 2006, 137:343-346.

16. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Philo JS, Arakawa T: Role of arginine in protein refolding, solubilization, and purification. *Biotechnol Prog* 2004, 20:1301-1308.

17. Pattnaik P, Babu JP, Verma SK, Tak V, Rao PV: Bacterially expressed and refolded envelope protein [domain III] of dengue virus type-4 binds heparan sulfate. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007, 846:184-194.

18. Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, Jones CT, Mukhopadhyay S, Chipman PR, Strauss EG, Baker TS, Strauss JH: Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* 2002, 108:717-725.

19. Staropoli I, Frenkiel MP, Megret F, Deubel V: Affinity-purified dengue-2 virus envelope glycoprotein induces neutralizing antibodies and protective immunity in mice. *Vaccine* 1997, 15:1946-1954.

20. Srivastava AK, Putnak JR, Warren RL, Hoke CH,Jr: Mice immunized with a dengue type 2 virus E and NS1 fusion protein made in *Escherichia coli* are protected against lethal dengue virus infection. *Vaccine* 1995, 13:1251-1258.

21. Staropoli I, Clement JM, Frenkiel MP, Hofnung M, Deubel V: Dengue virus envelope glycoprotein can be secreted from insect cells as a fusion with the maltose-binding protein. *J Virol Methods* 1996, 56:179-189.

22. Sugrue RJ, Cui T, Xu Q, Fu J, Chan YC: The production of recombinant dengue virus E protein using *Escherichia coli* and *Pichia pastoris*. *J Virol Methods* 1997, 69:159-169.

23. Das D, Gares SL, Nagata LP, Suresh MR: Evaluation of a Western Equine Encephalitis recombinant E1 protein for protective immunity and diagnostics. *Antiviral Res* 2004, 64:85-92.

24. Zhang ZS, Yan YS, Weng YW, Huang HL, Li SQ, He S, Zhang JM: High-level expression of recombinant dengue virus type 2 envelope domain III protein and induction of neutralizing antibodies in BALB/C mice. *J Virol Methods* 2007, 143:125- 131.

26. Lin B, Parrish CR, Murray JM, Wright PJ: Localization of a neutralizing epitope on the envelope protein of dengue virus type 2. *Virol* 1994, 202:885-890.

25. Kelly EP, Greene JJ, King AD, Innis BL: Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice. *Vaccine* 2000,18:2549-2559.

27. Trirawatanapong T, Chandran B, Putnak R, Padmanabhan R: Mapping of a region of dengue virus type-2 glycoprotein required for binding by a neutralizing monoclonal antibody. *Gene* 1992, 116:139-150.

28. Das D, Jacobs F, Feldmann H, Jones SM, Suresh MR: Differential expression of the Ebola virus GP [1,2] protein and its fragments in E.coli. *Protein Expr Purif* 2004, 54:117-125.

29. Kurucz I, Titus JA, Jost CR, Segal DM: Correct disulfide pairing and efficient refolding of detergent-solubilized single-chain Fv proteins from bacterial inclusion bodies. *Mol Immunol* 1995, 32:1443-1452.

30. Fath S, Bauer AP, Liss M, Spriestersbach A, Maertens B, Hahn P, Ludwig C, Schafer F, Graf M, Wagner R: Multiparameter RNA and codon optimization: A standardized tool to assess and enhance autologous mammalian gene expression. *PLoS ONE* 2011, 6[3]:e17596. doi:10.1371/journal.pone.0017596.

31. Jaiswal S, Khanna N, Swaminathan S: High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in Escherichia coli. *Protein Expr Purif* 2004, 33:80-91.

32. Guzman mL, Belin D, Carson MJ, Beckwith J: Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 1995, 177:4121-4130.

33. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, 72:248-254.

34. Kanai R, Kar K, Anthony K, Gould LH, Ledizet M, Fikrig E, Marasco W, Koski RA, Modis Y: Crystal Structure of west nile glycoprotein reveals viral surface epitopes. *Amer Soc Micro* 2006, 80:11000-11008.

35. Nayak V, Dessau M, Kucera K, Anthony K, Ledizet M, Modis Y: Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion. *J Virol* 2009, 83:4338-4344.

36. Kaufman BM, Summers PL, Dubois DR, Eckels KH: Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. *Am J Trop Med Hyg* 1987, 36:427-434.

37. Holbrook MR, Shope RE, Barrett AD: Use of recombinant E protein domain III-based enzyme-linked immunosorbent assays for differentiation of tick-borne encephalitis serocomplex flaviviruses from mosquito-borne flaviviruses. 2004, 42[9]:4101-4110

38. Bisht H, Chugh DA, Swaminathan S, Khanna N: Expression and purification of Dengue virus type 2 envelope protein as a fusion with hepatitis B surface antigen in Pichia pastoris. *Protein Expr Purif* 2001, 23:84-96.

39. Burleson FG, Chambers TM, Wiedbrauk DL: Virology: A Laboratory Manual. *Academic Press* [New York] 1992

8

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST DENGUE VIRUS ENVELOPE PROTEIN

Dengue is one of the foremost vector-borne diseases recognized in the world with more than 50 million dengue fever cases per year [1,2]. The vector, *Aedes aegypti* is most common in tropical regions [3,4] whereas the vector, *Aedes Albopictus*, is generally found in temperate environments, which result in dengue outbreaks [5]. The flavivirus Env glycoprotein is known to induce protective immunity. This protein is critical for binding to cellular receptors and endosomal membrane fusion. Based on the studies, the Env protein folds itself into three functional domains. Domain I comprises of both virus-specific and cross-reactive epitopes, which are generally non-neutralizing. Domain II contains a conserved 'fusion loop', which participates in structural rearrangements as well as several overlapping immune-dominant epitopes that induce neutralizing antibodies. Domain III is considered the most important as it contains the host cell receptor-recognition site, which elicits the production of virus-specific, highly protective neutralizing antibodies [5, 6, 7].

Four antigenically distinct dengue serotypes [DEN-1, DEN-2, DEN-3 and DEN-4] coexist in many dengue endemic areas [9,10]. Infection with one dengue serotype provides life-long immunity to that serotype but cannot offer immunity when infected with a different dengue serotype [11]. Most dengue infections remain asymptomatic and cause dengue fever [DF]. Dengue Hemorrhagic Fever [DHF] and Dengue Shock Syndrome [DSS] are the most severe forms of DF, which are often fatal [12, 13,14]. A person previously infected with a dengue serotype on subsequent infection with another dengue serotype leads to clinical disease for most serotype combinations, and is deemed a major risk for developing DHF or DSS [15]. In this study, we developed and characterized three anti-dengue Envelope mAbs, which were raised against the Dengue

type 1 Env recombinant protein. We describe mAb affinity towards purified Dengue virus as well as towards the env domain III region of all the serotypes. No neutralizing activity was observed *in vitro*. Due to its limited cross-reactivity with the four serotypes, the mAbs are ideal candidates for the development of reagents for investigating dengue pathogenesis. Moreover dengue-specific mAbs that recognize antigenically different serotypes are particularly significant for specific and sensitive dengue antigen-capture immunoassays for clinical dengue diagnosis.

BALB/c mice were immunized intra-peritonially with full-length dengue envprotein expressed in *E.coli,* following an established immunization protocol. Based on high antibody titers, splenocytes were isolated from the immunized mice and fused with freshly grown SP2/0 myeloma cells using polyethylene glycol. After a third recloning step, three stable clones were generated against Dengue env protein and designated P150.19, P150.28 and P150.30. Each anti dengue envelope hybridoma clone was cultured, and supernatants were purified by affinity chromatography. Immunoglobulin G [IgG] yields were between 20-28 mg/liter of cell culture supernatant. Isotyping of the three mAbs was carried out by commercially available isotyping strip [Roche]. The results indicated that the heavy chain of all three MAbs [P150.19, P150.28 and P150.30] was of the IgG1 class and the light chain was found to be kappa for all of the mAbs. The purity of the anti-dengue envelope mAbs was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE], and Western blot analysis showed the specificity of purified mAbs to the Dengue envelope antigen.

Binding studies with purified Dengue virus and dengue env domain III fragments from all the four serotypes were carried out. The recombinant protein fragments were selected

as they represent the active sites, which are the target for neutralizing epitopes. Also the env protein is known to elicit the primary immune response in primary dengue infections [19]. ELISA plates were coated with 20 ng of purified dengue virus [DENV1-4] or 200 ng of recombinant dengue env domain III [rDIII-D1 to D4] [in carbonate buffer at pH 9.6 for 2 h at room temperature and incubated with blocking buffer [0.05% TBS-T containing 3% skim milk or 3% normal goat serum] at 37^{o}C for 1 hr. Anti-dengue env mAbs [800 ng] diluted in blocking buffer were added for 1 h at 37°C followed by alkaline phosphatase-conjugated goat anti-human IgG [Sigma] for 1 h at 37°C. Lastly, p-nitrophenyl phosphate substrate [Sigma] was added to each well and the reaction was allowed to develop for 15 minutes before measuring absorbance at 405 nm on a spectrophotometer. Results indicated good binding particularly with dengue type 1 virus owing to the fact the antibodies were raised against the recombinant envelope protein from serotype 1. But the mAbs also showed considerable cross reactivity with the other serotypes [Fig 8.1]. ELISA data with recombinant dengue envelope domain III fragments also showed considerable binding [Fig 8.2]. It can be assumed from the results that the binding region of the mAbs lie in some overlapping regions between Domain III and the other domains of dengue envelope protein.

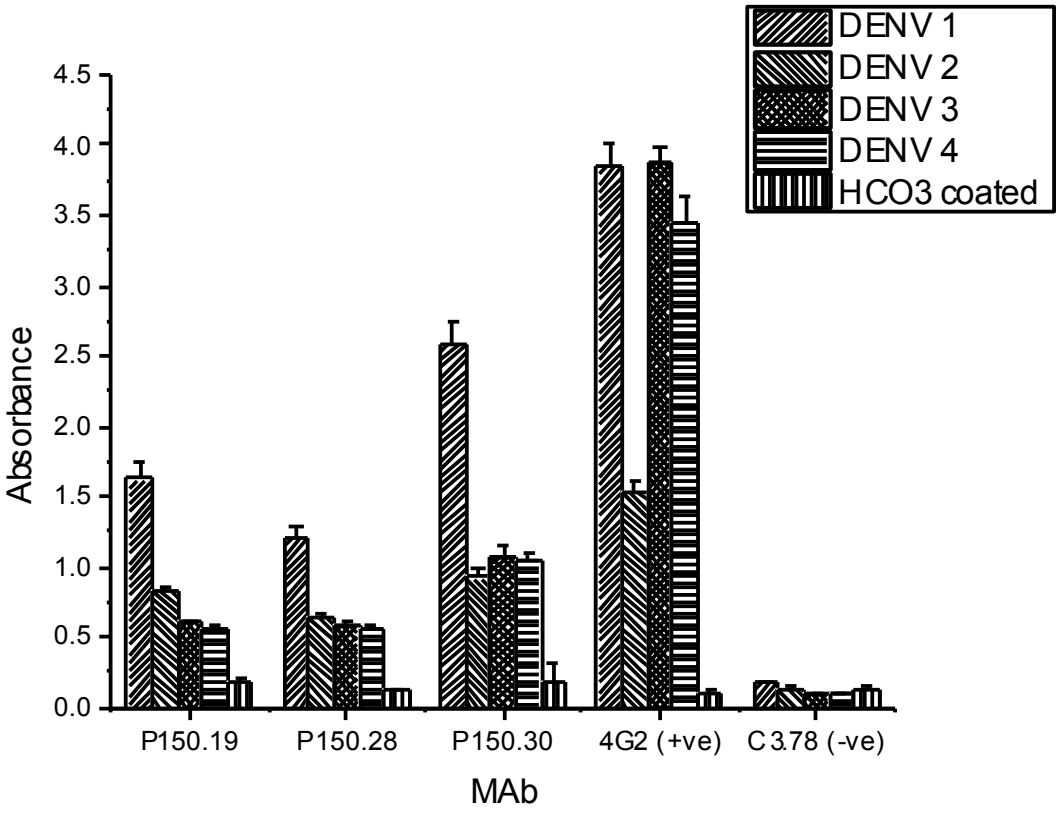


Fig 8.1: Binding analysis between mAbs and purified dengue virus of each serotype. Absorbance was measured at 405 nm

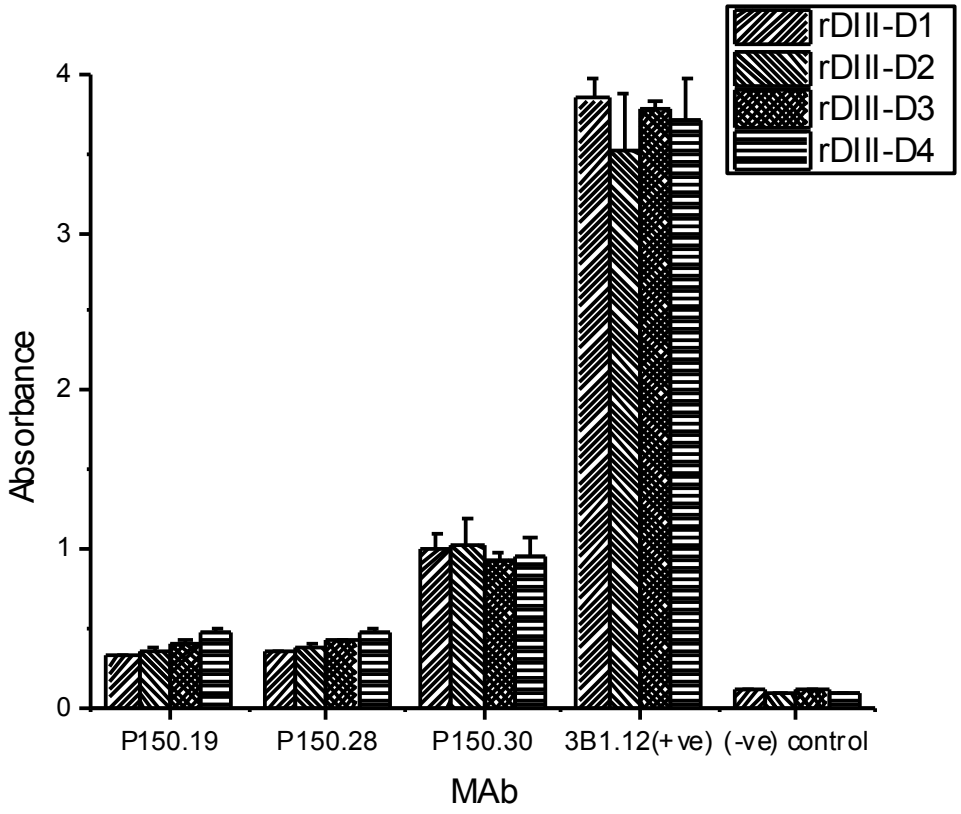
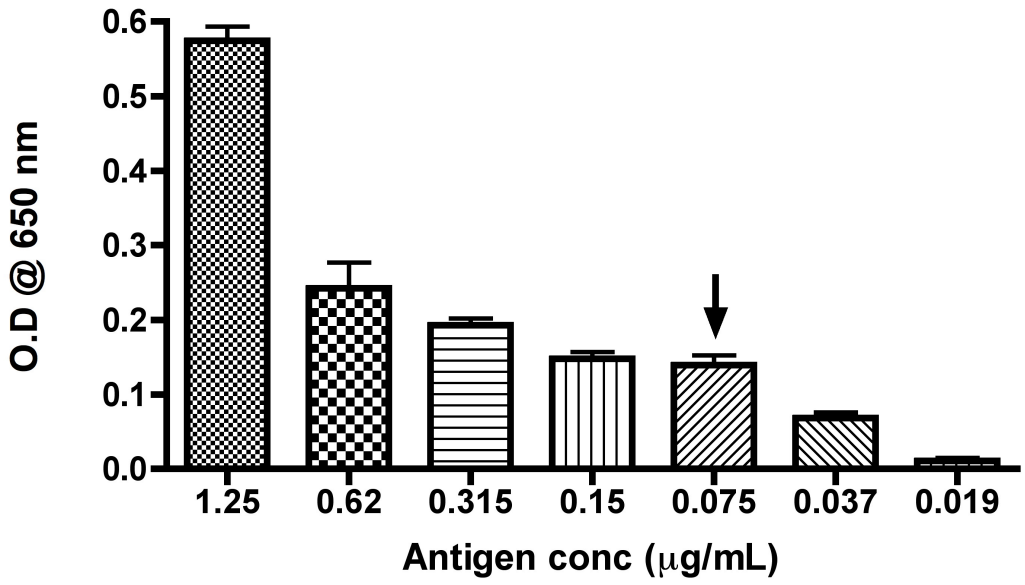


Fig 8.2: Binding analysis between the mAbs P150.19, P150.28, P150.30 and purified, recombinant enve Domain III protein from each Dengue serotype to measure binding affinity. Absorbance was measured at 405 nm

The neutralizing potential of the three anti-dengue env mAbs were measured by a focus reduction neutralization test [FRNT] with Vero cells or using a flow cytometry-based neutralization assay with the U937 human monocytic cell line stably transfected with DC-SIGN according to a previously established procedure [16]. All the three mAbs turned out to be non-neutralizing but appeared to be slightly enhancing supporting the phenomenon of antibody dependent enhancement. P150.30 was found to be the mAb with the strongest affinity against the purified virus as well as the protein fragments.

In the hetero-sandwich immunoassay, an optimal concentration of 5 μg/ml was used for the capture antibody and 1 μg/ml was used for the detection antibody. These concentrations were optimized in preliminary experiments using checkerboard titrations [17]. Full-length recombinant dengue env protein at different concentrations was used in the immunoassay. The immunoassay was specific and sensitive to detect nanogram levels of antigen [Fig 8.3].

[A]


O.D @ 650 nm
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1.25
0.62
0.315
0.15
0.075
0.037
0.019
Antigen conc (μg/mL)

[B]

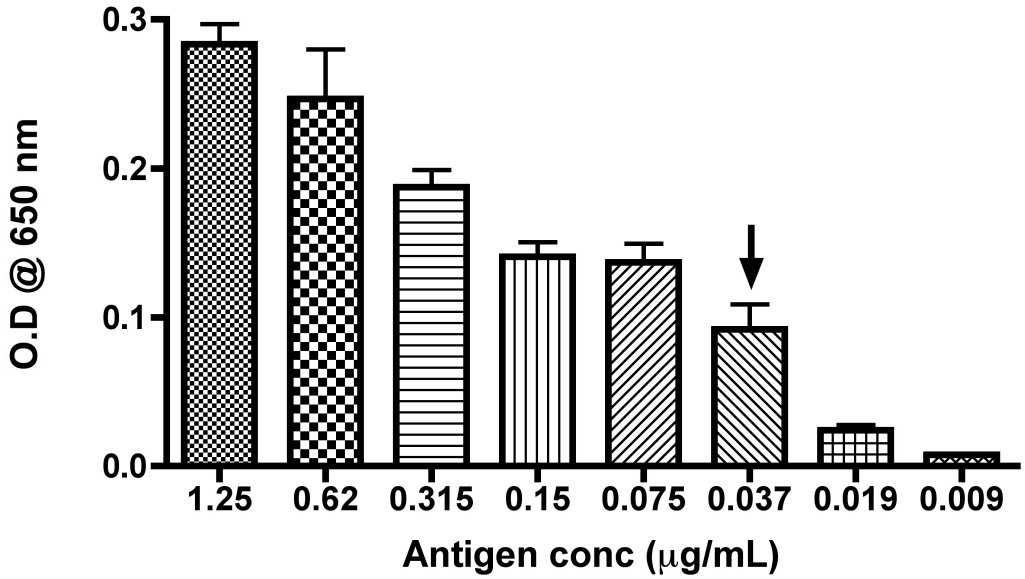


Fig 8.3: Evaluation of hetero-sandwich pairs of anti-dengue env mAbs at low antigen concentrations. P150.19 [A] and P150.30 [B] formed excellent hetero-sandwich pairs with P150.28 as the detecting mAb. Detection was carried out by the addition of streptavidin-horseradish peroxidase to the biotin labeled detecting antibodies after the env protein was captured by capture antibodies.

Hence, the anti-dengue envelope mAbs generated and characterized in this study can be used, as effective diagnostic reagents to detect dengue infection and also be used in immunological studies to better understand dengue pathogenesis.

8.1 References

1. Halstead SB [2007] Dengue. The Lancet 370: 1644–1652

2. Kautner I, Robinson MJ, Kuhnle U [1997] Dengue virus infection: Epidemiology, pathogenesis, clinical presentation, diagnosis, and prevention. The Journal of Pediatrics 131: 516–524

3. Gubler DJ, Clark GG [1996] Community involvement in the control of Aedes aegypti. Acta Trop 61: 169–179

4. Holmes EC, Twiddy SS [2003] The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol 3: 19–28

5. Grist NR [1993] Aedes albopictus: the tyre-travelling tiger. J Infect 27: 1–4

6. Grist NR, Burgess NRH [1994] Aedes and dengue. The Lancet 343: 477–477

7. Benedict MQ, Levine RS, Hawley WA, Lounibos LP [2007] Spread of the tiger: global risk of invasion by the mosquito Aedes albopictus. Vector Borne and Zoonotic Diseases 7: 76–85

8. Gratz NG [2004] Critical review of the vector status of Aedes albopictus. Med Vet Entomol 18: 215–227

9. Gibbons RV, Kalanarooj S, Jarman RG, Nisalak A, Vaughn DW, et al. [2007] Analysis of repeat hospital admissions for dengue to estimate the frequency of third or fourth dengue infections resulting in admissions and dengue hemorrhagic fever, and serotype sequences. Am J Trop Med Hyg 77: 910–913

10. Gubler DJ [1998] Dengue and Dengue Hemorrhagic Fever. Clin Microbiol Rev 11: 480–496.

11. Kumar K, Singh PK, Tomar J, Baijal S [2010] Dengue: epidemiology, prevention and pressing need for vaccine development. Asian Pac J Trop Med 3: 997–1000.

12. Deen JL, Harris E, Wills B, Balmaseda A, Hammond SN, et al. [2006] The WHO dengue classification and case definitions: time for a reassessment. Lancet 368: 170–173

13. Gubler DJ [2002] Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 10: 100–103.

14. Guha-Sapir D, Schimmer B [2005] Dengue fever: new paradigms for a changing epidemiology. Emerg Themes Epidemiol 2: 1

15. Guzman MG, Kouri G [2002] Dengue: an update. The Lancet Infectious Diseases 2: 33–42.

16. Kraus AA, Messer W, Haymore LB, de Silva AM. Comparison of Plaque- and Flow Cytometry- Based Methods for Measuring Dengue Virus Neutralization. J Clin Microbiol. 2007;45: 3777–3780

17. Shahhosseini, S., D. Das, X. Qiu, H. Feldmann, S. M. Jones, and M. R. Suresh. 2007. Production and characterization of monoclonal antibodies against different epitopes of Ebola virus antigens. J. Virol. Methods 143:29-37

18. Kammila, S., D. Das, P. K. Bhatnagar, H. H. Sunwoo, G. Zayas-Zamora, M. King, and M. R. Suresh. 2008. A rapid point of care immunoswab assay for SARS-CoV detection. J. Virol. Methods 152:77-84

19. Cuzzubbo AJ, Endy TP, Nisalak A, Kalayanarooj S, Vaughn DW, Ogata SA, Clements DE, Devine PL. 2001. Use of recombinant envelope proteins for serological diagnosis of Dengue virus infection in an immunochromatographic assay. Clin. Diagn. Lab. Immunol. 8:1150–1155

9

CONCLUSION AND FUTURE STUDIES

The non-structural protein 1 [NS1] of dengue virus is a useful target for developing diagnostics of dengue infection since the protein is abundantly circulating in blood during the acute phase of the disease during both primary and secondary infections. Prior work has established that secreted NS1 levels in plasma correlates with viremia levels and hence can also be used to diagnose patients at the risk for developing dengue hemorrhagic fever. Thus detection of non-structural dengue antigens may be of benefit for an early rapid diagnosis of dengue infection due to its long half-life in the blood. Here we describe a simple and efficient method for the expression of NS1 in *E. coli*, which could potentially be used to develop monoclonal and bispecific antibodies for point of care diagnostics. *E.coli* codon optimized synthetic full-length NS1 gene of dengue serotype 1 [DEN-1] was successfully cloned and expressed in very high-level as inclusion bodies. The NS1 protein was successfully affinity purified and refolded as a recombinant NS1 [rNS1] protein in *E.coli* and yield was 230– 250 mg/L of bacterial culture. The rNS1 protein was used to immunize mice for hybridoma development. The polyclonal antiserum from animals immunized with this rNS1 protein was found to specifically recognize the rNS1, thus demonstrating the immunogenic nature of the protein. The rNS1 protein purified from *E.coli* could be useful for developing a sensitive serum diagnostic assay to monitor dengue outbreaks.

We tried raising monoclonal antibodies and second-generation bispecific antibodies for dengue diagnostic research. A bridge ELISA was used to select the quadromas obtained for bispecific monoclonal antibody purification and characterization. Preliminary enzyme linked immunosorbent assays for dengue NS1 antigen detection was also carried out. Optimization of these novel reagents would help to develop simple and inexpensive dengue diagnostics like the quadroma-based capture enzyme-linked

immunosorbent assay for the diagnosis of the dengue virus nonstructural protein NS1. The hetero-sandwich assay involves using mAb and bsMAb as capture and detection antibodies, respectively. By using the bsMAb capture-antibody, the sensitivity of the sandwich ELISA assay developed, was significantly enhanced. We were able to establish a detection sensitivity of 31.25 pg/ ml for NS1 protein from dengue 1 virus. A significant feature of the assay is, that it reduces the number of steps required to determine detection thus reducing the cost of the diagnosis. The ELISA assay developed, because it is quicker, more sensitive and cheaper to administer, has the potential to become another useful diagnostic tool for dengue virus detection.

Another study was conducted to develop a quantitative detection system for dengue viral protein, targeting the dengue non-structural protein [NS1] to determine the presence of infection in suspected individuals using a combination of chicken IgY and mAbs. Full length NS1 was used to generate chicken polyclonal IgY antibodies for development of heterosandwich enzyme-linked immunosorbent assay for early diagnostic of dengue infection. The sensitivity of the developed heterosandwich ELISA can detect nanogram levels of recombinant NS1. This study illustrates an ultrasensitive ELISA using mAb as capture antibody and IgY as detecting antibody for the detection of dengue NS1 antigen. One of the most important findings was the use of inexpensive polyclonal IgY antibody to increase the sensitivity of the detection system for dengue at picogram level. Furthermore, the IgY based dengue NS1 antigen detection immunoassay developed could be an effective and sensitive method of diagnosing dengue suspected individuals during dengue outbreaks and screening people at ports of entry.

Yet another embodiment in the development of effective dengue diagnostics is the simple to use immunoswab based diagnostic procedure employing monoclonal antibodies and

the second-generation quadromas. The detection limit for NS1 has been established to be in the sub nanogram range. The assay is very sensitive, has a visual end point and also being extremely inexpensive. With this assay, screening time for a dengue-infected person would be very rapid.

Apart from the development of sensitive and specific dengue detection systems, it is imperative to simultaneously develop vaccine candidates and other constructs to understand the pathogenesis of dengue infection. Dengue virus infection is now a worldwide problem affecting nearly 2.5 billion people. The spread of the four-dengue virus serotypes had also led to the increased incidence of dengue haemorrhagic fever [DHF]. Endeavors towards developing safe and effective vaccines against dengue are a global priority. Dengue virus infection poses a serious public health threat and presently there is no licensed vaccine available. The dengue virus encoded envelope protein carries multiple conformation-dependent epitopes important for virus infectivity. We have expressed and purified the recombinant dengue virus type-1 antigen exploiting the codon optimized full-length envelope for increased yield in E.coli. A 6x His tag was inserted at the C terminus to facilitate purification. The purified protein was recognized in Western blot by monoclonal antibody specific for the tag. The *in vitro* refolded recombinant protein was used to immunize mice for the development of hybridomas and also analyzed for its biological functionality with heparan sulfate binding assay. The polyclonal anti-sera from the immunized mice were found to recognize the envelope protein thereby establishing the immunogenicity of the protein. Our recombinant envelope protein was able to successfully block dengue virus infection *in vitro*. The purified envelope protein was also used towards dengue diagnostics and vaccine development efforts.

Three monoclonal antibodies [mAbs] against recombinant dengue envelope protein were developed by hybridoma technology. Mapping by Western blot and ELISA studies with purified Dengue virus and recombinant dengue envelope domain III protein showed that these anti-dengue env mAbs bind to overlapping domains of dengue env proteins of all four dengue serotypes. These anti-dengue env mAbs, with their high specificity, are potentially ideal candidates for developing early and sensitive diagnostic assays for dengue as well as can be useful in studying dengue pathogenesis. The monoclonal antibodies were comprehensively characterized and it was observed its was able to significantly detect dengue virus type 1 and possessed limited cross reactivity to the other serotypes. These antibodies can be used in diagnostics as well as to better understand the pathogenesis of dengue.

We successfully proved all the hypotheses proposed in the thesis and beyond. We designed and produced the first of its kind second generation self-assembling bi-specific antibodies with binding specificities to dengue virus antigens NS1 in one paratope and HRPO in the second paratope and developed highly sensitive diagnostics for detection of Dengue NS1 antigen. The immunoassay thus developed possesses enhanced sensitivity in the bsMAb-based format in comparison to the conventional chemically conjugated mAb [biotin labeled] based format. The bsMab-based immunoassay was able to detect Dengue specific antigen at the point of care level. Another immunoassay involving chicken IgY as a diagnostic probe with good sensitivity and less expensive was also successfully developed as a supplementary diagnostic to detect Dengue infection. We also produced and characterized the dengue envelope antigen with higher yields and also developed monoclonal antibodies against the protein that were used to further substantiate dengue-screening assays. The Dengue envelope protein as a potential vaccine candidate was able

to neutralize viral infection.

Current studies are underway to address some of the limitations with our diagnostic assays. We are in the process of developing a tetravalent immunoassay that would be ideal for dengue screening particularly in endemic areas where there is simultaneous circulation of all the dengue serotypes. Sensitivity and specificity studies with dengue infected clinical samples are being planned to make our immunoswab assay more robust and effective. We are also addressing intra dengue serotype cross reactivity analysis for our assays maintaining precision parameters as well. Another limitation with the immunoswab assay in its present form could be the requirement of blood for analysis. Obtaining blood sample particularly from children may be difficult. To address this, we are in the process of developing a saliva-based assay that would be more user friendly. It should also be noted that another limitation of the assay, or any assay relying on a qualitative visual detection, is inter observer variations, which influences the sensitivity. To minimize the inter observer variability; the result of the assay should always be compared with a robust negative control. Future efforts involving development of targeted monovalent and tetravalent dengue DNA vaccines and exploiting of plant based expression of dengue antigens our discussed in the following sections.

9.1 Development of Low Dose Dendritic Cell Targeted Dengue DNA Vaccine

9.1.1 BACKGROUND

The enhanced transmission and global spread of dengue fever [DF] and its most severe presentations, dengue hemorrhagic fever [DHF] and dengue shock syndrome [DSS], make it one of the most crucial and challenging mosquito-borne viral disease of humans. Four distinct serotypes of dengue viruses are transmitted to humans through the bites of the mosquitoes. Currently, there is no vaccine or antiviral drug against dengue virus infections. Cross-protection between serotypes is limited and antibody dependent enhancement [ADE] contributes significantly to the severity of the disease. To address this severe human health hazard, we are investigating the *in vivo* efficacy of dendritic cell targeted [DEC-205] bifunctional fusion protein based tetravalent and monovalent dengue DNA vaccine formulations.

9.1.2 METHODS AND RESULTS

Based on consensus amino acid sequence, human codon optimized and RNA optimized constructs were obtained from GENEART, Germany. The codon-optimized sequence was further cloned into *pVAX* expression vector. The expression vectors are referred by their serotypes. Dengue-universal construct, pDENV-U encodes the combination all four DIII regions together as one open reading frame under CMV-promoter. In pDENV-U, DIII sequences are separated by endoproteolytic target sequences. The five DNA contructs were labelled with photobiotin acetate as per manufacturers instruction. Prior to

their application in vaccination, the ability of the monovalent as well as tetravalent DNA expressing plasmids to produce appropriate gene products was confirmed. Lipofectamine based transfection was done in HEK293T cells Western blot analyses was done with anti – Dengue MAbs to verify expression. The DC targeting vector was prepared using our established protocol. The pWET-7 vector was chemically transformed into BL21-CodonPlus® [DE3]-RIPL *E.coli* cells, cultured in 2xYT medium with antibiotics and induced with IPTG when OD reached 0.4 to 0.6. Following the induction the bacterial culture was grown at 26°C for 5 hr and harvested by centrifugation. Protein was extracted and lysate was cleared by centrifugation and loaded onto to the Ni-NTA matrix. The purification of periplasmic soluble protein was performed using immobilized metal affinity chromatography [IMAC]. A total of five mice per group are used for evaluating immune responses against vaccine construct. Mice are injected subcutaneously near the inguinal lymph node. The immunization protocol was based on our previous communicated data [1]. Serum was collected for analysis. The DIII regions of four subtypes were cloned individually as well as together under one open reading frame into the pVAX vector. In order to maximize expression as well as secretion into the blood stream efficiently, a highly efficient Ig-E leader sequence was fused in frame to the DIII domain coding sequence at its N-terminus. Similarly, the codon usage of this fusion gene was adapted to the codon bias of *Homo sapiens* genes to further enhance expression levels. Prior to mice immunization *in vitro* expression of the Dengue DIII fragments was confirmed in transfected HEK293T cells. After immunizing mice with vaccine constructs, serum was collected from each group and sent for analysis to our collaborators at the University of North Carolina, Chapel Hill, United States.

9.1.3 CONCLUSION

Four monovalent and one universal dengue DNA vaccine constructs were successfully made and functionality verified with *in vitro* expression. Mice were successfully vaccinated IM and serum analysis are underway. Future studies also include alternate models of antigen production particularly plant based systems which have been discussed in the next section.

9.1.4 REFERENCE

1. Welson W. Wang, Dipankar Das, and Mavanur R. Suresh, A Versatile Bifunctional Dendritic Cell Targeting Vaccine Vector, Molecular Pharmaceutics 2009 6 [1], 158-172

9.2 Overview of Plant-Derived Vaccine Antigens: Dengue Virus

9.2.1 BACKGROUND

The success story of any immunization programme depends upon the efficacy of the vaccine [1-4]. For many reasons, developing countries cannot afford to produce expensive vaccines [1, 2, 5, 6]. There are many issues associated with the production of vaccines that have been widely reviewed and discussed [1-4]. Traditional vaccines are produced by the application of fermentation technology in various cell culture systems. However, there are many limitations involved with fermentation so that development of alternative systems for the production of vaccines is timely [1-3, 5, 6]. Plants have been used as herbal drugs for millenia; they also play an important role in modern medicine. Recent advances in the area of plant biotechnology have revealed many achievements, including the production of desired vaccine antigens in plants [4,7-16]. The use of plants for the production of vaccines has many advantages, which have been previously discussed [1, 2, 4, 6, 13, 14, 15, 17-22]. Plant cells also play an important role in the accumulation of foreign proteins in specialized cell compartments, allowing for the retention of native biological activity [7, 21, 23-27]. There are many established and published protocols in the literature for the isolation of protein from plant tissues. Purification of an antigenic protein from plant cell culture is very simple since the antigenic protein is histidine-tagged and can be separated using an immomobilized-metal affinity chromatography [IMAC] system. Plants are considered to be effective alternative production systems for subunit vaccines as they are likely to contribute to all of these critical features of effective vaccines [12, 14, 28-33]. The strategy for the production of plant- derived vaccine antigen [PDVA] against human infectious diseases is

diagrammatically presented in Figure 1. Furthermore, plants are photoautotrophic and use light as their energy source, unlike mammalian or insect cell culture growth, resulting in a more robust and inert system for the production of subunit vaccines in different plant systems [4, 34]. Several plant-derived vaccines have been through Phase I and II clinical trials in humans and are increasingly developed [4, 13, 15]. Plant derived vaccines constitute a technology with proven worth as a cheap and easy means to scale-up the production of valuable materials [7, 15, 21, 26, 27, 35]. The production of antigenic protein in plants affects protein yield. The yields of PDVA can be as high as 45% of a plant cell's total protein compared to that of vaccines produced from a mammalian cell culture system [36]. Thus far, several plant species, including potato, tobacco, tomato, Arabidopsis, soybean, alfalfa, lettuce, lupin, rice, banana, grapes, watermelon, black-eyed bean, cowpea, and corn have been used for production of vaccine antigens against dengue, tuberculosis, human immune deficiency virus [HIV], foot and mouth disease, hepatitis virus B surface antigen, cholera toxin B, severe acute respiratory syndrome [SARS], Norwalk virus capsid protein, avian influenza [H5N1 subtype], swine fever, malaria, diabetes-type 1, human papilloma virus, rotavirus, and smallpox recombinant vaccine virus [3-5, 30- 33, 37, 38]. Separately, the use of a magnifection system increased the total soluble protein accumulation level of hepatitis B virus [HB core] in tobacco by more than 7% [6, 39-41]. Plant-derived vaccines [PDVs] could soon be on the market due to many advantages. This is mainly due to the established plant tissue culture protocols for many commercial plants, as well as established gene cloning and plant transformation technology [42]. A number of factors may modulate gene expression in plants, including: appropriate codon optimization popularized by Geneart Inc., Germany, Integrated DNA Technologies [IDT], USA, Biobasic Inc., Canada, Abnova Inc., Taiwan;

promoter, leader and polyadenylation signals. A very powerful tool in biotechnology is genetic transformation and transgenic plants represent a potentially stable and cheap propagation source for the production of a protein of pharmaceutical interest such as a vaccine [42-46]. This is achieved by the transfer of a foreign gene of interest into the nuclear or organelle [chloroplast] genome of the plant to generate transgenic plants that express the antigenic protein [3, 34, 47-48]. Transgenic plants are most commonly obtained by Agrobacterium-mediated gene transfer or by bombardment with DNA coated high velocity gold/tungsten particles [43, 49-53], both followed by an appropriate plant tissue culture regeneration methods either via organogenesis or somatic embryogenesis [54-65]. Therefore, the successful application of plant tissue culture techniques and genetic transformation plays an important role in the production of PDVs.

9.2.2 GLYCOENGINEERING

Plant and mammalian glycosylation are not similar [7, 12, 22, 66-67]. The covalent attachment of sugar molecules to proteins in glycosylation has improved folding capacity, biological activity and solubility [22- 23, 68-69]. Proteins produced by plants lack the terminal galactose and sialic acid residues commonly found in animals and have **α**-[1, 3] fucose and **β**- [1, 2] xylose which are absent in mammalian systems [7, 22, 70]. Recently these issues have been addressed by the application of genetic engineering techniques [22]. On the other hand, PDVAs have shown higher immunogenicity than other expression systems [7, 22, 38]. However, the immunogenicity of plant glycans is still of major concern in the context of plant proteins [7, 21, 22, 26, 27, 71]. Furthermore, 13 [1, 4]-galactosyltransferase is one of the key enzymes that plays an important role in the glycosylation pathway [7, 22, 71]. Production of transgenic tobacco resulted in 15% of

proteins expressing terminal [1, 4]-galactose residues [7, 21, 22, 71]. Various methods have been adopted to modify the N-glycosylation pattern in plants [68, 69, 72, 73]. Therefore, plants could be used for the production of glycosylated proteins for the development of vaccine antigens against human diseases [68, 69, 74].

9.2.3 COMMERCIALIZATION AND CLINICAL TRIALS

Plant-derived vaccines will likely replace traditional vaccines in the pharmaceutical industry in the future [6, 15, 38, 39, 68, 69]. A review article published by Tiwari and co-workers [15] highlighted the outcome and results of most clinical trials of plant-derived vaccines. Our paper updates that review with some more information on clinical trials. On the basis of published reports, PDVAs were successful during phase I clinical trials [15, 69]. There are many plant-derived vaccines that, during clinical trials, induced neutralizing antibodies following immunization [9, 15, 44-46, 68, 69, 75, 76, 77, 78]. A US-based company [Dow Agro Sciences LLC] received regulatory approval for a plant-made vaccine from the US Department of Agriculture [USDA] Centre for Veterinary Biologists in 2006 [15, 68]. This plant culture-derived veterinary vaccine antigen that protects poultry from Newcastle disease [www.thepoultrysite.com] also met the requirements of the FDA [15, 68, 69]. In another development, a Canadian-based company from Calgary, SemBioSys Inc., has completed Phase II trials of insulin produced in transgenic safflower [Carthamus tinctorius L.], and has filed an Investigational New Drug Application with the FDA, and submitted a Clinical Trial Application to European authorities [38, 79]. Medicago Inc. [Canada] are currently undergoing Phase II trials for their avian influenza vaccine produced transiently in tobacco [Nicotiana tobaccum], after receiving clearance from Health Canada [38, 80].

Taliglucerase alfa produced in stable carrot cell cultures is used to treat Gaucher disease. Protalix Bio-Therapeutics has just completed a Phase III trial and the product was approved by the FDA. Furthermore, the FDA has also accepted a New Drug Application and granted a Prescription Drug User Act action date in early 2011 [38, 81]. Therefore, results of clinical trials have already confirmed the potential of transgenic plant biotechnology in diagnostic and therapeutic industry.

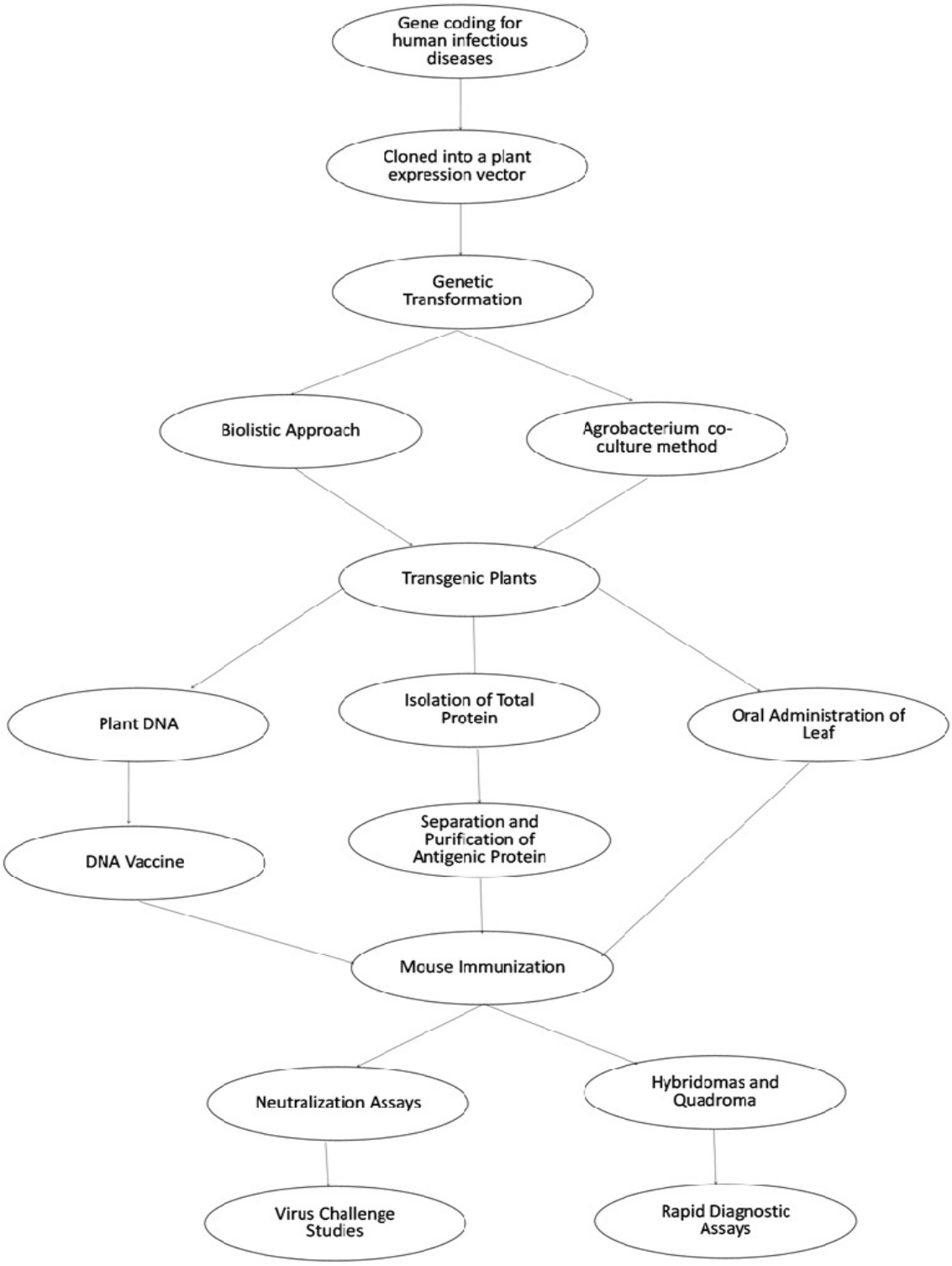


Fig 9.1 Schematic representation of strategies for the development of plant derived vaccines and diagnostics

9.2.4 DENGUE EXPRESSION SYSTEMS: CASE STUDIES

Dengue is one of the major causes of mosquito borne viral disease of humans reported in different regions of the world [82-87]. The World Health Organization [WHO] estimates that approximately more than 2.5 billion people are at risk of getting infected with dengue [82, 84, 86-91]. In some countries, dengue disease has become the leading cause of death among children [82-84, 92-98]. Different expression systems [bacterial, mammalian, baculovirus, and yeast] have been used for the production of dengue antigenic protein from various laboratories around the world. Bacterial expression is one of the most commonly employed expression systems for the production of recombinant proteins [99, 147]. A gene fragment from the structural envelope glycoprotein [Env] and the non-structural protein [NS1] of dengue virus serotype 2 [DEN-2] was expressed in *E.coli* as a fusion protein with Staphylococcal protein A [100]. The expressed protein was found to be immunogenic against dengue2 virus in a mouse model [100]. Hermida and co-workers [101] immunized Macaca fascicularis monkeys with two variants of these proteins [PD3 [insertion variant] and PD5 [fusion variant]] corresponding to serotype 2 of dengue disease. The results of this study confirmed the induction of immunity in a nonhuman primate model by using *E.coli* as the expression platform [101]. In another development, Khanam and coworkers [102] developed a recombinant adenovirus capable of expressing the E domain III [EDIII] of DEN-2 and evaluated its potential as a dengue vaccine [102]. This study also showed the induction of antibodies that specifically neutralized the infectivity of DEN-2 virus [102]. Furthermore, the dengue antigen specific antibody titers elicited by the fusion protein [Domain II of *Mycobacterium tuberculosis* [Mtb] heat shock protein 70 [HSP70], was covalently linked to a recently described synthetic dengue virus antigen], and successfully expressed in *E.coli* [103]. A

higher rate of anti-dengue antibodies were produced in mice than those induced by either the synthetic dengue antigen alone or a physical mixture of the dengue antigen plus Mtb HSP70 domain II protein [103]. A recombinant vaccine strain SL3261/pLT105 of attenuated aroA *Salmonella enterica serovar Typhimurium* SL3261 strain expressing a secreted [DEN-2] non-structural NS1 and *Yersinia pestis* F1 [Caf1] fusion protein, rNS1: Caf1, was developed to test its immunological potential via a prime boost vaccine regimen [104]. The addition of an antifungal antibiotic amphotericin B [AmB] to Salmonella vaccine further boosted the synergic effects of prime-boost vaccine regimen on the elicited NS1-specific serum IgG response and the protective efficacy [104]. Therefore, this study clearly demonstrated the potential of amphotericin B [AmB] as an effective strategy for dengue vaccine development [104]. Furthermore, a bacterial [*E.coli*] expression system has been utilized to study the immunogenicity and protective capacity of a recombinant capsid protein from dengue-2 virus [105]. The results of that study also demonstrated the protective capacity of the capsid protein of dengue virus indicating the existence of a protector mechanism totally independent of the antibodies [105]. The immunomodulatory potential of refolded dengue [D4EIII] protein in combination with various adjuvants [Freund's Complete adjuvant, Montanide ISA720, Alum] has been reported by Babu and coworkers [106]. All the formulations resulted in high antibody titers that neutralized the virus entry in vitro [106]. D4EIII, in combination with montanide ISA720 and Freund's complete adjuvant, gave highest antibody endpoint titers followed by alum [106]. Therefore, this study demonstrated the recombinant D4EIII protein expressed in *E.coli* induced neutralizing antibodies and cell-mediated immune response in immunized animals in combination with different adjuvants [106]. On the other hand, Sim and coworkers [107] noted that mucosal vaccines present several

advantages over conventional vaccines, including their ease of administration and low cost [107]. Their study showed that the antibody's response depended on the route of administration and on the mouse strain inoculated [107]. A simple and significant method for the expression of NS1 in *E.coli* has been demonstrated to develop monoclonal and bispecific antibodies for dengue point of care diagnostics [99]. An *E.coli* codon-optimized synthetic full-length NS1 gene of dengue serotype 1 [DEN-1] was successfully cloned and expressed at very high levels as inclusion bodies [99]. The rNS1 protein was used to immunize mice for hybridoma development [99]. The polyclonal antiserum from animals immunized with this rNS1 protein was found to specifically reorganize the rNS1, thus demonstrating the immunogenic nature of the protein [99]. The rNS1 protein purified from *E.coli* could be useful for developing a sensitive serum diagnostic assay to monitor dengue outbreaks [99]. Dengue NS1 antigen testing is one of the important tools for the early diagnosis of dengue infection after the onset of fever [99]. Commercially available dengue NS1 antigen capture ELISA has been evaluated for the detection of NS1 from patients in different stages. It is therefore, an important antigen for rapid viral diagnosis [99, 108]. Khanam and co-workers [109] successfully produced a tetravalent vaccine against dengue disease by mixing four monovalent vaccine components [109]. This vector induced effective immune responses and virus-neutralizing antibodies specific to each of the four dengue virus serotypes [DENVs] in mice [109]. Interestingly, anti-AdV5 antibodies did not suppress the induction of DENV-specific neutralizing antibodies [109]. Therefore, it could be an alternative approach for the development of a single component tetravalent vaccine that bypasses the complexities inherent in the currently adopted four-in-one physical mixture approach [109]. Very recently, Block and coworkers [110] evaluated the high yield insect cell expression, neutralizing and

enhancing antibody response to E domain III [dIII] proteins of dengue virus in which serotype-specific neutralizing determinants were concentrated [110]. Therefore, vaccine strategies directed to DENV-dIII-targeted neutralizing antibody production remain attractive [110]. In another development, Brandler and coworkers [111] evaluated for the first time a new strategy based on the expression of a single minimal tetravalent DV antigen by a single replicating viral vector derived from pediatric live attenuated measles vaccine [MV] [111]. That study reports the successful induction of neutralizing antibodies against DENVs [111].

Hence, this study concluded the possibility of a combined measles-dengue vaccine, which might be feasible to immunize infants against both diseases [111]. In another study by Batra and coworkers [112], biotinylated chimeric dengue antigens have been used to exploit the high affinity of the biotin–streptavidin interaction for the detection of anti-dengue antibodies [112]. On the other hand, Ramanathan and co-workers [113] highlighted two major problems in the development of a dengue vaccine: failure of a neutralization effect and the unequal presentation of antigens against DENVs [113]. This study also revealed the problems of allergic or varying levels of immune responses against dengue in different patients [113]. These problems have led many to consider the effectiveness of PDVAs and also DNA vaccines as a potential platform for the development of a dengue vaccine. Therefore, a PDVA against dengue would be immunogenic and offer protection from the disease. The ability to induce neutralizing antibodies against DENVs disease with a single immunogen is a significant advantage.

9.2.5 RELEVANCE OF PLANT-DERIVED VACCINE AGAINST DENGUE

Despite decades of efforts, no licensed vaccine for dengue disease is currently available

on the world market [84, 113, 114]. Vaccine development would be a major concern because dengue virus infections have led to the death of many people and now it is a major economic issue, especially in developing countries [84, 114, 115]. The development of a dengue virus vaccine is difficult, because every serotype provides life-long immunity, but infection with a heterologus virus enhances the disease severity [113, 114, 115]. This phenomenon is called ADE, and occurs often in children born to dengue-immune mothers [114]. A tetravalent vaccine with low reactogenicity would be an effective choice of treatment [113, 114, 115]. Several groups attempted to develop a vaccine, including live attenuated viruses, chimeric viruses, recombinant subunit antigens, vector-based vaccines and DNA vaccines. The Dengue E glycoprotein has been produced in several heterologous expression systems such as *E.coli* [116, 117], Pichia pastoris [118, 119], and baculovirus [120] with appropriate yields of the antigenic and immunogenic dengue 2E protein. However, the expression level of the full length or ectodomain of the dengue 2E protein is low in mammalian or insect cells or the expressed protein is easily degraded [115]. In addition, the existing expression systems have failed to show antibody neutralizing activity against DEN-2. To explore alternative expression systems, it is necessary to evaluate the production of dengue antigenic protein in plant cell culture and in transgenic plants. Recently a gene fragment-encoding domain III of the dengue 2 envelope protein [D2EIII] was successfully expressed in a model plant system *Nicotiana benthamiana* using a Tobacco mosaic virus [TMV]-based transient expression system [121-123]. The intramuscular immunization of mice with D2EIII induced the production of the antibodies against dengue virus [121-123]. The induced antibodies demonstrated neutralizing activity against DEN-2 [121, 122]. The results indicate that the plant system produces the dengue virus antigen, which possesses appropriate antigenicity

and immunogenicity [121, 122]. Therefore, transgenic plants demonstrate the feasibility of using PDVs to prevent infection by the dengue virus [121, 122]. Saejung and co-workers [121] reported the successful induction of anti-dengue virus antibody as well as an anti-D2EIII antibody production in mice by utilizing the N. benthamiana-recombinant TocJ, a TMV-based viral vector, as a bioreactor system to produce the D2EIII protein [121]. The yield of purified D2EIII protein was 0.28% of total soluble protein TSP [121]. The high expression level of D2EIII was achieved by a combination of several factors, including targeting the protein to the endoplasmic reticulum [ER] by the signal peptide and 5′ UTR [121]. During the study conducted by Saejung and co-workers [121], it was also noticed that the higher the level of anti-dengue virus antibody, the higher the neutralizing antibody activity in the sera of immunized mice, which was directly correlated with the anti-dengue virus antibody level and the neutralizing antibody activity of the immunized mouse antisera [121]. The level of induction of immunized-mouse antisera to the dengue virus antigen was low [121]. A low level of anti-dengue virus antibody induction might be due to the small antigenic fragments resulting in insufficient induction of high antibody production, even though an adjuvant was used in the experiment [121]. No antibody induction was detected when mice were immunized with plant produced D2EIII protein without adjuvant [121]. Immunized mice induced neutralizing antibodies, with plant-produced D2EIII protein with Titer Max Gold adjuvant many times and for a long period [121]. Therefore, vaccine development should focus on high-level induction of neutralizing antibodies [121]. Furthermore, the titer of the neutralizing antibody induced by the plant produced D2EIII protein was not high, but was quite promising [121]. The neutralizing antibody titer can be increased by changing the antigen dose, route of immunization, or by using other adjuvants [121]. The

advantages of using TocJ-TMV as an expression system are a high yield of a foreign protein up to 10% of TSP in infected leaves, a short time required for protein expression, and low toxicity [121]. In another study by Kim and co-workers [122], the plant-produced domain III of dengue virus E glycoprotein [EIII] was between 0.13 and 0.25% of the total soluble protein in transgenic N. tabacum L. cv. 'MD609' under the control of the 35S promoter [122]. This study demonstrated the feasibility of using plant-based vaccines to prevent infection by the dengue virus [122]. A truncated version of dengue virus env glycoprotein was designed and expressed alone and coexpressed with dengue virus structural proteins [122]. The recombinant proteins were produced in N. benthamiana plants and were reactive with the anti-env antibody [123]. The fusion was reactive with both anti-env and anti-HB core antibodies [123]. Some studies have shown that Flavivirus env glycoprotein produced in different expression systems undergoes proteolytic degradation [124]. However, Martínez and co-workers [123] reported the absence of any discernible low MW protein when expressed in an *N. benthamiana* system, indicating that there are no plant proteolytic degradation events [123]. They also demonstrated that the dengue virus env glycoprotein truncated version with a KDEL retention signal or co-expressed with other structural proteins in plants was not subjected to such a degradation process [123]. The level of protein accumulation was estimated and expressed protein equalled 0.6 mg/g FW leaf at 7 dpi, CMEt at 0.5 mg/g FW leaf at 10 dpi and HB core-dengue virus env glycoprotein at 0.4 mg/g FW leaf at 7 dpi [123]. Furthermore, the production of domain III of the DEN-2 env protein in tobacco plants using a TMV transient expression system is safe for humans and the environment [123]. N. benthamiana is a non-food crop in which foreign proteins are subsequently purified or processed to yield desirable products [34, 42, 121-123].

9.2.6 DENDRITIC CELL TARGETING STRATEGIES FOR DENGUE

Dendritic cells [DCs] have been identified as the most specialized and potent antigen presenting cells capable of initiating and directing immune responses following infection [125-133]. Wang and co-workers [131, 132] developed a bifunctional fusion protein [bsMAb] that can bind any class of biotinylated antigen proteins, peptides, carbohydrates, gangliosides and even naked DNA and target them to a DEC-205 receptor molecule which is expressed on DCs [131]. In another study, biologically active plant-derived medicines have been identified with DC-modulating properties [133]. Plant-derived medicines play an important role as immunomodulators of DCs for the maintenance of human health [133]. In many infectious and cancerous situations, priming immune system to a single antigen may not be sufficient for induction of effective immune responses [131, 132]. Therefore, a universal DC-targeting vehicle such as bispecific or bifunctional antibody [bsMAb or bfMAb, respectively] that can bind to a mixture of biotinylated plant-derived antigens would be the best approach to elicit the immune response against dengue [82, 131, 132,134, 135, 136, 137-146]. Therefore, PDVA presentation by DC targeting might play an important role in eliciting a good immune response with efficient neutralizing antibodies against dengue.

9.2.7 LIMITATIONS OF PDVAS

PDVAs have many challenges. The major limitation of this system is the relatively low protein yield, protein degradation and incorrect post-translational modifications of protein [3, 15, 22]. Recently, these issues have been addressed by improving the protein expression levels in plants by some important steps by using ER retention signal sequences like KDEL and HDEL, by the use of a proper promoter, choice of vectors, methods of transformation, and codon optimization [22]. Development of codon-optimized genes for plants during expression studies has improved protein yields up to 200 mg/g FW of tobacco tissue. Recently, the yield of purified dengue 2E III protein was 0.28% total soluble protein [TSP]. This high expression level was achieved by a combination of several factors viz. by inserting the plant signal peptide at the N-terminal end, and C-terminal ER retention sequence for plants [121]. Expression of protein in the ER has drastically reduced the degradation of expressed protein [antigen] and resulted in a higher yield of protein accumulation [12, 15, 22]. Gene silencing issues are another major problem during the expression of vaccine antigenic proteins in transgenic plants. This is resolved by the use of *Agrobacterium*, which tends to result in fewer copies of transgenes than biolistic transformation [15, 22, 43]. Biosafety, risk assessment, and public acceptance of transgenic plants producing vaccine antigens are other issues. These issues have been widely discussed by other researchers and are beyond the scope of this chapter. Furthermore, cross contamination of transgenic plants with other food crops was resolved by applying containment approach technology, thus limiting the environmental exposure of transgenic products [15, 22]. The slow growth of plants under in vitro conditions has also hindered the commercialization of PDVAs. This is particularly related

to the regeneration of transgenic plants under in vitro conditions. This issue is solved by the manipulation of media, growth conditions and plant growth regulator concentration to develop a reproducible plant tissue culture protocol before considering the right candidate for genetic transformation.

9.2.8 CONCLUSION

Production of a vaccine antigen against dengue, an infectious disease, is one of the serious problems due to many issues. There are many disadvantages associated with the traditional production of vaccine antigens. This situation has forced many scientists throughout the world for the production of PDVA against many human infectious diseases including dengue. Recently many private companies and government agencies throughout world have also joined hands and funded many projects on the PDVAs in order to meet current demands of the immunization programmes particularly in the developing countries. DC targeting approach using PDVA may play an important role. This would help in the early diagnostic tests in the patients suffering from dengue infection. On the other hand, in many instances, plants will replace the traditional mammalian system as a cost effective vaccine production system against dengue. This would also save of money in developing countries because of high budget of health care programmes. Therefore, the plants seem to be a superior expression system, and plant biotechnology has a bright future.

9.2.9 REFERENCES

1. Davoodi-Semiromi A, Samson N, Daniell H. The green vaccine: A global strategy to combat infectious and autoimmune diseases. Hum vaccines, 2009; 5[7]: 488-493.

2. Davoodi-Semiromi A, Schreiber M, Nallapali S, Verma D, Singh ND, Banks RK, Chakrabarti D, Daniell H. Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. Plant Biotech J, 2010; 8[2]: 223-242.

3. Daniell H, Singh ND, Mason H, Streatfield SJ. Plant-made vaccine antigens and biopharmaceuticals. Trend Plant Sci, 2009; 14[12]: 669-679.

4. Rybicki EP. Plant-made vaccines for humans and animals. Plant Biotech J, 2010; 8: 620-637.

5. Bock R, Warzecha H. Solar-powered factories for new vaccines and antibiotics. Trends Biotech, 2010; 28[5]: 246-252.

6. Gomez E, Zoth SC, Carrillo E, Berinstein A. Developments in plant-based vaccines against diseases of concern in developing countries. The Open Infect Dis J, 2010; 4: 55-62.

7. Ma JKC, Drake PMW, Chargelegue D, Obregon P, Prada A. Antibody processing and engineering in plants, and new strategies for vaccine production. Vaccine, 2005; 23: 1814-1818.

8. Obregon P, Chargelegue D, Drake PMW, Prada A, Nuttall J, Frigerio L, Ma JKC. HIV-1 p24– immunoglobulin fusion molecule: a new strategy or plant-based protein production. Plant Biotech J, 2006; 4: 195–207.

9. Thanavala Y, Yang YF, Lyons P, Mason HS, Arntzen C. Immunogenicity of

transgenic plantderived hepatitis B-surface antigen. Proc Nat Acad Sci USA, 1995; 92: 3358-3361.

10. Korban SS. Targeting and expression of antigenic proteins in transgenic plants for production of edible oral vaccines. In Vitro Cell Dev Biol - Plant, 2002; 38: 231-236.

11. Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. Nature, 1989; 342: 76-78.

12. Hellwig S, Drossard J, Twyman RM, Fischer R. Plant cell cultures for the production of recombinant proteins. Nat Biotech, 2004; 22 [11]: 1415-1422.

13. Vermij P, Waltz E. USDA approves the first plantbased vaccine. Nat Biotech, 2006; 24: 233-234.

14. Malabadi RB. Production of edible vaccines for oral immunization in transgenic plants, current and future prospective. J Phytol Res, 2008; 21[1]: 1-10.

15. Tiwari S, Verma PC, Singh PK, Tuli R. Plants as bioreactors for the production of vaccine antigens. Biotech Advan, 2009; 27: 449-467.

16. Malabadi RB, Parashar A, Ganguly A, Suresh MR. Expression of Dengue virus envelope protein in a different plant system. Faculty Research and Development day, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada, 19th November 2010. Abstract No-69, page no-31.

17. Walmsley AM, Arntzen CJ. Plant cell factories and mucosal vaccines. Curr Opin Biotech, 2003; 14:145-150.

18. Pascual DW. Vaccines are for dinner. Proc Nat Acad Sci USA, 2007; 104[26]: 10757-10758.

19. Lal P, Ramachandran VG, Goyal R, Sharma R. Edible vaccines: Current status and future. Ind J Med Microbiol, 2007; 25 [2]: 93-102.

20. Giddings G, Allison G, Brooks D, Carter A. Transgenic plants as factories for biopharmaceuticals. Nat Biotech, 2000; 18[11]: 1151-1155.

21. Ma S, Huang Y, Yin Z, Menassa R, Brandle JE, Jevnikar AM. Induction of oral tolerance to prevent diabetes with transgenic plants requires glutamic acid decarboxylase [GAD] and IL-4. Proc Nat Acad Sci USA, 2004; 101: 5680-5685.

22. Desai PN, Shrivastava N, Padh H. Production of heterologous proteins in plants: strategies for optimal expression. Biotech Adv, 2010; 28: 427- 435.

23. Lienard D, Sourrouille C, Gomord V, Faye L. Pharming and transgenic plants. Biotech Ann Rev, 2007; 13:115-147.

24. Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. Proc Nat Acad Sci USA, 1992; 89: 11745-11749.

25. Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proc Nat Acad Sci USA, 1996; 93: 5335-5340.

26. Ma S, Zhao DL, Yin ZQ, Mukherjee R, Singh B, Qin HY. Transgenic plants expressing auto antigens fed to mice to induce oral immune tolerance. Nat Med, 1997; 3: 793-796.

27. Ma JK, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L. Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. Nat Med, 1998; 4: 601–606.

28. Goldstein DA, Thomas JA. Biopharmaceuticals derived from genetically modified plants. Oxf J Med, 2004; 97: 705-716.

29. Haq TA, Mason HS, Clements JD, Arntz CJ. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science, 1995; 268: 714-716.

30. Chen X and Liu J. Generation and immunogenicity of transgenic potato expressing the GP5 protein of porcine reproductive and respiratory syndrome virus. J Virol Meth, 2011; 173[1]: 153-158.

31. Phoolcharoen W, Bhoo SH, Lai H, Ma J, Arntzen CJ, Chen Q, Mason HS. Expression of an immunogenic Ebola immune complex in Nicotiana benthamiana. Plant Biotech J, 2011; [still in press].

32. Farrance CE, Chichester JA, Musiychuk K, Shamloul M, Rhee A, Manceva SD, Jones RM, Mamedov T, Sharma S, Mett V, Streatfield SJ, Roeffen W, Vegte-Bolmer MV, Sauerwein RW, Wu Y, Muratova O, Miller L, Duffy P, Sinden R, Yusibov V. Antibodies to plant-produced Plasmodium falciparum sexual stage protein Pfs25 exhibit transmission blocking activity. Hum Vaccines, 2011; 7: 191-198.

33. Mathew LG, Maloney B, Takeda N, Mason HS. Spurious polyadenylation of Norovirus capsid protein mRNA in transgenic plants. Plant Mol Biol, 2011; 75[3]: 263-75.

34. Tremblay R, Wang D, Jevnikar AM, Ma S. Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. Biotech Adv, 2010; 28: 214-221.

35. Arakawa T, Chong DKX, Langridge WHR. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. Nat Biotech, 1998; 16: 292-297.

36. Hefferon KL. The mucosal immune response to plant-derived vaccines. Pharma Res, 2010; 27: 2040-2042.

37. Shoji Y, Chichester JA, Jones M, Manceva SD, Damon E, Mett V, Musiychuk K, Bi H, Farrance C, Shamloul M, Kushnir N, Sharma S, Yusibov V. Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. Hum Vaccine, 2011; 7: 41-50.

38. Penney CA, Thomas DR, Deen SS, Walmsley AM. Plant-made vaccines in support of the Millennium Development Goals. Plant Cell Rep, 2011; 30: 789- 798.

39. Yusibov V, Rabindran S. Recent progress in the development of plant derived vaccine. Exp. Rev. Vaccines, 2008; 7: 1173-1183.

40. Gleba Y, Klimyuk V, Marillonnet S. Magnifectiona new platform for expressing recombinant vaccines in plants. Vaccine, 2005; 23: 2042-2048.

41. Huang Z, Santi L, LePore K, Kilbourne J, Arntzen CJ, Mason HS. Rapid, high level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. Vaccine, 2006; 24: 2506-2513.

42. Sala F, Rigano MM, Barbante A, Basso B, Walmsley AM, Castiglione S. Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives. Vaccine, 2003; 21[7&8]: 803-808.

43. Malabadi RB, Nataraja K. Genetic transformation of conifers: Applications in and Impacts on commercially forestry. Trans Plant J, 2007a; 1[2]: 289-313.

44. Tacket CO. Plant derived vaccines against diarrheal diseases. Vaccine, 2005; 23: 1866-1869.

45. Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in transgenic potato. Nat Med, 1998; 4: 607-609.

46. Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ. Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. J Inf Dis, 2000; 182: 302-305.

47. Maliga P. Plastid transformation in higher plants. Ann Rev Plant Biol, 2004; 55: 289-313.

48. Bally J, Nadal M, Vitel M, Rolland A, Dumain R, Dubald M. Plant physiological adaptations to the massive foreign protein synthesis occurring in recombinant chloroplasts. Plant Physiol, 2009; 150: 1474-1481.

49. Malabadi RB, Nataraja K. Stable transformation and recovery of transgenic plants by particle bombardment in P. wallichiana A. B. Jacks [Himalayan blue pine]. Biotechnology, 2007b; 6: 105-111.

50. Malabadi RB, Teixeira da Silva JA, Nataraja K. Agrobacterium tumefaciens-mediated genetic transformation of P. kesiya Royle ex Gord [Khasi pine]. Asian Australasian J Plant Sci Biotech, 2008a; 2[1]: 7-14.

51. Malabadi RB, Teixeira da Silva JA, Nataraja K. Stable and consistant Agrobacterium-mediated genetic transformation in P. roxburghii [Chir pine]. Tree For Sci Biotech, 2008b; 2[1]: 7-13.

52. Malabadi RB,Teixeira da Silva JA, Nataraja K. A new approach involving salicyclic acid and thin cell layers for cloning mature trees of Pinus roxburghii [Chir Pine]. Am J Plant Sci Biotech, 2008c; 2[2]: 56-59.

53. Malabadi RB, Teixeira da Silva JA, Nataraja K. Green fluorescent protein in the genetic transformation of plants. Trans Plant J, 2008d; 2[2]: 86-109.

54. Malabadi RB, Nataraja K. In vitro plant regeneration in Clitoria ternatea. J Med Arom Plant Sci, 2002; 24: 733-737.

55. Malabadi RB, Choudhury H, Tandon P. Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in Pinus kesiya [Royle ex. Gord] promoted by partial desiccation and gellan gum. Sci Hortic, 2004; 102: 449-459.

56. Malabadi RB, Mulgund GS, Nataraja K. Micropropagation of Dendrobium nobile from shoot tip sections. J. Plant Physiol 2005; 162:473- 478.

57. Malabadi RB, van Staden J. Somatic embryogenesis from vegetative shoot apices of mature trees of P. patula. Tree Physiol, 2005a; 25: 11-16.

58. Malabadi RB, van Staden J. Role of antioxidants and amino acids on somatic embryogenesis of P. patula. In Vitro Cell Deve Biol-Plant, 2005b; 41: 181-186.

59. Malabadi RB, van Staden J. Storability and germination of sodium alginate encapsulated somatic embryos derived from vegetative shoot apices of mature Pinus patula trees. Plant Cell Tiss Org Cult, 2005c; 82: 259-265.

60. Malabadi RB, Nataraja K. Cryopreservation and plant regeneration via somatic embryogenesis using

shoot apical domes of mature Pinus roxburghii Sarg. Trees. In vitro Cell Dev Biol - Plant, 2006; 42 [2]: 152-159.

61. Malabadi RB, Teixeira da Silva JA. Thin cell layers: Application to forestry biotechnology. Tree For Sci Biotech, 2011; 5[SI1]: 14-18.

62. Malabadi RB, Teixeira da Silva JA, Mulgund GS. Induction of somatic embryogenesis in Pinus caribaea. Tree For Sci Biotech, 2011; 5[SI1]: 27- 32.

63. Malabadi RB, Mulgund GS, Nataraja K. Triacontanol induced somatic embryogenesis and plantlet regeneration in Catharanthus roseus. J Med Arom Plant Sci, 2009; 31: 147-151.

64. Malabadi RB, Mulgund GS, Vijaykumar S. Expression of WUSCHEL-gene promoting somatic embryogenesis in plants. J Phytol Res, 2009; 22 [1]: 103-106.

65. Malabadi RB, Teixeira da Silva JA, Mulgund GS. TDZ-induced in vitro shoot regeneration of Aerides maculosum Lindl. From shoot tip thin cell layers. Floriculture Ornamental Biotech, 2009; 3[1]: 35-39.

66. Palacpac NQ, Yoshida S, Sakai H, Kimura Y, Fujiyama K, Yoshida T, Seki T. Stable

expression of human b1, 4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns. Proc Nat Acad Sci USA, 1999; 96: 4692-4697.

67. Bosch D, Schots A. Plant glycans: friend or foe in vaccine development? Exp Rev Vaccine, 2010; 9[8]: 835–842.

68. Obembe OO,Popoola JO, Leelavathi S,Siva V. Reddy SV. Advances in plant molecular farming. Biotech Adv, 2011; 29[2]: 210-222.

69. Obembe OO.The plant biotechnology flight: Is Africa on board? Afr J Biotech, 2010; 9[28]: 4300- 4308.

70. Sethuraman N, Stadheim TA. Challenges in therapeutic glycoprotein production. Curr Opin Biotech, 2006; 17[4]: 341-346.

71. Bakker H, Bardor M, Molthoff JW, Gomord V, Elbers I, Stevens LH. Galactose-extended glycans of antibodies produced by transgenic plants. Proc Nat Acad Sci USA, 2001; 98: 2899-2904.

72. Koprivova A, Stemmer C, Altmann F, Hoffmann A, Kopriva S, Gorr G. Targeted knockouts of Physcomitrella lacking plant-specific immunogenic N-glycans. Plant Biotech J, 2004; 2: 517-523.

73. Kang JS, Frank J, Kang CH, Kajiura H, Vikram M, Ueda A. Salt tolerance of Arabidopsis thaliana requires maturation of N-glycosylated proteins in the Golgi apparatus. Proc Natl Acad Sci USA, 2008; 105: 5933-5938.

74. Castilho A, Strasser R, Stadlmann J, Grass J, Jez J, Gattinger P. In planta protein sialyation through overexpression of the respective mammalian pathway. J. Biol Chem, 2010; 285: 15923-15930.

75. Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O. A plant derived edible vaccine against hepatitis B virus. FASEB J, 1999; 13:1796-1799.

76. Lamphear BJ, Jilka JM, Kesi L, Welter M, Howard JA, Streatfield SJ. A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine, 2004. 22: 2420-2424.

77. Basaran P, Rodriguez-Cerezo E. Plant molecular farming; opportunities and challenges. Crit Rev Biotech, 2008; 28:153- 172.

78. McCormik AA, Palmer KE. Genetically engineered tobacco mosaic virus as nanoparticle vaccines. Exp. Rev. Vaccines, 2008; 7: 33-41.

79. SemBioSys. SemBioSys provides update on insulin program. Press release. Retrieved 12 Dec 2010. http://micronewswire.ca/release.cgi?rkey=17040 16279&view =36078-0& Start=30&htm=0.

80. Medicago. Medicago receives regulatory approval and commences Phase II clinical testing of its avian influenza vaccine. Press release. Retrieved 12 Dec 2010. http://www.medicago.com/ English/news/News-Releases/News-Release Details/2010/Medicago-Receives-Regulatory- Approval-and-CommencesPhase-II-Clinical- Testing-of-its-Avian-Influenza-Vaccine1123044/ default.aspx [Nov 1, 2010].

81. Protalix BioTherapeutics. ProtalixBio Therapeutics announces preliminary top-line positive data from Taliglucerase alfa switchover trial. Press release. Retrieved 12 Dec 2010. http://phx.corporate ir.net/phoenix.zhtml?c= 10116 1&p=irol-newsArticle&ID=1490071&highlight= [Nov 2, 2010].

82. Murrell S, Wu S-C, Butler M. Review of dengue virus and the development of a vaccine. Biotech Adv, 2011; 29: 239-247.

83. Schieffelin JS, Costin JM, Nicholson CO, Orgeron NM, Fontaine KA, Isern S, Michael SF, Robinson JE. Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived from a naturally infected patient. Virol J, 2010; 7:

[28] 1-11.

84. Shrivastava A, Dash PK, Tripathi NK, Sahni AK, Gopalan N, Lakshmana Rao PV Evaluation of a commercial Dengue NS1 enzyme-linked immunosorbent assay for early diagnosis of dengue infection. Ind J Med Mirobiol, 2011; 29[1]: 51-55.

85. Monath TP. Dengue: the risk to developed and developing countries. Proc Nat Acad Sci USA, 1994; 85: 7627-7631.

86. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science, 1988; 239[4839]: 476- 481.

87. Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenic cascade. Rev. Infect. Dis, 1989; 11[4]: S830-9.

88. World Health Organization. W.P. R. Dengue in the Western Pacific Region [online]. http.//www.wpro.who.int/health_topics/dengue/ove rview.htm 2010.

89. World Health Organization. Vector borne viral infections: dengue fever [online]. http.//www.who.int/vaccine_research/diseases/vect or/en/index1.html. 2009.

90. Gubler DJ, Meltzer M. Impact of dengue/dengue hemorrhagic fever on the developing world. Adv Virus Res, 1999; 53: 35-70.

91. Simasathien S, Watanaveeradej V. Dengue vaccine. J Med Assoc Thai, 2005; 88[3]: S363-77.

92. Holmes E, Twiddy SS. The origin, emergence and evolutionary genetics of dengue virus. Inf Gen Evol, 2003; 3: 19-28.

93. Rico-Hesse R. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology, 1990; 174[2]: 479-93.

94. Sabin AB. Research on dengue during World War II. Am J Trop Med Hyg, 1989; 1[1]: 30-50.

95. Henchal EA, Putnak JR. The dengue viruses. Clin Microb Rev, 1990; 3[4]:376-396.

96. Perez-Velez ME, Garcia-Nieves T, Colon-Sánchez C, Martínez I. Induction of neutralization antibodies

J Pharm Pharmaceut Sci [www.cspsCanada.org] 14[3] 400 - 413, 2011 411 in mice by dengue-2 envelope DNA vaccines. PR Health Sci J 2009; 28[3]: 239-250.

97. Swaminathan S, Batra G, Khanna N. Dengue vaccines: state of the art. Exp Opin Therap Pat, 2010; 20[6]: 819-835.

98. Cardosa MJ. Dengue vaccine design: issues and challenges. Brit Med Bull, 1998; 54 [2]: 395-405.

99. Das D, Mongkolanngkoon S, Suresh MR. Super induction of dengue virus NS1 protein in E.coli. Prot Exp Purification, 2009; 66: 66-72.

100. Srivastava AK, Putnak JR, Warren RL, Hoke Jr CH. Mice immunized with a dengue type-2 virus E and NS1 fusion protein made in E.coli are protected against lethal dengue virus infection. Vaccine, 1995; 13: 1251-1258.

101. Hermida L, Bernardo L, Martín J, Mayling Alvarez M, Prado I, López C, Sierra BDC, Martínez R, Rodríguez R, Zulueta A, Pérez AB, Lazo L, Rosario D, Guillén G, Guzmán MG. A recombinant fusion protein containing the domain III of the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. Vaccine, 2006; 24: 3165- 3171.

102. Khanam S, Khanna N, Swaminathan S. Induction of neutralizing antibodies and T cell responses by dengue virus type 2 envelope domain III encoded by plasmid and adenoviral vectors. Vaccine, 2006; 24: 6513-6525.

103. Kolli R, Khanam S, Jain M, Ganju L, Sai Ram M, Khanna N, Swaminathan S. A synthetic dengue virus antigen elicits enhanced antibody titers when linked to, but not

mixed with, Mycobacterium tuberculosis HSP70 domain II. Vaccine, 2006; 24: 4716-4726.

104. Liu WT, Lin WT, Tsai CC, Chuang CC, Liao CL, Lin HC, Hung YW, Huang SS, Liang CC, Hsu HL, Wang HJ, Liu YT. Enhanced immune response by amphotericin B following NS1 protein prime-oral recombinant Salmonella vaccine boost vaccination protects mice from dengue virus challenge. Vaccine, 2006; 24: 5852-5861.

105. Lazo L, Hermida L, Zulueta A, Sánchez J, Lopez C, Silva R, Guillen G, Guzmán MG. A recombinant capsid protein from Dengue-2 induces protection in mice against homologous virus. Vaccine, 2007; 25: 1064-1070.

106. Babu JP, Pattnaik P, Nimesh Gupta N, Shrivastava A, Khan M, Lakshmana PV, Rao L. Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice. Vaccine, 2008; 26: 4655-4663.

107. Sim ACN, Lin W, Tan GKX, Sim MST, Chow VTK, Alonso S. Induction of neutralizing antibodies against dengue virus type 2 upon mucosal administration of a recombinant Lactococcus lactis strain expressing envelope domain III antigen. Vaccine, 2008; 26: 1145- 1154.

108. Kumarasamy V, Wahb AH, C, Chua SK, Hassan Z, Chem YK, Mohamad M, Chua KB. Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J Virol Meth, 2007; 140: 75-79.

109. Khanam S, Pilankatta R, Khanna N, Swaminathan S. An adenovirus type 5 [AdV5] vector encoding an envelope domain III-based tetravalent antigen elicits immune responses against all four dengue viruses in the presence of prior AdV5 immunity. Vaccine, 2009; 27: 6011-6021.

110. Block OKT, Shanaka WW, Rodrigo I, Quinn M, Jin X, Rose RC, Schlesinger JJ. A

tetravalent recombinant dengue domain III protein vaccine stimulates neutralizing and enhancing antibodies in mice.

Vaccine, 2010; 28: 8085-8094.

111. Brandler S, Ruffie C, Najburg V, Frenkiel MP, Bedouelle H, Despres P, Frederic-Tangy F. Pediatric measles vaccine expressing a dengue tetravalent antigen elicits neutralizing antibodies against all four dengue viruses. Vaccine, 2010; 28: 6730-6739.

112. Batra G, Talha SM, Nemani SK, Dhar N, Swaminathan S, Khanna N. Expression, purification and characterization of in vivo biotinylated dengue virus envelope domain III based tetravalent antigen. Prot Exp Purification, 2010; 74: 99-105.

113. Ramanathan MP, Kuo YC, Selling BH, Li Q, Sardesai NY, Kim JJ, Weiner DB. Development of novel DNA SynConTM tetravalent dengue vaccine that elicts immune response against four seoryptes. Vaccine, 2009; 27: 6444-6453.

114. Alan MMF, Kaptein SJF, Burghgraeve TD, Balzarini J, Neyts J, Schols D. Antiviral activity of carbohydrate-binding agents and the role of DCSIGN in dengue virus infection. Virology, 2009; 387: 67-75.

115. Yap YK, Smith DR. Strategies for the plant-based expression of dengue subunit vaccines. Biotech Appl Biochem, 2010; 57[2]: 47-53.

116. Sugrue RJ, Cui T, Xu Q, Fu J, Chan YC. The production of recombinant dengue virus E protein using Escherichia coli and Pichia pastoris. J Virol Meth, 1997; 69[1/2]: 159-169.

117. Jaiswal S, Khanna N, Swaminathan S. High level expression and one step purification of recombinant dengue virus type 2 envelope domain III protein in Escherichia coli. Prot Exp Purification, 2004; 33 [1]: 80-91.

118. Wei H-Y, Jiang L-F, Xue Y-H, Fang D-Y, Guo HY. Secreted expression of dengue

virus type 2 fulllength envelope glycoprotein in Pichia pastoris. J Virol Meth, 2003; 109[1]: 17-23.

119. Bisht H, Chugh DA, Swaminathan S, Khanna N.Expression and purification of dengue virus type 2 envelope protein as a fusion with hepatitis B surface antigen in Pichia pastoris. Prot Express Purification, 2001; 23[1]: 84-96.

120. Kelly EP, Greene JJ, King AD, Innis BL. Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice. Vaccine, 2000; 18 [23]: 2549-2559.

121. Saejung W, Fujiyama K, Takasaki T, Ito M, Hori K, Malasit P, Watanabe Y, Kurane I, Seki T. Production of dengue 2 envelope domain III in plant using TMV-based vector system. Vaccine, 2007; 25: 6646-6654.

122. Kim MY, Yang MS, Kim TG. Expression of dengue virus E glycoprotein domain III in nonnicotine transgenic tobacco plants. Biotech Biop Eng, 2009; 14: 725-730.

123. Martinez CA, Topal E, Giulietti AM, Talou JR, Mason H. Exploring different strategies to express dengue virus envelope protein in a plant system. Biotech Lett, 2010; 32: 867-875.

124. Guirakhoo F, Heinz FX, Kunz C. Epitope model of tick borne encephalitis virus envelope glycoprotein E: analysis of structural properties, role of carbohydrate side chain and conformational changes occurring at acidic pH. Virology, 1989; 169: 90-99.

125. Inaba K, Swiggard WJ, Inaba M, Meltzer A, Sasagawa T, Nussenzweig MC, Steinman RM. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145: I. Expression on dendritic cells and other subsets of mouse leukocytes. Cell Immunol, 1995; 163: 148-153.

126. Jiang W, Swiggard WJ, Heufler C, Peng M, Mizra A, Steinman RM, Nussenzwig

MC. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature, 1995; 375: 151.

127. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature, 1998; 392: 245.

128. Den Haan JM, Lehar SM, Bevan MJ. CD8 [+] but not CD8 [-] dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med, 2000; 192: 1685-1689.

129. Nestle FO, Banchereau J, Hart D. Dendritic cells: on the move from bench to bedside. Nat Med, 2001; 7: 761.

130. Cerundolo V, Hermans IF, Salio M [2004] Dendritic cells: a journey from laboratory to clinic. Nat Immunol, 2004; 5: 7.

131. Wang WW, Das D, Tang XL, Budzynski W, Suresh MR. Antigen targeting to dendritic cells with bispecific antibodies. J Immunol Meth, 2005; 306: 80-92.

132. Wang WW, Das D, Suresh MR. A versatile bifunctional dendritic cell targeting vaccine vector. Mol Pharma, 2009; 6[1]: 158-172

133. Licciardi PV, Underwood JR. Plant-derived medicines: A novel class of immunological adjuvants. Int. Immunopharma, 2010; 11[3]: 390- 398.

134. Suresh MR, Cuello AC, Milstein C. Advantages of bispecific hybridomas in one step immunocytochemistry and immunoassays. Proc Nat Acad Sci USA, 1986a; 83: 7989-7993.

135. Suresh MR, Cuello AC, Milstein C. Bispecific monoclonal antibodies from hybrid hybridomas. Meth Enzymology, 1986b; 121: 210-215.

136. Husereau DR, Suresh MR. A general affinity method to purify peroxidase-tagged antibodies. J Imm Meth, 2001; 249: 33-41.

137. Cao Y, Suresh MR. Bispecific antibodies as novel bioconjugates. Biocon Chem,

1998; 9[6]:635-644.

138. Bhatnagar PK, Das D, Suresh MR. Sequential affinity purification of peroxidase tagged bispecific anti-SARS-CoV antibodies on phenylboronic acid agarose. J Chrom B, 2008; 863: 235-241.

139. Kammila S, Das D, Bhatnagar PK, Sunwoo HH, Zayas-Zamora G, King M, Suresh MR. A rapid point of care immunoswab assay for SARS-CoV detection. J Virol Meth, 2008; 152:77-84.

140. Kreutz FT, Suresh MR. Novel bispecific immunoprobe for rapid and sensitive detection of prostate-specific antigen. Clin Chem, 1997; 43[4]: 649-656.

141. Guttikonda S, Tang XL, Yang BM, Armstrong GD, Suresh MR. Monospecific and bispecific antibodies against E.coli 0157 for diagnostics. J Immunol Meth, 2007; 327:1-9.

142. Magee WC, Shahhosseini S, Lin YCJ, Suresh MR, Evans DH. Production and characterization of antibodies against vaccinia virus DNA polymerase. J Immunol Meth, 2009; 161:44-51.

143. Tang XL, Peppler MS, Irvin RT, Suresh MR. Use of bispecific antibodies in molecular Velcro assays whose specificity approaches the theoretical limit of immunodetection for Bordetella pertussis. Clin Diag Lab Immunol, 2004; 11[4]: 752-757.

144. Shahhosseini S, Das D, Qiu X, Feldmann H, Jones SM, Suresh MR. Production and characterization of monoclonal antibodies against different epitopes of Ebola virus antigens. J Immunol Meth, 2007; 143: 29-37.

145. Liu F, Guttikonda S, Suresh MR. Bispecific monoclonal antibodies against a viral and an enzyme: utilities in ultrasensitive virus ELISA and phage display technology. J Immunol Meth, 2003; 274: 115-127.

146. Cao Y, Christian S, Suresh MR. Development of a bispecific monoclonal antibody as a universal immunoprobe for detecting biotinylated macromolecules. J Immunol Meth, 1998; 220: 85-91.

147. Simmons LC, Reilly D, Klimowski L, Raju TS, Meng G, Sims P. Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. J Immunol Meth, 2002; 263: 133–147.