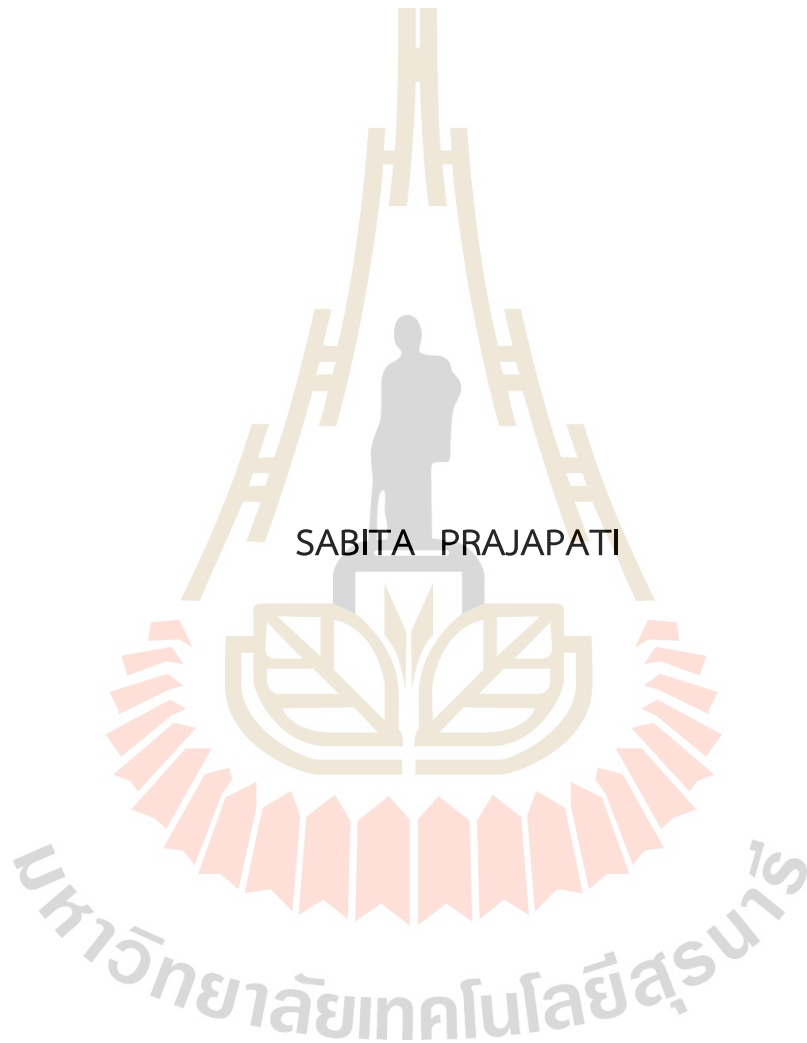


INVESTIGATION OF HUMAN IMMUNE RESPONSES TO DENGUE  
VIRUS AND THE APPLICATION OF PHAGE DISPLAY  
TECHNOLOGY IN DENGUE RESEARCH



A Thesis Submitted in Partial Fulfillment of the Requirements for the  
Degree of Doctor of Philosophy in Biotechnology  
Suranaree University of Technology  
Academic Year 2024

การศึกษาระบบภูมิคุ้มกันของมนุษย์ต่อไวรัสไข้เลือดออก และการประยุกต์ใช้  
เทคโนโลยีการแสดงผลโปรตีนบนผิวเฟจ ในการศึกษาวิจัย  
โรคไข้เลือดออก



นางสาวซาบิตา ประจาพะติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต  
สาขาวิชาเทคโนโลยีชีวภาพ  
มหาวิทยาลัยเทคโนโลยีสุรนารี  
ปีการศึกษา 2567



INVESTIGATION OF HUMAN IMMUNE RESPONSES TO DENGUE VIRUS  
AND THE APPLICATION OF PHAGE DISPLAY TECHNOLOGY  
IN DENGUE RESEARCH

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

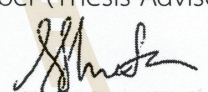


(Prof. Dr. Watchara Kasinrerak)

Chairperson

  
.....  
(Prof. Dr. Montarop Yamabhai)

Member (Thesis Advisor)

  
.....  
(Prof. Dr. Sujan Shresta)

Member (Thesis Co-advisor)

  
.....  
(Prof. Dr. Krishna Das Manandhar)

Member

  
.....  
(Asst. Prof. Dr. Mantana Jamklang)

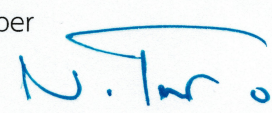
Member

  
.....  
(Dr. Annie Elong Ngono)

Member

  
.....  
(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)

Vice Rector for Academic Affairs  
and Quality Assurance

  
.....  
(Prof. Dr. Neung Teaumroong)

Dean of Institute of Agricultural  
Technology



ซาบิตา ประจาพะติ: การศึกษาระบบภูมิคุ้มกันของมนุษย์ต่อไวรัสไข้เลือดออก และการประยุกต์ใช้เทคโนโลยีการแสดงโปรตีนบนผิวเฟจ ในการศึกษาวิจัยโรคไข้เลือดออก  
(INVESTIGATION OF HUMAN IMMUNE RESPONSES TO DENGUE VIRUS AND THE APPLICATION OF PHAGE DISPLAY TECHNOLOGY IN DENGUE RESEARCH)  
อาจารย์ที่ปรึกษา: ศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 145 หน้า

คำสำคัญ: ไวรัสเดงกี/ฟลาวิไวรัส/ปฏิกิริยาข้าม/การวินิจฉัย/การบำบัด/การเพิ่มประสิทธิภาพที่ขึ้นอยู่กับแอนติบอดี/การแสดงออกของฟาจ/โดเมนของฉันทามติ III (cEDIII)/แอนติบอดีสลับสายฤทธิ์/อิมมูโนเซนเซอร์

ไวรัสเดงกี (DENV) เป็นไวรัสตระกูลฟลาวิวิรีเดที่มีอยู่เป็นพาหะ ซึ่งเป็นภัยคุกคามด้านสาธารณสุขระดับโลกโดยเฉพาะในพื้นที่เขตร้อนและกึ่งร้อน โดยก่อให้เกิดอาการเจ็บป่วยในระดับต่างๆ ตั้งแต่ไข้เล็กน้อยไปจนถึงไข้เลือดออกและภาวะช็อกที่รุนแรง ไวรัสเดงกีมี 4 สายพันธุ์ ได้แก่ เดงกี 1 ถึง เดงกี 4 การติดเชื้อสายพันธุ์หนึ่งจะให้ภูมิคุ้มกันแบบชั่วคราวต่อสายพันธุ์อื่น และอาจเพิ่มความเสี่ยงของการเกิดโรครุนแรงขึ้นเมื่อมีการติดเชื้อซ้ำซ้อนจากสายพันธุ์อื่นเนื่องจากมีกลไกการเสริมฤทธิ์ของแอนติบอดี การที่ยังไม่มีวัคซีนที่สามารถป้องกันได้ครอบคลุมทุกสายพันธุ์ ประกอบกับการแพร่กระจายของไวรัสเดงกีและยุงพาหะไปในวงกว้าง จึงมีความจำเป็นในการทำวิจัยอย่างต่อเนื่องเพื่อพัฒนาการวินิจฉัย, ยาต้านไวรัส, วัคซีนที่ให้การป้องกันได้อย่างครอบคลุม และแนวทางการรักษาที่มีประสิทธิภาพมากยิ่งขึ้น

การศึกษานี้มุ่งเน้นการวิเคราะห์ลักษณะทางโมเลกุลของภูมิคุ้มกันต่อการติดเชื้อไวรัสเดงกีในประเทศเนปาลและประเทศไทย การประเมินรูปแบบการเกิดภูมิคุ้มกันข้ามกับฟลาวิไวรัสชนิดอื่น ๆ การประยุกต์ใช้เทคโนโลยีการแสดงโปรตีนบนผิวเฟจ โดยใช้โปรตีนโดเมน III ส่วน envelope (cEDIII) เพื่อพัฒนาแอนติบอดีที่สามารถยับยั้งไวรัสและพัฒนาวิธีการวินิจฉัยใหม่ที่สามารถตรวจจับแอนติบอดีต่อเดงกีได้อย่างรวดเร็วและแม่นยำโดยใช้ cEDIII ในการศึกษาใช้วิธีการที่หลากหลายและเสริมกันในกลุ่มประชากรที่แตกต่างกัน ในประเทศเนปาลมีผู้ป่วยต้องสงสัยติดเชื้อเดงกีจำนวน 49 ราย ในฤดูระบาดปี 2017 โดยใช้การวิเคราะห์ทางซีรัมและพันธุกรรม ส่วนในประเทศไทยมีการเก็บตัวอย่างเลือดในระยะเฉียบพลัน จำนวน 60 ตัวอย่าง ตัวอย่างระยะพักฟื้น 25 ตัวอย่าง (28 - 45 วัน) และตัวอย่างในการติดตามระยะยาวจำนวน 6 ตัวอย่าง (1 ปี) จากโรงพยาบาลมหาวิทยาลัยเทคโนโลยีสุรนารีและโรงพยาบาลสิคิ้ว จังหวัดนครราชสีมา การวิเคราะห์ภูมิคุ้มกันทางโมเลกุลประกอบด้วย การตรวจหาแอนติเจน NS1 การตรวจหาแอนติบอดี IgM/IgG โดยวิธีอีไลซ่า การตรวจหาและแยกสายพันธุ์ของไวรัสด้วยเทคนิค qRT-PCR การเพาะเลี้ยงและแยกไวรัส การหาลำดับพันธุกรรมทั้งจีโนม และการทดสอบความสามารถในการยับยั้งไวรัสต่อฟลาวิไวรัสหลายชนิด (PRNT) สำหรับการพัฒนาแอนติบอดี ใช้เทคโนโลยีการแสดงโปรตีนบนผิวเฟจ โดยใช้โปรตีนโดเมน III ส่วน envelope เป็นเป้าหมายในการคัดกรองจากคลังเฟจเอชซีเอฟวี (scFv) นอกจากนี้ยังมีการพัฒนาอิมมูโนเซนเซอร์ชนิด impedimetric แบบไม่ต้องใช้สารติดฉลาก โดยใช้ขั้วไฟฟ้าทองคำที่เคลือบด้วยแอนติเจน cEDIII สำหรับตรวจแอนติบอดีได้อย่างรวดเร็ว



จากกลุ่มตัวอย่างในประเทศเนปาล พบว่าผู้เข้าร่วมการศึกษา 21 ราย จากทั้งหมด 49 ราย (43%) ตรวจพบแอนติเจน NS1 โดยมีลักษณะโปรไฟล์ของ IgM/IgG ที่หลากหลายซึ่งบ่งชี้ถึงการติดเชื้อทั้งแบบปฐมภูมิและทุติยภูมิ มีตัวอย่าง 7 รายที่ตรวจพบอาร์เอ็นเอ (RNA) ของไวรัสเดงกีด้วยเทคนิค RT-PCR (DENV-2 จำนวน 6 ตัวอย่าง และ DENV-3 จำนวน 1 ตัวอย่าง) โดย DENV-2 เป็นสายพันธุ์หลักที่พบ การวิเคราะห์ลำดับพันธุกรรมทั้งจีโนมพบความคล้ายคลึงกับสายพันธุ์ที่ระบาดในประเทศสิงคโปร์ (ปี 2016) และอินเดีย (ปี 2017) โดยพบแอนติบอดีที่สามารถยับยั้งไวรัสในซีรัมร้อยละ 66 และร้อยละ 58 มีภูมิคุ้มกันข้ามกับหลายสายพันธุ์ สำหรับกลุ่มตัวอย่างจากประเทศไทย ตัวอย่างในระยะเฉียบพลันจำนวน 34 รายจาก 60 ราย แสดงผลเป็นบวกต่อแอนติเจน NS1 21 ราย แสดงผลบวกต่อแอนติบอดี IgM ต่อไวรัสเดงกี 44 รายแสดงผลบวกต่อแอนติบอดี IgG และ 25 รายเป็นแสดงผลบวกเมื่อตรวจด้วยวิธี qRT-PCR ตรวจพบไวรัสเดงกีทั้งหมด 3 สายพันธุ์ (DENV-1 2 และ 4) โดยสามารถแยกไวรัส DENV-1 และ DENV-2 จากการเพาะเชื้อได้สำเร็จ พบการติดเชื้อทุติยภูมิมากกว่าการติดเชื้อปฐมภูมิ การทดสอบการยับยั้งไวรัส พบว่ามีแอนติบอดีต่อ DENV-1 และ DENV-3 ในระดับสูง อย่างไรก็ตามเป็นที่น่าประหลาดใจที่พบว่าแอนติบอดีต่อไวรัสซิกา (ZIKV) และไวรัสสมองอักเสบญี่ปุ่น (JEV) มากกว่าแอนติบอดีต่อ DENV-2 และ DENV-4 นอกจากนี้ยังพบแอนติบอดีที่ยับยั้งไวรัสไข้เหลือง (YFV) ซึ่งไม่ได้ระบาดในประเทศไทย แสดงถึงความคล้ายคลึงกันของแอนติเจนของไวรัสเดงกี อีกทั้งการคัดแยกด้วยเทคนิคการแสดงโปรตีนบนผิวเฟจ สามารถระบุแอนติบอดี IgG ชนิด cross-reactive ได้ 4 ชนิด ซึ่งสามารถยับยั้งไวรัส DENV-1 ถึง DENV-4 ได้ในระดับที่แตกต่างกัน นอกจากนี้ภูมิโนเซนเซอร์ที่พัฒนาขึ้นสามารถแยกแยะตัวอย่างผู้ป่วยได้อย่างชัดเจน มีศักยภาพสูงในด้านการวินิจฉัย ณ จุดให้บริการ

โดยสรุปการศึกษานี้เผยให้เห็นถึงรูปแบบการเกิดภูมิคุ้มกันข้ามระหว่างฟลาวิไวรัสในผู้ป่วยต้องสงสัยติดเชื้อเดงกีในพื้นที่ระบาดต่างๆซึ่งมีความเกี่ยวข้องโดยตรงกับความรุนแรงของโรคและความท้าทายในการวินิจฉัย ผลการศึกษาแสดงให้เห็นถึงการเกิดภูมิคุ้มกันข้ามในระดับสูงระหว่างเดงกีและฟลาวิไวรัสชนิดอื่นๆที่ระบาดร่วมกันในพื้นที่ เน้นย้ำถึงข้อจำกัดของวิธีการวินิจฉัยเพียงวิธีเดียวในพื้นที่ที่มีการระบาดของฟลาวิไวรัสหลายชนิดร่วมกัน ความสำเร็จในการใช้ cEDIII ทั้งในเทคโนโลยีการแสดงโปรตีนบนผิวเฟจเพื่อพัฒนาแอนติบอดี และในอุปกรณ์ไบโอเซนเซอร์สำหรับการวินิจฉัย แสดงให้เห็นถึงความหลากหลายของวิธีในงานวิจัยเดงกีอย่างครอบคลุม งานวิจัยนี้แสดงหลักฐานของการสัมผัสกับเดงกีหลายสายพันธุ์และฟลาวิไวรัสอื่นๆ ทั้งในอดีตและปัจจุบัน ซึ่งเน้นย้ำถึงความสำคัญของการผสมผสานการตรวจทางโมเลกุล, ซีรัม และการทดสอบ neutralization เพื่อให้การวินิจฉัยมีแม่นยำมากขึ้น เมื่อพิจารณาร่วมกันข้อมูลที่หลากหลายนี้เน้นถึงความจำเป็นเร่งด่วนในเพิ่มประสิทธิภาพในการเฝ้าระวังโรค การพัฒนาเครื่องมือวินิจฉัยที่ดีขึ้น และการคิดค้นแนวทางการรักษาที่มีประสิทธิภาพอย่างครอบคลุม พร้อมทั้งเน้นย้ำความเข้าใจในเรื่องภูมิคุ้มกันข้ามระหว่างฟลาวิไวรัส เพื่อการพัฒนาวัคซีนและตอบสนองทางด้านสาธารณสุขต่อการระบาดของเดงกีอย่างมีประสิทธิภาพ

สาขาวิชาเทคโนโลยีชีวภาพ  
ปีการศึกษา 2567

ลายมือชื่อนักศึกษา.....  
ลายมือชื่ออาจารย์ที่ปรึกษา.....  
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....



SABITA PRAJAPATI: INVESTIGATION OF HUMAN IMMUNE RESPONSES TO DENGUE VIRUS AND THE APPLICATION OF PHAGE DISPLAY TECHNOLOGY IN DENGUE RESEARCH. THESIS ADVISOR: PROF. MONTAROP YAMABHAI, Ph.D. 145 PP.

Keywords: Dengue virus/ flaviviruses/ cross-reactivity/ diagnostics/ therapeutics/ antibody-dependent enhancement/ phage display/ consensus envelope domain III (cEDIII)/ neutralizing antibodies/ immunosensor

Dengue virus (DENV), a mosquito-borne pathogen belonging to the *Flaviviridae* family, is a major global public health threat that is mostly found to be endemic in tropical and subtropical regions. It can cause a spectrum of illnesses from mild fever to severe hemorrhagic fever and shock syndrome. DENV exists as four distinct serotypes, namely DENV1 to DENV4. Infection by one provides only temporary cross-protection against others, while potentially increases the risk of severe disease upon subsequent infection from the phenomenon known as antibody-dependent enhancement. The lack of a universal vaccine, with expanding geographic distribution of DENV and its mosquito vectors necessitate ongoing research for improved diagnostics, antivirals, broadly protective vaccines and therapeutics.

This comprehensive study aimed to characterize the molecular profile of immunity to dengue virus infections in Nepal and Thailand, to evaluate cross-reactivity patterns with other flaviviruses, to explore the application of phage display technology utilizing consensus envelope domain III (cEDIII) for developing neutralizing antibodies, and to develop novel diagnostic approaches for rapid and sensitive detection of anti-dengue antibodies based on the reactivity to cEDIII. Multiple complementary approaches were employed across different study populations. In Nepal, 49 patients presenting suspected DENV infection during the 2017 dengue season were assessed using serological and genetic analyses. In Thailand, 60 acute samples, 25 convalescent samples (28-45 days), and 6 long-term follow-up samples (1 year) were collected from Suranaree University of Technology Hospital and Sikhio Hospital in the Nakhon Ratchasima province. The comprehensive immunomolecular analysis methods including: NS1 antigen detection, IgM/IgG enzyme-linked immunosorbent assays (ELISA), real time reverse transcription-polymerase chain reaction (qRT-PCR) for serotyping, virus culture and isolation, whole genome sequencing, and plaque reduction neutralization tests (PRNT) against multiple flaviviruses. For antibody development, phage display was applied using consensus envelope domain III as a target to screen on the single-chain variable fragment (scFv) libraries. Additionally, a label-free impedimetric immunosensor was developed using gold electrodes modified with self-assembled monolayers of cEDIII antigen for rapid antibody detection.



In the Nepalese cohort, 21 of 49 subjects (43%) were positive for DENV NS1 antigen, with varying IgM/IgG profiles, indicating both primary and secondary infections. Seven samples were RNA-positive by RT-PCR (six DENV-2, one DENV-3), with DENV-2 being the dominant serotype. Whole genome sequencing revealed similarity with strains circulating in Singapore (2016) and India (2017). Neutralizing antibodies were present in 66% of sera tested, with 58% showing cross-reactivity against multiple serotypes. In the Thai cohort, 34 of 60 acute samples were NS1-positive, 21 were anti-DENV IgM-positive, 44 were anti-DENV IgG-positive, and 25 were positive by qRT-PCR. Three serotypes (DENV-1, 2, and 4) were detected, with successful isolation of DENV-1 and DENV-2 from virus culture. Secondary infections were predominant over primary infections. Neutralization assays revealed high neutralizing antibodies against DENV-1 and DENV-3, while surprisingly, antibodies against ZIKV and JEV were higher than those against DENV-2 and DENV-4. Neutralizing antibodies against YFV were also detected despite its non-endemic status in Thailand, suggesting shared antigenic similarity with DENV. Phage display successfully identified four different cross-reactive IgGs capable of neutralizing DENV-1 to DENV-4 to varying extents. The impedimetric immunosensor demonstrated significant analytical differences between patient samples, showing promising potential for point-of-care diagnostics.

Conclusively, the study reveals complex flavivirus cross-reactivity patterns in dengue-suspected cases across different endemic regions, with direct correlations to disease severity and diagnostic challenges. The findings demonstrated substantial serological cross-reactivity between dengue and other co-circulating flaviviruses, emphasizing the limitations of single diagnostic approaches in flavivirus-endemic areas. The successful application of consensus envelope domain III in both phage display for antibody development and biosensor platforms for diagnostics highlights the versatility of this approach for comprehensive DENV research. The study provided evidence for current and past exposure to multiple DENV serotypes and other flaviviruses, underscoring the importance of integrating molecular, serological, and neutralization-based assays for accurate diagnosis. Collectively, these multidisciplinary findings emphasize the urgent need for enhanced surveillance, improved diagnostic tools, and broadly effective therapeutic strategies. They also underline the importance of understanding immune cross-reactivity among flaviviruses to refine vaccine development and public health responses to DENV outbreaks.

School of Biotechnology  
Academic Year 2024

Student's Signature.....  
Advisor's Signature.....  
Co-Advisor's Signature.....



## ACKNOWLEDGEMENT

Writing this thesis has been a truly enriching and fulfilling experience. I am deeply grateful to many individuals whose support made this work possible.

First and foremost, I would like to express my heartfelt appreciation to my supervisor, Prof. Montarop Yamabhai, for her unwavering support throughout my thesis and research journey. Her patience, encouragement, and profound knowledge have been invaluable to me. Her insightful guidance consistently steered me through every phase of the research and writing process. Whenever I encountered difficulties or had questions, she was always there to provide clarity and direction. Like a true mentor and guardian, she has supported me unconditionally.

I would also like to extend my sincere gratitude to my co-advisor, Prof. Sujan Shresta, La Jolla Institute for Immunology, for her valuable insights, thoughtful feedback, and constant encouragement throughout this research. Her expertise in the field and constructive suggestions greatly enriched the quality of this thesis. She has always been approachable and supportive, offering clarity and perspective whenever I faced research challenges. I truly appreciate her dedication and the time she invested in guiding me. It has been an honor to work under her co-supervision. I am equally grateful to Prof. Meng Ling Moi, The University of Tokyo, for her generous support, encouragement, and academic guidance throughout the course of my research. I would also like to thank her for inviting me to work in her lab and guiding me very closely throughout my time there. Her insightful suggestions and meticulous attention to detail played a crucial role in shaping the direction and clarity of this thesis. I truly appreciate her willingness to share her expertise and provide constructive feedback at every stage. Working under her guidance has been a valuable learning experience, and I am sincerely thankful for her involvement in my academic journey.

I would like to extend my gratitude to Prof. Watchara Kasinrer, Chairperson of the thesis committee for his valuable time, thoughtful evaluations, and constructive critiques. His perspectives and suggestions provided meaningful direction and helped strengthen my research. I am deeply grateful to Prof. Krishna Das

Manandhar, a respected member of my thesis committee, for his thoughtful critiques, academic wisdom, and constant encouragement. His feedback helped me broaden my perspective and refine key aspects of this work. I truly appreciate his generosity with time and knowledge, and his long-standing dedication to academic excellence has been a great source of inspiration. I also wish to extend my sincere thanks to Dr. Annie Elong Ngono for her involvement as a committee member. Her expertise in the field, keen observations, and insightful recommendations provided significant value to my research. Her questions and suggestions during the evaluation process challenged me to think more critically and helped enhance the scientific rigor of my thesis. I would also like to extend my sincere thanks to Asst. Prof. Dr. Mantana Jamklang for her involvement as a member of my thesis committee. Her valuable expertise, thoughtful observations, and constructive feedback greatly enriched the quality of my work.

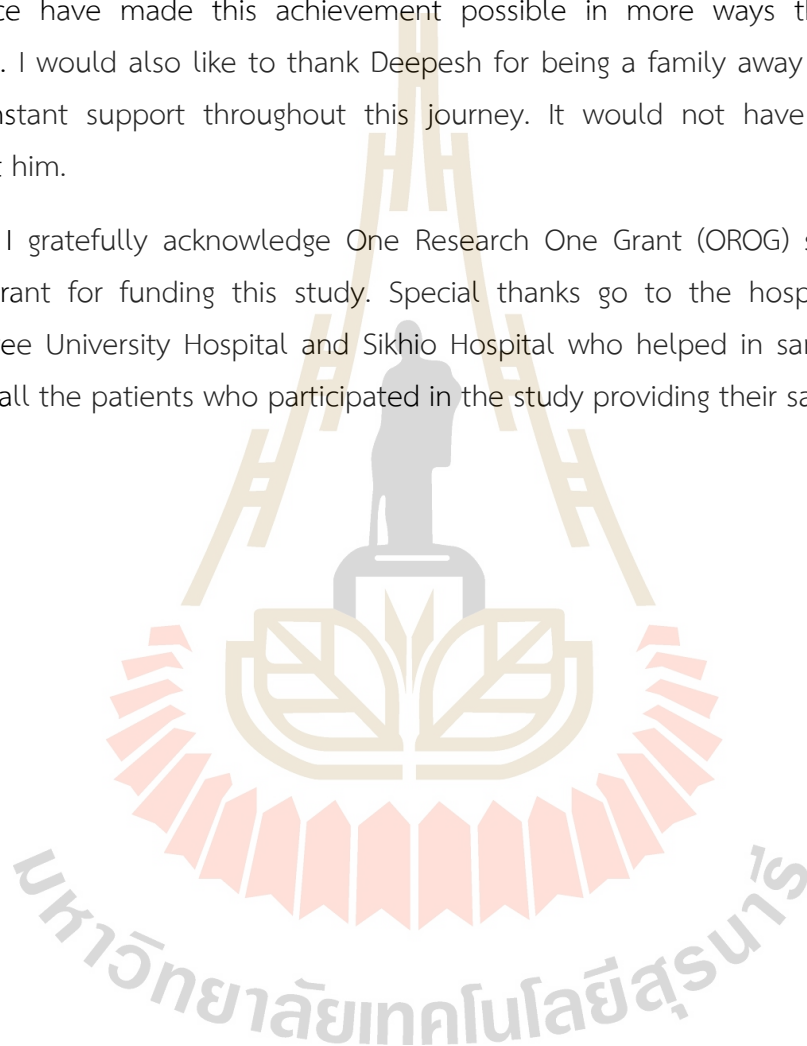
I would also like to express my sincere gratitude to all the professors, faculty members and staffs of School of Biotechnology at the University who have contributed to my academic development over the years. I would also like to thank Asst. Prof. Pinyut Pinyou, School of Chemistry for her guidance to carry out experiments on biosensor.

I would also like to extend my heartfelt appreciation to all MY lab members, seniors, and juniors, whose support and companionship made this journey both productive and enjoyable. I am especially grateful to my seniors, Drs. Kuntalee Rangnoi, Sararat Hattakam, Thitima Sumphanapai, Munthipa Khampio, Thae Thae Min, Nepelean Bonaparte for their guidance, encouragement, and for always being willing to share their knowledge and experience. Your mentorship has helped me overcome many challenges and shaped my approach to research. To my labmates Polina, Wahreni, Golf and Cao, thank you for the collaborative spirit, meaningful discussions, and all the moments both in and out of the lab that made this experience memorable. Your feedback, motivation, and friendship made a significant difference. To the juniors, Chetana, Siru, Biru, Thet, Tanveer I'm thankful for your enthusiasm, assistance with experiments, and fresh perspectives. I would also like to thank Haga-San, Adeline Sensei for guiding me and also all lab members from University of Tokyo, for supporting me during my time there.

I hold a profound sense of gratitude in my heart for my late parents, whose values, love, and vision continue to guide me. Their memory remains a lasting source of strength and inspiration, shaping the person I am today and reminding me of what truly matters in life. I am also deeply thankful to my brother and sister, whose unwavering support, spiritual presence, and constant encouragement have been a pillar of strength throughout this journey. Their belief in me and their quiet, enduring presence have made this achievement possible in more ways than words can express. I would also like to thank Deepesh for being a family away from family for his constant support throughout this journey. It would not have been possible without him.

I gratefully acknowledge One Research One Grant (OROG) scholarship and eAsia grant for funding this study. Special thanks go to the hospital staffs from Suranaree University Hospital and Sikhio Hospital who helped in sample collection and to all the patients who participated in the study providing their samples.

Sabita Prajapati





## TABLE OF CONTENTS

|                                                           | Page     |
|-----------------------------------------------------------|----------|
| ABSTRACT (THAI).....                                      | I        |
| ABSTRACT (ENGLISH).....                                   | III      |
| ACKNOWLEDGEMENT.....                                      | V        |
| TABLE OF CONTENTS .....                                   | VIII     |
| LIST OF TABLES .....                                      | XII      |
| LIST OF FIGURES.....                                      | XIII     |
| LIST OF ABBREVIATIONS.....                                | XV       |
| <b>CHAPTER I</b> .....                                    | <b>1</b> |
| 1.1 Significance of Study .....                           | 2        |
| 1.2 Hypothesis.....                                       | 4        |
| 1.3 Research Objectives .....                             | 4        |
| 1.3.1 General Objectives.....                             | 4        |
| 1.3.2 Specific Objectives.....                            | 4        |
| 1.4 References.....                                       | 5        |
| <b>CHAPTER II</b> .....                                   | <b>6</b> |
| 2.1 Serotypes and Genotypes of DENV.....                  | 6        |
| 2.2 Structure of DENV .....                               | 8        |
| 2.3 Life cycle of DENV, virus entry and replication ..... | 10       |
| 2.4 Diagnosis of DENV infection .....                     | 12       |
| 2.5 Host Immunity .....                                   | 13       |
| 2.5.1 Innate immune response .....                        | 13       |
| 2.5.2 Adaptive immune response.....                       | 14       |
| 2.6 Role of adaptive immunity in viral pathogenesis.....  | 15       |
| 2.6.1 Antibody responses against DENV .....               | 15       |
| 2.6.2 T cell responses against DENV .....                 | 19       |
| 2.7 Impact of cross-reactive immunity.....                | 24       |
| 2.7.1 Effect of prior ZIKV immunity on DENV.....          | 24       |
| 2.7.2 Effect of prior DENV immunity on ZIKV.....          | 25       |
| 2.7.3 Effect of prior JEV immunity on DENV.....           | 28       |
| 2.7.4 Effect of prior DENV immunity on JEV.....           | 29       |



## TABLE OF CONTENTS (Continued)

|                                                                              | Page |
|------------------------------------------------------------------------------|------|
| 2.7.5 Cross reactivity of DENV and YFV .....                                 | 29   |
| 2.8 DENV evolution .....                                                     | 30   |
| 2.9 References .....                                                         | 35   |
| <b>CHAPTER III</b> .....                                                     | 50   |
| 3.1 Abstract .....                                                           | 50   |
| 3.2 Introduction .....                                                       | 51   |
| 3.3 Material and Methods .....                                               | 53   |
| 3.3.1 Ethics statement .....                                                 | 53   |
| 3.3.2 Sample collection .....                                                | 53   |
| 3.3.3 Enzyme-linked immunosorbent assays .....                               | 53   |
| 3.3.4 DENV serotyping by RT-PCR .....                                        | 53   |
| 3.3.5 Whole genome DENV sequencing .....                                     | 53   |
| 3.3.6 Phylogenetic analysis .....                                            | 54   |
| 3.3.7 Flow cytometry-based DENV1-4 neutralization assay .....                | 54   |
| 3.4 Results .....                                                            | 55   |
| 3.4.1 Patient demographics and disease association with platelet count... 55 | 55   |
| 3.4.2 DENV serostatus and serotypes .....                                    | 60   |
| 3.4.3 Neutralizing Ab response against DENV1-4 serotypes .....               | 60   |
| 3.4.4 Phylogenetic and nucleotide analysis of DENV isolates .....            | 61   |
| 3.5 Discussion .....                                                         | 64   |
| 3.7 References .....                                                         | 68   |
| <b>CHAPTER IV</b> .....                                                      | 73   |
| 4.1 Abstract .....                                                           | 73   |
| 4.2 Introduction .....                                                       | 74   |
| 4.3 Material and Methods .....                                               | 77   |
| 4.3.1 Ethics, Sample collection, Processing and Storage .....                | 77   |
| 4.3.2 Plasma separation and PBMC Isolation .....                             | 77   |
| 4.3.3 Detection of DENV Antigen and Antibody .....                           | 78   |
| 4.3.4 Virus culture .....                                                    | 78   |
| 4.3.4 RNA extraction and serotyping .....                                    | 78   |
| 4.3.5 Neutralization test using Microneutralization Assay (MNT) .....        | 78   |
| 4.4 Results .....                                                            | 79   |
| 4.4.1 Patient demographics .....                                             | 79   |



## TABLE OF CONTENTS (Continued)

|                                                                                                           | Page       |
|-----------------------------------------------------------------------------------------------------------|------------|
| 4.4.2 DENV antigen detection and serology.....                                                            | 80         |
| 4.4.3 Serotyping by real time reverse transcriptase PCR.....                                              | 81         |
| 4.4.4 DENV case classification.....                                                                       | 82         |
| 4.4.5 Neutralizing antibody response against DENV1-4 and other<br>flaviviruses .....                      | 83         |
| 4.4.6 Neutralizing antibody response based on DENV case classification....                                | 83         |
| 4.4.7 Neutralization titer of the samples against DENV1 .....                                             | 86         |
| 4.5 Discussion .....                                                                                      | 92         |
| 4.6 References .....                                                                                      | 97         |
| <b>CHAPTER V .....</b>                                                                                    | <b>101</b> |
| 5.1 Abstract .....                                                                                        | 101        |
| 5.2 Introduction.....                                                                                     | 101        |
| 5.3 Material and Methods.....                                                                             | 103        |
| 5.3.1 cEDIII production.....                                                                              | 103        |
| 5.3.2 Affinity selection.....                                                                             | 103        |
| 5.3.3 Affinity determination by ELISA.....                                                                | 104        |
| 5.3.4 scFv expression and purification.....                                                               | 105        |
| 5.3.5 Expression of recombinant full-length anti-human<br>monoclonal IgGs antibody and purification ..... | 106        |
| 5.3.6 Affinity determination by ELISA.....                                                                | 108        |
| 5.3.7 Neutralization test using Microneutralization Assay (MNT) .....                                     | 108        |
| 5.4 Results.....                                                                                          | 108        |
| 5.4.1 Expression and Purification of target protein.....                                                  | 108        |
| 5.4.2 Affinity selection of cEDIII and Immunogenetic analysis.....                                        | 109        |
| 5.4.3 Expression of full-length anti-human monoclonal IgGs<br>antibody and purification .....             | 111        |
| 5.4.4 Check binding of IgGs by ELISA .....                                                                | 113        |
| 5.4.5 Neutralization activity check by using MNT.....                                                     | 113        |
| 5.5 Discussion .....                                                                                      | 114        |
| 5.6 References .....                                                                                      | 116        |
| <b>CHAPTER VI .....</b>                                                                                   | <b>118</b> |
| 6.1 Abstract .....                                                                                        | 118        |
| 6.2 Introduction.....                                                                                     | 118        |

## TABLE OF CONTENTS (Continued)

|                                                                                                     | Page |
|-----------------------------------------------------------------------------------------------------|------|
| 6.3 Material and Methods.....                                                                       | 120  |
| 6.3.1 Chemicals, target and samples .....                                                           | 120  |
| 6.3.2 Immunosensor fabrication .....                                                                | 121  |
| 6.3.3 Cyclic Voltammetry (CV) and Electrochemical Impedance<br>Spectroscopy (EIS) Measurements..... | 121  |
| 6.4 Results and Discussion.....                                                                     | 122  |
| 6.4.1 Production of target protein.....                                                             | 122  |
| 6.4.2 Sample details.....                                                                           | 122  |
| 6.4.3 Electrochemical characterization of the immunosensor.....                                     | 123  |
| 6.4.4 Impedance responses of the immunosensor toward anti-dengue<br>antibodies detection .....      | 126  |
| 6.6 References .....                                                                                | 127  |
| <b>CHAPTER VII</b> .....                                                                            | 128  |
| <b>APPENDIX</b> .....                                                                               | 130  |
| List of publications:.....                                                                          | 130  |
| <b>BIOGRAPHY</b> .....                                                                              | 145  |

มหาวิทยาลัยเทคโนโลยีสุรนารี



## LIST OF TABLES

| Table | Page                                                                                                       |
|-------|------------------------------------------------------------------------------------------------------------|
| 3.1   | Characteristics of study participants..... 55                                                              |
| 3.2   | Platelet counts and DENV serology, serotyping, neutralization<br>titers, and infection status..... 57      |
| 4.1   | Detail of the virus strains used..... 79                                                                   |
| 4.2   | Summary of serological tests performed..... 80                                                             |
| 4.3   | CT values for the PCR positive RNA samples from plasma..... 81                                             |
| 4.4   | CT values for the PCR positive RNA samples from virus culture<br>supernatant..... 82                       |
| 4.5   | Statistical analysis for presence of neutralizing antibodies against<br>DENV1-4, ZIKV, JEV and YFV..... 84 |
| 4.6   | Neutralization titer for confirmed DENV samples..... 88                                                    |
| 4.7   | Neutralization titer for only IgG positive samples..... 90                                                 |
| 4.8   | Neutralization titer for negative samples..... 91                                                          |
| 5.1   | Primers for construction of IgG vectors..... 107                                                           |
| 5.2   | Analysis of V-gene germline family..... 110                                                                |
| 6.1   | ELISA results of the samples..... 123                                                                      |

## LIST OF FIGURES

| Figure                                                                                                                                                                          | Page |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 2.1 Structure and Serotypes of DENV.....                                                                                                                                        | 8    |
| 2.2 Diagrammatic representation of genome organization of DENV.....                                                                                                             | 10   |
| 2.3 Dengue virus replication.....                                                                                                                                               | 12   |
| 2.4 Diagnosis of dengue.....                                                                                                                                                    | 13   |
| 2.5 Outcomes influenced by antibody behavior due to ADE, protection,<br>or minimal impact.....                                                                                  | 18   |
| 2.6 T cell original antigenic sin and possible effects of combined<br>humoral and T cell immunity.....                                                                          | 23   |
| 2.7 The trade-off hypothesis of dengue virus quasi-species transmission.....                                                                                                    | 32   |
| 2.8 Factors and forces that influence the evolution of dengue viruses<br>and the possible effects on dengue<br>epidemiology.....                                                | 34   |
| 3.1 Geographic distribution of the home residences of the study subjects.....                                                                                                   | 56   |
| 3.2 Distribution of nAb titers in samples collected during the 2017<br>outbreak.....                                                                                            | 61   |
| 3.3 Phylogenetic analysis of full-genome sequences of the Nepal 2017 DENV<br>isolates generated here and other DENV2 isolates.....                                              | 62   |
| 3.4 Phylogenetic analysis of E gene sequences from the 2017 DENV2<br>genomes from Nepal.....                                                                                    | 63   |
| 4.1 Demographic distribution of cases by age and sex.....                                                                                                                       | 80   |
| 4.2 Percentage neutralization of active DENV cases against DENV1-4,<br>ZIKV, JEV and YFV.....                                                                                   | 83   |
| 4.3 Percentage neutralization of convalescent phase of active DENV<br>cases against DENV1-4, ZIKV, JEV and YFV. A- 28-45 days<br>convalescence and B. 1 year convalescence..... | 85   |
| 4.4 Comparison of neutralization activity between paired acute,<br>convalescence 28-45 days (n=20) and 1 year (n=6) of active<br>DENV cases.....                                | 85   |
| 4.5 Comparison of neutralization activity between active DENV,<br>probable past DENV and negative samples.....                                                                  | 86   |



## LIST OF FIGURES (Continued)

| Figure                                                                                                                   | Page |
|--------------------------------------------------------------------------------------------------------------------------|------|
| 5.1 Cloning Consensus Envelope Domain III (cEDIII) in pET21d vector<br>for expression in <i>E. coli</i> .....            | 103  |
| 5.2 Representation of a biopanning process using Phage display<br>antibody library.....                                  | 104  |
| 5.3 Representative steps for scFv expression, purification and binding<br>analysis.....                                  | 106  |
| 5.4 Representative figure for IgG expression.....                                                                        | 107  |
| 5.5 SDS-PAGE (16%) for consensus envelope domain III (cEDIII) after<br>purification.....                                 | 109  |
| 5.6 ELISA showing binding of the scFv clones with cEDIII and DENV1-4.....                                                | 111  |
| 5.7 Representative figure showing cloning of scFv VH and VL to heavy<br>chain and light chain vectors respectively.....  | 112  |
| 5.8 SDS-PAGE of the expressed recombinant monoclonal IgGs.....                                                           | 112  |
| 5.9 ELISA showing binding of the recombinant monoclonal<br>antibodies with cEDIII and DENV1-4.....                       | 113  |
| 5.10 Percentage neutralization activity of recombinant antibodies.....                                                   | 114  |
| 6.1 Schematic representation of cEDIII based dengue biosensor.....                                                       | 122  |
| 6.2 Cyclic voltammograms of the immunosensor (SPE+cEDIII)<br>measured after each stage of electrode<br>modification..... | 123  |
| 6.3 EIS Nyquist diagram showing effect of incubation temperature<br>on binding between cEDIII and YE11.....              | 124  |
| 6.4 Bar graph showing the comparison of YE11 against delta charge<br>transfer resistance value.....                      | 125  |
| 6.5 EIS Nyquist diagram for cEDIII/plasma/SPE.....                                                                       | 125  |
| 6.6 Comparative analysis of the difference in ohm for negative and<br>positive sample.....                               | 126  |

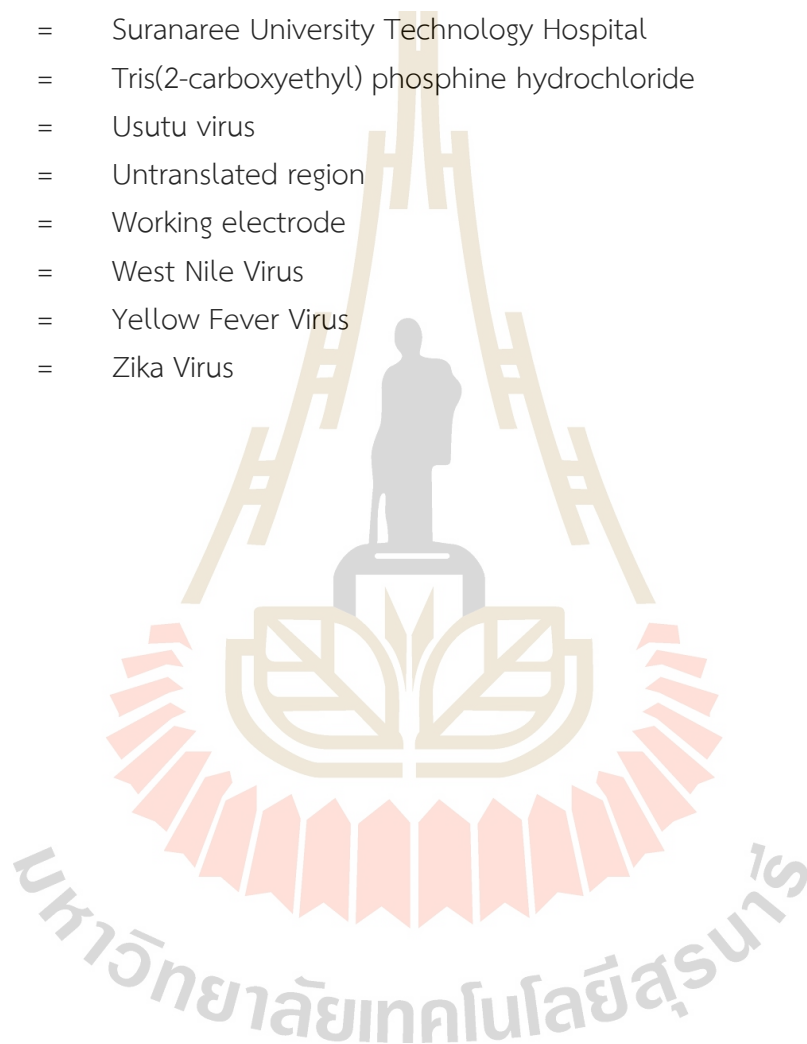
## LIST OF ABBREVIATIONS

|        |   |                                               |
|--------|---|-----------------------------------------------|
| ADE    | = | Antibody dependent enhancement                |
| C      | = | Capsid protein                                |
| CAC    | = | Combined avidity and concentration            |
| CE     | = | Counter electrode                             |
| cEDIII | = | Consensus envelope domain III                 |
| CV     | = | Cyclic voltammetry                            |
| DENV   | = | Dengue Virus                                  |
| DF     | = | Dengue fever                                  |
| DHF    | = | Dengue hemorrhagic fever                      |
| DSP    | = | Dithiobis-succinimidyl propionate             |
| DSS    | = | Dengue shock syndrome                         |
| E      | = | Envelope                                      |
| EIS    | = | Electrochemical impedance spectroscopy        |
| ER     | = | Endoplasmic reticulum                         |
| HC     | = | Heavy chain                                   |
| HLA    | = | Human Leucocyte Antigen                       |
| IgG    | = | Immunoglobulin                                |
| JEV    | = | Japanese Encephalitis Virus                   |
| LC     | = | Light chain                                   |
| mAbs   | = | Monoclonal antibodies                         |
| MVEV   | = | Murray Valley Encephalitis virus              |
| nAb    | = | Neutralizing antibodies                       |
| NCBI   | = | National Center for Biotechnology Information |
| NGS    | = | Next generation sequencing                    |
| NS     | = | Non-structural                                |
| ORF    | = | Open reading frame                            |
| PBMC   | = | Peripheral blood mononuclear cells            |
| PBS    | = | Phosphate Buffer Saline                       |
| PCR    | = | Polymerase chain reaction                     |
| prM    | = | Pre-membrane protein                          |
| RdRp   | = | RNA dependent RNA polymerase                  |
| scFv   | = | Single chain variable fragment                |



## LIST OF ABBREVIATIONS (Continued)

|      |   |                                              |
|------|---|----------------------------------------------|
| SH   | = | Sikhio Hospital                              |
| SLEV | = | St Louis Encephalitis virus                  |
| SNV  | = | Single nucleotide variations                 |
| SPE  | = | Screen printed electrode                     |
| SUTH | = | Suranaree University Technology Hospital     |
| TCEP | = | Tris(2-carboxyethyl) phosphine hydrochloride |
| USUV | = | Usutu virus                                  |
| UTR  | = | Untranslated region                          |
| WE   | = | Working electrode                            |
| WNV  | = | West Nile Virus                              |
| YFV  | = | Yellow Fever Virus                           |
| ZIKV | = | Zika Virus                                   |



## CHAPTER I

### INTRODUCTION

Dengue fever is one of the most important tropical diseases in the world (Guzman & Harris, 2015) with its incidence greater than 30 folds along with the geographical expansion of *Aedes* vector and dengue viruses (DENVs) (WHO, 2009a). The term “dengue virus” encompasses four genetically and antigenically related viruses, classified as serotypes DENV-1 to DENV-4. These serotypes are further divided into genotypes, which are characterized by the limited ability of antibodies produced against one to neutralize the others (Kyle & Harris, 2008). Infection by any of the four serotypes can result in a range of clinical manifestations for which the timing or sequence of infections can be an important determinant of disease severity and course. Dengue illness is clinically classified as either dengue with or without warning signs or severe dengue (WHO, 2009a). This classification was launched by WHO in 2009 for the purpose of improving clinical management. The assessment of warning signs is designed to permit the early identification of patients with more-severe disease manifestations who require supportive therapy.

Dengue virus (DENV) belongs to the *Flavivirus* genus, which also includes viruses such as Japanese encephalitis (JEV), Zika (ZIKV), and Yellow Fever (YFV). While infections with DENV, JEV, and ZIKV are often asymptomatic, each of these viruses has the potential to cause severe and life-threatening illness (Theodore C Pierson & Michael S Diamond, 2020). The global morbidity and mortality associated with these viral infections, together with the extensive spread and epidemic transmission of multiple flaviviruses, underscore the urgent need to understand mechanisms of flaviviral pathogenesis and immunity. In particular, DENV infects an estimated 400 million people each year, and over a quarter of world's population lives in DENV-endemic areas (Bhatt et al., 2013). Despite extensive research over many years, the immune mechanisms that control the balance between protective immunity and disease-causing processes during DENV infections are still not well understood. DENV-immune individuals experiencing secondary DENV infection by a different DENV serotype may develop a more severe disease that is mediated by waning immunity to



a previous serotype, and among other factors, by antibody dependent enhancement (ADE) of infection (Halstead, 2014). While the impact of pre-existing immunity to different serotypes of DENV on later DENV transmission and disease has been thoroughly investigated, there is limited understanding of how prior immunity to other related flaviviruses influences DENV transmission and the severity of the disease. Sporadic cases and cohort studies suggest an increase in dengue severity in JEV-immune patients (Anderson et al., 2011). Recent human and animal studies from multiple groups have shown that pre-existing immunity to DENV can either protect against or exacerbate a subsequent ZIKV infection, and vice versa (Ngono & Shrestha, 2018).

### 1.1 Significance of Study

There is not yet an approved antiviral medication to treat dengue infections. It is commonly known that dengue patients have a brief period of viremia; however, since high viremia has been linked to a severe onset of the illness, administering antivirals early on may prevent the disease's progression and hasten patients' recovery (de la Guardia, Quijada, & Lleonart, 2017). Researchers have spent many years developing vaccines, antibody-based therapeutics, and antiviral medications to fight dengue, yet no vaccine has successfully offered complete protection against all four dengue virus serotypes. Currently, the only licensed dengue vaccine is Sanofi's Dengvaxia, available in 20 dengue endemic countries. However, this vaccine doesn't offer equal protection against all four dengue serotypes. While it protects individuals who have had a prior dengue infection, it is not effective in those who have never been infected. In fact, vaccinating previously unexposed individuals can heighten their risk of severe dengue requiring hospitalization if they later contract the virus (Sridhar et al., 2018). Recently, a vaccine called QDENGAR (developed by Takeda) has been approved for both dengue-naïve and previously infected individuals; however, its duration of long-term protection remains limited (Thomas, 2023). Another tetravalent vaccine Butantan-DV (developed by Instituto Butantan and NIAID) demonstrated strong protection against DENV1 but only moderate effectiveness against DENV2 in its phase 3 clinical trial (Kallas et al., 2024). Because of their significant antigenic similarity, finding effective treatments and sensitive, specific diagnostic testing remains extremely difficult.

In regions with tropical and subtropical climates, especially in Thailand, the epidemiology of dengue is made more complex by ADE. This phenomenon not only impacts the clinical progression of the disease but also plays a significant role in shaping the patterns of serotype circulation within communities. Additionally, Thailand's climate and environment create ideal conditions for the spread of several arboviruses, such as Chikungunya, Zika, and Yellow Fever, all of which are transmitted by *Aedes* mosquitoes. These viruses frequently circulate in the same areas, contributing to intricate interactions and competition among them. Gaining insights into how different dengue serotypes compete and interact with other co-existing arboviruses is key to understanding transmission dynamics and implementing effective prevention and control measures (Perez et al., 2025). Therefore, it is crucial to gain a comprehensive understanding of whether prior immunity to JEV, ZIKV, or YFV might have harmful or beneficial impacts on dengue virus infection. The presence of both JEV and ZIKV make Thailand unique geographic location to study the contribution of pre-existing anti-JEV or -ZIKV immunity on DENV incidence, transmission dynamics, and disease severity. Even though, YFV is not currently prevalent in Thailand, studies of yellow fever and dengue vaccine co-administration have demonstrated immunological interference, with reduced antibody responses when vaccines are given concomitantly. Hence, understanding DENV cross-reactive antibodies together with ZIKV, JEV and YFV will help to establish the protective thresholds for neutralizing antibodies more accurately.

Producing recombinant antibodies capable of neutralization can lead to effective treatments, while searching for serological biomarkers using monoclonal antibodies (mAbs) that recognize specific viral regions can improve differential diagnosis. Among methods for selecting human mAbs with high affinity for specific targets, the Phage Display technique stands out. Consequently, this technology offers a faster approach for developing specialized diagnostic tools as well as safe and effective therapies against flavivirus infections.

Therefore, this thesis explores the DENV infection in Nepal and Thailand, two geographically and climatically distinct countries. For samples from Thailand, cross reactive immune responses against ZIKV, JEV and YFV were also tested. Also, this study delves into the diagnostics and therapeutics aspect against DENV using phage display technology. Altogether, this thesis is divided into six main sections. In the beginning, the literature review is in chapter II, where different aspects of dengue are discussed



altogether with its cross reactivity with other flaviviruses. Also, the perspectives of antibody development against dengue are discussed. After that, chapter III explains about the study of Genomic sequencing and neutralizing serological profiles during acute dengue infection: A 2017 cohort study in Nepal. Third in chapter IV, immune molecular aspects of DENV from samples collected from suspected DENV cases in Thailand and their cross reactivity with other flaviviruses are discussed. In chapter V, phage display antibody library was used to get antibodies against DENV which was evaluated in vitro. Lastly in chapter VI, impedimetric immunosensor has been employed to detect anti-dengue antibodies in plasma from the patients.

## 1.2 Hypothesis

- Host immune responses play major role in dengue disease outcome.
- Different levels of pre-existing immunity to circulating flaviviruses, including DENV1-4, JEV, ZIKV and YFV have potential role in dengue disease outcome.
- Phage display technology can identify viral protein-specific antigenic targets and therapeutic antibodies, enabling improved diagnostics and treatments for dengue.

## 1.3 Research Objectives

### 1.3.1 General Objectives

- Investigation of human immune responses to DENV and/or other flaviviruses
- Exploration of the potential applications of using Phage display technology to study DENV

### 1.3.2 Specific Objectives

1. Establish cohorts for sample collection from suspected DENV cases
2. Immunological and molecular characterization of the suspected DENV samples
3. Culture of virus from patient samples and their characterization
4. Study of neutralizing capabilities of the antibodies from infected patients against all serotypes of DENV and Cross-reactivity analysis with other flaviviruses including ZIKV, JEV and YFV
5. Production of antibodies against DENV using phage display antibody technology and characterize their property
6. Development of impedimetric immunosensor for detection of anti-dengue antibodies in plasma

## 1.4 References

- Anderson, K. B., Gibbons, R. V., Thomas, S. J., Rothman, A. L., Nisalak, A., Berkelman, R. L., . . . Endy, T. P. (2011). Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. *PLoS Negl Trop Dis*, 5(10), e1311. doi:10.1371/journal.pntd.0001311
- Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., . . . Hay, S. I. (2013). The global distribution and burden of dengue. *Nature*, 496(7446), 504-507. doi:10.1038/nature12060
- de la Guardia, C., Quijada, M., & Lleonart, R. (2017). Phage-Displayed Peptides Selected to Bind Envelope Glycoprotein Show Antiviral Activity against Dengue Virus Serotype 2. *Adv Virol*, 2017, 1827341. doi:10.1155/2017/1827341
- Guzman, M. G., & Harris, E. (2015). Dengue. *Lancet*, 385(9966), 453-465. doi:10.1016/S0140-6736(14)60572-9
- Halstead, S. B. (2014). Dengue Antibody-Dependent Enhancement: Knowns and Unknowns. *Microbiol Spectr*, 2(6). doi:10.1128/microbiolspec.AID-0022-2014
- Kallas, E. G., Cintra, M. A. T., Moreira, J. A., Patino, E. G., Braga, P. E., Tenorio, J. C. V., . . . Nogueira, M. L. (2024). Live, Attenuated, Tetravalent Butantan-Dengue Vaccine in Children and Adults. *N Engl J Med*, 390(5), 397-408. doi:10.1056/NEJMoa2301790
- Kyle, J. L., & Harris, E. (2008). Global spread and persistence of dengue. *Annu Rev Microbiol*, 62, 71-92. doi:10.1146/annurev.micro.62.081307.163005
- Ngono, A. E., & Shrestha, S. (2018). Immune Response to Dengue and Zika. *Annu Rev Immunol*, 36, 279-308. doi:10.1146/annurev-immunol-042617-053142
- Perez, L. J., Yamaguchi, J., Weiss, S., Carlos, C., Meyer, T. V., Rodgers, M. A., . . . Berg, M. G. (2025). Climate, inter-serotype competition and arboviral interactions shape dengue dynamics in Thailand. *Commun Biol*, 8(1), 601. doi:10.1038/s42003-025-07999-9
- Pierson, T. C., & Diamond, M. S. (2020). The continued threat of emerging flaviviruses. *Nature microbiology*, 5(6), 796-812.
- Sridhar, S., Luedtke, A., Langevin, E., Zhu, M., Bonaparte, M., Machabert, T., . . . DiazGranados, C. A. (2018). Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy. *N Engl J Med*, 379(4), 327-340. doi:10.1056/NEJMoa1800820
- Thomas, S. J. (2023). Is new dengue vaccine efficacy data a relief or cause for concern? *NPJ Vaccines*, 8(1), 55. doi:10.1038/s41541-023-00658-2
- WHO. (2009). In *Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition*. Geneva.

## CHAPTER II

### LITERATURE REVIEW

Dengue virus (DENV) is an enveloped, single-stranded positive-sense RNA virus with a genome of about 11 kb. Its genome encodes the three structural proteins, a core nucleocapsid (C), a membrane-associated protein (M), and an envelope (E) glycoprotein, along with seven non-structural (NS) proteins. Dengue fever, caused by DENV, is a major arthropod-borne tropical viral disease, with an estimated 390 million infections occurring each year (Bhatt et al., 2013). Dengue is the most prevalent and widely distributed arboviral infection in tropical and subtropical areas, causing substantial illness and death (Tang et al., 2017). The clinical presentation of dengue varies from mild dengue fever (DF) to severe forms such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which can be fatal. While a primary infection with any one serotype typically results in fever, severe disease usually occurs during a secondary infection with a different serotype, especially in the presence of non-neutralizing antibodies. This process is referred to as Antibody Dependent Enhancement (ADE) (Halstead, 2014). The existence of four distinct classical dengue virus serotypes, which do not provide cross-protective immunity in infected individuals, significantly complicates the disease. Molecular evidence reveals considerable genetic diversity among dengue viruses. Although the four serotypes are often treated as a single virus and their associated illnesses are regarded as the same disease, the genetic differences between these serotypes actually exceed the genetic distances observed between many recognized virus species within the same genus, such as Japanese Encephalitis virus (JEV), West Nile virus (WNV), Murray Valley Encephalitis virus (MVEV), Usutu virus (USUV), and St. Louis Encephalitis virus (SLEV).

#### 2.1 Serotypes and Genotypes of DENV

Dengue virus (DENV) has four distinct serotypes, all of which are transmitted by *Aedes* mosquitoes. These serotypes typically cause similar clinical manifestations and exhibit comparable patterns of systemic dissemination. However, subtle biological differences exist among the serotypes that can influence specific manifestations and disease characteristics (Bara, Clark, & Remold, 2013; Messer et al., 2012). While certain



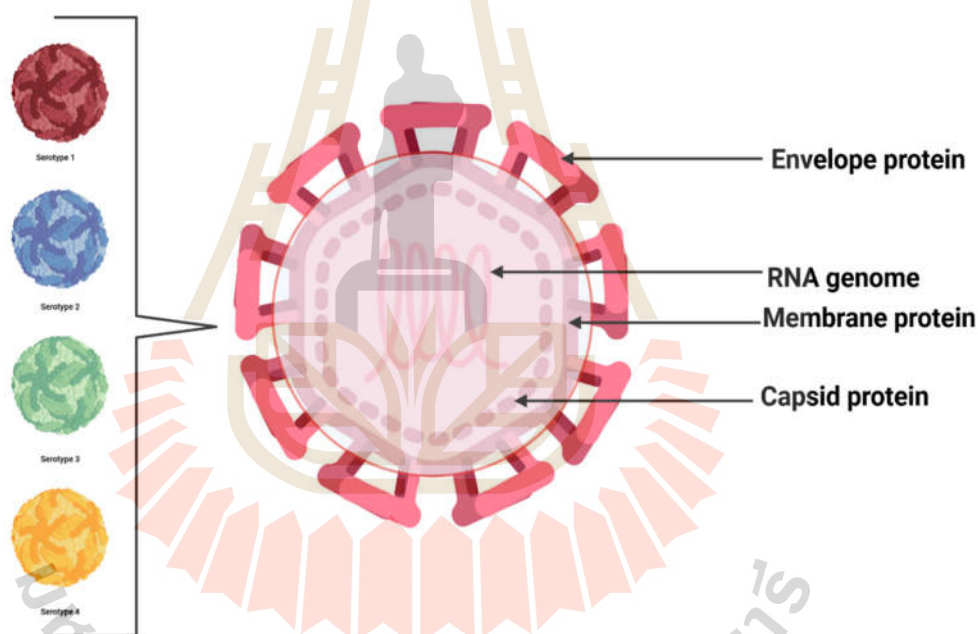
serotypes or genotypes of DENV have been linked to variations in disease severity, outbreak potential, and transmission efficiency, these relationships may also be affected by external factors beyond the virus's inherent properties. These include the host's immune status, the mosquito vector's capacity to acquire and transmit the virus, and environmental or ecological conditions that favor the replacement of one genotype by another (Halstead, 2008; Rico-Hesse, 2003).

These serotypes can be further subdivided into multiple genetic groups known as genotypes (or subtypes), based on differences in their genetic sequences. Initially, genotype was defined as a group of viruses with no more than 6% sequence variation within a 240-nucleotide segment of the E/NS1 junction for DENV-1 and DENV-2. However, the specific genome regions and lengths used for sequencing have varied widely among research groups, ranging from single gene sequences to the entire DENV genome. Today, genotypes are classified primarily through phylogenetic analysis rather than relying on fixed sequence divergence thresholds (Holmes & Twiddy, 2003; Rico-Hesse, 1990). Five genotypes of DENV-1 (Genotypes I, II, III, IV, and V); six genotypes of DENV-2 (Asian I, Asian II, Cosmopolitan, American, Southeast Asian/American, and Sylvatic); four genotypes of DENV-3 (Genotypes I, II, III, and IV); and four genotypes of DENV-4 (Genotypes I, II, III, and IV) are currently recognized (Weaver & Vasilakis, 2009).

Although DENV serotypes show greater heterogeneity in the structure of viral proteins, there is also antigenic similarity between DENV serotypes and, for some peptide sequences, between flaviviruses. Phylogeny studies of virus sequences, by estimating the antigenic distance between them, concluded that serotypes 1 and 3 are the most similar, serotype 2 was the second to diverge evolutionarily, and serotype 4 is the one that presents greater genetic difference. There is a 32% difference in the structure of protein E of the four DENV serotypes. Specific mutations in the genome result in the antigenic variability found in each serotype (Bell, Katzelnick, & Bedford, 2019; Wen & Shresta, 2019).

## 2.2 Structure of DENV

The dengue virus particle, similar to other flaviviruses, is spherical with a diameter of 40-50 nm. It consists of a nucleocapsid about 30 nm wide, which houses the viral capsid and RNA genome, surrounded by a lipid envelope. This envelope includes a lipid bilayer, an envelope protein weighing between 51,000 and 59,000 daltons that facilitate attachment, fusion, and entry into host cells, and a small internal matrix protein of roughly 8,500 daltons that is non-glycosylated. The envelope protein is glycosylated in most flaviviruses and is prominently displayed on the virion surface, as demonstrated by electron microscopy (Dwivedi, Tripathi, Tripathi, Bharadwaj, & Mishra, 2017).



**Figure 2.1** Structure and Serotypes of DENV (Dhole et al., 2024)

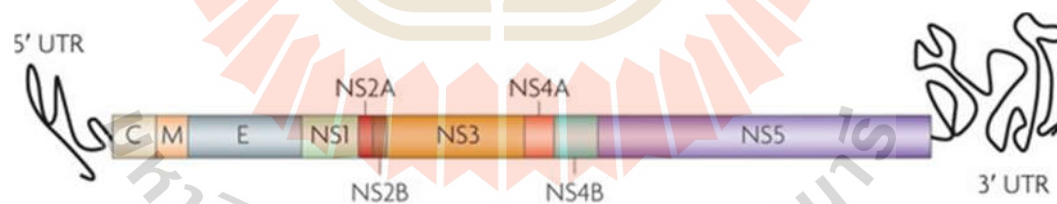
The dengue virus (DENV) genome is about 11 kb long and encodes a polyprotein that produces three structural proteins: capsid (C), envelope (E), and precursor membrane (prM), alongside seven non-structural proteins and two untranslated regions. The RNA genome is packaged inside a nucleocapsid formed by multiple capsid proteins, which is surrounded by a host-derived lipid envelope containing prM/M and E proteins arranged in an icosahedral pattern. The M protein is a cleaved form of prM. The E glycoprotein, a Class II fusion protein, mediates viral attachment and membrane fusion during infection (Stiasny & Heinz, 2006). The dengue

virus E protein monomer is a 53 kDa glycoprotein made up of three  $\beta$ -barrel structural domains: domain I (DI), domain II (DII), and domain III (DIII). On the virus surface, it forms 90 "head-to-tail" homodimers. DI is centrally located between DII, the dimerization domain, and DIII, which resembles an immunoglobulin-like structure. DII is an elongated, finger-like domain containing a hydrophobic fusion loop at its tip essential for membrane fusion. DIII contains the receptor-binding site responsible for virus attachment to host cells. As a class II fusion protein, the E protein can reorganize under acidic conditions to form a fusogenic homotrimer that facilitates viral membrane fusion with the host cell (Modis, Ogata, Clements, & Harrison, 2004). This process is believed to occur within the endosome, allowing the viral nucleocapsid to be released into the cytoplasm, where it initiates RNA and protein synthesis. Likewise, the prM protein is believed to act as a chaperone for the E glycoprotein during viral maturation, stabilizing its structure until the viral assembly is finished and the virion exits the acidic exocytic vesicles (Stiasny & Heinz, 2006). These structural domains I, II, and III are defined as the antigenic domains. Domain III (EDIII) contains type specific neutralization sites unlike domain I and II, hence induces a type specific neutralizing response with a low potential for inducing cross-reactive antibodies and thereby potentially avoiding Antibody Dependent Enhancement (ADE). A study analyzing a large panel of anti-E monoclonal antibodies showed that antibodies bound to DIII were strong blockers of DENV (Crill & Roehrig, 2001). On the other hand, antibodies targeting either domain I or II are weakly neutralizing and strongly cross-reactive with several flaviviruses (Oliphant et al., 2006).

The seven non-structural (NS) proteins—NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 play major roles in initiating viral replication, assembling new virus particles, and modulating the host immune response. The NS1 protein (46 kDa) exists in three distinct forms: a secreted hexamer, a membrane-bound homodimer, and an intracellular monomer. The soluble form of NS1, once secreted, circulates in the bloodstream and binds to the human complement regulatory protein clusterin (Clu), which normally inhibits the terminal complement pathway to protect cells from complement attack. The interaction between NS1 and Clu is thought to reduce Clu levels, leading to complement activation and contributing to the vascular leakage characteristic of dengue hemorrhagic fever (Kurosu, Chaichana, Yamate, Anantapreecha, & Ikuta, 2007). Additionally, NS1 binds to lipid rafts on the surface of infected cells, facilitating DENV



entry and enhancing its infectivity (Alcon-LePoder et al., 2005; Noisakran et al., 2008). The hydrophobic membrane proteins NS2A, NS4A, and NS4B serve as structural scaffolds for the viral replication complex. NS2A, a transmembrane protein, is associated with the endoplasmic reticulum (ER) (Xie, Gayen, Kang, Yuan, & Shi, 2013), is crucial for viral RNA replication and the assembly of new virions (Xie, Zou, Puttikhunt, Yuan, & Shi, 2015). The NS2B protein binds with NS3, serving as a cofactor that activates the protease function of NS3 (Niyomrattanakit et al., 2004). NS3 possesses protease activity, while its C-terminal region functions as a nucleotide triphosphatase and RNA helicase (Kadare & Haenni, 1997). The N-terminal domain displays a six-stranded  $\beta$ -barrel structure characteristic of chymotrypsin-like serine proteases (Murthy, Clum, & Padmanabhan, 1999). The integral membrane protein NS4A is embedded in the ER membrane and facilitates membrane remodeling to support the formation of the replication complex. NS4B is found alongside double-stranded RNA (dsRNA) and is essential for replication of the virus (S. Miller, Sparacio, & Bartenschlager, 2006). NS4B also suppresses the interferon  $\alpha/\beta$  response (Munoz-Jordan et al., 2005). NS5 possesses two major functions: RNA-dependent RNA polymerase (RdRp) and methyltransferase. The methyltransferase function resides in the N-terminal third of NS5 and is essential for adding the viral RNA cap and for internal methylation of the RNA (Dong et al., 2012; Ray et al., 2006). Additionally, NS5 also have a role in evasion of innate immune response (Ashour, Laurent-Rolle, Shi, & Garcia-Sastre, 2009).



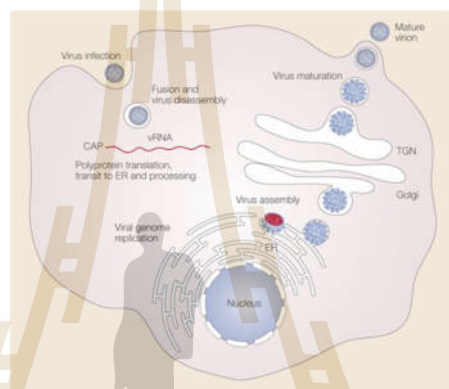
**Figure 2.2** Diagrammatic representation of genome organization of DENV (C: Core protein; PrM & M: Membrane associated protein; E: Enveloped protein; NS1-5: Non-Structural protein 1-5) (Guzman et al., 2010)

### 2.3 Life cycle of DENV, virus entry and replication

After being bitten by a mosquito infected with DENV, the initial immune cells encountered in the skin are Langerhans cells, a form of dendritic cells (DCs) (Wu et al., 2000). Infection of these cells leads to three major outcomes. Firstly, dendritic cells (DCs) recognize the pathogen through the cell-surface receptor DC-SIGN (CD209), which

activates them and triggers the release of inflammatory cytokines and chemokines. Secondly, the infected DCs migrate to lymphoid organs such as the spleen, lymph nodes, and bone marrow, where viral replication takes place and the infection is transmitted to monocytes, macrophages, and other DCs. Thirdly, the DCs present DENV antigens to activate and prime naive T-cells (Tassaneetrithep et al., 2003). In addition, monocytes and macrophages present in peripheral blood, lymphoid, and non-lymphoid tissues are also susceptible to DENV infection. Furthermore, DENV utilizes the DC-SIGN homolog, L-SIGN, to infect liver endothelial cells (Dejnirattisai et al., 2011). The receptors for DENV on monocytes include CD14, HSP70/HSP90, and Fc receptors, while macrophages express the mannose receptor (MR; CD206) as a DENV receptor (Y. C. Chen, Wang, & King, 1999; J. L. Miller et al., 2008; Reyes-Del Valle, Chavez-Salinas, Medina, & Del Angel, 2005). Additionally, mammalian non-immune cells, including liver and kidney cells, can also be infected by DENV. The human hepatoma cell line HepG2 were used to study DENV receptors, which are serotype-specific: GRP78 serves as a receptor for DENV serotype 2, while laminin receptors are specific to serotype 1 (Jindadamrongwech, Thepparit, & Smith, 2004; Thepparit & Smith, 2004). Monkey kidney cell lines, such as Vero and LLC-MK2, along with the hamster kidney cell line (BHK), utilize heparan sulfate and glycosphingolipids as their receptors (Aoki et al., 2006; Germi et al., 2002; Wichit et al., 2011). *A. albopictus* mosquito cell line, C6/36 identifies laminin as the receptor for DENV (Sakoonwatanyoo, Boonsanay, & Smith, 2006). In cells that do not express selectin-type receptors, research demonstrated that DENV exploits the transmembrane receptors TIM and TAM, which play a role in the phosphatidylserine-mediated clearance of apoptotic cells (Meertens et al., 2012). After the DENV envelope binds to the host receptor, the virus particles are taken inside through internalization and fusing with endosomal membrane. The acidic conditions inside the endosome induce a structural change in the virus, prompting the DII fusion loops to embed into the endosomal membrane. This action promotes uncoating and allows the viral RNA genome to be released into the cytoplasm (Smit, Moesker, Rodenhuis-Zybert, & Wilschut, 2011). The viral RNA functions as mRNA, the DENV genome associates with the rough ER, where it undergoes translation. Subsequently, NS2A, NS4A, and NS4B are then incorporated into the ER membrane, forming the viral replication complex essential for assembly of the virus. The positive-sense RNA genome of DENV is first copied into a negative-sense RNA strand, which serves as a template for synthesizing numerous new positive-sense RNA genomes. These newly

formed RNA strands can either be translated into viral proteins or encapsulated with capsid proteins to form nucleocapsids. These nucleocapsids then associate with membrane and envelope proteins at the replication complex, resulting in the formation of immature viral particles within the lumen of the endoplasmic reticulum (ER). The immature virions are transported in vesicles to the Golgi apparatus, where they undergo glycosylation. As they move through the secretory pathway, the prM protein is cleaved by the enzyme furin into the M protein, leading to the formation of mature virions, which are ultimately released from the host cell.



**Figure 2.3** Dengue virus replication, © 2005 [Nature Publishing Group](#),

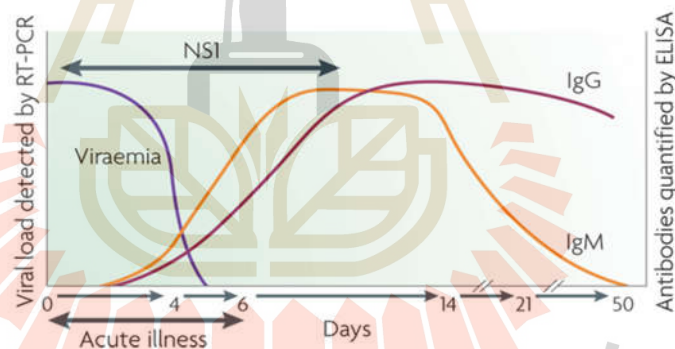
(Mukhopadhyay, Kuhn, & Rossmann, 2005)

## 2.4 Diagnosis of DENV infection

Diagnosis of dengue virus infection in laboratory can be confirmed through various methods, including detection of the virus itself, viral nucleic acids, antigens, antibodies, or a combination of these approaches. The virus can be identified in serum, plasma, circulating blood cells, and other tissues within 4–5 days after the onset of symptoms. In the early phase of the illness, diagnosis can be made using virus isolation, nucleic acid detection, or antigen detection techniques. As the infection progresses to the later acute phase, serological testing becomes the preferred diagnostic method (WHO, 2009b). Virological, molecular, and serological methods are used to confirm DENV infection for purposes of epidemiological surveillance and clinical diagnosis. Among these, the detection of anti-DENV IgM antibodies is the most frequently utilized test in routine settings. During the acute phase of the disease, measuring IgM antibody levels in patient serum helps to confirm infection serologically. In contrast, patients in the convalescent stage are identified by observing IgM and IgG



seroconversion through comparison of antibody levels in paired acute and convalescent serum samples (Gubler et al., 2014). For dengue suspected patients, a preliminary diagnosis can be established by detecting anti-IgM antibodies in samples taken on the sixth day of acute symptoms. There are commercially available diagnostic kits for detecting IgM or IgG antibodies using enzyme-linked immunosorbent assay (ELISA) as well as less sensitive rapid test formats (Hunsperger et al., 2009). Reverse transcription PCR (RT-PCR), real-time RT-PCR, virus isolation in mosquito cell lines, and mosquito inoculation are techniques used for virological confirmation and identification of DENV. While virus isolation and identification offer high specificity, these methods tend to be less sensitive, resource-intensive, and time-consuming. In contrast, detecting DENV RNA provides a faster, more sensitive and specific approach for virological diagnosis. Detection of the NS1 protein offers a valuable opportunity for early etiological diagnosis. The sensitivity and specificity of DENV NS1 detection vary depending on factors such as the infecting serotype, the timing of sample collection, whether the infection is primary or secondary, and the test method used (Hunsperger et al., 2014).



**Figure 2.4** Diagnosis of dengue. Viraemia, non-structural 1 (NS1) antigen and antibodies change over time; thus, different diagnostic tests will be appropriate depending on the stage of infection. ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription (Guzman et al., 2010)

## 2.5 Host Immunity

### 2.5.1 Innate immune response

After attaching to receptors and being internalized into endosomes, DENV triggers the innate immune response. The human innate immune system serves as the initial defense against invading pathogens by mobilizing leukocytes such as natural

killer (NK) cells, mast cells, and phagocytic cells like monocytes, neutrophils, macrophages, and dendritic cells (DCs) (Navarro-Sanchez, Despres, & Cedillo-Barron, 2005). They inhibit viral replication by promoting the production of type I interferons (IFN- $\alpha$  and IFN- $\beta$ ) as well as type II interferon (IFN- $\gamma$ ). This interferon expression is initiated when the virus binds to pattern recognition receptors, particularly toll-like receptors (TLR) 3, 7, and 8, which are located inside endosomes. Activation of this TLR-dependent pathway has been shown to stimulate the release of pro-inflammatory cytokines, including IL-8, IL-12, IFN- $\alpha$ , and IFN- $\gamma$ , in the monocytic THP-1 cell line (Chareonsirisuthigul, Kalayanarooj, & Ubol, 2007). The production of interferons (IFNs) boosts the generation of nitric oxide radicals (NOs). Combined, IFNs and NOs establish an antiviral state in neighboring cells and suppress DENV replication inside infected cells (Ubol, Chareonsirisuthigul, Kasisith, & Klungthong, 2008).

### 2.5.2 Adaptive immune response

In addition to producing interferons as part of the innate antiviral response, the virus also activates the host's adaptive immune system through the maturation of dendritic cells (DCs). DCs stimulate antibody production both with and without T-cell involvement. Infected DCs present DENV antigens on MHC II molecules, which are recognized by helper T-cells that play a crucial role in activating B-cells to produce antibodies against DENV. Even without assistance from T-cell, DENV antigens displayed on DCs' MHC II molecules can directly interact with receptors on B-cells to trigger the primary antibody response. These antibodies typically develop around six days after infection. Antibodies target the DENV virions, specifically binding to the structural proteins E and prM, neutralizing the virus's ability to infect. Neutralization happens via two primary mechanisms: stopping the virus from binding to cell receptors and blocking the fusion process by either attaching antibodies to the fusion loop of the E protein or preventing the conformational changes in the E protein required for membrane fusion. Antibodies that target proteins other than the E glycoprotein, such as anti-NS1 antibodies, can also offer protective effects against the virus, though these effects do not involve direct interaction with the virion itself. Humoral immunity is crucial in the dengue immune response by producing neutralizing antibodies against the DENV envelope glycoprotein. After DENV infection, the body produces IgM, IgG, and IgA antibodies. The IgM response begins early, often before symptoms arise, and is usually detectable in serological tests about 7–8 days after symptoms start. IgA

antibodies are also present and have a half-life comparable to that of IgM. IgG antibodies appear soon after infection and can remain for several years. Immunity developed against one DENV serotype protects against that particular serotype but does not provide long-term defense against infections from other serotypes (St John & Rathore, 2019).

## **2.6 Role of adaptive immunity in viral pathogenesis**

Re-infection with a different dengue serotype can lead to dengue fever (DF) or more severe forms of the disease. Several hypotheses have been proposed to explain this increased severity following heterotypic secondary infection, including original antigenic sin by T cells and ADE. These mechanisms are believed to elevate viral load inducing activation of complement system and cytokine storm, ultimately contributing to severe dengue.

### **2.6.1 Antibody responses against DENV**

In case of DENV infection, DENV-specific IgM antibodies appear first, followed by IgG antibodies, primarily IgG1 and IgG3, which can persist for extended periods even as their levels gradually decline. Studies have shown that antibodies can either neutralize or enhance DENV infection. Mouse monoclonal antibodies (mAbs) have been primarily used by researchers to investigate interactions between antibodies and DENV. According to these investigations, DENV antibody neutralization is a "multi-hit" phenomenon that necessitates the attachment of multiple antibodies in order to neutralize a virion. The dengue envelope (E) protein's domain III surface-exposed epitopes are where the strongest mouse mAbs for neutralizing dengue attach. The polyclonal response to primary and secondary infections with four different DENV serotypes makes the human antibody response complicated (Wahala & Silva, 2011). Detectable DENV antibodies, which show partial cross-reactivity with all four serotypes and other flaviviruses, have been observed in infected individuals for decades. A primary DENV infection generates long-lasting immunity against the same serotype; however, the resulting cross-reactive antibodies typically have low avidity and limited neutralizing ability. While reinfections are relatively rare, a secondary DENV infection often elicits cross-reactive antibodies with higher avidity and strong neutralizing capacity in vitro (Tsai et al., 2015; Tsai et al., 2013).

Neutralizing antibodies are considered as prime factor for defense against DENV infection. In terms of pathogenicity, DENV-targeting antibodies have two conflicting



functions: virus neutralization and infection enhancement. Antibodies neutralize DENV at greater avidities, whereas lower avidities promote DENV infection and prevent virus neutralization. Antibodies that neutralize viruses are thought to be essential for defense against DENV infection. Regarding pathogenicity, antibodies targeting DENV play dual and opposing roles: they can either neutralize the virus or enhance infection. High-avidity antibodies effectively neutralize DENV, while low-avidity antibodies may facilitate viral entry and hinder neutralization. The idea was supported by research using serum samples from dengue patients, where neutralizing activity against several DENV serotypes was detected using non-Fc $\gamma$ R-expressing cells, but ADE activity that lowers the overall neutralizing activity was present to the currently infecting serotypes (DENV-1 and DENV-3) as observed in Fc $\gamma$ R-expressing cells (Moi, Lim, Chua, Takasaki, & Kurane, 2012). In individuals with a primary DENV infection, antibodies exhibit strong neutralizing activity against the same serotype after the initial cross-protection phase, but lack neutralizing effects against other serotypes. These strongly neutralizing antibodies to the original serotype can also cross-react with other serotypes and may contribute to enhanced infection (Kutsuna et al., 2015). After an acute DENV infection, most antibodies are directed against the envelope (E) protein, although some also recognize the prM and NS1 proteins. The envelope and membrane proteins form the lipid envelope of the virus particle. Because the majority of antibodies target the E protein, neutralizing its epitopes is likely essential for effective viral neutralization. While antibodies consistently recognize the E protein, their reactivity to its domains I, II, and III varies among individuals. This variability suggests that multiple epitopes across these domains may contribute to viral neutralization (Beltramello et al., 2010; Tsai et al., 2013). Co-circulation of several serotypes in DENV hyperendemic areas illustrates an epidemiological pattern in which outbreaks arise from a dominant serotype in a 2-4-year cycle. These cycles continue because infection with one serotype provides only partial immunity and may be driven by low immunity to each of the dengue serotypes allowing the virus to persist in the region. Most individuals in hyperendemic areas exhibit seroconversion and are exposed to two or more serotypes (Moi, Takasaki, & Kurane, 2016).

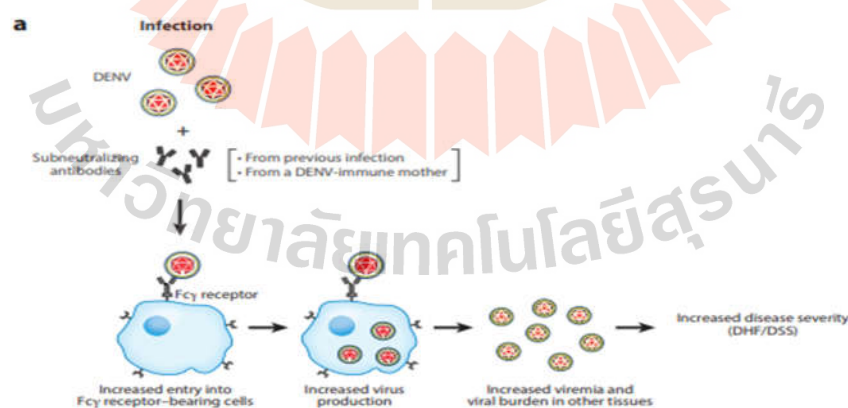
Besides offering protection, the antibody response to DENV can also play a role in increased viral replication and disease severity during a secondary infection with a different serotype, through a process known as Antibody-Dependent Enhancement (ADE). According to epidemiological data, two categories of people i.e. people with

declining homotypic immunity and children whose moms have immunity to dengue are more likely to experience severe dengue (Kliks, Nimmanitya, Nisalak, & Burke, 1988; Kliks, Nisalak, Brandt, Wahl, & Burke, 1989). The ADE theory suggests that complexes formed between DENV and antibodies bind to Fc $\gamma$  receptors on cells like macrophages, promoting virus entry and replication. This enhanced viral load leads to the release of inflammatory mediators, which in turn increase vascular permeability (Figure 2.5). Numerous studies now provide evidence supporting ADE. For instance, mothers of infants suffering from DHF/DSS possess DENV-specific antibodies from previous primary infections, suggesting that antibodies generated during initial infections or acquired passively play a role in causing DHF/DSS in infants. The most convincing evidence for the function of anti-DENV antibodies in disease progression is the absence of DHF/DSS in infants born to mothers who have never been infected with DENV (Halstead, 1988). Secondly, research on nonhuman animals showed ADE of DENV replication *in vivo*. In particular, monkeys with heterologous DENV infections experience higher levels and longer-lasting viremia than those with primary DENV infection with the same virus (Halstead, Shotwell, & Casals, 1973), and monkeys passively transferred with DENV-immune human sera (Halstead, 1979) or a humanized IgG1 monoclonal antibody have higher viral loads compared to control monkeys (Goncalvez, Engle, St Claire, Purcell, & Lai, 2007). Thirdly, research involving mouse models lacking type I interferon receptor signaling has demonstrated that ADE increases both DENV replication within the host and the severity of the resulting illness (Balsitis et al., 2010; Ng et al., 2014).

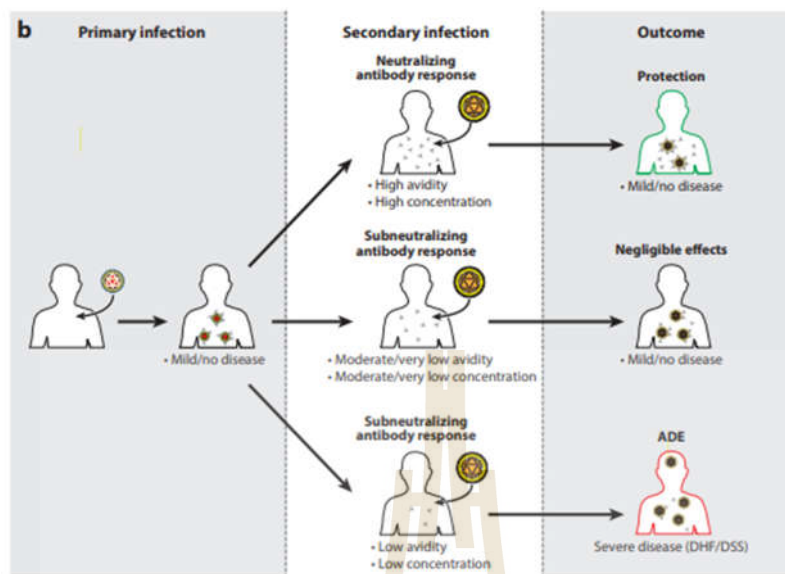
Studies using different monoclonal antibodies have shown that all neutralizing antibodies are capable of promoting ADE *in vitro* when present at sub-neutralizing levels (Morens & Halstead, 1987; Pierson et al., 2007; Yamanaka, Kosugi, & Konishi, 2008). This pertains to the idea that, in the absence of the viral occupancy threshold necessary for neutralization, a neutralizing antibody can cause ADE (Pierson et al., 2007). The outcome of whether an antibody neutralizes or enhances the virus depends on the stoichiometry of its binding to viral particles (Yamanaka et al., 2008). An antibody must have a sufficient concentration and a strong enough affinity to successfully block the appropriate virus surface epitopes for neutralization to occur. Hence, even neutralizing antibodies only work when present in sufficiently high concentrations. If the antibody levels are too low to achieve neutralization but enough to cause ADE, the antibodies may facilitate ADE; however, if conditions do not favor

either ADE or neutralization, the antibodies may have little to no effect (Figure 2.5b). The precise features and molecular processes behind ADE are still unclear. Research suggests that the activation of different signaling pathways might influence whether antibodies have a strong or minimal effect. For example, the infectivity of DENV immune complexes is variably increased depending on whether the Fc $\gamma$ RI and Fc $\gamma$ RII receptors involved are capable of signaling or not (Rodrigo, Jin, Blackley, Rose, & Schlesinger, 2006). Additionally, co-ligation of the inhibitory receptor Fc $\gamma$ RIIB requiring high antibody concentrations can suppress ADE (Boonnak, Slike, Donofrio, & Marovich, 2013; Chan et al., 2011). ADE also reduces functions of antiviral molecules like IRF-1 and STAT-1 (Chareonsirisuthigul et al., 2007) while simultaneously increasing the production of specific cytokines like TNF, IL-6, and IL-10 (Boonnak et al., 2008; Chareonsirisuthigul et al., 2007). This evasion of the antiviral response through ADE is seemingly caused by the co-ligation of LILRB1, which suppresses the expression of interferon-stimulated genes (ISGs) that are normally activated by the Fc $\gamma$ R signaling pathway (Chan et al., 2014). According to recent research, non-fucosylated Fc glycans and specific IgG subclasses may play a role in promoting ADE, which is consistent with the significant role that Fc $\gamma$ R signaling plays in mediating ADE (T. T. Wang et al., 2017).

In summary, these findings point to the complicated interactions among various signaling pathways as modulators of ADE, offering a framework for additional research on mechanisms of ADE using appropriate cultured human cells and experimental animal models.







**Figure 2.5** Outcomes influenced by antibody behavior due to ADE, protection, or minimal impact.

(a) Following ADE hypothesis, sub neutralizing antibodies either in quality or quantity bind to a cross-reactive virus, enabling the immune complex to enter permissive cells through the Fc $\gamma$  receptor. This results in increased virus replication and viremia, leading to severe disease such as DHF/DSS. (b) Following a primary infection, antibodies are generated. During a subsequent infection with a related virus, the combined antibody avidity and concentration (CAC) can lead to three different clinical outcomes based on four CAC levels. In case CAC is high, the second infection is neutralized without symptoms. If CAC is moderate or low, antibodies have little effect, and the infection is controlled by other mechanisms. However, insufficient CAC leads to ADE, resulting in severe disease (Ngono & Shresta, 2018).

### 2.6.2 T cell responses against DENV

During viral infection, T cells play a protective role. Mostly, CD8 T cells help control viral infections through various methods, including directly killing infected cells and producing pro-inflammatory cytokines like IFN- $\gamma$  and TNF- $\alpha$ . Similarly, CD4 T cells are thought to combat viral infections by supporting B cell and CD8 T cell activity, releasing both inflammatory and antiviral cytokines, exhibiting cytotoxic effects, and aiding in the development of memory immune responses. Two host-specific factors regulate human antigen-specific T cell immune reactions. First, antigen-derived peptides must be able to bind to and be presented by HLA class I and II molecules.

Second, the immunogenicity of these peptides determines their ability to be recognized by T cells, which is essential for triggering T cell-specific immune responses.

While T cells play a protective role in fighting viral infections, reports indicate that in DENV infection, T cells can have both harmful and beneficial effects. In dengue, the concept of T cell original antigenic sin suggests that, regardless of ADE, T cells initially primed against the first DENV serotype (the “original antigen”) may disproportionately dominate the immune response during a secondary infection with a different serotype, which could potentially exacerbate the severity of the secondary infection (Figure 2.6). Clinical studies have shown that patients with DHF/DSS have a higher number of cross-reactive CD8 T cells with low affinity for different DENV serotypes in their blood compared to those suffering from dengue fever (Mongkolsapaya et al., 2003). Additionally, the cytokine profiles of DENV-specific CD8 T cells were observed to be altered in patients with DHF/DSS (Duangchinda et al., 2010; Rothman, 2011). Tumor necrosis factor (TNF), a key element of the cytokine storm seen in DHF/DSS patients, is produced in higher amounts by DENV-specific CD4 T cells when they respond to heterotypic antigens compared to homotypic antigens (Mangada et al., 2002; Mangada & Rothman, 2005). However, there is currently no definitive evidence directly connecting T cells to the development of DHF/DSS.

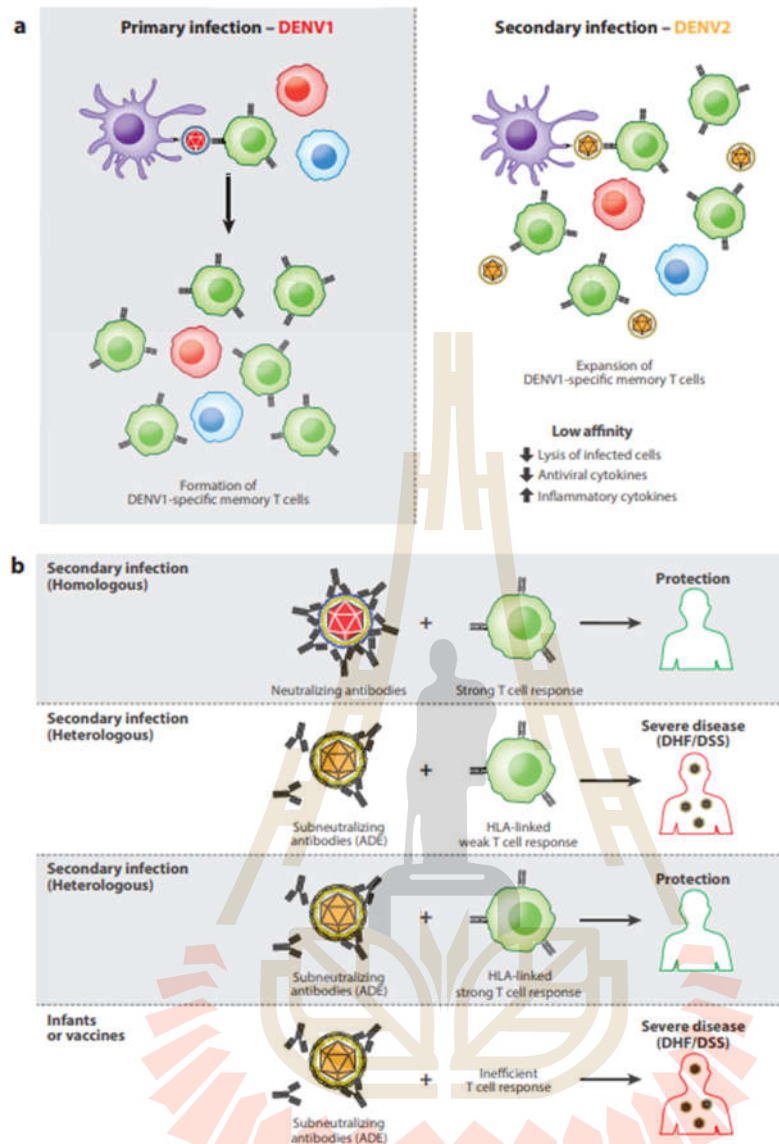
Although limited evidence suggests a pathogenic role for T cells, recent findings indicate that both serotype-specific and cross-reactive T cells may provide protection against DENV infection. The main targets of the CD8 T cell response to DENV are the nonstructural proteins NS3, NS4B, and NS5 (Weiskopf et al., 2013). Although T cell specificity tends to favor conserved epitopes, research analyzing epitope-specific CD8 T cell responses in blood samples from individuals previously exposed to DENV and those vaccinated with a live-attenuated tetravalent DENV vaccine has demonstrated that this preference does not result in a weakened immune response (Weiskopf, Angelo, et al., 2015; Weiskopf et al., 2013; Weiskopf, Cerpa, et al., 2015). In fact, studies found that the strength and diversity of the CD8 T cell response were linked to specific HLA alleles associated with disease susceptibility in individuals previously exposed to DENV, suggesting that a robust and broad CD8 T cell response is an indicator of protective immunity (de Alwis et al., 2016; Weiskopf et al., 2013). DENV-specific CD8 T cell responses linked to protective HLA alleles were multifunctional, producing both IFN- $\gamma$  and TNF- $\alpha$ . Most of these cells were effector memory T cells, including both the CD45RA<sup>-</sup>CCR7<sup>-</sup> T effector memory and CD45RA<sup>+</sup>CCR7<sup>-</sup> T effector

memory RA subsets. Additionally, these cells exhibited high expression of the programmed death 1 (PD-1) receptor (de Alwis et al., 2016; Weiskopf et al., 2013). During an acute DENV infection, DENV-specific CD8 T cells in the bloodstream can also produce CXCR3, CCR5, and the skin-homing marker known as cutaneous lymphocyte-associated antigen (CLA) (Rivino et al., 2015). Furthermore, children with subclinical secondary DENV infection exhibited higher frequencies of DENV-specific CD8 T cells producing IFN- $\gamma$  compared to those who developed symptomatic secondary infections (Hatch et al., 2011). Moreover, children who experienced subclinical secondary DENV infection showed increased levels of DENV-specific CD8 T cells that produce IFN- $\gamma$  compared to children with symptomatic secondary infections (Rivino et al., 2013). Similar to CD8 T cells, CD4 T cells can also contribute to protection against DENV. Phase I human trials with a live-attenuated vaccine suggest that multifunctional CD4 T cells producing proinflammatory cytokines may play a role in controlling DENV replication or facilitating viral clearance (Lindow et al., 2012). Additionally, the CD45RA+CCR7- subset of CD4 T cells was elevated in individuals experiencing secondary (multiple) DENV infections, particularly among those carrying an HLA allele associated with protection against severe disease (Weiskopf, Bangs, et al., 2015). Based on the presence of granzyme B, perforin, CD107a, and DENV-specific cytolytic activity observed ex vivo, these CD4 T cells are likely cytotoxic effectors whose expansion depends on an individual's prior DENV infection history and HLA genotype. Therefore, both CD4 and CD8 T cells may play significant roles as cytotoxic effectors in defending against DENV infection, with HLA alleles influencing the strength and scope of responses from both cell types. Supporting human data, mouse studies have demonstrated the essential role of T cells in combating DENV infection. Specifically, experiments with mice lacking type I and/or type II interferon receptors, conditions that facilitate enhanced DENV replication compared to wild-type mice indicate that CD8 T cells are more critical than CD4 T cells for protection against both primary and secondary DENV infections (Elong Ngonu et al., 2016; Prestwood et al., 2012; Yauch et al., 2010; Zellweger et al., 2015; Zompi, Santich, Beatty, & Harris, 2012). These studies have specifically demonstrated that primary DENV infection triggers the development of multifunctional effector CD8 T cells that produce IFN- $\gamma$ , TNF, and CD107a, playing a key role in clearing the virus (Yauch et al., 2009); CD8 T cells are essential for clearing DENV from the central nervous system (Prestwood et al., 2012) preventing ADE (Zellweger, Eddy, Tang, Miller, & Shrestha, 2014). The studies also indicate that a vaccine



candidate based on the DENV E protein provides protection through the action of CD8 T cells (Zellweger et al., 2013); CD8 T cells are crucial for protection against heterotypic infection (Zellweger et al., 2015) and viral clearance is aided by cross-reactive CD8 T cells that respond to heterotypic infection with lower avidity and magnitude (Elong Ngonu et al., 2016). Even though, CD4 T cells don't seem to be necessary for preventing DENV infection, their induction by immunization leads to viral clearance leading to protection, most likely due to their cytotoxic actions (Yauch et al., 2010). Various studies using HLA transgenic mice have confirmed T cell-mediated protection in mice, which is likely applicable to human DENV infection. Additionally, the epitopes identified in these mice were also recognized by T cells from humans infected with DENV (Elong Ngonu et al., 2016; Weiskopf et al., 2014) and both in mice and in humans, a predominance of HLA B\*0702-restricted responses was found (Weiskopf et al., 2013). Additional research has demonstrated that while CD8 T cell responses against DENV3 target both structural and nonstructural proteins, responses against the other three DENV serotypes primarily focus on nonstructural proteins in both mice and humans (Weiskopf, Angelo, et al., 2015; Weiskopf et al., 2014; Weiskopf, Cerpa, et al., 2015). After primary and heterotypic secondary DENV infections, CD8 T cell responses were broad in both mice and humans. However, CD8 T cell responses following heterotypic secondary DENV infections in mice were different (Weiskopf et al., 2014), vaccination with tetravalent live attenuated DENV (Chu, George, Stinchcomb, Osorio, & Partidos, 2015; Weiskopf, Angelo, et al., 2015), and natural reinfections in humans (Duangchinda et al., 2010; Rivino et al., 2015; Weiskopf et al., 2013) were directed towards the conserved nonstructural proteins. Humans have confirmed the protective function of cellular immunity that was seen in animal models. A new study compared infections in children with no symptoms to infections in children showing clinical symptoms. The former group had a significant increase in T cell-mediated apoptosis, which aided in the clearance of DENV (Simon-Loriere et al., 2017). The distinction between T cells from patients with mild or severe syndromes was the focus of another research. These T cells, which targeted NS3 and NS5, substantially increased IFN- $\gamma$  and TNF- $\alpha$  levels in patients who had mild illnesses, indicating that the immune response mediated by T cells may have an impact on the severity of disease (Wijeratne et al., 2019).

In conclusion, both neutralizing antibodies and T cells are essential components of a successful immune response. Both would be essential for the host's defenses to function properly, and their removal would be a serious breach.



**Figure 2.6** T cell original antigenic sin and possible effects of combined humoral and T cell immunity.

a) Following the concept of T cell original antigenic sin, T cells initially exposed to a virus undergo clonal expansion. However, during a subsequent infection where cross-reactive viral antigens are presented, these previously expanded T cell populations which have lower affinity for the new infecting virus are reactivated. This leads to inadequate elimination of infected cells, resulting in abnormal and potentially harmful immune responses. b) Current evidence supports the idea that T cells generally provide protection during both primary and secondary infections, even in the presence of antibody-dependent enhancement (ADE). Nonetheless, host factors such as HLA type can influence the nature of T cell responses (Ngono & Shrestha, 2018).

## 2.7 Impact of cross-reactive immunity

Similar to the dengue virus, other flaviviruses transmitted by mosquito also tend to cause strong immune responses that cross-react with each other and can affect one another (Hou, Chen, Gao, & An, 2022). Mosquito-borne flaviviruses are primarily spread by *Aedes* mosquitoes (including DENV and ZIKV) or *Culex* mosquitoes (such as JEV). Because these vectors influence the geographic distribution of the viruses, the epidemic regions of these flaviviruses often overlap, significantly complicating efforts to prevent and control their spread (Theodore C Pierson & Michael S Diamond, 2020).

### 2.7.1 Effect of prior ZIKV immunity on DENV

Since Zika virus (ZIKV) emerged in DENV endemic areas, questions are raised whether existing immunity could impact these related flaviviruses. Hence, detailed study has to be performed in these closely related flaviviruses.

According to a study by Fowler et al., mice with circulating maternal Abs against ZIKV at birth developed severe disease when exposed to DENV. Pups from ZIKV-immune mice missing the type I interferon receptor in myeloid cells ( $\text{LysMCre}^+ \text{Ifnar1}^{\text{fl/fl}}$ ) show worse disease and viremia after DENV infection compared to pups from naive mothers. Passive transfer of IgG from mice born to ZIKV-immune mothers showed increased viremia in naive recipient mice. The survival of DENV-infected mice born to ZIKV-immune mothers was improved by treatment with Abs blocking the inflammatory cytokine TNF related to DENV disease or Abs preventing DENV entry. Therefore, the maternal Ab reaction to ZIKV infection or vaccination may put infants at risk for developing severe dengue disease (Fowler et al., 2018). In another study by Watanabe et al., they found that in a mouse model ( $\text{ifnar}^{-/-} \text{Sv/129}$ ), when exposed to DENV2 after first being infected with ZIKV, the mice had lower death rates and lower levels of the virus in their bodies compared to a control group. This suggests that the immune response triggered by ZIKV may provide some protection against DENV infection. But in the  $\text{ifnar}^{-/-} \text{ifngr}^{-/-}$  mouse model, experiments showed that increasing the level of immunity ZIKV through transferring protective antibodies and vaccinating with an inactivated ZIKV actually increased the mortality rate when the mice were later infected with DENV2. This suggests that the Zika virus-specific humoral immunity can negatively impact the outcome of a dengue virus infection in mice (Watanabe, Tan, Chan, & Vasudevan, 2019).



Studies show that even though C57BL/6 mice and rhesus monkeys that had been previously exposed to ZIKV had some level of neutralizing antibodies against DENV, these levels were low. When the mice were infected with ZIKV a second time, no cross-reactive neutralizing antibodies were detected (Breitbach et al., 2019; Kawiecki & Christofferson, 2016). A comparable unexpected finding was seen in the ELISA antibody test for DENV when comparing individuals with a primary ZIKV infection to those who later experienced infections with both DENV and ZIKV (Katzelnick et al., 2021). Langerak et al., conducted a study in which they collected blood samples from a group of people who had previously been infected with the ZIKV and found that the immunity developed from the Zika virus did not protect against the DENV (Langerak et al., 2021). Instead, sera from ZIKV-immune mice promote antibody-dependent enhancement (ADE) of DENV infection in THP-1 cells (Kawiecki & Christofferson, 2016). Another study also showed that convalescent sera from ZIKV-infected patients can induce ADE of DENV (Stettler et al., 2016). According to one study using the rhesus macaque model, it is observed that prior Zika virus infection significantly increases the DENV2 viraemia and is accompanied by neutropenia, lymphocytosis, hyperglycemia, higher reticulocyte counts, together with the activation of pro-inflammatory monocyte subsets, and release of inflammatory mediators. The findings revealed that ZIKV infection triggered detectable serum IgG responses that cross-react with DENV, which were significantly boosted following DENV2 infection. Even before exposure to DENV2, serum from animals immune to Zika virus notably enhanced antibody-dependent enhancement (ADE) across all four DENV serotypes, suggesting that pre-existing immunity to ZIKV may promote the transmission of different DENV serotypes. This in vivo evidence indicates that prior ZIKV infection can intensify DENV infection, highlighting important considerations for understanding disease mechanisms and vaccine development (George et al., 2017).

### **2.7.2 Effect of prior DENV immunity on ZIKV**

DENV and ZIKV are closely related flaviviruses sharing common vector for transmission (*Aedes aegypti*) and co-circulating in overlapping geographical regions. These viruses share 52-57% total amino acid homology and share cross-reactivity in antibody and T cell responses (Barba-Spaeth et al., 2016). Different studies have been performed to study how pre-existing dengue immunity impacts subsequent ZIKV infection which is described as follows:

a) In-vitro serological experiment

Only a small number of cross-reactive neutralizing antibodies (nAbs) against ZIKV are generated following DENV infection. Data from Swanstrom and colleagues show that 73% of sera from people with dengue fever could not neutralize ZIKV, 18% demonstrated minimal cross-neutralization, and just 9% exhibited significant neutralization (Swanstrom et al., 2016). Out of 16 dengue convalescent sera samples collected by Dejnirattisai et al., only 3 showed a strong ability to neutralize ZIKV. Instead, ADE of ZIKV could be induced in U937 cells by serum or monoclonal FL epitope antibodies obtained from dengue fever convalescent patients (Dejnirattisai et al., 2016). Breitbach et al. showed that in cynomolgus monkeys, DENV2-4 immune sera were also unable to eliminate ZIKV, and that only DENV1-immune sera displayed ZIKV reactive Nabs (Breitbach et al., 2019).

b) The in vivo mouse model

Other than through the use of cross-reactive antibodies, mice with a DENV immune background may be cross-protective against ZIKV infection. In the *ifnar*<sup>-/-</sup> Sv/129 mouse model studied by Watanabe et al., all mice inoculated with DENV2 survived after being exposed to ZIKV. However, when DENV2-immune human or mouse sera were passively transferred to *ifnar*<sup>-/-</sup> *ifngr*<sup>-/-</sup> mice, there was no impact on mortality or viremia levels following ZIKV challenge (Watanabe et al., 2019). *Stat2*<sup>-/-</sup> mice that received passive transfer of DENV-immune human sera exhibited increased ZIKV viremia levels, indicating that prior DENV humoral immunity can enhance ZIKV infection (Bardina et al., 2017). Regarding the cellular immune response, numerous studies have demonstrated that DENV and ZIKV T cell immunity are cross-reactive (Andrade et al., 2020; Serrano-Collazo et al., 2020; Wen et al., 2017; Wen et al., 2020). In the *ifnar1*<sup>-/-</sup> mouse model, removing CD8+ T cells led to increased ZIKV infection in mice immune to DENV2, while transferring DENV2-immune CD8+ T cells into naive mice provided protection against ZIKV infection (Wen et al., 2017). Vaccination with cross-reactive DENV and ZIKV CD4+ T cell epitopes significantly reduced ZIKV infection in an HLA-transgenic mouse model, highlighting the protective function of CD4+ T cells in cross-reactive immunity (Wen et al., 2020). Prior DENV immunity may protect fetal mice from ZIKV infection. When pregnant female mice were exposed to ZIKV, fetuses from DENV-immune C57BL/6 mice maintained normal size and showed reduced viral loads in both the placenta and fetal tissues, while fetuses from non-immune mice experienced atrophy and weight loss (Regla-Nava et al., 2018). The outcomes were

similar in the *ifnar1*<sup>-/-</sup> mouse model and the *ifnar1* transient blockaded mouse model, indicating that previous DENV immunity had a protective effect on the fetuses. Cross-reactive CD8+ T cells generated by DENV infection were activated by ZIKV and accumulated in the placenta, acting as a protective factor, as shown by experiments involving CD8+ T cell depletion and passive transfer. Furthermore, CD4+ T cells also contributed supportively to this protective effect (Regla-Nava et al., 2018). Thus, cellular immunity may provide greater protection than humoral immunity during sequential infections with DENV and ZIKV.

c) In vivo non-human primate animal model

In non-human primate models, ZIKV infection had no significant impact on body weight, blood parameters, or body temperature. To better understand cross-reactivity, other measures like RNA levels and immune responses may be utilized (Breitbach et al., 2019; Larocca et al., 2021; Pantoja et al., 2017). The severity of subsequent ZIKV infection in cynomolgus macaques did not improve or worsen within a year of the initial DENV infection. (Breitbach et al., 2019). Similarly, Pantoja et al. found that in rhesus macaques, prior DENV immunity did not lead to more severe ZIKV disease but did promote ADE in vitro. The pre-existing DENV immunity influenced the immune response, leading to a shorter viremia period and reduced activation of B cells, CD4+ T cells, and CD8+ T cells compared to naive controls (Pantoja et al., 2017).

d) Study in humans

Cohort studies showed that individuals with DENV-immune backgrounds can develop neonatal microcephaly caused by ZIKV (Reynolds et al., 2020). Another hypothesis proposed by an ecological study suggested that extending the interval between DENV and ZIKV infections could shift the risk of neonatal microcephaly from being protective to becoming harmful (Carvalho, Freitas, Cruz, Brasil, & Bastos, 2020). Despite some ambiguous findings, it is more convincing that multiple DENV infections provide newborns with protection against ZIKV infection. Pedroso et al. collected serum samples from 29 mothers who gave birth to infants with congenital malformations caused by ZIKV and from 108 mothers who had healthy babies despite ZIKV infection, serving as the control group. Their study confirmed that the seroprevalence of all DENV serotypes was lower in mothers of babies with microcephaly compared to the control group, suggesting that prior infection with two or more DENV serotypes may offer protection against congenital ZIKV syndrome (Pedroso et al., 2019). Similarly, repeated exposure to DENV led to stronger cross-



reactive neutralizing antibody responses against ZIKV compared to a single DENV infection (S. Hattakam, A. Elong Ngono, M. McCauley, S. Shresta, & M. Yamabhai, 2021). Another study performed in a cohort of 1453 urban residents of Brazilian urban community showed that pre-existing immunity to dengue virus and spatial variation influenced Zika virus transmission (Rodriguez-Barraquer et al., 2019). Similar study in Taiwan suggested that pre-existing dengue immunity facilitates ZIKV infection and that the continuous importation of ZIKV infection may pose a threat to indigenous Zika emergence in Southern Taiwan (W. H. Wang et al., 2020).

### **2.7.3 Effect of prior JEV immunity on DENV**

Research has shown that vaccination against JEV can induce high levels of cross-reactive antibodies against DENV1-4 in both humans and mice (Saito et al., 2016; Saron et al., 2018). Li et al., demonstrated that human sera from individuals vaccinated with either the live attenuated (LAV) or inactivated (INV) JE vaccines produced high-titer neutralizing antibodies against DENV2 and DENV3, while immune sera from vaccinated mice showed modest cross-neutralization against all four DENV serotypes (Li et al., 2016). Saron et al., also found that human serum from individuals immune to JEV contained high levels of cross-reactive neutralizing antibodies against DENV1 (Saron et al., 2018).

Prior immunity to JEV offered protection against DENV in immunocompetent mouse models (Li et al., 2016; Saron et al., 2018). A study demonstrated that three doses of JE LAV or INV vaccination decreased mortality in BALB/c mice exposed to all DENV serotypes (Li et al., 2016). In another study, when C57B/6NTac mice were first infected with JEV and then with DENV1, lower viral loads of DENV1 were observed in their lymph nodes compared to the non-immunized group (Saron et al., 2018).

Previous immunity to JEV through vaccination appeared to enhance both the production of cross-reactive neutralizing antibodies and cytokines during a subsequent infection with dengue virus (DENV), indicating that prior JEV has a positive effect on the body's defense against DENV (Komiya, Honda-Okubo, Baldwin, & Petrovsky, 2021; Saron et al., 2018). However, passive transfer of anti-JEV sera led to an increased DENV1 viral load in mouse lymph nodes, the transfer of T cells from JEV-immunized mice had no impact on DENV1 infection (Saron et al., 2018). These results demonstrate that prior JEV protective immunity could protect against both JEV and DENV1 viruses cannot be attained solely through either the body's antibodies or T cells. Both types of immunity need to work together in order to provide protection.

A study conducted in Thailand showed that having previous immunity to JEV increases the likelihood of developing symptoms of dengue fever. This was found by observing patients with dengue fever who did not have previous exposure to dengue virus but had anti-JEV neutralizing antibodies (Anderson et al., 2011). Additionally, in children younger than 10 years, who have been infected with multiple serotypes of DENV, there is a higher proportion of severe dengue cases in those who have anti-JEV neutralizing antibodies (Anderson et al., 2011). Additionally, a Japanese traveler vaccinated against JEV who visited Thailand developed dengue hemorrhagic fever during their first DENV infection (Sato et al., 2015) suggesting that pre-existing JEV antibodies may have contributed to DHF through a mechanism such as ADE.

#### **2.7.4 Effect of prior DENV immunity on JEV**

Saron et al., evaluated the IgG and nAbs in the sera of flavivirus-immune immunocompetent mice to assess the quality of cross-reactive antibodies produced by different flaviviruses. They found that DENV-immune sera showed slight cross-reactivity against JEV but lacked cross-neutralizing capability (Saron et al., 2018). In a human study of 182 JE cases, patients who tested positive for dengue IgG experienced better clinical outcomes and prognoses compared to those who were dengue IgG-negative, suggesting that prior DENV immunity provided cross-protection against JEV infection (Verma, Jain, Kumar, Agarwal, & Kumar, 2018).

#### **2.7.5 Cross reactivity of DENV and YFV**

Cross reactivity between DENV and YFV is well observed, involving both humoral and cellular immune responses. This cross-reactivity can complicate diagnosis and influence vaccine efficacy and disease severity.

For instance, studies have demonstrated that antibodies generated following YFV vaccination or natural infection can cross-react with DENV, potentially leading to false-positive results in diagnostic assays (Houghton-Trivino, Montana, & Castellanos, 2008). This cross-reactivity can complicate the interpretation of serological data, particularly in regions where both viruses co-circulate.

Conversely, the immunological response to YFV vaccination may also be impacted by a history of DENV infection. Studies suggests that pre-existing DENV immunity might enhance the neutralizing antibody response to YFV vaccination, potentially conferring a more robust and long-lasting protection (Gotuzzo, Yactayo, & Cordova, 2013). However, other studies have suggested that a history of DENV infection may similarly raise the chance of negative outcomes after receiving YFV immunization.

Additionally, efforts are being made to investigate the molecular mechanisms that underlie this cross-reactivity. The envelope proteins of YFV and DENV, which are important targets of neutralizing antibodies, show structural similarities (Modis et al., 2004). The observed cross-reactivity is probably caused by these common epitopes. It is essential to comprehend the unique characteristics of these shared epitopes in order to create better targeted diagnostic tests and vaccinations that can produce broadly protective immunity against both viruses without increasing the likelihood of side effects. The intricate relationship between DENV and YFV immunity and its consequences for public health initiatives require more investigation.

## 2.8 DENV evolution

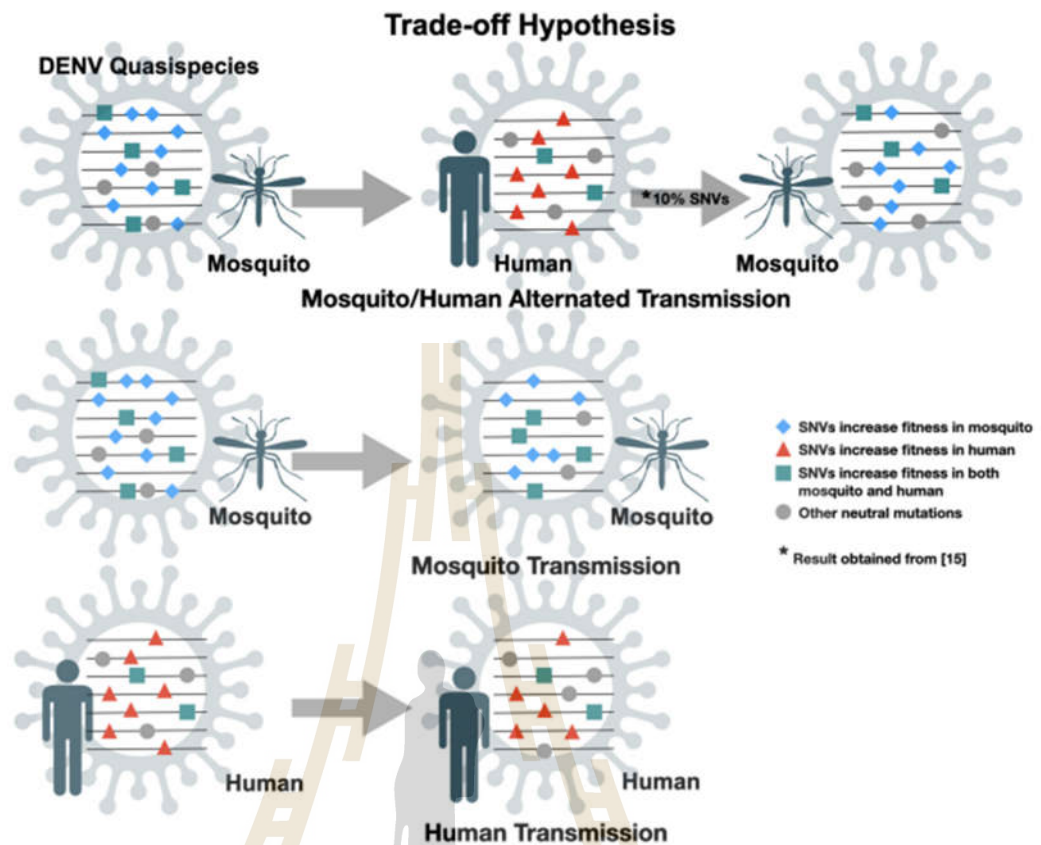
DENV is an RNA virus belonging to *Flaviviridae* family and is currently among the most significant mosquito-borne viruses linked to human illness and have steadily expanded their geographic distribution. It is important to sequence dengue genome to understand about various factors. Whole genome sequencing reveals the complete DNA make-up of an organism, enabling us to better understand variations both within and between species. We can monitor the spread of a virus, how it is changing, and how those changes may impact public health by comparing sequences from specimens using genome sequencing. Epidemiological data shows a correlation between the frequency of severe dengue cases and exposure to new DENV serotypes, connecting viral genomics with dengue epidemics. Therefore, ongoing virological surveillance through sequencing circulating variants can help illustrate and predict new epidemics and their severity.

The DENV genome contains a single open reading frame (ORF) bordered by 5' and 3' untranslated regions (UTRs). This ORF is translated into one long polypeptide, which is then processed post-translationally by proteases from both the host and the virus, producing three structural proteins—capsid (C), pre-membrane (prM), and envelope (E)—as well as seven non-structural (NS) proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (Chambers, Hahn, Galler, & Rice, 1990). The RNA-dependent RNA polymerase (RdRp) lacks a proofreading ability, resulting in random mutations in the genome with each viral replication cycle. Consequently, RNA viruses often exhibit a high mutation rate, generating a diverse population of genomic sequences within an infected individual, referred to as intra-host genetic variants or quasi-species. Like other

RNA viruses, DENV is continuously evolving. The viral population is shaped by various factors, including genetic mechanisms like mutations and recombination (Holmes, Worobey, & Rambaut, 1999), demographic processes, such as genetic drift (Lequime, Fontaine, Ar Gouilh, Moltini-Conclois, & Lambrechts, 2016), and natural selection (Bennett et al., 2003; Holmes & Twiddy, 2003). Unlike other single-stranded RNA viruses such as poliovirus, DENV evolves at a notably slower pace. Non-synonymous mutations occur more frequently within individual hosts than between different hosts, suggesting strong negative or purifying selection acting on DENV. This conclusion was reached by comparing non-synonymous mutation variations both within hosts and between hosts using conventional Sanger consensus sequencing (Holmes & Twiddy, 2003). This discrepancy has been theorized to be explained by the "trade-off" hypothesis (Figure 2.7) (Hanley, Azar, Campos, Vasilakis, & Rossi, 2019; Sim et al., 2015; Weaver & Vasilakis, 2009). The alternating replication cycle between vertebrate and arthropod hosts limits arbovirus evolution because the virus must adapt to the distinct physiological conditions and fitness landscapes of these two different hosts.

The four serotypes of DENV are composed of several genotypes, which further consists of various clades. The evolution of DENV is characterized by lineage turnover in which an entire clade of circulating viruses is replaced by a new clade. It has been shown that certain genotypes within DENV serotypes and certain clades within genotypes are more commonly linked to the development of severe disease. Fitter viruses defined by the ability to replicate better in a given environment and replicate more efficiently are often hypothesized to be more pathogenic in the host. In support of this concept, high levels of virus in patient sera (viremia) have been linked to paradigm in which fitter viruses are more virulent. Additionally, Asian DENV2 viruses, which are more frequently linked to severe disease, are less sensitive to antibody-mediated neutralization by sera from people who have already contracted DENV1 than American genotype DENV2 viruses, which suggests that mutations that could change the neutralization profile of some DENV genotypes may lead to increased fitness in comparison to other strains of the same serotype (OhAinle et al., 2011).



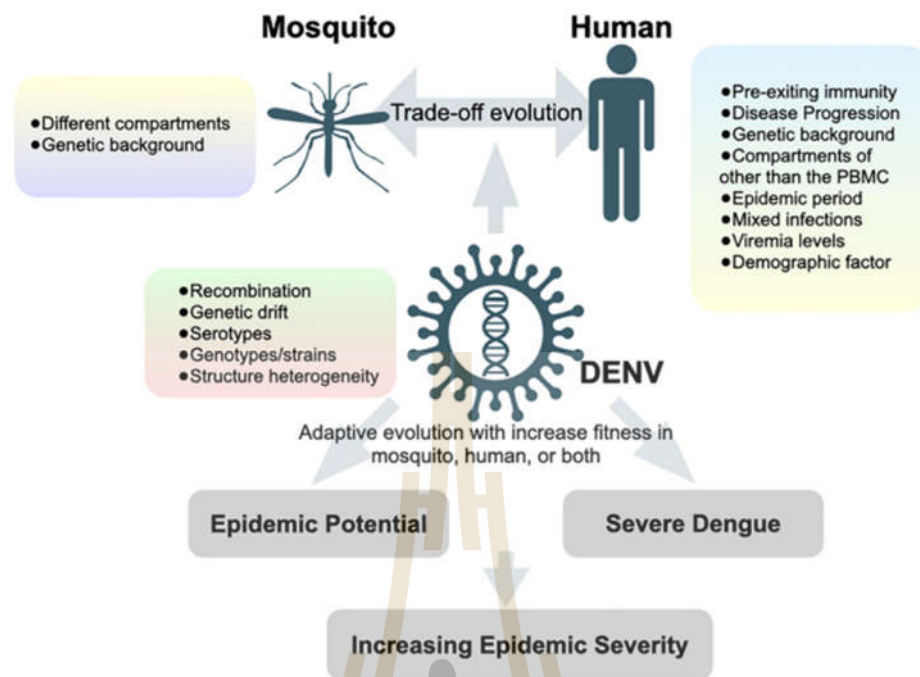


**Figure 2.7** The trade-off hypothesis of dengue virus quasi-species transmission. Random mutations arise in the viral genome during replication. Transmission occurs through the transfer of minor viral populations containing genomic variants, typically detected as single nucleotide variants (SNVs). Only those SNVs that enhance fitness in both mosquito and human hosts are maintained following alternating transmission between these two species (Ko, Salem, Chang, & Chao, 2020).

Nowadays, next generation sequencing (NGS) is being used to understand the evolution of DENV in both mosquitoes and humans. Although research using conventional Sanger sequencing has demonstrated that DENV is present in mosquito vectors as heterogeneous populations, these population may change as the virus passes through various mosquito organs (Lequime et al., 2016). Anatomical barriers can cause population bottlenecks during viral transmission, and with advancements in deep sequencing technology, it is now possible to study the diversity of viral populations within a host by trailing various genetic variants as they move over the mosquito vector's anatomical barriers. By infecting mosquitoes with diverse genetic backgrounds, Lequime et al., examined the evolution of a wild-type dengue virus

isolate within the mosquito (Lequime et al., 2016). The overall genetic diversity of the viral population during infection was mainly shaped by purifying selection, as demonstrated by deep sequencing of full-length viral genomes from different mosquito organs collected at various time points post-infection. Although the initial bottleneck size was similar, the genetic diversity differed significantly among mosquitoes with different genetic backgrounds. Following a random reduction in genetic variation, the dengue virus population size and diversity rapidly rebounded, showing only minor genetic changes as the virus passed through different anatomical compartments and eventually reached the saliva tissue. Ultimately, the "trade-off" hypothesis was supported by the observed fitness loss when a virus derived from the salivary gland was inoculated into Vero mammalian cell cultures. Although tissue-specific sequence-dependent small interfering RNA (siRNA) may contribute to viral diversification during the virus's transition from the mosquito midgut to saliva (Brackney, Beane, & Ebel, 2009), it remains uncertain how other factors—such as different viral genotypes, strains, or serotypes (Ko et al., 2018; Parameswaran et al., 2017) and the mosquito's genetic background (Grubaugh et al., 2016; Sessions et al., 2015) interact with tissue-specific siRNA. These interactions could influence the genetic diversity of the virus population and its relationship to viral fitness (Figure 2.8).

Next-generation sequencing (NGS) has also been utilized to trace transmission pathways and track minor viral variants by analyzing single nucleotide variants (SNVs) within the intra-host genetic diversity of RNA viruses shared among different hosts or individuals (Nasheri, Petronella, Ronholm, Bidawid, & Corneau, 2017; Plummer et al., 2015). Sim et al., successfully tracked genetic variations transmitted from humans to mosquitoes in a laboratory setting by feeding *Aedes aegypti* mosquitoes with viremic blood samples from twelve Vietnamese dengue virus patients. (Sim et al., 2015). By employing whole-genome deep sequencing technology, viral RNA from both patient blood and mosquitoes was analyzed. These findings showed that the frequencies of dengue virus genetic variations, identified through various SNV-based methods, were comparable between humans and mosquitoes. However, only about 10% of the SNVs (out of a total of 267) detected in humans were consistently found in mosquitoes. This suggests that most mutations present in viremic patients are either harmful or confer low fitness when transmitted to mosquitoes.



**Figure 2.8** Factors and forces that influence the evolution of dengue viruses and the possible effects on dengue epidemiology (Ko et al., 2020)

In summary, despite the sequential transmission bottlenecks causing the loss of many variants, there is a rapid appearance and persistence of single nucleotide variants (SNVs) during dengue virus transmission from viremic humans to mosquitoes, which could play a role in the emergence of new clades or lineages driving dengue virus evolution.

## 2.9 References

- Alcon-LePoder, S., Drouet, M. T., Roux, P., Frenkiel, M. P., Arborio, M., Durand-Schneider, A. M., . . . Flamand, M. (2005). The secreted form of dengue virus nonstructural protein NS1 is endocytosed by hepatocytes and accumulates in late endosomes: implications for viral infectivity. *J Virol*, 79(17), 11403-11411. doi:10.1128/JVI.79.17.11403-11411.2005
- Anderson, K. B., Gibbons, R. V., Thomas, S. J., Rothman, A. L., Nisalak, A., Berkelman, R. L., . . . Endy, T. P. (2011). Preexisting Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. *PLoS Negl Trop Dis*, 5(10), e1311. doi:10.1371/journal.pntd.0001311
- Andrade, P., Narvekar, P., Montoya, M., Michlmayr, D., Balmaseda, A., Coloma, J., & Harris, E. (2020). Primary and Secondary Dengue Virus Infections Elicit Similar Memory B-Cell Responses, but Breadth to Other Serotypes and Cross-Reactivity to Zika Virus Is Higher in Secondary Dengue. *J Infect Dis*, 222(4), 590-600. doi:10.1093/infdis/jiaa120
- Aoki, C., Hidari, K. I., Itonori, S., Yamada, A., Takahashi, N., Kasama, T., . . . Suzuki, Y. (2006). Identification and characterization of carbohydrate molecules in mammalian cells recognized by dengue virus type 2. *J Biochem*, 139(3), 607-614. doi:10.1093/jb/mvj067
- Ashour, J., Laurent-Rolle, M., Shi, P. Y., & Garcia-Sastre, A. (2009). NS5 of dengue virus mediates STAT2 binding and degradation. *J Virol*, 83(11), 5408-5418. doi:10.1128/JVI.02188-08
- Balsitis, S. J., Williams, K. L., Lachica, R., Flores, D., Kyle, J. L., Mehlhop, E., . . . Harris, E. (2010). Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. *PLoS Pathog*, 6(2), e1000790. doi:10.1371/journal.ppat.1000790
- Bara, J. J., Clark, T. M., & Remold, S. K. (2013). Susceptibility of larval *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) to dengue virus. *J Med Entomol*, 50(1), 179-184. doi:10.1603/me12140
- Barba-Spaeth, G., Dejnirattisai, W., Rouvinski, A., Vaney, M. C., Medits, I., Sharma, A., . . . Rey, F. A. (2016). Structural basis of potent Zika-dengue virus antibody cross-neutralization. *Nature*, 536(7614), 48-53. doi:10.1038/nature18938
- Bardina, S. V., Bunduc, P., Tripathi, S., Duehr, J., Frere, J. J., Brown, J. A., . . . Lim, J. K. (2017). Enhancement of Zika virus pathogenesis by preexisting ant flavivirus immunity. *Science*, 356(6334), 175-180. doi:10.1126/science.aal4365



- Bell, S. M., Katzelnick, L., & Bedford, T. (2019). Dengue genetic divergence generates within-serotype antigenic variation, but serotypes dominate evolutionary dynamics. *Elife*, 8. doi:10.7554/eLife.42496
- Beltramello, M., Williams, K. L., Simmons, C. P., Macagno, A., Simonelli, L., Quyen, N. T., . . . Sallusto, F. (2010). The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. *Cell Host Microbe*, 8(3), 271-283. doi:10.1016/j.chom.2010.08.007
- Bennett, S. N., Holmes, E. C., Chirivella, M., Rodriguez, D. M., Beltran, M., Vorndam, V., . . . McMillan, W. O. (2003). Selection-driven evolution of emergent dengue virus. *Mol Biol Evol*, 20(10), 1650-1658. doi:10.1093/molbev/msg182
- Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., . . . Hay, S. I. (2013). The global distribution and burden of dengue. *Nature*, 496(7446), 504-507. doi:10.1038/nature12060
- Boonnak, K., Slike, B. M., Burgess, T. H., Mason, R. M., Wu, S. J., Sun, P., . . . Marovich, M. A. (2008). Role of dendritic cells in antibody-dependent enhancement of dengue virus infection. *J Virol*, 82(8), 3939-3951. doi:10.1128/JVI.02484-07
- Boonnak, K., Slike, B. M., Donofrio, G. C., & Marovich, M. A. (2013). Human FcγRIII cytoplasmic domains differentially influence antibody-mediated dengue virus infection. *J Immunol*, 190(11), 5659-5665. doi:10.4049/jimmunol.1203052
- Brackney, D. E., Beane, J. E., & Ebel, G. D. (2009). RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. *PLoS Pathog*, 5(7), e1000502. doi:10.1371/journal.ppat.1000502
- Breitbach, M. E., Newman, C. M., Dudley, D. M., Stewart, L. M., Aliota, M. T., Koenig, M. R., . . . O'Connor, D. H. (2019). Primary infection with dengue or Zika virus does not affect the severity of heterologous secondary infection in macaques. *PLoS Pathog*, 15(8), e1007766. doi:10.1371/journal.ppat.1007766
- Carvalho, M. S., Freitas, L. P., Cruz, O. G., Brasil, P., & Bastos, L. S. (2020). Association of past dengue fever epidemics with the risk of Zika microcephaly at the population level in Brazil. *Sci Rep*, 10(1), 1752. doi:10.1038/s41598-020-58407-7
- Chambers, T. J., Hahn, C. S., Galler, R., & Rice, C. M. (1990). Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol*, 44, 649-688. doi:10.1146/annurev.mi.44.100190.003245
- Chan, K. R., Ong, E. Z., Tan, H. C., Zhang, S. L., Zhang, Q., Tang, K. F., . . . Ooi, E. E. (2014). Leukocyte immunoglobulin-like receptor B1 is critical for antibody-dependent dengue. *Proc Natl Acad Sci U S A*, 111(7), 2722-2727. doi:10.1073/pnas.1317454111

- Chan, K. R., Zhang, S. L., Tan, H. C., Chan, Y. K., Chow, A., Lim, A. P., . . . Ooi, E. E. (2011). Ligation of Fc gamma receptor IIB inhibits antibody-dependent enhancement of dengue virus infection. *Proc Natl Acad Sci U S A*, 108(30), 12479-12484. doi:10.1073/pnas.1106568108
- Chareonsirisuthigul, T., Kalayanarooj, S., & Ubol, S. (2007). Dengue virus (DENV) antibody-dependent enhancement of infection upregulates the production of anti-inflammatory cytokines, but suppresses anti-DENV free radical and pro-inflammatory cytokine production, in THP-1 cells. *J Gen Virol*, 88(Pt 2), 365-375. doi:10.1099/vir.0.82537-0
- Chen, Y. C., Wang, S. Y., & King, C. C. (1999). Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. *J Virol*, 73(4), 2650-2657. doi:10.1128/JVI.73.4.2650-2657.1999
- Chu, H., George, S. L., Stinchcomb, D. T., Osorio, J. E., & Partidos, C. D. (2015). CD8+ T-cell Responses in Flavivirus-Naive Individuals Following Immunization with a Live-Attenuated Tetravalent Dengue Vaccine Candidate. *J Infect Dis*, 212(10), 1618-1628. doi:10.1093/infdis/jiv258
- Crill, W. D., & Roehrig, J. T. (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(16), 7769-7773. doi:10.1128/JVI.75.16.7769-7773.2001
- de Alwis, R., Bangs, D. J., Angelo, M. A., Cerpas, C., Fernando, A., Sidney, J., . . . Weiskopf, D. (2016). Immunodominant Dengue Virus-Specific CD8+ T Cell Responses Are Associated with a Memory PD-1+ Phenotype. *J Virol*, 90(9), 4771-4779. doi:10.1128/JVI.02892-15
- Dejnirattisai, W., Supasa, P., Wongwiwat, W., Rouvinski, A., Barba-Spaeth, G., Duangchinda, T., . . . Screaton, G. R. (2016). Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with Zika virus. *Nat Immunol*, 17(9), 1102-1108. doi:10.1038/ni.3515
- Dejnirattisai, W., Webb, A. I., Chan, V., Jumnainsong, A., Davidson, A., Mongkolsapaya, J., & Screaton, G. (2011). Lectin switching during dengue virus infection. *J Infect Dis*, 203(12), 1775-1783. doi:10.1093/infdis/jir173
- Dhole, P., Zaidi, A., Nariya, H. K., Sinha, S., Jinesh, S., & Srivastava, S. (2024). Host Immune Response to Dengue Virus Infection: Friend or Foe? *Immuno*, 4(4), 549-577. Retrieved from <https://www.mdpi.com/2673-5601/4/4/33>
- Dong, H., Chang, D. C., Hua, M. H., Lim, S. P., Chionh, Y. H., Hia, F., . . . Shi, P. Y. (2012). 2'-O methylation of internal adenosine by flavivirus NS5 methyltransferase. *PLoS Pathog*, 8(4), e1002642. doi:10.1371/journal.ppat.1002642

- Duangchinda, T., Dejnirattisai, W., Vasanawathana, S., Limpitikul, W., Tangthawornchaikul, N., Malasit, P., . . . Screaton, G. (2010). Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. *Proc Natl Acad Sci U S A*, 107(39), 16922-16927. doi:10.1073/pnas.1010867107
- Dwivedi, V. D., Tripathi, I. P., Tripathi, R. C., Bharadwaj, S., & Mishra, S. K. (2017). Genomics, proteomics and evolution of dengue virus. *Brief Funct Genomics*, 16(4), 217-227. doi:10.1093/bfpg/elw040
- Elong Ngono, A., Chen, H. W., Tang, W. W., Joo, Y., King, K., Weiskopf, D., . . . Shresta, S. (2016). Protective Role of Cross-Reactive CD8 T Cells Against Dengue Virus Infection. *EBioMedicine*, 13, 284-293. doi:10.1016/j.ebiom.2016.10.006
- Fowler, A. M., Tang, W. W., Young, M. P., Mamidi, A., Viramontes, K. M., McCauley, M. D., . . . Shresta, S. (2018). Maternally Acquired Zika Antibodies Enhance Dengue Disease Severity in Mice. *Cell Host Microbe*, 24(5), 743-750 e745. doi:10.1016/j.chom.2018.09.015
- George, J., Valiant, W. G., Mattapallil, M. J., Walker, M., Huang, Y. S., Vanlandingham, D. L., . . . Mattapallil, J. J. (2017). Prior Exposure to Zika Virus Significantly Enhances Peak Dengue-2 Viremia in Rhesus Macaques. *Sci Rep*, 7(1), 10498. doi:10.1038/s41598-017-10901-1
- Germi, R., Crance, J. M., Garin, D., Guimet, J., Lortat-Jacob, H., Ruigrok, R. W., . . . Drouet, E. (2002). Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus. *Virology*, 292(1), 162-168. doi:10.1006/viro.2001.1232
- Goncalvez, A. P., Engle, R. E., St Claire, M., Purcell, R. H., & Lai, C. J. (2007). Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. *Proc Natl Acad Sci U S A*, 104(22), 9422-9427. doi:10.1073/pnas.0703498104
- Gotuzzo, E., Yactayo, S., & Cordova, E. (2013). Efficacy and duration of immunity after yellow fever vaccination: systematic review on the need for a booster every 10 years. *Am J Trop Med Hyg*, 89(3), 434-444. doi:10.4269/ajtmh.13-0264
- Grubbaugh, N. D., Weger-Lucarelli, J., Murrieta, R. A., Fauver, J. R., Garcia-Luna, S. M., Prasad, A. N., . . . Ebel, G. D. (2016). Genetic Drift during Systemic Arbovirus Infection of Mosquito Vectors Leads to Decreased Relative Fitness during Host Switching. *Cell Host Microbe*, 19(4), 481-492. doi:10.1016/j.chom.2016.03.002
- Guzman, M. G., Halstead, S. B., Artsob, H., Buchy, P., Farrar, J., Gubler, D. J., . . . Peeling, R. W. (2010). Dengue: a continuing global threat. *Nat Rev Microbiol*, 8(12 Suppl), S7-16. doi:10.1038/nrmicro2460

- Halstead, S. B. (1979). In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J Infect Dis*, 140(4), 527-533. doi:10.1093/infdis/140.4.527
- Halstead, S. B. (1988). Pathogenesis of dengue: challenges to molecular biology. *Science*, 239(4839), 476-481. doi:10.1126/science.3277268
- Halstead, S. B. (2008). Dengue virus-mosquito interactions. *Annu Rev Entomol*, 53, 273-291. doi:10.1146/annurev.ento.53.103106.093326
- Halstead, S. B. (2014). Dengue Antibody-Dependent Enhancement: Knowns and Unknowns. *Microbiol Spectr*, 2(6). doi:10.1128/microbiolspec.AID-0022-2014
- Halstead, S. B., Shotwell, H., & Casals, J. (1973). Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. *J Infect Dis*, 128(1), 15-22. doi:10.1093/infdis/128.1.15
- Hanley, K. A., Azar, S. R., Campos, R. K., Vasilakis, N., & Rossi, S. L. (2019). Support for the Transmission-Clearance Trade-Off Hypothesis from a Study of Zika Virus Delivered by Mosquito Bite to Mice. *Viruses*, 11(11). doi:10.3390/v11111072
- Hatch, S., Endy, T. P., Thomas, S., Mathew, A., Potts, J., Pazoles, P., . . . Rothman, A. L. (2011). Intracellular cytokine production by dengue virus-specific T cells correlates with subclinical secondary infection. *J Infect Dis*, 203(9), 1282-1291. doi:10.1093/infdis/jir012
- Hattakam, S., Elong Ngonu, A., McCauley, M., Shrestha, S., & Yamabhai, M. (2021). Repeated exposure to dengue virus elicits robust cross neutralizing antibodies against Zika virus in residents of Northeastern Thailand. *Sci Rep*, 11(1), 9634. doi:10.1038/s41598-021-88933-x
- Holmes, E. C., & Twiddy, S. S. (2003). The origin, emergence and evolutionary genetics of dengue virus. *Infect Genet Evol*, 3(1), 19-28. doi:10.1016/s1567-1348(03)00004-2
- Holmes, E. C., Worobey, M., & Rambaut, A. (1999). Phylogenetic evidence for recombination in dengue virus. *Mol Biol Evol*, 16(3), 405-409. doi:10.1093/oxfordjournals.molbev.a026121
- Hou, B., Chen, H., Gao, N., & An, J. (2022). Cross-Reactive Immunity among Five Medically Important Mosquito-Borne Flaviviruses Related to Human Diseases. *Viruses*, 14(6). doi:10.3390/v14061213
- Houghton-Trivino, N., Montana, D., & Castellanos, J. (2008). Dengue-yellow fever sera cross-reactivity; challenges for diagnosis. *Rev Salud Publica (Bogota)*, 10(2), 299-307. doi:10.1590/s0124-00642008000200010



- Jindadamrongwech, S., Thepparit, C., & Smith, D. R. (2004). Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. *Arch Virol*, 149(5), 915-927. doi:10.1007/s00705-003-0263-x
- Kadare, G., & Haenni, A. L. (1997). Virus-encoded RNA helicases. *J Virol*, 71(4), 2583-2590. doi:10.1128/JVI.71.4.2583-2590.1997
- Katzelnick, L. C., Zambrana, J. V., Elizondo, D., Collado, D., Garcia, N., Arguello, S., . . . Harris, E. (2021). Dengue and Zika virus infections in children elicit cross-reactive protective and enhancing antibodies that persist long term. *Sci Transl Med*, 13(614), eabg9478. doi:10.1126/scitranslmed.abg9478
- Kawiecki, A. B., & Christofferson, R. C. (2016). Zika Virus-Induced Antibody Response Enhances Dengue Virus Serotype 2 Replication In Vitro. *J Infect Dis*, 214(9), 1357-1360. doi:10.1093/infdis/jiw377
- Kliks, S. C., Nimmanitya, S., Nisalak, A., & Burke, D. S. (1988). Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. *Am J Trop Med Hyg*, 38(2), 411-419. doi:10.4269/ajtmh.1988.38.411
- Kliks, S. C., Nisalak, A., Brandt, W. E., Wahl, L., & Burke, D. S. (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(4), 444-451. doi:10.4269/ajtmh.1989.40.444
- Ko, H. Y., Li, Y. T., Chao, D. Y., Chang, Y. C., Li, Z. T., Wang, M., . . . King, C. C. (2018). Inter- and intra-host sequence diversity reveal the emergence of viral variants during an overwintering epidemic caused by dengue virus serotype 2 in southern Taiwan. *PLoS Negl Trop Dis*, 12(10), e0006827. doi:10.1371/journal.pntd.0006827
- Ko, H. Y., Salem, G. M., Chang, G. J., & Chao, D. Y. (2020). Application of Next-Generation Sequencing to Reveal How Evolutionary Dynamics of Viral Population Shape Dengue Epidemiology. *Front Microbiol*, 11, 1371. doi:10.3389/fmicb.2020.01371
- Komiya, T., Honda-Okubo, Y., Baldwin, J., & Petrovsky, N. (2021). An Advax-Adjuvanted Inactivated Cell-Culture Derived Japanese Encephalitis Vaccine Induces Broadly Neutralising Anti-Flavivirus Antibodies, Robust Cellular Immunity and Provides Single Dose Protection. *Vaccines (Basel)*, 9(11). doi:10.3390/vaccines9111235
- Kurosu, T., Chaichana, P., Yamate, M., Anantapreecha, S., & Ikuta, K. (2007). Secreted complement regulatory protein clusterin interacts with dengue virus nonstructural protein 1. *Biochem Biophys Res Commun*, 362(4), 1051-1056. doi:10.1016/j.bbrc.2007.08.137

- Kutsuna, S., Kato, Y., Moi, M. L., Kotaki, A., Ota, M., Shinohara, K., . . . Ohmagari, N. (2015). Autochthonous dengue fever, Tokyo, Japan, 2014. *Emerg Infect Dis*, 21(3), 517-520. doi:10.3201/eid2103/141662
- Langerak, T., Kasbergen, L. M. R., Chandler, F., Brinkman, T., Faerber, Z., Phalai, K., . . . Vreden, S. (2021). Zika Virus Antibody Titers Three Years after Confirmed Infection. *Viruses*, 13(7). doi:10.3390/v13071345
- Larocca, R. A., Abbink, P., Ventura, J. D., Chandrashekar, A., Mercado, N., Li, Z., . . . Barouch, D. H. (2021). Impact of prior Dengue immunity on Zika vaccine protection in rhesus macaques and mice. *PLoS Pathog*, 17(6), e1009673. doi:10.1371/journal.ppat.1009673
- Lequime, S., Fontaine, A., Ar Gouilh, M., Moltini-Conclois, I., & Lambrechts, L. (2016). Genetic Drift, Purifying Selection and Vector Genotype Shape Dengue Virus Intra-host Genetic Diversity in Mosquitoes. *PLoS Genet*, 12(6), e1006111. doi:10.1371/journal.pgen.1006111
- Li, J., Gao, N., Fan, D., Chen, H., Sheng, Z., Fu, S., . . . An, J. (2016). Cross-protection induced by Japanese encephalitis vaccines against different genotypes of Dengue viruses in mice. *Sci Rep*, 6, 19953. doi:10.1038/srep19953
- Lindow, J. C., Borochoff-Porte, N., Durbin, A. P., Whitehead, S. S., Fimlaid, K. A., Bunn, J. Y., & Kirkpatrick, B. D. (2012). Primary vaccination with low dose live dengue 1 virus generates a proinflammatory, multifunctional T cell response in humans. *PLoS Negl Trop Dis*, 6(7), e1742. doi:10.1371/journal.pntd.0001742
- Mangada, M. M., Endy, T. P., Nisalak, A., Chunsuttiwat, S., Vaughn, D. W., Libraty, D. H., . . . Rothman, A. L. (2002). Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. *J Infect Dis*, 185(12), 1697-1703. doi:10.1086/340822
- Mangada, M. M., & Rothman, A. L. (2005). Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. *J Immunol*, 175(4), 2676-2683. doi:10.4049/jimmunol.175.4.2676
- Meertens, L., Carnec, X., Lecoine, M. P., Ramdasi, R., Guivel-Benhassine, F., Lew, E., . . . Amara, A. (2012). The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. *Cell Host Microbe*, 12(4), 544-557. doi:10.1016/j.chom.2012.08.009
- Messer, W. B., Yount, B., Hacker, K. E., Donaldson, E. F., Huynh, J. P., de Silva, A. M., & Baric, R. S. (2012). Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS Negl Trop Dis*, 6(2), e1486. doi:10.1371/journal.pntd.0001486

- Miller, J. L., de Wet, B. J., Martinez-Pomares, L., Radcliffe, C. M., Dwek, R. A., Rudd, P. M., & Gordon, S. (2008). The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathog*, 4(2), e17. doi:10.1371/journal.ppat.0040017
- Miller, S., Sparacio, S., & Bartenschlager, R. (2006). Subcellular localization and membrane topology of the Dengue virus type 2 Non-structural protein 4B. *J Biol Chem*, 281(13), 8854-8863. doi:10.1074/jbc.M512697200
- Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature*, 427(6972), 313-319. doi:10.1038/nature02165
- Moi, M. L., Lim, C. K., Chua, K. B., Takasaki, T., & Kurane, I. (2012). Dengue virus infection-enhancing activity in serum samples with neutralizing activity as determined by using FcγR-expressing cells. *PLoS Negl Trop Dis*, 6(2), e1536. doi:10.1371/journal.pntd.0001536
- Moi, M. L., Takasaki, T., & Kurane, I. (2016). Human antibody response to dengue virus: implications for dengue vaccine design. *Trop Med Health*, 44, 1. doi:10.1186/s41182-016-0004-y
- Mongkolsapaya, J., Dejnirattisai, W., Xu, X. N., Vasanawathana, S., Tangthawornchaikul, N., Chairunsri, A., . . . Screaton, G. (2003). Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med*, 9(7), 921-927. doi:10.1038/nm887
- Morens, D. M., & Halstead, S. B. (1987). Disease severity-related antigenic differences in dengue 2 strains detected by dengue 4 monoclonal antibodies. *J Med Virol*, 22(2), 169-174. doi:10.1002/jmv.1890220208
- Mukhopadhyay, S., Kuhn, R. J., & Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol*, 3(1), 13-22. doi:10.1038/nrmicro1067
- Munoz-Jordan, J. L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W. I., & Garcia-Sastre, A. (2005). Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *J Virol*, 79(13), 8004-8013. doi:10.1128/JVI.79.13.8004-8013.2005
- Murthy, H. M., Clum, S., & Padmanabhan, R. (1999). Dengue virus NS3 serine protease. Crystal structure and insights into interaction of the active site with substrates by molecular modeling and structural analysis of mutational effects. *J Biol Chem*, 274(9), 5573-5580. doi:10.1074/jbc.274.9.5573
- Nasheri, N., Petronella, N., Ronholm, J., Bidawid, S., & Corneau, N. (2017). Characterization of the Genomic Diversity of Norovirus in Linked Patients Using a Metagenomic Deep Sequencing Approach. *Front Microbiol*, 8, 73. doi:10.3389/fmicb.2017.00073

- Navarro-Sanchez, E., Despres, P., & Cedillo-Barron, L. (2005). Innate immune responses to dengue virus. *Arch Med Res*, 36(5), 425-435. doi:10.1016/j.arcmed.2005.04.007
- Ng, J. K., Zhang, S. L., Tan, H. C., Yan, B., Martinez, J. M., Tan, W. Y., . . . Alonso, S. (2014). First experimental in vivo model of enhanced dengue disease severity through maternally acquired heterotypic dengue antibodies. *PLoS Pathog*, 10(4), e1004031. doi:10.1371/journal.ppat.1004031
- Ngono, A. E., & Shresta, S. (2018). Immune Response to Dengue and Zika. *Annu Rev Immunol*, 36, 279-308. doi:10.1146/annurev-immunol-042617-053142
- Niyomrattanakit, P., Winoyanu Wattikun, P., Chanprapaph, S., Angsuthanasombat, C., Panyim, S., & Katzenmeier, G. (2004). Identification of residues in the dengue virus type 2 NS2B cofactor that are critical for NS3 protease activation. *J Virol*, 78(24), 13708-13716. doi:10.1128/JVI.78.24.13708-13716.2004
- Noisakran, S., Dechtawewat, T., Avirutnan, P., Kinoshita, T., Siripanyaphinyo, U., Puttikhunt, C., . . . Sittisombut, N. (2008). Association of dengue virus NS1 protein with lipid rafts. *J Gen Virol*, 89(Pt 10), 2492-2500. doi:10.1099/vir.0.83620-0
- OhAinle, M., Balmaseda, A., Macalalad, A. R., Tellez, Y., Zody, M. C., Saborio, S., . . . Harris, E. (2011). Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. *Sci Transl Med*, 3(114), 114ra128. doi:10.1126/scitranslmed.3003084
- Oliphant, T., Nybakken, G. E., Engle, M., Xu, Q., Nelson, C. A., Sukupolvi-Petty, S., . . . Diamond, M. S. (2006). Antibody recognition and neutralization determinants on domains I and II of West Nile Virus envelope protein. *J Virol*, 80(24), 12149-12159. doi:10.1128/JVI.01732-06
- Pantoja, P., Perez-Guzman, E. X., Rodriguez, I. V., White, L. J., Gonzalez, O., Serrano, C., . . . Sario, C. A. (2017). Zika virus pathogenesis in rhesus macaques is unaffected by pre-existing immunity to dengue virus. *Nat Commun*, 8, 15674. doi:10.1038/ncomms15674
- Parameswaran, P., Wang, C., Trivedi, S. B., Eswarappa, M., Montoya, M., Balmaseda, A., & Harris, E. (2017). Intrahost Selection Pressures Drive Rapid Dengue Virus Microevolution in Acute Human Infections. *Cell Host Microbe*, 22(3), 400-410 e405. doi:10.1016/j.chom.2017.08.003
- Pedroso, C., Fischer, C., Feldmann, M., Sarno, M., Luz, E., Moreira-Soto, A., . . . Drexler, J. F. (2019). Cross-Protection of Dengue Virus Infection against Congenital Zika Syndrome, Northeastern Brazil. *Emerg Infect Dis*, 25(8), 1485-1493. doi:10.3201/eid2508.190113



- Pierson, T. C., & Diamond, M. S. (2020). The continued threat of emerging flaviviruses. *Nature microbiology*, 5(6), 796-812. doi:10.1038/s41564-020-0714-0
- Pierson, T. C., Xu, Q., Nelson, S., Oliphant, T., Nybakken, G. E., Fremont, D. H., & Diamond, M. S. (2007). The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection. *Cell Host Microbe*, 1(2), 135-145. doi:10.1016/j.chom.2007.03.002
- Plummer, E., Buck, M. D., Sanchez, M., Greenbaum, J. A., Turner, J., Grewal, R., . . . Shrestha, S. (2015). Dengue Virus Evolution under a Host-Targeted Antiviral. *J Virol*, 89(10), 5592-5601. doi:10.1128/JVI.00028-15
- Prestwood, T. R., Morar, M. M., Zellweger, R. M., Miller, R., May, M. M., Yauch, L. E., . . . Shrestha, S. (2012). Gamma interferon (IFN-gamma) receptor restricts systemic dengue virus replication and prevents paralysis in IFN-alpha/beta receptor-deficient mice. *J Virol*, 86(23), 12561-12570. doi:10.1128/JVI.06743-11
- Ray, D., Shah, A., Tilgner, M., Guo, Y., Zhao, Y., Dong, H., . . . Shi, P. Y. (2006). West Nile virus 5'-cap structure is formed by sequential guanine N-7 and ribose 2'-O methylations by nonstructural protein 5. *J Virol*, 80(17), 8362-8370. doi:10.1128/JVI.00814-06
- Regla-Nava, J. A., Elong Ngono, A., Viramontes, K. M., Huynh, A. T., Wang, Y. T., Nguyen, A. T., . . . Shrestha, S. (2018). Cross-reactive Dengue virus-specific CD8(+) T cells protect against Zika virus during pregnancy. *Nat Commun*, 9(1), 3042. doi:10.1038/s41467-018-05458-0
- Reyes-Del Valle, J., Chavez-Salinas, S., Medina, F., & Del Angel, R. M. (2005). Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. *J Virol*, 79(8), 4557-4567. doi:10.1128/JVI.79.8.4557-4567.2005
- Reynolds, C. J., Watber, P., Santos, C. N. O., Ribeiro, D. R., Alves, J. C., Fonseca, A. B. L., . . . Altmann, D. M. (2020). Strong CD4 T Cell Responses to Zika Virus Antigens in a Cohort of Dengue Virus Immune Mothers of Congenital Zika Virus Syndrome Infants. *Front Immunol*, 11, 185. doi:10.3389/fimmu.2020.00185
- Rico-Hesse, R. (1990). Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology*, 174(2), 479-493. doi:10.1016/0042-6822(90)90102-w
- Rico-Hesse, R. (2003). Microevolution and virulence of dengue viruses. *Adv Virus Res*, 59, 315-341. doi:10.1016/s0065-3527(03)59009-1
- Rivino, L., Kumaran, E. A., Jovanovic, V., Nadua, K., Teo, E. W., Pang, S. W., . . . MacAry, P. A. (2013). Differential targeting of viral components by CD4+ versus CD8+ T lymphocytes in dengue virus infection. *J Virol*, 87(5), 2693-2706. doi:10.1128/JVI.02675-12

- Rivino, L., Kumaran, E. A., Thein, T. L., Too, C. T., Gan, V. C., Hanson, B. J., . . . MacAry, P. A. (2015). Virus-specific T lymphocytes home to the skin during natural dengue infection. *Sci Transl Med*, 7(278), 278ra235. doi:10.1126/scitranslmed.aaa0526
- Rodrigo, W. W., Jin, X., Blackley, S. D., Rose, R. C., & Schlesinger, J. J. (2006). Differential enhancement of dengue virus immune complex infectivity mediated by signaling-competent and signaling-incompetent human FcγRIIA (CD64) or FcγRIIA (CD32). *J Virol*, 80(20), 10128-10138. doi:10.1128/JVI.00792-06
- Rodriguez-Barraquer, I., Costa, F., Nascimento, E. J. M., Nery, N. J., Castanha, P. M. S., Sacramento, G. A., . . . Ko, A. I. (2019). Impact of preexisting dengue immunity on Zika virus emergence in a dengue endemic region. *Science*, 363(6427), 607-610. doi:10.1126/science.aav6618
- Rothman, A. L. (2011). Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat Rev Immunol*, 11(8), 532-543. doi:10.1038/nri3014
- Saito, Y., Moi, M. L., Takeshita, N., Lim, C. K., Shiba, H., Hosono, K., . . . Takasaki, T. (2016). Japanese encephalitis vaccine-facilitated dengue virus infection-enhancement antibody in adults. *BMC Infect Dis*, 16(1), 578. doi:10.1186/s12879-016-1873-8
- Sakoonwatanyoo, P., Boonsanay, V., & Smith, D. R. (2006). Growth and production of the dengue virus in C6/36 cells and identification of a laminin-binding protein as a candidate serotype 3 and 4 receptor protein. *Intervirology*, 49(3), 161-172. doi:10.1159/000089377
- Saron, W. A. A., Rathore, A. P. S., Ting, L., Ooi, E. E., Low, J., Abraham, S. N., & St John, A. L. (2018). Flavivirus serocomplex cross-reactive immunity is protective by activating heterologous memory CD4 T cells. *Sci Adv*, 4(7), eaar4297. doi:10.1126/sciadv.aar4297
- Sato, R., Hamada, N., Kashiwagi, T., Imamura, Y., Hara, K., Nishimura, M., . . . Koga, T. (2015). Dengue Hemorrhagic Fever in a Japanese Traveler with Pre-existing Japanese Encephalitis Virus Antibody. *Trop Med Health*, 43(2), 85-88. doi:10.2149/tmh.2014-34
- Serrano-Collazo, C., Perez-Guzman, E. X., Pantoja, P., Hassert, M. A., Rodriguez, I. V., Giavedoni, L., . . . Sariol, C. A. (2020). Effective control of early Zika virus replication by Dengue immunity is associated to the length of time between the 2 infections but not mediated by antibodies. *PLoS Negl Trop Dis*, 14(5), e0008285. doi:10.1371/journal.pntd.0008285
- Sessions, O. M., Wilm, A., Kamaraj, U. S., Choy, M. M., Chow, A., Chong, Y., . . . Ooi, E. E. (2015). Analysis of Dengue Virus Genetic Diversity during Human and Mosquito

- Infection Reveals Genetic Constraints. *PLoS Negl Trop Dis*, 9(9), e0004044. doi:10.1371/journal.pntd.0004044
- Sim, S., Aw, P. P., Wilm, A., Teoh, G., Hue, K. D., Nguyen, N. M., . . . Hibberd, M. L. (2015). Tracking Dengue Virus Intra-host Genetic Diversity during Human-to-Mosquito Transmission. *PLoS Negl Trop Dis*, 9(9), e0004052. doi:10.1371/journal.pntd.0004052
- Simon-Loriere, E., Duong, V., Tawfik, A., Ung, S., Ly, S., Casademont, I., . . . Sakuntabhai, A. (2017). Increased adaptive immune responses and proper feedback regulation protect against clinical dengue. *Sci Transl Med*, 9(405). doi:10.1126/scitranslmed.aal5088
- Smit, J. M., Moesker, B., Rodenhuis-Zybert, I., & Wilschut, J. (2011). Flavivirus cell entry and membrane fusion. *Viruses*, 3(2), 160-171. doi:10.3390/v3020160
- St John, A. L., & Rathore, A. P. S. (2019). Adaptive immune responses to primary and secondary dengue virus infections. *Nat Rev Immunol*, 19(4), 218-230. doi:10.1038/s41577-019-0123-x
- Stettler, K., Beltramello, M., Espinosa, D. A., Graham, V., Cassotta, A., Bianchi, S., . . . Corti, D. (2016). Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. *Science*, 353(6301), 823-826. doi:10.1126/science.aaf8505
- Stiasny, K., & Heinz, F. X. (2006). Flavivirus membrane fusion. *J Gen Virol*, 87(Pt 10), 2755-2766. doi:10.1099/vir.0.82210-0
- Swanstrom, J. A., Plante, J. A., Plante, K. S., Young, E. F., McGowan, E., Gallichotte, E. N., . . . Baric, R. S. (2016). Dengue Virus Envelope Dimer Epitope Monoclonal Antibodies Isolated from Dengue Patients Are Protective against Zika Virus. *mBio*, 7(4). doi:10.1128/mBio.01123-16
- Tang, T. H., Alonso, S., Ng, L. F., Thein, T. L., Pang, V. J., Leo, Y. S., . . . Yeo, T. W. (2017). Increased Serum Hyaluronic Acid and Heparan Sulfate in Dengue Fever: Association with Plasma Leakage and Disease Severity. *Sci Rep*, 7, 46191. doi:10.1038/srep46191
- Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., . . . Marovich, M. A. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med*, 197(7), 823-829. doi:10.1084/jem.20021840
- Thepparit, C., & Smith, D. R. (2004). Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. *J Virol*, 78(22), 12647-12656. doi:10.1128/JVI.78.22.12647-12656.2004
- Tsai, W. Y., Durbin, A., Tsai, J. J., Hsieh, S. C., Whitehead, S., & Wang, W. K. (2015). Complexity of Neutralizing Antibodies against Multiple Dengue Virus Serotypes

- after Heterotypic Immunization and Secondary Infection Revealed by In-Depth Analysis of Cross-Reactive Antibodies. *J Virol*, 89(14), 7348-7362.  
doi:10.1128/JVI.00273-15
- Tsai, W. Y., Lai, C. Y., Wu, Y. C., Lin, H. E., Edwards, C., Jumnainsong, A., . . . Wang, W. K. (2013). High-avidity and potentially neutralizing cross-reactive human monoclonal antibodies derived from secondary dengue virus infection. *J Virol*, 87(23), 12562-12575. doi:10.1128/JVI.00871-13
- Ubol, S., Chareonsirisuthigul, T., Kasisith, J., & Klungthong, C. (2008). Clinical isolates of dengue virus with distinctive susceptibility to nitric oxide radical induce differential gene responses in THP-1 cells. *Virology*, 376(2), 290-296. doi:10.1016/j.virol.2008.03.030
- Verma, A., Jain, A., Kumar, C., Agarwal, M., & Kumar, R. (2018). Effect of prior dengue infection on severity and outcome of Japanese encephalitis. *Eur J Clin Microbiol Infect Dis*, 37(3), 519-525. doi:10.1007/s10096-018-3207-9
- Wahala, W. M., & Silva, A. M. (2011). The human antibody response to dengue virus infection. *Viruses*, 3(12), 2374-2395. doi:10.3390/v3122374
- Wang, T. T., Sewatanon, J., Memoli, M. J., Wrammert, J., Bournazos, S., Bhaumik, S. K., . . . Ravetch, J. V. (2017). IgG antibodies to dengue enhanced for FcγRIIIA binding determine disease severity. *Science*, 355(6323), 395-398. doi:10.1126/science.aai8128
- Wang, W. H., Urbina, A. N., Wu, C. C., Lin, C. Y., Thitithanyanont, A., Assavalapsakul, W., . . . Wang, S. F. (2020). An epidemiological survey of the current status of Zika and the immune interaction between dengue and Zika infection in Southern Taiwan. *Int J Infect Dis*, 93, 151-159. doi:10.1016/j.ijid.2020.01.031
- Watanabe, S., Tan, N. W. W., Chan, K. W. K., & Vasudevan, S. G. (2019). Dengue Virus and Zika Virus Serological Cross-reactivity and Their Impact on Pathogenesis in Mice. *J Infect Dis*, 219(2), 223-233. doi:10.1093/infdis/jiy482
- Weaver, S. C., & Vasilakis, N. (2009). Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. *Infect Genet Evol*, 9(4), 523-540. doi:10.1016/j.meegid.2009.02.003
- Weiskopf, D., Angelo, M. A., Bangs, D. J., Sidney, J., Paul, S., Peters, B., . . . Sette, A. (2015). The human CD8<sup>+</sup> T cell responses induced by a live attenuated tetravalent dengue vaccine are directed against highly conserved epitopes. *J Virol*, 89(1), 120-128. doi:10.1128/JVI.02129-14
- Weiskopf, D., Angelo, M. A., de Azeredo, E. L., Sidney, J., Greenbaum, J. A., Fernando, A. N., . . . Sette, A. (2013). Comprehensive analysis of dengue virus-specific



- responses supports an HLA-linked protective role for CD8+ T cells. *Proc Natl Acad Sci U S A*, 110(22), E2046-2053. doi:10.1073/pnas.1305227110
- Weiskopf, D., Angelo, M. A., Sidney, J., Peters, B., Shrestha, S., & Sette, A. (2014). Immunodominance changes as a function of the infecting dengue virus serotype and primary versus secondary infection. *J Virol*, 88(19), 11383-11394. doi:10.1128/JVI.01108-14
- Weiskopf, D., Bangs, D. J., Sidney, J., Kolla, R. V., De Silva, A. D., de Silva, A. M., . . . Sette, A. (2015). Dengue virus infection elicits highly polarized CX3CR1+ cytotoxic CD4+ T cells associated with protective immunity. *Proc Natl Acad Sci U S A*, 112(31), E4256-4263. doi:10.1073/pnas.1505956112
- Weiskopf, D., Cerpas, C., Angelo, M. A., Bangs, D. J., Sidney, J., Paul, S., . . . Sette, A. (2015). Human CD8+ T-Cell Responses Against the 4 Dengue Virus Serotypes Are Associated With Distinct Patterns of Protein Targets. *J Infect Dis*, 212(11), 1743-1751. doi:10.1093/infdis/jiv289
- Wen, J., Elong Ngono, A., Regla-Nava, J. A., Kim, K., Gorman, M. J., Diamond, M. S., & Shrestha, S. (2017). Dengue virus-reactive CD8(+) T cells mediate cross-protection against subsequent Zika virus challenge. *Nat Commun*, 8(1), 1459. doi:10.1038/s41467-017-01669-z
- Wen, J., & Shrestha, S. (2019). Antigenic cross-reactivity between Zika and dengue viruses: is it time to develop a universal vaccine? *Curr Opin Immunol*, 59, 1-8. doi:10.1016/j.coi.2019.02.001
- Wen, J., Wang, Y. T., Valentine, K. M., Dos Santos Alves, R. P., Xu, Z., Regla-Nava, J. A., . . . Shrestha, S. (2020). CD4(+) T Cells Cross-Reactive with Dengue and Zika Viruses Protect against Zika Virus Infection. *Cell Rep*, 31(4), 107566. doi:10.1016/j.celrep.2020.107566
- WHO. (2009). Dengue and dengue haemorrhagic fever Factsheet No 117. Retrieved from Geneva, Switzerland:
- Wichit, S., Jittmittraphap, A., Hidari, K. I., Thaisomboonsuk, B., Petmitr, S., Ubol, S., . . . Jampangern, W. (2011). Dengue virus type 2 recognizes the carbohydrate moiety of neutral glycosphingolipids in mammalian and mosquito cells. *Microbiol Immunol*, 55(2), 135-140. doi:10.1111/j.1348-0421.2010.00293.x
- Wijeratne, D. T., Fernando, S., Gomes, L., Jeewandara, C., Jayarathna, G., Perera, Y., . . . Malavige, G. N. (2019). Association of dengue virus-specific polyfunctional T-cell responses with clinical disease severity in acute dengue infection. *Immun Inflamm Dis*, 7(4), 276-285. doi:10.1002/iid3.271

- Wu, S. J., Grouard-Vogel, G., Sun, W., Mascola, J. R., Brachtel, E., Putvatana, R., . . . Frankel, S. S. (2000). Human skin Langerhans cells are targets of dengue virus infection. *Nat Med*, 6(7), 816-820. doi:10.1038/77553
- Xie, X., Gayen, S., Kang, C., Yuan, Z., & Shi, P. Y. (2013). Membrane topology and function of dengue virus NS2A protein. *J Virol*, 87(8), 4609-4622. doi:10.1128/JVI.02424-12
- Xie, X., Zou, J., Puttikhunt, C., Yuan, Z., & Shi, P. Y. (2015). Two distinct sets of NS2A molecules are responsible for dengue virus RNA synthesis and virion assembly. *J Virol*, 89(2), 1298-1313. doi:10.1128/JVI.02882-14
- Yamanaka, A., Kosugi, S., & Konishi, E. (2008). Infection-enhancing and -neutralizing activities of mouse monoclonal antibodies against dengue type 2 and 4 viruses are controlled by complement levels. *J Virol*, 82(2), 927-937. doi:10.1128/JVI.00992-07
- Yauch, L. E., Prestwood, T. R., May, M. M., Morar, M. M., Zellweger, R. M., Peters, B., . . . Shresta, S. (2010). CD4+ T cells are not required for the induction of dengue virus-specific CD8+ T cell or antibody responses but contribute to protection after vaccination. *J Immunol*, 185(9), 5405-5416. doi:10.4049/jimmunol.1001709
- Yauch, L. E., Zellweger, R. M., Kotturi, M. F., Qutubuddin, A., Sidney, J., Peters, B., . . . Shresta, S. (2009). A protective role for dengue virus-specific CD8+ T cells. *J Immunol*, 182(8), 4865-4873. doi:10.4049/jimmunol.0801974
- Zellweger, R. M., Eddy, W. E., Tang, W. W., Miller, R., & Shresta, S. (2014). CD8+ T cells prevent antigen-induced antibody-dependent enhancement of dengue disease in mice. *J Immunol*, 193(8), 4117-4124. doi:10.4049/jimmunol.1401597
- Zellweger, R. M., Miller, R., Eddy, W. E., White, L. J., Johnston, R. E., & Shresta, S. (2013). Role of humoral versus cellular responses induced by a protective dengue vaccine candidate. *PLoS Pathog*, 9(10), e1003723. doi:10.1371/journal.ppat.1003723
- Zellweger, R. M., Tang, W. W., Eddy, W. E., King, K., Sanchez, M. C., & Shresta, S. (2015). CD8+ T Cells Can Mediate Short-Term Protection against Heterotypic Dengue Virus Reinfection in Mice. *J Virol*, 89(12), 6494-6505. doi:10.1128/JVI.00036-15
- Zompi, S., Santich, B. H., Beatty, P. R., & Harris, E. (2012). Protection from secondary dengue virus infection in a mouse model reveals the role of serotype cross-reactive B and T cells. *J Immunol*, 188(1), 404-416. doi:10.4049/jimmunol.1102124

# CHAPTER III

## GENOMIC SEQUENCING AND NEUTRALIZING SEROLOGICAL PROFILES DURING ACUTE DENGUE INFECTION: A 2017 COHORT STUDY IN NEPAL

### 3.1 Abstract

Dengue virus (DENV) is a mosquito-borne flavivirus that poses a threat to nearly 50% of the global population. DENV has been endemic in Nepal since 2006; however, little is known about how DENV is evolving or the prevalence of anti-DENV immunity within the Nepalese population. To begin to address these gaps, we performed a serologic and genetic study of 49 patients from across Nepal who presented at central hospitals during the 2017 dengue season with suspected DENV infection. Of the 49 subjects assessed, 21 (43%) were positive for DENV NS1 antigen; of these; 5 were also anti-DENV IgM<sup>+</sup> IgG<sup>+</sup>; 7 were DENV IgM<sup>+</sup> IgG<sup>-</sup>, 2 were IgM<sup>-</sup> IgG<sup>+</sup>, and 7 were IgM<sup>-</sup> IgG<sup>-</sup> by specific ELISAs. Seven of the 21 NS1 positive sera were RNA positive by RT-PCR (six DENV2, one DENV3), suggesting that DENV2 was the dominant serotype in our cohort. Whole-genome sequencing of two DENV2 isolates showed similarity with strains circulating in Singapore in 2016, and the envelope genes were also similar to strains circulating in India in 2017. DENV-neutralizing antibodies (nAbs) were present in 31 of 47 sera tested (66%); among these, 20, 24, 26, and 12 sera contained nAbs against DENV1, 2, 3, and 4 serotypes, respectively. Additionally, 27 (58%) samples had nAbs against multiple serotypes (2 or more). Serology analysis suggested that 12 (26%) and 19 (40%) of the 47 subjects were experiencing primary and secondary DENV infections, respectively. Collectively, our results provided evidence for current and/or past exposure to multiple DENV serotypes in our cohort. These data suggest that expanded local surveillance of circulating DENV genotypes and population immunity will be important to effectively manage and mitigate future dengue outbreaks in Nepal.

### 3.2 Introduction

Dengue virus (DENV) is a positive-sense single-stranded RNA virus of the *Flavivirus* genus, which also includes Japanese encephalitis, Zika, yellow fever, and West Nile viruses (Guzman & Harris, 2015; T. C. Pierson & M. S. Diamond, 2020), all of which are predominantly transmitted by infected *Aedes* spp. mosquitoes (Bhatt et al., 2013; M. WHO, 2019). The four antigenically distinct serotypes of DENV (DENV1–4) are responsible for about 400 million reported DENV infections per year (Bhatt et al., 2013; M. WHO, 2019), which may be asymptomatic or have symptoms ranging from self-limiting dengue fever to severe disease characterized by hemorrhagic fever, shock and, in some cases, death (Guzman & Harris, 2015).

DENV infection provides long lasting immunity against the same serotype but provides only limited and short-lived protection against different serotypes. In some instances, a secondary infection with a heterologous serotype can trigger severe dengue due to antibody-dependent enhancement (Halstead, 2014; Katzelnick, Bos, & Harris, 2020; Ngono & Shrestha, 2018). As of now, there are no DENV vaccines that offer reliable, long-term protection against all four DENV serotypes for individuals of all ages, regardless of their prior exposure to the virus. The first licensed dengue vaccine, DengVaxia, is recommended only for individuals with a history of natural DENV infection (Sridhar et al., 2018), as it has been shown to increase the risk of severe dengue in DENV-naïve individuals who are then exposed naturally after vaccination. A more recent vaccine, QDenga (Takeda) has been approved for use in both DENV-naïve and previously exposed individuals in countries such as Indonesia, Thailand, Argentina, Brazil, and several European countries, however it provides robust long-term protection against only selected DENV serotypes (Aguar & Stollenwerk, 2020; Patel, Rauscher, Kudela, & Pang, 2023; Thomas, 2023). A phase 3 trial of the tetravalent vaccine Butantan-DV (developed by Instituto Butantan/NIAID) showed strong protection against DENV1, but was only moderately efficacy against DENV2 (Kallas et al., 2024). These findings emphasize the ongoing difficulty in creating a dengue vaccine that offers broad and effective immunity against all four serotypes, regardless of an individual's previous exposure. Additionally, due to the potential risks associated with vaccination, it is essential to have up-to-date data on the circulating DENV serotypes and the population's immune profile. This information is crucial for governments to make well-



informed decisions when selecting the most appropriate vaccine for deployment each year (OhAinle et al., 2011).

In Nepal, the first documented DENV infection occurred in 2004 in a Japanese traveler, from whom DENV2 was isolated (Takasaki et al., 2008). By 2006, all 4 DENV serotypes had been identified in the country (Malla et al., 2008). Since then, dengue cases have appeared sporadically during the annual dengue season (September to December), with major outbreaks roughly every 3 years starting in 2010 (Rijal et al., 2021). Each successive outbreak has shown increases in the number of cases, geographic spread, severity, and death toll (EDCD, Services, & Division, 2022; Organization, 2022; Rimal et al., 2023). In 2017, Nepal recorded 2,111 cases from 28 out of 77 districts, while in 2019, the number surged to 17,992 cases and 6 deaths across 68 districts, a 140-fold rise in incidence over two years (Adhikari & Subedi, 2020; Poudyal et al., 2021; Rijal et al., 2021), representing a 140-fold increase in incidence in just 2 years (Rijal et al., 2021). This upward trend continued in the 2022 outbreak, which saw 54,784 reported cases and 88 deaths, affecting all 77 districts (EDCD et al., 2022). These patterns suggest that Nepal may face another significant dengue outbreak in 2025.

To date, limited research has been conducted on the virology and immunology of DENV within the Nepalese population. This is largely due to Nepal being a low-income country with limited scientific infrastructure. For instance, since the first confirmed DENV case in 2006, only a small number of complete genome sequences from circulating strains have been obtained (Manandhar et al., 2021). The present study was conducted as part of a long-term process of enabling local scientists/clinicians to effectively surveil the circulation of, and population exposure to, DENV serotypes in Nepal. To this end, we describe here serological and genomic analyses of sera from 49 residents of districts across Nepal who presented with suspected dengue at hospitals in Kathmandu and Chitwan, two geographically and climatically distinct districts. All sera were analyzed for DENV-NS1 antigen, DENV-specific anti-IgM/IgG, and neutralizing antibody (nAb) response against all four DENV serotypes. A subset of NS1 positive sera were further serotyped by RT-PCR and subjected to whole-genome sequencing. The knowledge gained from this study has already facilitated the expansion of DENV genomic surveillance studies by local scientists/clinicians with the goal of mitigating the dengue outbreak predicted to occur in 2025.

### **3.3 Material and Methods**

#### **3.3.1 Ethics statement**

Ethical approval was obtained from the Nepal Health Research Council (Reg. no. 378/2016). Patients were provided with a verbal explanation about the study, its potential significance, the tests to be performed on their samples, including genetic testing, and potential risks associated with drawing blood. The adult patient (or parent/guardian for patients under 18 years of age) then provided written informed consent to be enrolled in the study.

#### **3.3.2 Sample collection**

The study cohort consisted of 49 febrile patients who presented with suspected dengue at Sukraraj Tropical and Infectious Disease Hospital in Kathmandu and at Chitwan Medical College and Teaching Hospital in Chitwan during the 2017 dengue outbreak (September 2017 through January 2018). A diagnosis of suspected dengue was made by attending physicians. Venous blood samples (5 mL) were collected into EDTA tubes and demographic and clinicopathological information was recorded. All steps were performed by the Nepal-based authors and teams, with advice from the authors based in the US, Japan, and Thailand.

#### **3.3.3 Enzyme-linked immunosorbent assays**

Plasma was isolated from the blood samples and stored at  $-80^{\circ}\text{C}$  until analyzed. Levels of DENV NS1 antigen, anti-DENV IgM, and anti-DENV IgG were quantified using commercial ELISA kits (InBios: DNS1-R, DDMS-1 and DDGS-R respectively). Assay procedures, calculation of immune status ratios, and classification as seronegative or seropositive were all performed according to the manufacturer's instructions.

#### **3.3.4 DENV serotyping by RT-PCR**

Viral RNA was isolated from plasma samples using a QIAamp Viral RNA Mini kit (Qiagen, 52906). DENV1–4 serotypes were identified by multiplex RT-PCR as described previously (Prajapati et al., 2020; Santiago et al., 2013; Waggoner et al., 2013) using US Centers for Disease Control and Prevention real-time RT-PCR assay kits (KK0128).

#### **3.3.5 Whole genome DENV sequencing**

All procedures were conducted according to the kit manufacturer's recommendations. Illumina libraries were constructed from total RNA using the NEB

Next Ultra Directional RNA Library Prep Kit (New England Biolabs, E7760) per the manufacturer's instructions. Libraries of 400–600 nucleotides were obtained using Mag-Bind RxnPure Plus beads (Omega Bio-Tek, M1386-01), purified with the MinElute PCR Purification Kit (Qiagen, 28004), and quantified using a Bioanalyzer High-Sensitivity DNA Assay (Agilent Technologies, 5067-4626). Targeted DENV genome enrichment was achieved using custom-designed biotinylated 120-mer xGen Lockdown baits (Integrated DNA Technologies) with complementarity to DENV1–4 serotypes, as previously described (Kamaraj et al., 2019), following manufacturer's instructions. The genome assembly was done using the VIPR4 pipeline (<https://github.com/nf-core/vipr/>).

### 3.3.6 Phylogenetic analysis

Multiple sequence alignment of DENV2 full-genome sequences from the present study (n=2) and the National Center for Biotechnology Information (NCBI) (n=1777) was carried out using a fast Fourier transformation method in MAFFT v7.490. An approximately maximum likelihood phylogenetic tree was generated using a generalized time-reversible model of nucleotide evolution in FastTree v2.1.11, which uses SH-like local supports with 1000 resamples to estimate and validate the reliability of each split in the tree. The branch containing the 2 sequences from the 2017 Nepal outbreak and 29 complete genome sequences from the NCBI were selected, and a more robust maximum likelihood phylogenetic tree created using RAXML v8.2.11 and the GTR GAMMA model with 1000 bootstrap replications. Trees were visualized using FigTree v1.4.4.

### 3.3.7 Flow cytometry-based DENV1-4 neutralization assay

Neutralization assays were conducted as previously described (de Alwis & de Silva, 2014), utilizing a flow cytometry method based on U937 DC-SIGN cells. Plasma samples were serially diluted 5-fold starting from 1:40 and incubated with pre-titrated DENV1–4 serotypes (WHO reference strains) for 1 hour at 37°C. The plasma-virus mixtures were then added to U937 DC-SIGN cells and incubated for 2 hours at 37°C, followed by washing and a second incubation for 16 hours. Control samples included cells exposed to virus without plasma to determine baseline infection. After incubation, cells were stained on the surface with anti-CD209-PE antibody (DC-SIGN marker) and internally with anti-FITC-labeled 4G2 antibody targeting the pan-flavivirus

envelope (E) protein. Data were acquired using a FACSCanto LSR flow cytometer and analyzed using FlowJo V9 software (BD Biosciences).

Samples were considered to have neutralizing activity if they showed  $\geq 90\%$  reduction in infection at a plasma dilution greater than 1:40 (cutoff =  $NT_{90} > 40$ ).  $NT_{90}$  values were further used to distinguish between primary infection (first infection with a single serotype) and secondary infection (prior infections with other serotypes), as previously described (S. Hattakam et al., 2021). Briefly, primary infection was defined as (i) an  $NT_{90} \geq 40$  against a single DENV serotype and  $< 40$  against all other serotypes, or (ii) a response against a single DENV serotype, with an  $NT_{90}$  4-fold higher than against the other serotypes. Secondary infection was defined as (i) an  $NT_{90} \geq 40$  against at least two DENV serotypes, and/or (ii) a response with an  $NT_{90} \leq 4$ -fold higher than the next highest response (S. Hattakam et al., 2021).

### 3.4 Results

#### 3.4.1 Patient demographics and disease association with platelet count

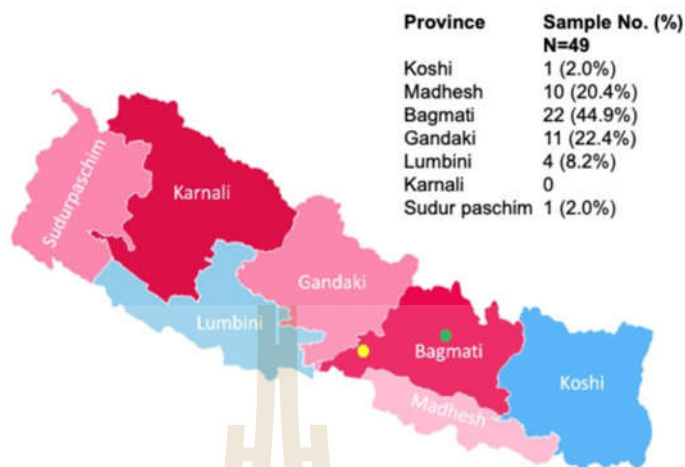
The study cohort consisted of 49 patients who presented with suspected dengue at two major hospitals in Kathmandu and Chitwan, Nepal during the 2017 dengue season (September 2017 to January 2018) (Nepal, 2012/2013 and 2013/2014). The majority of the subjects were male (71%, 35/49) and were aged 20 to 59 years (63%, 31/49), with a median age of 34 years (range 13–81) (Table 3.1). Although the 49 subjects presented to the two major hospitals in Kathmandu and Chitwan, they originated from 6 of Nepal's 7 provinces (Figure 3.1).

**Table 3.1** Characteristics of study participants

| Age range (years) | Male (N=35) | Female (N=14) | Total (N=49) |
|-------------------|-------------|---------------|--------------|
| 13–19             | 8           | 3             | 11           |
| 20–59             | 24          | 7             | 31           |
| 60–81             | 3           | 4             | 7            |

Abbreviation: N, number.





**Figure 3.1** Geographic distribution of the home residences of the study subjects.

Patients originated from across Nepal and were seen at hospitals in Kathmandu (green dot) and Chitwan (yellow dot). White borders demarcate the seven provinces in Nepal. Abbreviation: No, number. Image modified from Wikipedia ([https://en.m.wikipedia.org/wiki/File:Provinces\\_of\\_Nepal.png](https://en.m.wikipedia.org/wiki/File:Provinces_of_Nepal.png)).

All patients were symptomatic at the time of sample collection, although the time between symptom onset and blood collection was not recorded. Thrombocytopenia is a potential indicator of dengue disease severity (Jayashree, Manasa, Pallavi, & Manjunath, 2011), and platelet counts of  $41\text{--}100 \times 10^3/\mu\text{L}$ ,  $21\text{--}40 \times 10^3/\mu\text{L}$ , and  $\leq 20 \times 10^3/\mu\text{L}$  are considered to reflect low, moderate, and high risk, respectively, of bleeding associated with severe dengue (normal platelet counts:  $100\text{--}450 \times 10^3/\mu\text{L}$ ) (Ojha et al., 2017). Most samples (31/48) had normal platelet counts, with 13, 2, and 2 samples having counts within the low-, moderate-, and high-risk categories, respectively (Table 3.2). These results are consistent with the majority of our study cohort having mild dengue disease.

**Table 3.2** Platelet counts and DENV serology, serotyping, neutralization titers, and infection status

| Patient ID | Location  | Platelets<br>( $\times 10^3/\mu\text{L}$ ) <sup>a</sup> | DENV<br>NS1 | Anti-DENV |     | DENV<br>Serotype <sup>c</sup> | NT90 titer <sup>d</sup> |        |         |       | DENV<br>infection<br>status |
|------------|-----------|---------------------------------------------------------|-------------|-----------|-----|-------------------------------|-------------------------|--------|---------|-------|-----------------------------|
|            |           |                                                         |             | IgM       | IgG |                               | DENV1                   | DENV2  | DENV3   | DENV4 |                             |
| 1          | Kathmandu | 101                                                     | +           | +         | +   | –                             | <40                     | 1708   | 1735    | <40   | Sec                         |
| 2          |           | 128                                                     | +           | +         | –   | –                             | 42                      | 1010   | 99      | 89    | Prim                        |
| 3          |           | 94                                                      | +           | +         | –   | DENV2                         | <40                     | 82     | <40     | <40   | Prim                        |
| 4          |           | 20                                                      | –           | +         | +   | NE                            | 4070                    | 51,382 | 140,960 | <40   | Sec                         |
| 5          |           | 80                                                      | –           | Equ       | +   | NE                            | 120,158                 | 46,411 | 158,838 | <40   | Sec                         |
| 6          |           | 76                                                      | +           | +         | –   | DENV2                         | 245                     | 16,286 | 23,817  | <40   | Sec                         |
| 7          |           | 196                                                     | +           | –         | Equ | DENV3                         | <40                     | <40    | <40     | <40   | –                           |
| 8          |           | 111                                                     | +           | +         | +   | –                             | <40                     | 2523   | <40     | <40   | Prim                        |
| 9          |           | 53                                                      | +           | –         | –   | DENV2                         | <40                     | <40    | <40     | <40   | –                           |
| 10         |           | 210                                                     | +           | –         | –   | –                             | <40                     | <40    | <40     | 125   | Prim                        |
| 11         |           | 143                                                     | –           | –         | +   | NE                            | <40                     | 68     | 54      | 52    | Sec                         |
| 12         |           | 66                                                      | +           | +         | –   | –                             | 56                      | 67     | 721     | <40   | Prim                        |
| 13         |           | 61                                                      | +           | +         | –   | –                             | <40                     | <40    | <40     | <40   | –                           |
| 14         |           | 76                                                      | –           | Equ       | +   | NE                            | 16,887                  | 19,645 | 9985    | 352   | Sec                         |
| 15         |           | 69                                                      | +           | +         | +   | –                             | 50,072                  | 18,730 | 14,242  | 722   | Sec                         |
| 16         |           | 136                                                     | +           | –         | +   | DENV2                         | 202                     | 12,665 | 226     | 200   | Sec                         |

|    |         |     |   |   |   |       |        |      |      |     |      |
|----|---------|-----|---|---|---|-------|--------|------|------|-----|------|
| 17 |         | 365 | – | + | + | NE    | 57     | 238  | 1251 | <40 | Prim |
| 18 |         | 244 | – | – | – | NE    | <40    | <40  | <40  | <40 | –    |
| 19 |         | 51  | + | – | – | –     | <40    | <40  | <40  | <40 | –    |
| 20 |         | 91  | + | – | + | –     | 14,584 | 7496 | <40  | 914 | Sec  |
| 21 |         | 97  | + | + | – | –     | 45,639 | 384  | <40  | 87  | Prim |
| 22 |         | 21  | + | + | + | –     | NA     | NA   | NA   | NA  | NA   |
| 23 |         | 50  | + | + | + | DENV2 | NA     | NA   | NA   | NA  | NA   |
| 24 |         | 159 | + | – | – | DENV2 | <40    | <40  | <40  | <40 | –    |
| 25 |         | 124 | – | – | + | NE    | 94     | <40  | 60   | 102 | Sec  |
| 26 |         | 95  | + | + | – | –     | <40    | 3190 | 108  | 118 | Prim |
| 27 |         | 165 | + | – | – | –     | <40    | 74   | 308  | <40 | Prim |
| 28 |         | 206 | – | – | + | NE    | <40    | 58   | 75   | <40 | Sec  |
| 29 | Chitwan | 194 | – | – | – | NE    | <40    | <40  | <40  | <40 | –    |
| 30 |         | 121 | – | – | – | NE    | 49     | <40  | 55   | <40 | Sec  |
| 31 |         | 113 | – | – | – | NE    | <40    | <40  | <40  | <40 | –    |
| 32 |         | 231 | – | – | + | NE    | <40    | 45   | 73   | <40 | Sec  |
| 33 |         | 189 | + | – | – | –     | <40    | <40  | <40  | <40 | –    |
| 34 |         | N/D | – | – | – | NE    | <40    | <40  | <40  | <40 | –    |
| 35 |         | 106 | – | – | – | NE    | <40    | <40  | <40  | <40 | –    |
| 36 |         | 189 | – | – | – | NE    | 65     | <40  | 44   | <40 | Sec  |

|    |     |   |   |   |    |      |     |     |     |      |
|----|-----|---|---|---|----|------|-----|-----|-----|------|
| 37 | 357 | – | – | – | NE | <40  | <40 | <40 | <40 | –    |
| 38 | 120 | – | – | + | NE | <40  | <40 | 68  | <40 | Prim |
| 39 | 135 | – | – | – | NE | <40  | <40 | <40 | <40 | –    |
| 40 | 100 | – | – | – | NE | 60   | <40 | 53  | <40 | Sec  |
| 41 | 30  | – | – | + | NE | <40  | 118 | 77  | 143 | Sec  |
| 42 | 245 | – | – | – | NE | 46   | <40 | 49  | <40 | Sec  |
| 43 | 130 | – | – | – | NE | <40  | <40 | <40 | <40 | –    |
| 44 | 107 | – | – | + | NE | 356  | 128 | 81  | <40 | Sec  |
| 45 | 108 | – | – | + | NE | 137  | 46  | 84  | <40 | Sec  |
| 46 | 205 | – | – | – | NE | <40  | <40 | <40 | <40 | –    |
| 47 | 140 | – | – | + | NE | 1245 | 318 | 132 | <40 | Prim |
| 48 | 176 | – | – | – | NE | <40  | <40 | <40 | <40 | –    |
| 49 | 15  | – | – | + | NE | 89   | 193 | 940 | 74  | Prim |

Abbreviations: Equ, equivocal (inconclusive after 2 tests); NA, not available (insufficient plasma); NE, not evaluated (only NS1+ samples were evaluated by RT-PCR); NT<sub>90</sub>, 90% neutralization titer; Prim, primary; Sec, secondary.

<sup>a</sup>Platelet counts ( $\times 10^3/\mu\text{L}$ ) and dengue risk: low, 41–100; moderate, 21–40; high, <20 (normal, 100–450  $\times 10^3/\mu\text{L}$ ).

<sup>b</sup> By ELISA

<sup>c</sup> By serotype-specific RT-PCR

<sup>d</sup> Lowest dilution (1:40) was the designated cut-off value for positivity.

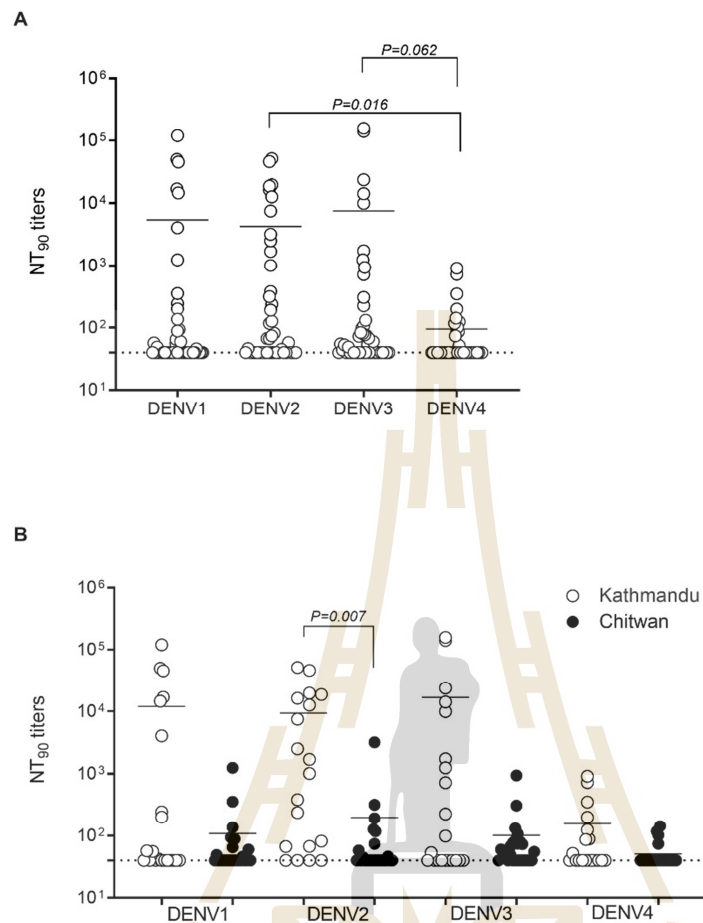


### 3.4.2 DENV serostatus and serotypes

NS1 antigen was detected in 43% (21/49) of samples, anti-DENV IgM Ab in 29% (14/49) and anti-DENV IgG Ab in 43% (21/49). Thus, 47% (23/49) of our cohort were considered to have active dengue at the time of sample collection based on the presence of  $\geq 1$  of the following 5 criteria: NS1<sup>+</sup> (14%, n=7), NS1<sup>+</sup>IgM<sup>+</sup> (14%, n=7), NS1<sup>+</sup>IgG<sup>+</sup> (4%, n=2), NS1<sup>+</sup>IgM<sup>+</sup>IgG<sup>+</sup> (10%, n=5) and IgM<sup>+</sup> IgG<sup>+</sup> (4%, n=2). Evidence of previous dengue infections were observed in subjects with IgG alone (24%, n=12) (Table 2). Samples from 29% (14/49) of subjects showed no evidence of current or prior infection (NS1<sup>-</sup>, IgM<sup>-</sup>, and IgG<sup>-</sup>). The 21 NS1 positive sera were further analyzed for DENV serotype by RT-PCR, 7 (33%) were found to be positive for DENV RNA, with 6 samples serotyped as DENV2 and 1 as DENV3 (Table 3.2). Thus, despite the small number of RT-PCR RNA-positive samples, DENV2 appears to have been the dominant circulating serotype in our 49-subject cohort at the time of the 2017 outbreak.

### 3.4.3 Neutralizing Ab response against DENV1-4 serotypes

Of the 49 samples collected, 47 were evaluated in cell-based neutralizing assays (Figure 3.2A). The mean NT<sub>90</sub> titers for DENV1, DENV2, and DENV3 were similar, whereas the mean NT<sub>90</sub> titer for DENV4 was much lower overall, and significantly lower when compared with DENV2 (Figure 3.2A). Anti-DENV4 nAbs were undetectable in 35 samples (74%) (Figure 3.2A). Strikingly, 3 of the 7 samples positive for DENV RNA by RT-PCR were negative for nAbs against any of the DENV serotypes (Table 3.2). By evaluating anti-DENV nAb activity, it is possible to infer whether DENV infection is primary or secondary for both single- and multiple-serotype infections (Nguyen et al., 2018). Using this approach, 26% (12/47) and 40% (19/47) of samples were from patients experiencing primary and secondary infections, respectively (Table 3.2). The remaining 34% (16/47) of samples had NT<sub>90</sub> values  $< 40$  for all 4 serotypes; of these, 10 were NS1<sup>-</sup> IgM<sup>-</sup> IgG<sup>-</sup> and an additional 4 were IgM<sup>-</sup> IgG<sup>-</sup> (Table 3.2). Thus, our study cohort consisted of patients who experienced both primary and secondary DENV exposure during the 2017 outbreak, with a higher prevalence of secondary infections.



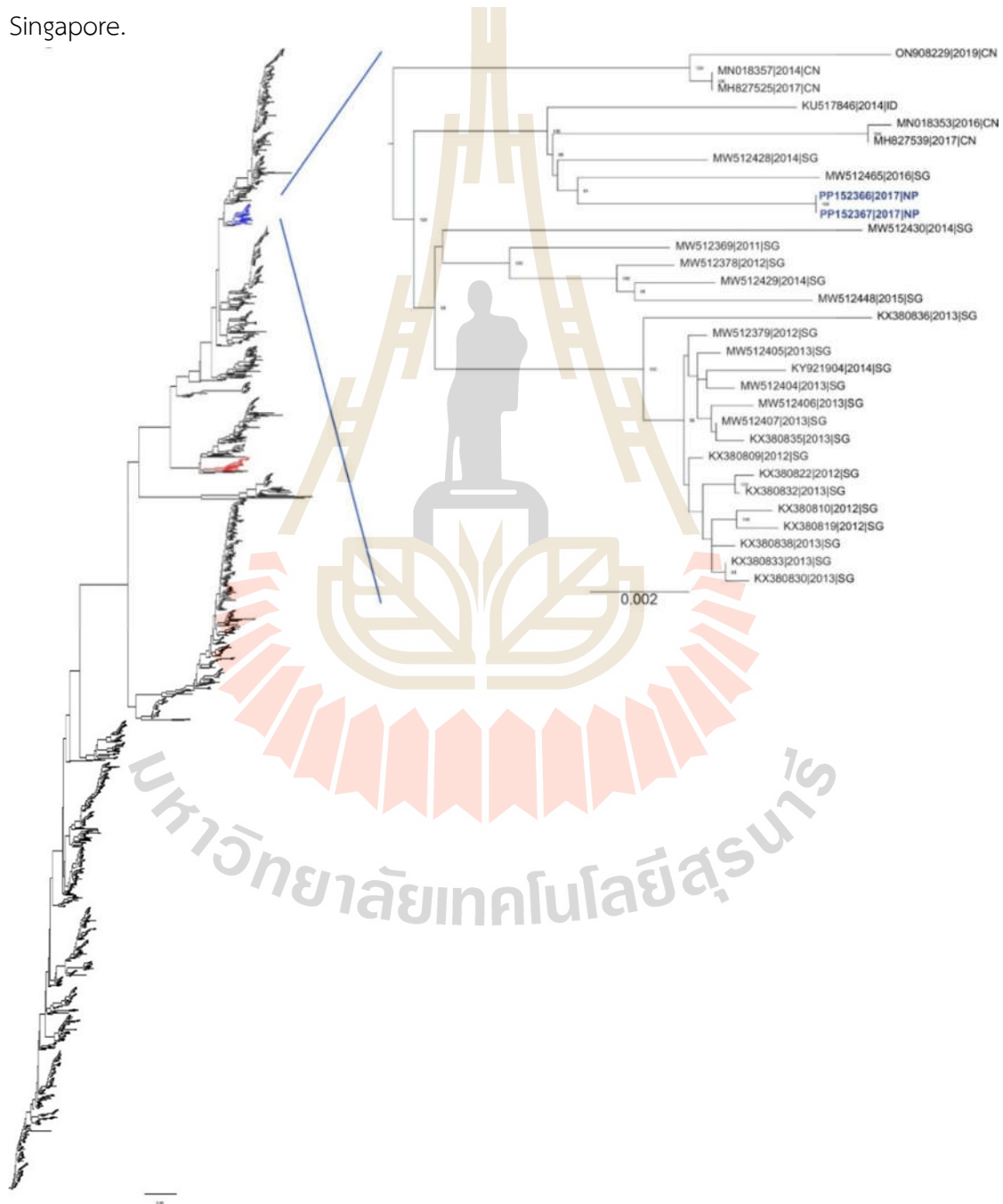
**Figure 3.2** Distribution of nAb titers in samples collected during the 2017 outbreak.

(A and B) NT<sub>90</sub> titers were determined according to DENV1–4 reference serotypes (A) and sample collection site (B). Mean values for  $n=47$  ( $n=21$  Kathmandu,  $n=26$  Chitwan) with circles representing individual samples. Dashed lines represent the cutoff value for nAb positivity (NT<sub>90</sub>=40). Mean values were compared using the non-parametric Kruskal–Wallis test.

#### 3.4.4 Phylogenetic and nucleotide analysis of DENV isolates

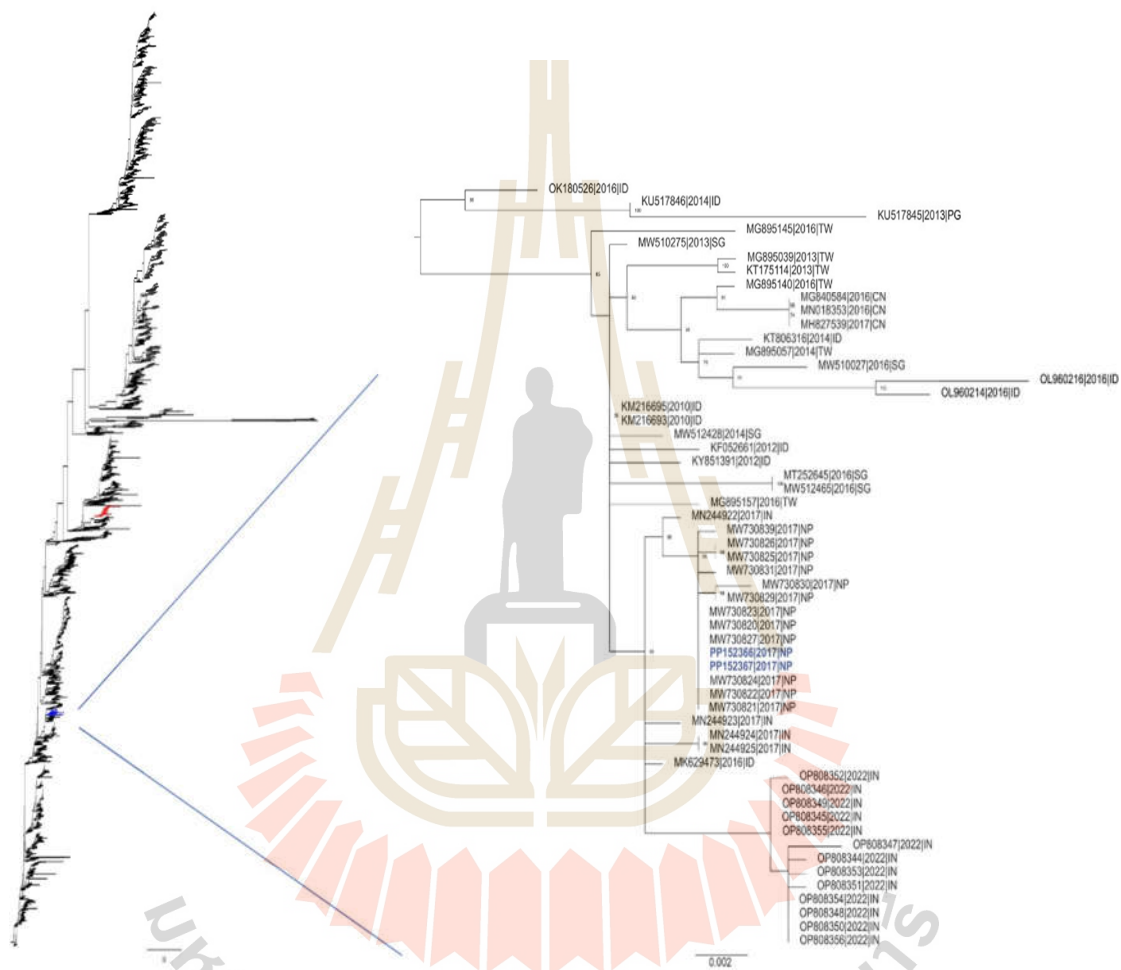
Full-genome sequences were obtained from the 7 serum samples shown to be positive for DENV RNA by RT-PCR (Table 3.2). Of the 7 genomes, 2 complete sequences and 5 incomplete sequences were obtained. Whole-genome phylogenetic analysis of the complete DENV2 genomes (PP152366 and PP152367, NCBI database) indicated that they belong to the Cosmopolitan Genotype, with the closest relatives being DENV2 sequences from Singapore in 2016 and 2014 (MW512465 and MW512428,

respectively) (Figure 3.3). Because of the scarcity of full-genome sequences from Nepal (n=2), we also analyzed the phylogeny of the E gene of PP152366 and PP152367 and E gene sequences from global DENV2 isolates. Close relationships were detected between PP152366 and PP152367 and E genes from 12 additional DENV2 sequences isolated in 2017 from Nepal (Ngwe Tun et al., 2021) and 4 were from India in 2017 (Figure 3.4). Hence, the 2017 DENV2 strains from Nepal and India likely originated from Singapore.



**Figure 3.3** Phylogenetic analysis of full-genome sequences of the Nepal 2017 DENV2 isolates generated here and other DENV2 isolates.

Phylogenetic tree comparing the DENV2 isolates from this study (blue, PP152366|2017|NP and PP152367|2017|NP), with other DENV2 full-genome sequences present in NCBI. Strains are labeled by GenBank ID, as well as year and country of isolation (CN, China; SG, Singapore; NP, Nepal).



**Figure 3.4** Phylogenetic analysis of E gene sequences from the 2017 DENV2 genomes from Nepal obtained in the present study shows a close relationship with other 2017 DENV2 isolates from Nepal and India.

Phylogenetic tree comparing E gene sequences of the two 2017 DENV2 strains from Nepal in the current study (blue font, PP152366|2017|NP and PP152367|2017|NP) with other E gene sequences in NCBI. Strains are labeled by GenBank ID, followed by the year and country of isolation (CN, China; ID, Indonesia; IN, India; NP, Nepal; PG, Papua New Guinea; SG, Singapore; TW, Taiwan).



### 3.5 Discussion

Over the past decade, Nepal has experienced increasingly severe outbreaks of dengue in approximately 3-year cycles, and cases were reported from all 77 districts during the 2019 outbreak (Gupta, Tuladhar, Kurmi, & Manandhar, 2018; Khetan et al., 2018; Prajapati et al., 2020). Despite growing public health impact, there has been limited information on the specific DENV serotypes and genotypes involved in each outbreak, as well as on the serological status of the population. This gap in knowledge is largely due to insufficient infrastructure and a lack of technical expertise required for systematic data collection and analysis. This study was initiated to begin addressing these gaps in collaboration with local researchers and physicians. The broader aim is to build a robust foundation of data that will support both local and national governments in preparing for future outbreaks and, ultimately, in guiding the deployment of effective DENV vaccines.

This study presents an analysis of samples collected during the 2017 dengue season from 49 patients who sought care at major hospitals in Kathmandu and Chitwan, located in central and southern Nepal, respectively. The majority of participants were male, which aligns with findings from other clinical studies conducted during the 2017 outbreak (Ngwe Tun et al., 2021; Rauniyar et al., 2023) as well as previous major DENV outbreaks in Nepal since 2006 (Gupta, Haselbeck, Kim, Marks, & Saluja, 2018; Gupta, Lamsal, et al., 2018; Gupta, Tuladhar, et al., 2018; Manandhar et al., 2021; Prajapati et al., 2020). The higher proportion of male patients may reflect greater healthcare-seeking behavior among men, as well as occupational exposures that increase their risk of contact with *Aedes* mosquitoes.

It is the first study in Nepal to evaluate the DENV-neutralizing capacity in individuals with suspected dengue infection. To improve specificity, we used a higher threshold for positivity (NT<sub>90</sub> vs standard NT<sub>50</sub>) considering the likelihood of cross-reactive neutralizing antibodies due to prior exposure to different DENV serotypes ((WHO), 2007). Our observation that most patient samples lacked DENV4-specific neutralizing antibodies aligns with earlier studies indicating that DENV4 has played only a limited role in past dengue outbreaks in Nepal (Manandhar et al., 2021; Prajapati et al., 2020; Rauniyar et al., 2023). Conversely, DENV1 and DENV2 have been the dominant serotypes during major outbreaks historically, with DENV1 prevailing in 2010 and 2016 (Pandey et al., 2013), and DENV2 in 2013 and 2019 (Khetan et al., 2018;

Poudyal et al., 2021; S. Singh, Gupta, Manakkadan, Das Manandhar, & Sreekumar, 2015). Among the seven samples successfully serotyped by RT-PCR in this study, six were identified as DENV2 and one as DENV3, indicating that the dominant serotype may vary even during periods between large-scale outbreaks. Notably, DENV3 has been rarely reported in Nepal since it was first identified in 2006 (Malla et al., 2008). The detection and possible re-emergence of DENV3 raises important public health concerns for Nepal. Notably, DENV3 was among the three main serotypes—alongside DENV1 and DENV2—circulating during the significant 2022 outbreak, which resulted in higher rates of hospitalizations, severe dengue cases, and fatalities compared to previous years (Rimal et al., 2023). Remarkably, 2022 marked the first documented instance where DENV1 and DENV3 were co-dominant. These findings highlight the critical need for consistent, year-round surveillance and reporting of circulating DENV serotypes, even between major outbreaks. Given that prior antibody responses can reduce the severity of dengue illness (Katzelnick, Montoya, Gresh, Balmaseda, & Harris, 2016), and growing evidence points to the importance of serotype-specific neutralizing antibodies in preventing infection (Bos et al., 2024), our results suggest that a future outbreak dominated by DENV4 in Nepal where immunity to this serotype is low could result in more severe disease outcomes.

Our whole-genome sequencing efforts were constrained by the fact that five out of the seven DENV RNA-positive samples yielded only partial sequences. Phylogenetic analysis of the two complete DENV2 genomes revealed a close genetic relationship with strains linked to Singapore's 2016 dengue outbreak (Yenamandra et al., 2021). Further analysis of the E gene sequences showed strong similarity to additional DENV strains collected from Nepal and India in 2017. As only a limited number of full-genome DENV sequences from Nepal are available, our findings suggest that cross-border movement, particularly due to Nepal's open borders, may significantly influence DENV transmission patterns. The genetic similarity between Nepalese and Singaporean isolates may reflect virus introduction via a Nepalese individual returning from Singapore. Collecting patient travel history as part of routine surveillance could improve our ability to trace the origins of such introductions. Previous studies also found strong genetic links between Nepal's major 2017 DENV isolates and strains from Singapore (2014, 2016), China (2016, 2017), and Indonesia (2014) (Ngwe Tun et al., 2021). Thus, our whole-genome and E gene sequence data

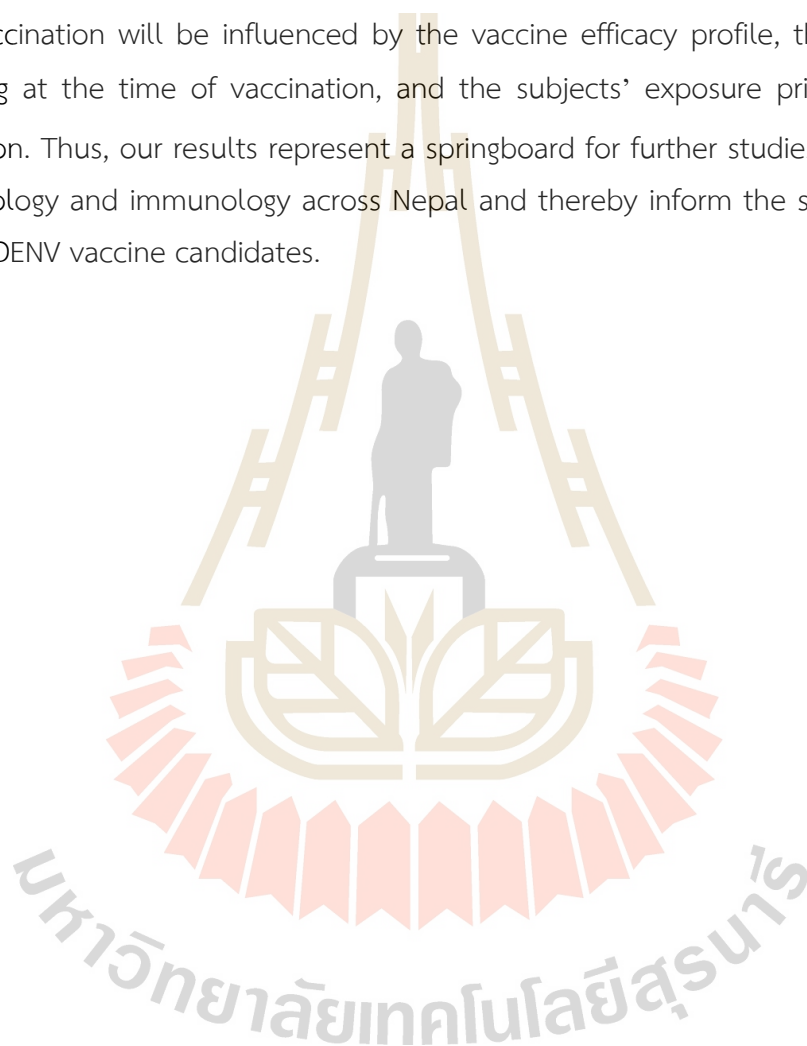
suggest that the DENV2 2017 Nepal lineage has been circulating in South Asia for at least 5 years. These results imply that the DENV2 lineage present in Nepal in 2017 may have been circulating in South Asia for at least five years. Interestingly, DENV RNA was detected by RT-PCR in only 7 out of 21 samples that tested positive for NS1 antigen by ELISA. This discrepancy may be due to the longer persistence of NS1 protein in serum compared to viral RNA (Alcon et al., 2002; Huhtamo et al., 2010; M. P. Singh et al., 2010), possible mutations in the viral RNA that hinder primer binding during RT-PCR (Alm, Lindegren, Falk, & Lagerqvist, 2015), and/or degradation of RNA during international sample transport. The latter issue further emphasizes the importance of building in-country infrastructure in Nepal for timely DENV surveillance and analysis.

The relatively small size of our study cohort limits the extent to which our findings can be generalized. Furthermore, the lack of complete clinical information and outcome data for all participants restricted our ability to draw meaningful correlations between specific DENV serotypes and clinical markers. Since all samples were collected at a single time point during the acute phase of illness rather than paired acute and convalescent samples, we could only infer, rather than definitively confirm, each subjects' dengue infection status (Collins et al., 2017).

Similar cohort study was further continued in Thailand (explained in detail in Chapter IV). Thailand has a long history of hyperendemicity for dengue, with all four serotypes circulating and frequent outbreaks. This provides ample opportunities for studying disease transmission, clinical manifestations, vector dynamics, and the effectiveness of control measures. In contrast, dengue is a relatively recent but rapidly growing public health concern in Nepal, with increasing outbreaks and geographic expansion. Thailand offers a mature setting for studying dengue in a hyperendemic context, with robust research infrastructure and ongoing control programs. Nepal, on the other hand, presents a valuable opportunity to investigate the dynamics of an emerging dengue epidemic in a unique geographic and climatic setting. Both countries offer valuable contributions to the global understanding of dengue and other flaviviruses, and collaborative research efforts between the two could be particularly fruitful.

In summary, despite its limited size, our study not only sheds light on the prevalence of DENV serotypes and nAbs in a Nepalese cohort during the 2017 dengue season, but also marks a significant advance in establishing, for the first time, the

scientific infrastructure to perform genomic and immunologic surveillance of DENV in Nepal. Our analysis demonstrates that a DENV2 genome closely related to strains from Singapore in 2016 was most likely the dominant serotype circulating in Nepal in 2017. The shift in dominant DENV serotypes between outbreaks and the prevalence of both primary and secondary DENV infections underscore the need for sustained surveillance of DENV at virologic and immunologic levels. As noted earlier, the clinical outcome of DENV vaccination will be influenced by the vaccine efficacy profile, the DENV strains circulating at the time of vaccination, and the subjects' exposure prior to and after vaccination. Thus, our results represent a springboard for further studies to understand DENV virology and immunology across Nepal and thereby inform the selection of one or more DENV vaccine candidates.





### 3.6 References

- Adhikari, N., & Subedi, D. (2020). The alarming outbreaks of dengue in Nepal. *Trop Med Health*, 48, 5. doi:10.1186/s41182-020-0194-1
- Aguiar, M., & Stollenwerk, N. (2020). The Impact of Serotype Cross-Protection on Vaccine Trials: DENVax as a Case Study. *Vaccines (Basel)*, 8(4). doi:10.3390/vaccines8040674
- 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(2), 376-381. doi:10.1128/JCM.40.02.376-381.2002
- Alm, E., Lindegren, G., Falk, K. I., & Lagerqvist, N. (2015). One-step real-time RT-PCR assays for serotyping dengue virus in clinical samples. *BMC Infect Dis*, 15, 493. doi:10.1186/s12879-015-1226-z
- Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., . . . Hay, S. I. (2013). The global distribution and burden of dengue. *Nature*, 496(7446), 504-507. doi:10.1038/nature12060
- Bos, S., Graber, A. L., Cardona-Ospina, J. A., Duarte, E. M., Zambrana, J. V., Ruíz Salinas, J. A., . . . Harris, E. (2024). Protection against symptomatic dengue infection by neutralizing antibodies varies by infection history and infecting serotype. *Nature Communications*, 15(1), 382. doi:10.1038/s41467-023-44330-8
- Collins, M. H., McGowan, E., Jadi, R., Young, E., Lopez, C. A., Baric, R. S., . . . de Silva, A. M. (2017). Lack of Durable Cross-Neutralizing Antibodies Against Zika Virus from Dengue Virus Infection. *Emerg Infect Dis*, 23(5), 773-781. doi:10.3201/eid2305.161630
- de Alwis, R., & de Silva, A. M. (2014). Measuring antibody neutralization of dengue virus (DENV) using a flow cytometry-based technique. *Methods Mol Biol*, 1138, 27-39. doi:10.1007/978-1-4939-0348-1\_3
- EDCD, M. o. H. a. P., Services, D. o. H., & Division, E. a. D. C. (2022). *Situation update of Dengue 2022 (As of 31th Dec,2022)*. Retrieved from <https://edcd.ekbana.info/news/download/situation-updates-of-dengue-as-of-30-nov-2022>
- Gupta, B. P., Haselbeck, A., Kim, J. H., Marks, F., & Saluja, T. (2018). The Dengue virus in Nepal: gaps in diagnosis and surveillance. *Ann Clin Microbiol Antimicrob*, 17(1), 32. doi:10.1186/s12941-018-0284-7

- Gupta, B. P., Lamsal, M., Chaulagain, S., Rauniyar, R., Malla, R., Shrestha, S., . . . Das Manandhar, K. (2018). Emergence of dengue in Nepal. *Virusdisease*, 29(2), 129-133. doi:10.1007/s13337-018-0439-3
- Gupta, B. P., Tuladhar, R., Kurmi, R., & Manandhar, K. D. (2018). Dengue periodic outbreaks and epidemiological trends in Nepal. *Ann Clin Microbiol Antimicrob*, 17(1), 6. doi:10.1186/s12941-018-0258-9
- Guzman, M. G., & Harris, E. (2015). Dengue. *Lancet*, 385(9966), 453-465. doi:10.1016/S0140-6736(14)60572-9
- Halstead, S. B. (2014). Dengue Antibody-Dependent Enhancement: Knowns and Unknowns. *Microbiol Spectr*, 2(6). doi:10.1128/microbiolspec.AID-0022-2014
- Hattakam, S., Elong Ngono, A., McCauley, M., Shresta, S., & Yamabhai, M. (2021). Repeated exposure to dengue virus elicits robust cross neutralizing antibodies against Zika virus in residents of Northeastern Thailand. *Sci Rep*, 11(1), 9634. doi:10.1038/s41598-021-88933-x
- Huhtamo, E., Hasu, E., Uzcategui, N. Y., Erra, E., Nikkari, S., Kantele, A., . . . Piiparinen, H. (2010). Early diagnosis of dengue in travelers: comparison of a novel real-time RT-PCR, NS1 antigen detection and serology. *J Clin Virol*, 47(1), 49-53. doi:10.1016/j.jcv.2009.11.001
- Jayashree, K., Manasa, G. C., Pallavi, P., & Manjunath, G. V. (2011). Evaluation of platelets as predictive parameters in dengue Fever. *Indian J Hematol Blood Transfus*, 27(3), 127-130. doi:10.1007/s12288-011-0075-1
- Kallas, E. G., Cintra, M. A. T., Moreira, J. A., Patino, E. G., Braga, P. E., Tenorio, J. C. V., . . . Nogueira, M. L. (2024). Live, Attenuated, Tetravalent Butantan-Dengue Vaccine in Children and Adults. *N Engl J Med*, 390(5), 397-408. doi:10.1056/NEJMoa2301790
- Kamaraj, U. S., Tan, J. H., Xin Mei, O., Pan, L., Chawla, T., Uehara, A., . . . Sessions, O. M. (2019). Application of a targeted-enrichment methodology for full-genome sequencing of Dengue 1-4, Chikungunya and Zika viruses directly from patient samples. *PLoS Negl Trop Dis*, 13(4), e0007184. doi:10.1371/journal.pntd.0007184
- Katzelnick, L. C., Bos, S., & Harris, E. (2020). Protective and enhancing interactions among dengue viruses 1-4 and Zika virus. *Curr Opin Virol*, 43, 59-70. doi:10.1016/j.coviro.2020.08.006
- Katzelnick, L. C., Montoya, M., Gresh, L., Balmaseda, A., & Harris, E. (2016). Neutralizing antibody titers against dengue virus correlate with protection from

- symptomatic infection in a longitudinal cohort. *Proc Natl Acad Sci U S A*, 113(3), 728-733. doi:10.1073/pnas.1522136113
- Khetan, R. P., Stein, D. A., Chaudhary, S. K., Rauniyar, R., Upadhyay, B. P., Gupta, U. P., & Gupta, B. P. (2018). Profile of the 2016 dengue outbreak in Nepal. *BMC Res Notes*, 11(1), 423. doi:10.1186/s13104-018-3514-3
- Malla, S., Thakur, G. D., Shrestha, S. K., Banjeree, M. K., Thapa, L. B., Gongal, G., . . . Gibbons, R. V. (2008). Identification of all dengue serotypes in Nepal. *Emerg Infect Dis*, 14(10), 1669-1670. doi:10.3201/eid1410.080432
- Manandhar, K. D., McCauley, M., Gupta, B. P., Kurmi, R., Adhikari, A., Nguyen, A. V., . . . Shrestha, S. (2021). Whole Genome Sequencing of Dengue Virus Serotype 2 from Two Clinical Isolates and Serological Profile of Dengue in the 2015-2016 Nepal Outbreak. *Am J Trop Med Hyg*, 104(1), 115-120. doi:10.4269/ajtmh.20-0163
- Nepal, G. o. (2012/2013 and 2013/2014). Annual Report, Department of Health Services, Ministry of Health & Population.
- Ngono, A. E., & Shrestha, S. (2018). Immune Response to Dengue and Zika. *Annu Rev Immunol*, 36, 279-308. doi:10.1146/annurev-immunol-042617-053142
- Nguyen, T. H. T., Clapham, H. E., Phung, K. L., Nguyen, T. K., TT, D. I., Nguyen, T. H. Q., . . . Wills, B. (2018). Methods to discriminate primary from secondary dengue during acute symptomatic infection. *BMC Infect Dis*, 18(1), 375. doi:10.1186/s12879-018-3274-7
- Ngwe Tun, M. M., Pandey, K., Nabeshima, T., Kyaw, A. K., Adhikari, M., Raini, S. K., . . . Morita, K. (2021). An Outbreak of Dengue Virus Serotype 2 Cosmopolitan Genotype in Nepal, 2017. *Viruses*, 13(8). doi:10.3390/v13081444
- OhAinle, M., Balmaseda, A., Macalalad, A. R., Tellez, Y., Zody, M. C., Saborio, S., . . . Harris, E. (2011). Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. *Sci Transl Med*, 3(114), 114ra128. doi:10.1126/scitranslmed.3003084
- Ojha, A., Nandi, D., Batra, H., Singhal, R., Annarapu, G. K., Bhattacharyya, S., . . . Guchhait, P. (2017). Platelet activation determines the severity of thrombocytopenia in dengue infection. *Sci Rep*, 7, 41697. doi:10.1038/srep41697
- Organization, W. H. (2022). Dengue-Nepal. In.
- Pandey, B. D., Nabeshima, T., Pandey, K., Rajendra, S. P., Shah, Y., Adhikari, B. R., . . . Morita, K. (2013). First isolation of dengue virus from the 2010 epidemic in Nepal. *Trop Med Health*, 41(3), 103-111. doi:10.2149/tmh.2012-17

- Patel, S. S., Rauscher, M., Kudela, M., & Pang, H. (2023). Clinical Safety Experience of TAK-003 for Dengue Fever: A New Tetravalent Live Attenuated Vaccine Candidate. *Clin Infect Dis*, 76(3), e1350-e1359. doi:10.1093/cid/ciac418
- Pierson, T. C., & Diamond, M. S. (2020). The continued threat of emerging flaviviruses. *Nat Microbiol*, 5(6), 796-812. doi:10.1038/s41564-020-0714-0
- Poudyal, P., Sharma, K., Dumre, S. P., Bastola, A., Chalise, B. S., Shrestha, B., . . . Pandey, K. (2021). Molecular study of 2019 dengue fever outbreaks in Nepal. *Trans R Soc Trop Med Hyg*, 115(6), 619-626. doi:10.1093/trstmh/traa096
- Prajapati, S., Napit, R., Bastola, A., Rauniyar, R., Shrestha, S., Lamsal, M., . . . Manandhar, K. D. (2020). Molecular phylogeny and distribution of dengue virus serotypes circulating in Nepal in 2017. *PLoS One*, 15(7), e0234929. doi:10.1371/journal.pone.0234929
- Rauniyar, R., Prajapati, S., Manandhar, B., Bastola, A., Chalise, B. S., Shrestha, S., . . . Das Manandhar, K. (2023). Dengue virus infection during window period of consecutive outbreaks in Nepal and assessment of clinical parameters. *Sci Rep*, 13(1), 9262. doi:10.1038/s41598-023-35928-5
- Rijal, K. R., Adhikari, B., Ghimire, B., Dhungel, B., Pyakurel, U. R., Shah, P., . . . Ghimire, P. (2021). Epidemiology of dengue virus infections in Nepal, 2006-2019. *Infect Dis Poverty*, 10(1), 52. doi:10.1186/s40249-021-00837-0
- Rimal, S., Shrestha, S., Pandey, K., Nguyen, T. V., Bhandari, P., Shah, Y., . . . Dumre, S. P. (2023). Co-Circulation of Dengue Virus Serotypes 1, 2, and 3 during the 2022 Dengue Outbreak in Nepal: A Cross-Sectional Study. *Viruses*, 15(2). doi:10.3390/v15020507
- Santiago, G. A., Vergne, E., Quiles, Y., Cosme, J., Vazquez, J., Medina, J. F., . . . Munoz-Jordan, J. L. (2013). Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus. *PLoS Negl Trop Dis*, 7(7), e2311. doi:10.1371/journal.pntd.0002311
- Singh, M. P., Majumdar, M., Singh, G., Goyal, K., Preet, K., Sarwal, A., . . . Ratho, R. K. (2010). NS1 antigen as an early diagnostic marker in dengue: report from India. *Diagn Microbiol Infect Dis*, 68(1), 50-54. doi:10.1016/j.diagmicrobio.2010.04.004
- Singh, S., Gupta, B. P., Manakkadan, A., Das Manandhar, K., & Sreekumar, E. (2015). Phylogenetic study reveals co-circulation of Asian II and Cosmopolitan genotypes of Dengue virus serotype 2 in Nepal during 2013. *Infect Genet Evol*, 34, 402-409. doi:10.1016/j.meegid.2015.07.006
- Sridhar, S., Luedtke, A., Langevin, E., Zhu, M., Bonaparte, M., Machabert, T., . . . DiazGranados, C. A. (2018). Effect of Dengue Serostatus on Dengue Vaccine



- Safety and Efficacy. *N Engl J Med*, 379(4), 327-340. doi:10.1056/NEJMoa1800820
- Takasaki, T., Kotaki, A., Nishimura, K., Sato, Y., Tokuda, A., Lim, C. K., . . . Kurane, I. (2008). Dengue virus type 2 isolated from an imported dengue patient in Japan: first isolation of dengue virus from Nepal. *J Travel Med*, 15(1), 46-49. doi:10.1111/j.1708-8305.2007.00165.x
- Thomas, S. J. (2023). Is new dengue vaccine efficacy data a relief or cause for concern? *NPJ Vaccines*, 8(1), 55. doi:10.1038/s41541-023-00658-2
- Waggoner, J. J., Abeynayake, J., Sahoo, M. K., Gresh, L., Tellez, Y., Gonzalez, K., . . . Pinsky, B. A. (2013). Single-reaction, multiplex, real-time rt-PCR for the detection, quantitation, and serotyping of dengue viruses. *PLoS Negl Trop Dis*, 7(4), e2116. doi:10.1371/journal.pntd.0002116
- WHO, M. (2019). Dengue and severe dengue. *Geneva*.
- WHO (2007). Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses (Vol. WHO/IVB/07.07).
- Yenamandra, S. P., Koo, C., Chiang, S., Lim, H. S. J., Yeo, Z. Y., Ng, L. C., & Hapuarachchi, H. C. (2021). Evolution, heterogeneity and global dispersal of cosmopolitan genotype of Dengue virus type 2. *Sci Rep*, 11(1), 13496. doi:10.1038/s41598-021-92783-y



## CHAPTER IV

### IMMUNOMOLECULAR STUDY OF DENGUE VIRUS AND ITS CROSSREACTIVITY WITH OTHER FLAVIVIRUSES

#### 4.1 Abstract

Dengue virus (DENV) belonging to *Flaviviridae* family is a major global public health burden affecting tropical and subtropical regions of the world including Thailand. Along with DENV, other flaviviruses including Japanese encephalitis virus (JEV), Zika virus (ZIKV), etc. also circulates here creating significant diagnostic challenges due to serological cross-reactivity due to shared antigenic structures. In this study, we aimed to perform an immune-molecular characterization of dengue virus infections and assess their serological cross-reactivity with other flaviviruses in suspected dengue cases. A total of 60 acute samples, 25 (28-45 days) and 6 (1 year) convalescent samples were collected from Suranaree University of technology Hospital and Sikhio Hospital, Nakhon Ratchasima which is northeastern region of Thailand. Serological assays, including ELISA and micro neutralization tests, were employed to determine the prevalence of DENV infection and assess the potential for cross-reactivity with other flaviviruses such as ZIKV, JEV and yellow fever virus (YFV). Serotyping was performed by qRT-PCR from plasma sample as well as virus culture supernatant to determine the prevalent serotypes in our cohort. Of the 60 acute samples, 34 were serologically positive for DENV NS1, 21 for anti-DENV IgM, 44 for anti-DENV IgG and 25 were positive by qRT-PCR. The three serotypes DENV-1, 2 and 4 were detected and there was no DENV-3. From virus culture, we were able to isolate only DENV1 and 2. Secondary infection was higher than primary infection. Regarding neutralization assay, we were able to see very high neutralizing antibodies against DENV1 followed by DENV3. Surprisingly antibodies against ZIKV and JEV were higher than for DENV2 and 4. Though YFV was not endemic in Thailand, we were able to see neutralizing antibodies against YFV as well which may be because of shared antigenic similarity with DENV. This immune-molecular investigation reveals complex flavivirus cross-reactivity patterns in dengue suspected cases which is directly correlated with disease severity. These findings have implications for improved diagnosis, surveillance, and development of effective control strategies for dengue and other flaviviral diseases in Thailand.

## 4.2 Introduction

Dengue virus (DENV) causing dengue fever, is still a major worldwide health concern, especially in tropical and subtropical areas (Guzman & Harris, 2015). With four distinct serotypes (DENV-1 to DENV-4), the virus shows high genetic diversity, which further complicates vaccine development and immunological responses (Bhatt et al., 2013). Understanding of viral genetics and host immune responses has been greatly enhanced by recent advances in molecular biology and immunology, which are major factors for designing effective interventions.

DENV is transmitted through the bite of infected *Aedes* mosquito species primarily *aegyptii* and secondarily *albopictus*. The incubation period usually lasts for 3-14 days (Chong, Leow, Abdul Majeed, & Leow, 2019). While 20% of people infected with DENV may experience self-limiting symptoms such as high fever, headache, body aches, nausea, and rash that last for one to two weeks, the other 80% of people are asymptomatic. Hepatosplenomegaly, mucosal bleeding, edema, lethargy, abdominal pain, and recurrent vomiting are some of the warning indications of serious disease that a tiny percentage of patients may exhibit (2009). Between 0.5-5% of infected individuals develop severe dengue with clinical complications including plasma leakage causing dengue shock syndrome or respiratory distress, severe bleeding, or severe organ impairment (Jiravejchakul et al., 2025).

The immune response involves both innate and adaptive pathways, which are responsible for the production of neutralizing antibodies and T-cell-mediated immunity. However, ADE can result in increased disease severity during secondary infections with different serotypes (Halstead, 2014). Hence, understanding the molecular and immunological basis of these responses is vital for improving vaccine efficacy and therapeutic strategies.

Similar to the dengue virus, other flaviviruses transmitted by mosquito also tend to cause strong immune responses that are cross-reactive (Hou et al., 2022). Mosquito borne flaviviruses are mainly transmitted by *Aedes* mosquitoes (such as DENV, ZIKV and YFV) or *Culex* (such as JEV). Since these vectors affect viral distribution, so epidemic regions of these flaviviruses are overlapping, which greatly increases the difficulty in prevention and control of flaviviruses (Theodore C Pierson & Michael S Diamond, 2020). The structural and genetic similarities among these flaviviruses often lead to cross-reactive immune responses, which can influence diagnostic accuracy and

disease progression. Hence, cross-reactivity presents both challenges and opportunities in flavivirus immunity research, especially regarding vaccine development and immune diagnostics.

Thailand represents a critical dengue disease epicenter that demands understanding of the environmental and evolutionary pressures sustaining DENV transmission, as unlike most affected countries experiencing recurrent outbreaks of the same serotype or replacement of one serotype for another. Thailand serves as an ecological niche for all four serotypes with stable, endemic circulation of genotypes. With approximately 100,000 annual cases and nearly 100 deaths reported each year, dengue poses a substantial burden on Thailand's healthcare system and represents one of the most rapidly spreading mosquito-borne viral diseases in the region (Perez et al., 2025). Recent epidemiological data indicates a concerning trend, with 84,000 cases reported by October 2024 and a 123% increase in cases compared to the same period in 2023, affecting all age groups but predominantly impacting individuals aged 5-24 years. The epidemiological landscape of dengue in Thailand has undergone significant demographic transitions, with the mean age of dengue hemorrhagic fever cases increasing considerably from 8.1 to 24.3 years between 1981 and 2017, reflecting complex interactions between population demographics, infection hazards, and reporting practices (A. T. Huang et al., 2022).

Vaccination remains the main preventive strategy for DENV. In Thailand, currently two tetravalent dengue vaccines are available. The first vaccine, Dengvaxia® (CYD-TDV), contains genes from all four DENV serotypes, three of which are isolates from Thailand, and has been widely adopted in many countries (Jain, Vimal, Angmo, Sengupta, & Thangaraj, 2023; Tully & Griffiths, 2021). However, this vaccine is limited to individuals with prior DENV infection only and living in endemic areas (Sridhar et al., 2018). Furthermore, its effectiveness varies across serotypes, offering greater protection against DENV-3 and 4, which are less common in Thailand (Tully & Griffiths, 2021). The second vaccine, Qdenga® (TAK-003), is also a tetravalent vaccine with multi-phase clinical trials conducted in Thailand for safety and efficacy. Both sero-positive and sero-negative individuals showed the best vaccine efficacy against DENV-2, however sero-negative patients showed unclear efficacy against DENV-4 (Biswal et al., 2020). Furthermore, hospitalization rates for the DENV-3 virus strain were higher in sero-



negative patients, raising concerns that sero-status may possibly impact overall safety (Jain et al., 2023; Rivera et al., 2022).

Thailand is endemic for several other flavivirus infections together with DENV such as JEV, ZIKV. The country's tropical environment supports year-round mosquito breeding across diverse regions, from the mountainous north and central rice paddies to the rocky coastlines of its southern peninsula. Zika virus circulation in Thailand has been documented with significant underreporting. Recent molecular epidemiological studies from 2020-2023 found that among 751 hospitalized patients initially testing negative for dengue and chikungunya, 12% (90 patients) were subsequently confirmed to have ZIKV infection, suggesting substantial underdiagnosis. Current surveillance shows 181 Zika cases across 31 provinces in 2024, with most cases (71) occurring in the Central Region (Saba Villarroel et al., 2024). Japanese encephalitis remains a significant cause of viral encephalitis in Thailand, with surveillance data from 2003-2019 showing 9,566 acute encephalitis syndrome patients and 642 confirmed JE cases (6.7%) with 16 deaths. Hospital-based studies found that 15% of encephalitis patients had JEV infection, with higher rates in southern Thailand (28%) compared to Bangkok (10%). JEV is endemic throughout Thailand outside Bangkok, with year-round transmission and seasonal epidemics occurring in Northern provinces during May-October. The disease particularly affects areas where rice cultivation and pig farming coexist (Maksikharin, Prommalikit, & Thisyakorn, 2023). Although YFV is not currently endemic in Thailand, studying the virus is crucial for early detection of imported cases, understanding flavivirus immune interactions, strengthening surveillance and preparedness, guiding vaccination policies, and contributing to global health security amid increasing yellow fever outbreaks worldwide. Altogether, Thailand's diverse arboviral ecosystem provides an outstanding real-world setting for investigating how multiple pathogens interact, how immune responses may interfere with each other, and how viruses evolve when circulating together.

In this study, we aimed to characterize various aspects of DENV infection in Northeastern region of Thailand by sampling 60 patients who presented with suspected dengue disease at two of our collaborating hospitals. We also tried to collect 28-45 days and 1 year convalescence samples. For the samples, we not only checked neutralizing antibodies against DENV, we also checked the antibodies against ZIKV, JEV and YFV which are closely related flaviviruses. Understanding cross-reactivity

mechanisms can inform the development of diagnostic tools capable of distinguishing between infections and aid in creating vaccines that elicit effective protective responses without causing ADE.

## 4.3 Material and Methods

### 4.3.1 Ethics, Sample collection, Processing and Storage

The study has been approved by ethics committee of Suranaree University of technology (EC-64-55). Samples having suspected dengue were collected from patients visiting Suranaree University of Technology (SUTH) and Sikhio Hospital (SH). Blood samples were collected only from the patients who consented for the study. Samples were also collected in PAXgene tubes which will be used for csRNA Seq identifies cell-type-specific gene regulatory elements and their underlying transcription factor networks. The tubes are stored at  $-80^{\circ}\text{C}$  till future use. Clinical symptoms and demographics were also recorded by the attending nurse during sample collection. The patients were given Patient ID based on the time the acute samples were collected serially and Sub ID based on the hospital the samples were collected.

### 4.3.2 Plasma separation and PBMC Isolation

Firstly, plasma was separated from the blood by centrifugation at 300-350g for 10 minutes. After plasma separation, from remaining blood, PBMC isolation was performed using Ficoll-gradient technique. Firstly, blood was diluted equal volume in a 50ml falcon tube with 1X PBS having 0.1% glucose and mixed gently. Then diluted blood can be slowly overlayed in Ficoll reagent such that a curtain is formed between two. After that, they are centrifuged at 400g, without brakes for 30-40 mins at  $20^{\circ}\text{C}$ . After centrifugation, plasma layer was removed and PBMC ring was carefully transferred to a new falcon tube. PBMC layer was then washed with equal volume of PBS-glucose and centrifuged at 350g, with brakes on for 7 mins at  $4^{\circ}\text{C}$ , twice. The cells were then resuspended in 2-5ml 1X PBS and counted by using 0.4% trypan blue. The cells were then resuspended in cold FBS ( $1 \times 10^7$  cells/ml) and then resuspended in equal volume of 20% DMSO in FBS ( $5 \times 10^6$  cells/ml) and distributed in cryovials and stored in liquid nitrogen tank for future research purposes.

#### **4.3.3 Detection of DENV Antigen and Antibody**

The samples were tested for NS1 antigen using Dengue Ag Rapid Test Device (Cat No. D-DAGD20) against DENV at the hospitals where they were collected. Anti-IgM and anti-IgG antibody were further tested in our lab. Anti-IgM has been tested from Focus Diagnostics Kit (Cat No. EL1500M) and Serion kit (Cat No. ESR114M). For, anti-IgG Serion kit (ESR114G) was used.

#### **4.3.4 Virus culture**

Virus was isolated from the plasma sample. At first the plasma sample was diluted and then added to the cultured cells. The plates were then incubated at 37°C, 5% CO<sub>2</sub> for 5-7 days. The cells were observed for cytopathic effect and then culture supernatant was harvested. RNA extraction was further performed for these culture supernatants and the virus isolation was confirmed by PCR.

#### **4.3.4 RNA extraction and serotyping**

Viral RNA was isolated from extracted from plasma or culture supernatant for all acute samples using QIAamp viral RNA Mini kit using spin protocol (Cat. No. 52904) or Quick-RNA™ Viral Kit (Cat. No. R1035) according to the manufacturer's instructions. Thus, extracted RNA was used for serotyping by using real time PCR (Ito et al., 2004).

#### **4.3.5 Neutralization test using Microneutralization Assay (MNT)**

The Vero cells were cultured and seeded in 96 well plate and incubated overnight to get 80-100% confluency. All of the samples used in the assay were first heat inactivated at 56°C for 30 minutes. The plasma samples were then diluted and mixed with virus at a 1:1 ratio and incubated at 37°C. The resulting mixture was added to Vero cells seeded in 96-well plates. Following a 1-hour adsorption period, the plates were incubated at 37°C for 5 to 7 days. Cells were then fixed with methanol (Wako Pure Chemical Industries) for 10 minutes at room temperature and stained with 0.1% crystal violet (Wako Pure Chemical Industries) for 15 minutes. After washing off the excess stain, 1% sodium dodecyl sulfate (SDS) was used to dissolve the cells. Absorbance was measured at a wavelength of 595 nm. The neutralizing antibody titer was defined as the reciprocal of the highest serum dilution that resulted in absorbance below 50% of the established cut-off, indicating at least 50% neutralization (Haga, Chen, Himeno, Majima, & Moi, 2023). The assay was performed on DENV1-4, ZIKV, JEV

and YFV. Titration of the viruses was performed to determine the concentration and days for incubation before the assay so as the virus can at least kill the cells by 50% (Table 4.1).

**Table 4.1** Detail of the virus strains used

| S. N. | Type  | Strain   | Titer                     | Final Titer              | Incubation |
|-------|-------|----------|---------------------------|--------------------------|------------|
| 1     | DENV1 | 01-44    | $4.25 \times 10^6$ pfu/mL | $1.3 \times 10^3$ pfu/mL | 5 days     |
| 2     | DENV2 | 00St22A  | $1.8 \times 10^5$ pfu/mL  | $1.8 \times 10^3$ pfu/mL | 5 days     |
| 3     | DENV3 | TL18     | $2.2 \times 10^5$ pfu/mL  | $6.0 \times 10^4$ pfu/mL | 5 days     |
| 4     | DENV4 | TVP360   | $1.5 \times 10^5$ pfu/mL  | $1.5 \times 10^2$ pfu/mL | 5 days     |
| 5     | ZIKV  | PRVABC59 | $1.5 \times 10^7$ pfu/mL  | $1.5 \times 10^3$ pfu/mL | 3 days     |
| 6     | JEV   | OH0566   | $1 \times 10^7$ pfu/mL    | $1 \times 10^2$ pfu/mL   | 4 days     |
| 7     | YFV   | YFD-17D  | $7 \times 10^6$ pfu/mL    | $7 \times 10^2$ pfu/mL   | 4 days     |

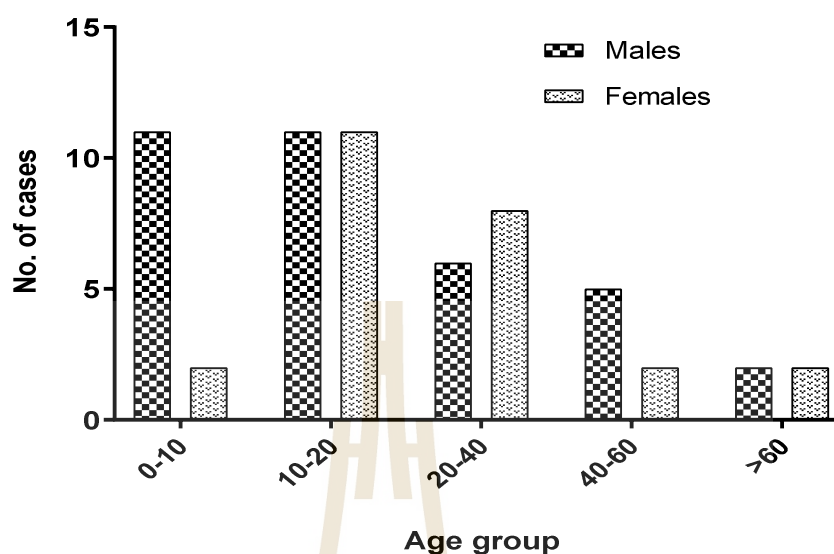
## 4.4 Results

### 4.4.1 Patient demographics

A total of 16 acute samples from Suranaree University Technology Hospital (SUTH) and 44 acute samples from Sikhio Hospital have been collected. After 28-45 days of the first sample collection, 25 convalescent samples have been collected and 6 convalescent samples after one year has been collected so far. In total, there are 91 samples.

Though the sample size is small, there is huge variation in the age group of samples. The lowest age ranged to 4 months and the highest age was 79 years. The ratio of males to females was 1.4:1 (males, 35/60, 58%). The majority of cases were in age group 10-20 years with equal number of males and females, followed closely by 20-40 and 0-10 years. Surprisingly, affected number of male children was very high in age group 0-10 years (males: 11 and females: 2).





**Figure 4.1** Demographic distribution of cases by age and sex.

#### 4.4.2 DENV antigen detection and serology

Out of 60 acute samples, 34 were positive and 24 were negative for NS1 antigen detection test. For 2 samples the test was not performed for NS1. For anti-DENV IgM ELISA, 21 were positive, 37 were negative and 2 were in the borderline. In the case of anti-DENV IgG ELISA, 44 were positive, 13 were negative and 3 were in the borderline. The detail of the serological status of the patients is shown in Table 4.2.

**Table 4.2** Summary of serological tests performed

| Serological Status | No. of samples |
|--------------------|----------------|
| NS1+               | 34             |
| IgM+               | 21             |
| IgG+               | 44             |
| Only NS1+          | 8              |
| Only IgM+          | None           |
| Only IgG+          | 15             |
| NS1+/IgM+          | 16             |
| NS1+/IgG+          | 24             |
| IgM+/IgG+          | 14             |
| All Positive       | 14             |
| All Negative       | 6              |

#### 4.4.3 Serotyping by real time reverse transcriptase PCR

RNA was extracted from plasma directly or virus culture supernatant. From virus culture, only 8 samples were positive (7 DENV1 and 1 DENV2). From plasma samples, 42% (25/60) samples were positive by real time PCR. Of the 25 PCR positive samples, 18 were DENV1, 4 were DENV2 and 3 were DENV4. None of the samples were infected by multiple serotypes and no DENV3 was detected. In our study, even if the sample size was small, we were able to detect multiple serotypes, DENV1 being the dominant serotype. The CT values of the positive samples and from the virus culture supernatant is shown in Table 4.3 and 4.4.

**Table 4.3** CT values for the PCR positive RNA samples from plasma

| Patient No. | Sub ID    | Gender | Year     | DENV1 | DENV2 | DENV3 | DENV4 |
|-------------|-----------|--------|----------|-------|-------|-------|-------|
| 6           | SUTH/06/A | M      | 2        | 26.50 | N/A   | N/A   | N/A   |
| 7           | SUTH/07/A | F      | 31       | 37.00 | N/A   | N/A   | N/A   |
| 8           | SUTH/08/A | M      | 4        | 33.24 | N/A   | N/A   | N/A   |
| 9           | SUTH/09/A | M      | 14       | 24.67 | N/A   | N/A   | N/A   |
| 10          | SUTH/10/A | M      | 11       | 29.33 | N/A   | N/A   | N/A   |
| 11          | SUTH/11/A | M      | 7        | 18.16 | N/A   | N/A   | N/A   |
| 12          | SUTH/12/A | F      | 14       | 34.32 | N/A   | N/A   | N/A   |
| 13          | SUTH/13/A | F      | 11       | 25.06 | N/A   | N/A   | N/A   |
| 14          | SUTH/14/A | M      | 8 months | 33.21 | N/A   | N/A   | N/A   |
| 16          | SUTH/16/A | M      | 14       | 35.04 | N/A   | N/A   | N/A   |
| 29          | SH/13/A   | F      | 11       | 20.21 | N/A   | N/A   | N/A   |
| 38          | SH/22/A   | M      | 13       | 34.39 | N/A   | N/A   | N/A   |
| 39          | SH/23/A   | M      | 5        | 35.02 | N/A   | N/A   | N/A   |
| 41          | SH/25/A   | F      | 15       | 16.16 | N/A   | N/A   | N/A   |
| 43          | SH/27/A   | M      | 15       | 21.19 | N/A   | N/A   | N/A   |
| 53          | SH/37/A   | F      | 11       | 34.12 | N/A   | N/A   | N/A   |
| 57          | SH/41/A   | M      | 13       | 32.03 | N/A   | N/A   | N/A   |
| 58          | SH/42/A   | F      | 8        | 28.25 | N/A   | N/A   | N/A   |
| 2           | SUTH/02/A | F      | 36       | N/A   | 31.27 | N/A   | N/A   |
| 45          | SH/29/A   | M      | 11       | N/A   | 33.39 | N/A   | N/A   |

| Patient No. | Sub ID    | Gender | Year | DENV1 | DENV2 | DENV3 | DENV4 |
|-------------|-----------|--------|------|-------|-------|-------|-------|
| 47          | SH/31/A   | F      | 14   | N/A   | 16.47 | N/A   | N/A   |
| 48          | SH/32/A   | F      | 31   | N/A   | 32.61 | N/A   | N/A   |
| 1           | SUTH/01/A | M      | 7    | N/A   | N/A   | N/A   | 29.11 |
| 4           | SUTH/04/A | M      | 50   | N/A   | N/A   | N/A   | 23.98 |
| 15          | SUTH/15/A | F      | 11   | N/A   | N/A   | N/A   | 29.33 |

**Table 4.4** CT values for the PCR positive RNA samples from virus culture supernatant

| Patient No. | Sub ID    | Gender | Year | DENV1 | DENV2 |
|-------------|-----------|--------|------|-------|-------|
| 6           | SUTH/06/A | M      | 2    | 13.75 | N/A   |
| 9           | SUTH/09/A | M      | 14   | 36.31 | N/A   |
| 11          | SUTH/11/A | M      | 7    | 14.00 | N/A   |
| 29          | SH/13/A   | F      | 11   | 13.61 | N/A   |
| 41          | SH/25/A   | F      | 15   | 14.21 | N/A   |
| 43          | SH/27/A   | M      | 15   | 21.19 | N/A   |
| 53          | SH/37/A   | F      | 11   | 13.91 | N/A   |
| 47          | SH/31/A   | F      | 14   | N/A   | 18.52 |

#### 4.4.4 DENV case classification

After different serological tests and PCR performed in our laboratory, the suspected cases were further classified as active/acute DENV infection as samples which were either/or PCR<sup>+</sup>/NS1<sup>+</sup>/IgM<sup>+</sup>. The samples which were only IgG<sup>+</sup> were considered as probable past DENV infection. The samples which were negative to all the tests performed were considered as other febrile illness. Out of 60 suspected cases, 39 were active DENV, 16 were probable past DENV and 6 were considered as other febrile illness.

The positive DENV cases were further classified as primary and secondary cases based of IgM: IgG ratio. For the samples diluted in 1:100 ratio, if IgM/IgG is <1.2, the cases are said to be secondary infection and if ≥1.2 they were classified as primary infection. Out of 39 confirmed DENV cases, only 3 were primary infections whereas 36 cases were secondary infections.

#### 4.4.5 Neutralizing antibody response against DENV1-4 and other flaviviruses

Initially all of the samples were screened for presence of neutralizing antibodies against DENV1-4. The plasma was diluted 1:40 and after mixing with virus in 1:1 ratio, the final concentration was 1:80. For the total of 60 acute samples, neutralizing antibodies were present in highest percentage against DENV1 (95%, 57/60) followed by DENV3 (75%, 45/60). Surprisingly the neutralizing antibodies against ZIKV and JEV were higher than that for DENV2 and DENV4. Some of the samples also showed response against YFV (15%, 9/60). More than half of the population had antibodies against ZIKV as well (52%, 31/60).

#### 4.4.6. Neutralizing antibody response based on DENV case classification

After the cases were classified as active DENV, probable DENV and negative DENV or other febrile illness, we further checked the neutralization activity in only DENV active cases (n=39) for all 7 viruses. It was clearly seen that almost all of the samples had neutralizing activity against DENV1 (38/39) followed by DENV3 (28/39). Surprising the neutralization against ZIKV and JEV was higher than that for DENV2 and 4 (Figure 4.2). Further Dunn's multiple comparisons test was performed and significant difference was seen between DENV1 and all other virus strains used except for DENV3. DENV3 however showed significant difference with other viruses. DENV4 also showed significant difference with ZIKV and YFV (Table 4.5).

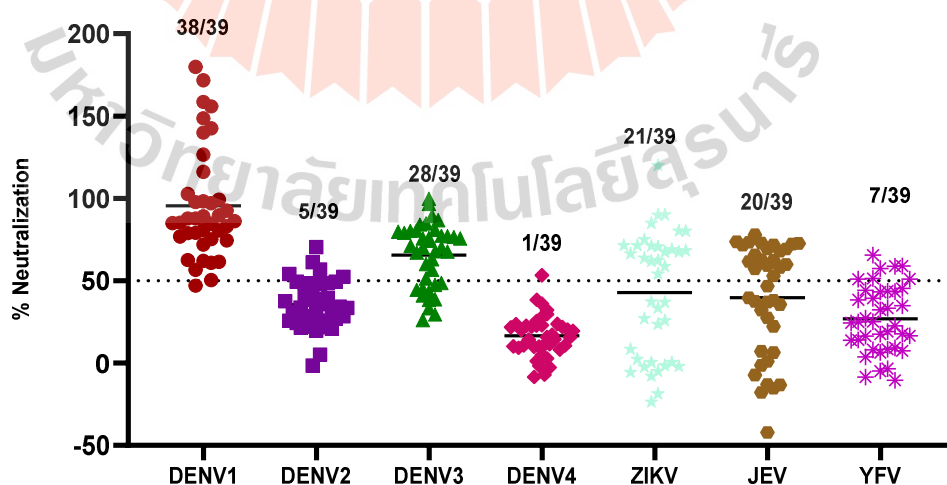


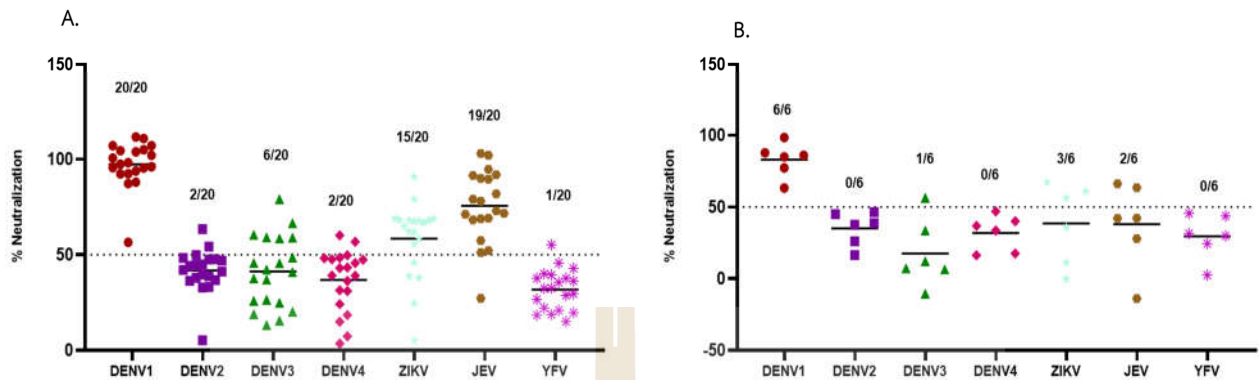
Figure 4.2 Percentage neutralization of active DENV cases against DENV1-4, ZIKV, JEV and YFV



**Table 4.5** Statistical analysis for presence of neutralizing antibodies against DENV1-4, ZIKV, JEV and YFV

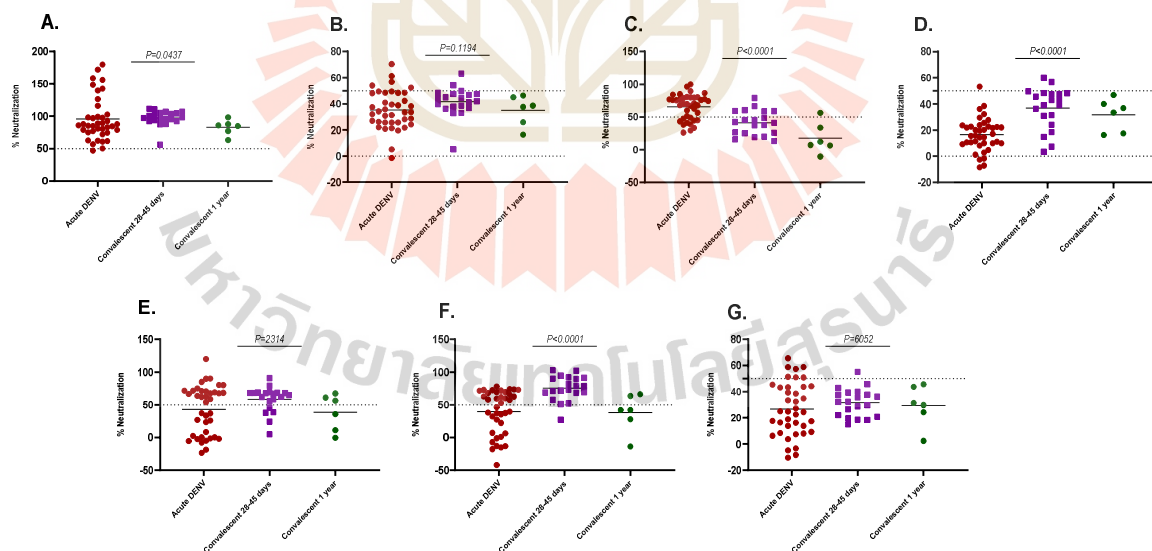
| Dunn's multiple comparisons test | Significance | Summary | Adjusted P Value |
|----------------------------------|--------------|---------|------------------|
| DENV1 vs. DENV2                  | Yes          | ****    | <0.0001          |
| DENV1 vs. DENV3                  | No           | ns      | 0.3278           |
| DENV1 vs. DENV4                  | Yes          | ****    | <0.0001          |
| DENV1 vs. ZIKV                   | Yes          | ****    | <0.0001          |
| DENV1 vs. JEV                    | Yes          | ****    | <0.0001          |
| DENV1 vs. YFV                    | Yes          | ****    | <0.0001          |
| DENV2 vs. DENV3                  | Yes          | ***     | 0.0002           |
| DENV2 vs. DENV4                  | No           | ns      | 0.0871           |
| DENV2 vs. ZIKV                   | No           | ns      | >0.9999          |
| DENV2 vs. JEV                    | No           | ns      | >0.9999          |
| DENV2 vs. YFV                    | No           | ns      | >0.9999          |
| DENV3 vs. DENV4                  | Yes          | ****    | <0.0001          |
| DENV3 vs. ZIKV                   | Yes          | *       | 0.0215           |
| DENV3 vs. JEV                    | Yes          | *       | 0.0103           |
| DENV3 vs. YFV                    | Yes          | ****    | <0.0001          |
| DENV4 vs. ZIKV                   | Yes          | **      | 0.0013           |
| DENV4 vs. JEV                    | Yes          | **      | 0.0031           |
| DENV4 vs. YFV                    | No           | ns      | >0.9999          |
| ZIKV vs. JEV                     | No           | ns      | >0.9999          |
| ZIKV vs. YFV                     | No           | ns      | 0.3545           |
| JEV vs. YFV                      | No           | ns      | 0.6035           |

Similar analysis was performed with the convalescent samples as well. In convalescent samples which were available, all of the samples had high neutralization against DENV1 followed by JEV and ZIKV rather than that with other serotypes of DENV.



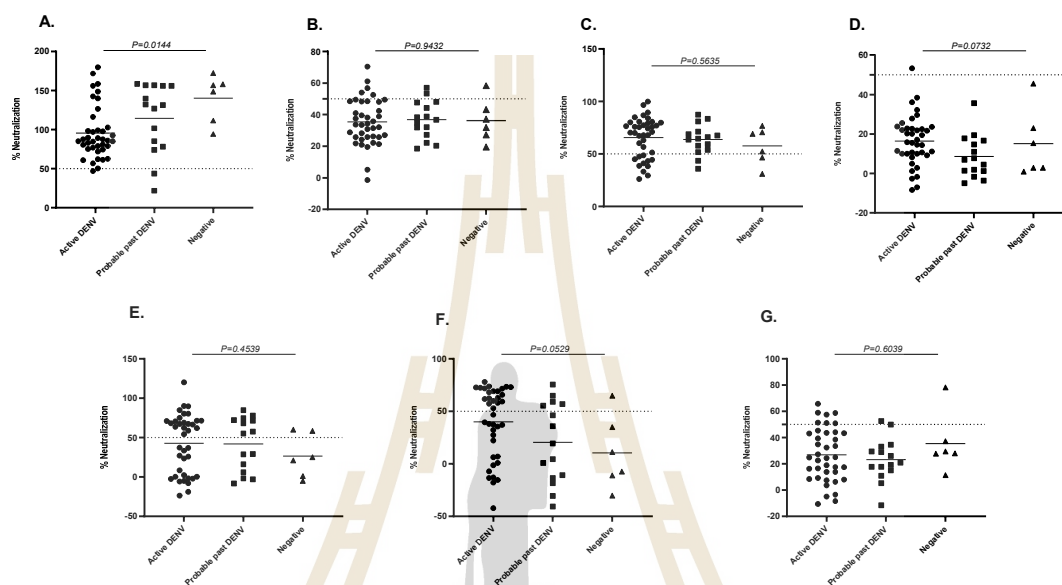
**Figure 4.3** Percentage neutralization of convalescent phase of active DENV cases against DENV1-4, ZIKV, JEV and YFV. A- 28-45 days convalescence and B. 1 year convalescence.

For the active DENV cases, we had paired 20 samples for 28-45 days convalescence and 6 after 1 year. So, it was further compared using Ordinary one-way ANOVA. Significant difference was observed in case of DENV1, 3, 4 and JEV. It could be seen that there was higher neutralization in the paired sample against DENV1 compared to other viruses. The trend was increase during first convalescent time point and then decrease during second convalescent time point (Figure 4.4).



**Figure 4.4** Comparison of neutralization activity between paired acute, convalescence 28-45 days (n=20) and 1 year (n=6). A-D: DENV1-4, E: ZIKV, F: JEV and G: YFV.

Further after the samples were classified as active DENV, probable past DENV and negative, non-parametric Kruskal-Wallis test was performed as the sample size is small. Significant difference was observed in case of DENV1 only ( $P$  value= 0.0144). Since the dominant serotype was likely DENV1 in the cohort, so the neutralizing antibody titer was higher against DENV1.



**Figure 4.5** Comparison of neutralization activity between active DENV, probable past DENV and negative samples. A-D: DENV1-4, E: ZIKV, F: JEV and G: YFV.

#### 4.4.7 Neutralization titer of the samples against DENV1

After the initial screening, it was seen that the patient samples had high percentage of neutralization against DENV1. So, the samples were further diluted from 1:80 through 1:10240 following two-fold dilution. After mixing with the virus, the final concentrations would be 1:160 to 1:20480. The serial dilution was performed for all of the 91 samples irrespective of their previous result and  $NT_{50}$  and  $NT_{90}$  titer was calculated as the inverse dilution which could neutralize the virus by 50% or 90% respectively. Some samples had higher titers than 20480. These samples need to be diluted further to get the exact  $NT_{50}$  values.

The  $NT_{50}$  values were found to be high for all of the confirmed DENV positive samples irrespective of their serotype. The titer increased even more in the samples collected after 28-45 days but were decreased after a year. In the 3 paired samples infected by DENV2 (Patient no. 2, 35 and 41), the  $NT_{50}$  values are very high and increased after convalescence. We do not have a record of severity at the

convalescence sample collection time point, so we are unable to determine the severity of the cases. Similarly in the 3 DENV4 positive samples (Patient no. 1, 4 and 15) as well, we could see high  $NT_{50}$  in both acute and convalescence state with significant increase in titer after 28-45 days. For the samples infected with DENV1, the  $NT_{50}$  values ranged from  $<80$  to  $>20480$ . However, the  $NT_{50}$  titer increased during convalescence after 28-45 days and then decreased in all of the available convalescent samples after 1 year except one sample from a 14-year-old boy (Patient no. 16) whose  $NT_{50}$  titer was highest during acute phase, decreased after 28-45 days and increased again after a year. For 14 samples, in which we could not detect the infecting serotype, we could see  $>20480$   $NT_{50}$  titer in 3 of these samples. In the available convalescent samples, we could not see significant increase or decrease in these samples. We also analyzed  $NT_{90}$  titer as it is more specific. The  $NT_{90}$  titer ranged from  $<80$  to 19539.31 following similar trend with  $NT_{50}$  values. The details of the neutralization titers for the confirmed DENV samples are shown in Table 4.6.

Similar analysis was performed on probable past DENV infection and samples which were negative as well. In only IgG positive samples ( $n=15$ ), two of the samples had  $<80$   $NT_{50}$  values. One 14-year-old boy had  $>20480$  in acute phase. However, it decreased during convalescence. For the negative samples, the titer was lesser which decreased further in two paired convalescence samples. No 1-year convalescent samples were available for analysis for these cases. The detail values have been shown in the following tables 4.7 and 4.8.



**Table 4.6** Neutralization titer for confirmed DENV samples

| Patient No. | Sub ID    | Gender | Age  | Serotype | NT50 Titer |            |         | NT90 Titer |            |         |
|-------------|-----------|--------|------|----------|------------|------------|---------|------------|------------|---------|
|             |           |        |      |          | Acute      | 28-45 days | 1 year  | Acute      | 28-45 days | 1 year  |
| 6           | SUTH/06/A | M      | 2    | DENV1    | 84.00      | 2232.61    | na      | <80        | 211.69     | na      |
| 7           | SUTH/07/A | F      | 31   | DENV1    | >20480     | 6664.33    | na      | <160       | 2983.64    | na      |
| 8           | SUTH/08/A | M      | 4    | DENV1    | 666.13     | 2255.29    | 418.77  | 178.63     | 744.47     | <160    |
| 9           | SUTH/09/A | M      | 14   | DENV1    | 1017.49    | 9496.62    | 331.33  | 214.68     | 1785.01    | <160    |
| 10          | SUTH/10/A | M      | 11   | DENV1    | 666.83     | 1452.88    | na      | 298.11     | 257.87     | na      |
| 11          | SUTH/11/A | M      | 7    | DENV1    | 160.00     | 1110.18    | na      | <160       | 264.93     | na      |
| 12          | SUTH/12/A | F      | 14   | DENV1    | <80        | 1573.21    | na      | <160       | 173.60     | na      |
| 13          | SUTH/13/A | F      | 11   | DENV1    | 273.20     | 10798.30   | 957.00  | 246.93     | 2689.57    | 445.23  |
| 14          | SUTH/14/A | M      | 8 mo | DENV1    | 620.22     | na         | na      | 237.78     | na         | na      |
| 16          | SUTH/16/A | M      | 14   | DENV1    | 9756.89    | 4412.01    | 6133.72 | 161.32     | 2102.34    | 2464.75 |
| 29          | SH/13/A   | F      | 11   | DENV1    | 294.89     | 14557.12   | na      | 46.14      | 1625.58    | na      |
| 38          | SH/22/A   | M      | 13   | DENV1    | 10071.27   | na         | na      | 4859.52    | na         | na      |
| 39          | SH/23/A   | M      | 5    | DENV1    | 676.68     | na         | na      | 224.15     | na         | na      |
| 41          | SH/25/A   | F      | 15   | DENV1    | 185.00     | na         | na      | <160       | na         | na      |
| 43          | SH/27/A   | M      | 15   | DENV1    | 196.70     | na         | na      | <160       | na         | na      |

|    |           |   |    |       |          |          |         |         |         |        |
|----|-----------|---|----|-------|----------|----------|---------|---------|---------|--------|
| 53 | SH/37/A   | F | 11 | DENV1 | 1317.42  | na       | na      | 525.89  | na      | na     |
| 57 | SH/41/A   | M | 8  | DENV1 | 344.77   | na       | na      | 185.68  | na      | na     |
| 58 | SH/42/A   | F | 8  | DENV1 | 6154.62  | na       | na      | 2086.77 | na      | na     |
| 2  | SUTH/02/A | F | 36 | DENV2 | 13593.67 | >20480   | na      | 3869.42 | 27.02   | na     |
| 45 | SH/29/A   | M | 11 | DENV2 | >20480   | >20480   | na      | 537.25  | 5130.48 | na     |
| 47 | SH/31/A   | F | 14 | DENV2 | 166.30   | 11557.34 | na      | <160    | 2168.07 | na     |
| 48 | SH/32/A   | F | 31 | DENV2 | 5332.02  | na       | na      | 3688.27 | na      | na     |
| 1  | SUTH/01/A | M | 7  | DENV4 | 3917.71  | >20480   | 2060.00 | 180.46  | 2577.54 | 990.28 |
| 4  | SUTH/04/A | M | 50 | DENV4 | 1463.13  | na       | na      | 976.51  | na      | na     |
| 15 | SUTH/15/A | F | 11 | DENV4 | 661.54   | 4526.39  | 328.62  | 235.41  | 1301.79 | <160   |
| 3  | SUTH/03/A | M | 32 | N/A   | >20480   | na       | na      | 7525.25 | na      | na     |
| 18 | SH/02/A   | M | 57 | N/A   | 322.35   | na       | na      | 121.69  | na      | na     |
| 20 | SH/04/A   | F | 14 | N/A   | 13072.48 | 11629.00 | na      | 4396.22 | 3139.80 | na     |
| 34 | SH/18/A   | F | 64 | N/A   | 1529.84  | na       | na      | 523.37  | na      | na     |
| 37 | SH/21/A   | F | 14 | N/A   | 424.87   | na       | na      | 203.52  | na      | na     |
| 40 | SH/24/A   | M | 31 | N/A   | >20480   | na       | na      | 2215.19 | na      | na     |
| 44 | SH/28/A   | M | 13 | N/A   | 864.20   | 991.47   | na      | 354.86  | 623.37  | na     |
| 46 | SH/30/A   | M | 11 | N/A   | 1349.80  | 1327.31  | na      | 719.65  | 488.16  | na     |

|    |         |   |    |     |          |          |    |          |         |    |
|----|---------|---|----|-----|----------|----------|----|----------|---------|----|
| 49 | SH/33/A | M | 31 | N/A | 7305.51  | na       | na | 3104.23  | na      | na |
| 50 | SH/34/A | F | 15 | N/A | 5635.77  | na       | na | 2551.37  | na      | na |
| 52 | SH/36/A | M | 7  | N/A | 173.60   | na       | na | 99.63    | na      | na |
| 54 | SH/38/A | M | 24 | N/A | 18976.29 | na       | na | 3091.01  | na      | na |
| 56 | SH/40/A | M | 13 | N/A | 160.80   | 256.72   | na | <160     | 26.47   | na |
| 59 | SH/43/A | M | 9  | N/A | >20480   | 18159.51 | na | 19539.31 | 6612.65 | na |

mo: months; N/A: Not amplified in PCR; na: not available for analysis

**Table 4.7** Neutralization titer for only IgG positive samples

| Patient No. | Sub ID    | Gender | Age | NT50 Titer |            | NT90 Titer |            |
|-------------|-----------|--------|-----|------------|------------|------------|------------|
|             |           |        |     | Acute      | 28-45 days | Acute      | 28-45 days |
| 5           | SUTH/05/A | M      | 5   | <80        | na         | <80        | na         |
| 17          | SH/01/A   | F      | 23  | 7002.79    | 18499.89   | 1907.77    | 3228.02    |
| 19          | SH/03/A   | F      | 26  | 880.51     | 657.40     | 396.35     | 295.30     |
| 21          | SH/05/A   | M      | 44  | 817.92     | na         | 464.39     | na         |
| 22          | SH/06/A   | M      | 49  | 1309.61    | na         | 602.80     | na         |
| 25          | SH/09/A   | F      | 28  | 579.49     | na         | 142.19     | na         |
| 26          | SH/10/A   | F      | 6   | <80        | na         | <80        | na         |

|    |         |   |    |         |          |         |         |
|----|---------|---|----|---------|----------|---------|---------|
| 27 | SH/11/A | F | 61 | 2553.52 | na       | 1380.00 | na      |
| 30 | SH/14/A | F | 33 | 1097.45 | na       | 349.88  | na      |
| 32 | SH/16/A | M | 73 | 182.84  | na       | <160    | na      |
| 33 | SH/17/A | M | 79 | 835.50  | na       | 421.43  | na      |
| 36 | SH/20/A | M | 24 | 3386.90 | na       | 1681.67 | na      |
| 42 | SH/26/A | F | 55 | 1109.11 | na       | 461.56  | na      |
| 51 | SH/35/A | M | 14 | >20480  | 12351.28 | 2532.83 | 6009.15 |
| 55 | SH/39/A | F | 24 | 169.29  | na       | <160    | na      |

na: not available for analysis

**Table 4.8** Neutralization titer for negative samples

| Patient No. | Sub ID  | Gender | Age  | NT50 Titer |            | NT90 Titer |            |
|-------------|---------|--------|------|------------|------------|------------|------------|
|             |         |        |      | Acute      | 28-45 days | Acute      | 28-45 days |
| 23          | SH/07/A | M      | 27   | 570.20     | 577.41     | 139.02     | 276.28     |
| 24          | SH/08/A | F      | 47   | 711.93     | na         | 245.25     | na         |
| 28          | SH/12/A | M      | 52   | 206.72     | na         | <160       | na         |
| 31          | SH/15/A | M      | 4 mo | 319.39     | na         | <160       | na         |
| 35          | SH/19/A | M      | 14   | 217.51     | na         | <160       | na         |
| 60          | SH/44/A | F      | 18   | 810.48     | 181.37     | 503.88     | <160       |

mo: months; na: not available for analysis



## 4.5 Discussion

DENV infections are a global health concern together with other flavivirus infections like ZIKV and JEV. Thailand being a tropical country is endemic to these flaviviruses. In this study, we have analyzed 60 acute DENV suspected DENV samples together with 25 convalescent samples collected within 28-45 days convalescence and 6 within 1 year convalescence from two hospitals of Nakhon Ratchasima province which lies in northeastern region of Thailand. Though the study cohort is small, it was diverse with the age group ranging from 4 months to 79 years. Dengue is the disease mainly affecting children and young adults in Thailand, which was similar to our cohort in which we found that more than 50% of the cohort belonged to less than 20 years. Also, there was more prevalence of secondary infections. Most hospitalized patients, even younger children, had secondary infection suggesting that more severe illness is associated with re-exposure to DENV (Lim et al., 2021).

There is co-circulation of all four DENV serotypes in nearly all regions throughout Thailand making it a hotspot for DENV evolution and transmission (Hamel et al., 2019) which is also observed in our cohort. In such a small cohort, we were able to see co-circulation of three serotypes of DENV, 1, 2 and 4, with DENV1 being the dominant serotype. However, we were able to isolate virus for only 6 samples: DENV1 (n=5) and DENV2 (n=1). Further phylogenetic analysis of the samples should be performed to know about the evolutionary details of these viruses. From a clinical standpoint, different DENV serotypes can influence the severity of illness, particularly during secondary infections. A modeling study based on longitudinal pediatric dengue data from Bangkok (1994–2014) revealed that the risk of hospitalization was highest in cases of primary DENV-2 infection followed by a secondary DENV-1 infection. In contrast, secondary infections with DENV-4 were associated with a lower overall risk of severe disease. The study also indicated that the greatest hospitalization risk occurred when there was an intermediate level of antigenic difference between the initial and subsequent infecting strains (L. Wang et al., 2024). When the viruses were too similar, the immune response from the first infection tended to offer protection against the second. Conversely, when the viruses were highly dissimilar, the immune system lacked sufficient antigenic recognition to mount an effective response. These findings imply that both cross-protection and cross-reactivity between viral strains play crucial roles in determining disease outcomes during secondary dengue infections.

A significant number of dengue infections are asymptomatic, making them difficult to detect through standard surveillance systems, which typically depend on case reporting from major hospitals (Hamins-Puertolas et al., 2024; Ribeiro Dos Santos et al., 2022). In hyperendemic regions like Thailand and Southeast Asia, dengue has traditionally been studied as a pediatric illness, resulting in limited data on asymptomatic infections among older individuals who may have experienced multiple infections. Since 2015, a longitudinal, prospective cohort-cluster study has been conducted in multigenerational households in Khamphaeng Phet, Central Thailand, to gather data on dengue exposure and transmission patterns. Early findings indicated that up to 90% of dengue infections identified in the study were subclinical, with the highest incidence of symptomatic illness occurring in individuals aged 15 to 25 (Anderson, Buddhari, et al., 2020). In addition to household studies, research into asymptomatic dengue has extended to blood donor screening. One nationwide study analyzing samples from blood centers across five regions of Thailand found that 89.1% of donors were dengue IgG positive, indicating previous infection, while 2.1% tested positive for IgM, suggesting ongoing or recent infection. As expected, IgG prevalence increased with age, reflecting cumulative exposure to different DENV serotypes over time (Limothai et al., 2024). These findings underscore the challenges of dengue surveillance in endemic settings like Thailand, where asymptomatic carriers can sustain transmission undetected. Enhanced surveillance strategies, including predictive modeling based on climatic conditions, mosquito density, and historical data, are essential. Broader population screening in high-risk areas may improve detection and control of ongoing viral transmission.

We further characterized the neutralizing antibody (nAb) response of all of the samples against DENV1-4 and other flaviviruses which include ZIKV, JEV and YFV. We could see high nAb titers against the dominant serotype DENV1 present in the cohort rather than the infecting serotype as well. Also, the titers were high against ZIKV and JEV rather than heterologous serotypes of DENV as opposed to studies by *Montoya et al., 2018* (Montoya et al., 2018). In Thailand, the incidence of JEV has decreased significantly due to vaccination and improved vector control. Thailand initiated a JEV vaccination program in 1990 using a two primary doses of mouse brain-derived vaccine (MBDV; JEVAX) in children aged 18-24 months (Sudjaritruk et al., 2022). A third dose was added in 2000, achieving 95% effectiveness (Asawapaithulsert, Ngamprasertchai, & Kitro, 2023; Sudjaritruk et al., 2022). This three-dose regimen was replaced in 2016 with

a two-dose live-attenuated vaccine either the SA14-14-2 strain (CD.JEVAX) or the chimeric JE-CV (IMOJEV) which is the prM and E proteins of SA14-14-2 integrated into the yellow fever 17D virus genome (Chotpitayasunondh, Suntarattiwong, & Yoksan, 2024). The first dose is administered at 9-12 months and the second dose approximately a year later. Hence, this may be the reason that our study cohort consisted of high neutralizing antibody response against JEV. Similarly, ZIKV infection in Thailand dates back to the 1950s through serological surveys, with ZIKV circulating at sustained levels likely from 2002 (Ruchusatsawat et al., 2019). ZIKV IgG prevalence in Thailand held steady at roughly 25-27% in young adults enrolled in a large clinical trial throughout the country during 1997-2017 (Choyrum et al., 2022). Although Thailand experienced ZIKV outbreaks between 2016 and 2018 concurrent with those in the Americas, the overall antibody prevalence remained lower than in Central and South America (Saba Villarroel et al., 2024). A study from January 2016 to December 2017, identified 368 ZIKV infections out of 1,717 individuals tested, with clustered cases within districts suggesting sustained local transmission and a shift towards endemic circulation (Ruchusatsawat et al., 2019). A study from Nakhon Ratchasima confirmed co-circulation of ZIKV in this region and concluded that residents of DENV-endemic regions with repeated exposure to DENV have higher titers of nAbs against ZIKV than individuals with only a single DENV exposure (Sararat Hattakam, Annie Elong Ngono, Melanie McCauley, Sujun Shrestha, & Montarop Yamabhai, 2021) which further supports our finding of higher neutralizing antibodies against ZIKV in our study cohort. The primary reason for the presence of neutralizing antibodies against YFV in patients from Thailand which is a non-endemic country for YFV may be due to the antigenic cross-reactivity between YFV and DENV, as both belong to the *Flavivirus* genus. All flaviviruses are antigenically related and can induce broadly flavivirus cross-reactive antibodies (Monath, 2007).

Immunological cross-reactivity among flaviviruses is a well-documented phenomenon that complicates serological diagnosis, particularly in countries like Thailand where DENV, JEV, and ZIKV co-circulate (Hou et al., 2022). One study analyzing 147 serum samples from healthy donors across four Thai provinces, Nakhon Sawan and Uthai Thani in the north, Rayong in the central region, and Phuket in the south—reported that 70% of the samples exhibited neutralizing activity against multiple flaviviruses, including DENV, JEV, and ZIKV (Yamanaka et al., 2021). However, the study did not specify whether the detected neutralizing antibodies resulted from

natural infection or prior vaccination. Another serological investigation conducted on adult ZIKV cases in Nonthaburi, Central Thailand, between December 2016 and September 2018, revealed cross-reactivity with anti-DENV antibodies: 25% of the samples were positive for IgM and 12.5% for IgG (Wongsawat et al., 2021). Interestingly, pediatric ZIKV cases in the same study showed no evidence of such cross-reactivity, likely reflecting their limited exposure to multiple flavivirus infections due to younger age.

Multiple studies have demonstrated a link between the presence of cross-reactive antibodies and potential protection against other flaviviruses. Research involving asymptomatic individuals from a DENV-endemic region in Thailand showed that a significant portion of participants possessed antibodies capable of neutralizing both ZIKV and at least one DENV serotype (Sararat Hattakam et al., 2021). Importantly, repeated exposure to DENV was correlated with higher neutralizing antibody titers and broader cross-reactivity against ZIKV. Similarly, a seroprevalence study among healthy adolescents in Chiang Mai, Northern Thailand, found that prior DENV infection was strongly associated with protective immunity against JEV (Sudjaritruk et al., 2022). On the other hand, widespread JEV vaccination among Thai children may contribute to the observed lower rates of primary DENV infections in this population compared to children of similar age in Ecuador (Anderson, Stewart-Ibarra, et al., 2020). These findings highlight the need for further research to distinguish the effects of natural infection versus vaccination on cross-reactive immune responses, and to determine whether initial infections with DENV, JEV, or ZIKV offer protective immunity against subsequent flaviviral infections.

The  $NT_{50}$  response against DENV1 was calculated further and we could see higher  $NT_{50}$  values against DENV1 for almost all of the samples against DENV1 irrespective of the serotype they were infected. Higher  $NT_{50}$  titers were observed in convalescent samples as well which decreased after a year.  $NT_{90}$  titer against these samples were also calculated against DENV1. While  $NT_{50}$  is considered as standard for neutralization, higher threshold like  $NT_{90}$  could improve the specificity for the samples from a country like Thailand where there is co-circulation of multiple flaviviruses (WHO, 2007). Further experiments need to be performed against other serotypes of DENV, ZIKV, JEV and YFV to determine the neutralization titers and both

NT<sub>50</sub> and NT<sub>90</sub> values can be considered to set up the threshold for positivity of these samples.

Compared with Nepal study, Thailand study had patients from more diverse age group with higher number of cases in children. Also, the IgG seroprevalence was higher in Thailand and more cases of secondary infection. In future, genome sequencing of the virus from Thailand could help more to understand the evolution of virus sequences from Thailand and we could compare with Nepal genome sequences to understand their phylogenetic similarity.

In conclusion, in this study we were able to collect 60 acute samples and then tried to collect convalescence samples as well. We were able to collect 25 (28-45 days) and 6 (1 year) convalescent samples. The samples were further characterized based on ELISA and PCR results. Further neutralizing activity of the patient samples were performed against DENV1-4, ZIKV, JEV and YFV. Hence, from this study even with small sample size, we were able to see co-circulation of 3 different serotypes of DENV with varied neutralizing antibody response against DENV1-4, ZIKV, JEV and YFV. These flaviviruses are all endemic in Thailand, and their incidence has been on the rise in recent years, largely driven by factors such as climate change and increasing urbanization. While vaccination campaigns have successfully reduced the number of JEV cases, the uptake of dengue vaccines in Thailand remains limited, particularly in the context of growing vaccine hesitancy. Simultaneous outbreaks of these diseases place additional strain on already burdened healthcare systems, potentially undermining their future capacity for effective surveillance, diagnosis, and care. To strengthen public health response, increased investment in affordable diagnostic tools is essential, alongside further research into flavivirus cross-reactivity to guide more effective vaccination strategies.



## 4.6 References

- WHO (2009). In Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. Geneva.
- WHO (2007). Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses (Vol. WHO/IVB/07.07).
- Anderson, K. B., Buddhari, D., Srikiatkachorn, A., Gromowski, G. D., Iamsirithaworn, S., Weg, A. L., . . . Endy, T. P. (2020). An Innovative, Prospective, Hybrid Cohort-Cluster Study Design to Characterize Dengue Virus Transmission in Multigenerational Households in Kamphaeng Phet, Thailand. *Am J Epidemiol*, 189(7), 648-659. doi:10.1093/aje/kwaa008
- Anderson, K. B., Stewart-Ibarra, A. M., Buddhari, D., Beltran Ayala, E. F., Sippy, R. J., Iamsirithaworn, S., . . . Endy, T. P. (2020). Key Findings and Comparisons From Analogous Case-Cluster Studies for Dengue Virus Infection Conducted in Machala, Ecuador, and Kamphaeng Phet, Thailand. *Front Public Health*, 8, 2. doi:10.3389/fpubh.2020.00002
- Asawapaithulsert, P., Ngamprasertchai, T., & Kitro, A. (2023). Japanese Encephalitis Vaccine Acceptance and Strategies for Travelers: Insights from a Scoping Review and Practitioners in Endemic Countries. *Vaccines (Basel)*, 11(11). doi:10.3390/vaccines11111683
- Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., . . . Hay, S. I. (2013). The global distribution and burden of dengue. *Nature*, 496(7446), 504-507. doi:10.1038/nature12060
- Biswal, S., Borja-Tabora, C., Martinez Vargas, L., Velasquez, H., Theresa Alera, M., Sierra, V., . . . group, T. s. (2020). Efficacy of a tetravalent dengue vaccine in healthy children aged 4-16 years: a randomised, placebo-controlled, phase 3 trial. *Lancet*, 395(10234), 1423-1433. doi:10.1016/S0140-6736(20)30414-1
- Chong, H. Y., Leow, C. Y., Abdul Majeed, A. B., & Leow, C. H. (2019). Flavivirus infection-A review of immunopathogenesis, immunological response, and immunodiagnosis. *Virus Res*, 274, 197770. doi:10.1016/j.virusres.2019.197770
- Chotpitayasunondh, T., Suntarattiwong, P., & Yoksan, S. (2024). Long-term immunogenicity of the SA14-14-2 Japanese encephalitis (JE) vaccine (CD.JEVAX(R)) booster following chimeric JE (IMOJEV(R)) vaccine priming in Thai children. *Hum Vaccin Immunother*, 20(1), 2407663. doi:10.1080/21645515.2024.2407663
- Choyrum, S., Wangsaeng, N., Nechba, A., Salvadori, N., Saisom, R., Achalapong, J., . . . Khamduang, W. (2022). Zika Virus Immunoglobulin G Seroprevalence among

- Young Adults Living with HIV or without HIV in Thailand from 1997 to 2017. *Viruses*, 14(2). doi:10.3390/v14020368
- Guzman, M. G., & Harris, E. (2015). Dengue. *Lancet*, 385(9966), 453-465. doi:10.1016/S0140-6736(14)60572-9
- Haga, K., Chen, Z. N., Himeno, M., Majima, R., & Moi, M. L. (2023). Utility of an In-Vitro Micro-Neutralizing Test in Comparison to a Plaque Reduction Neutralization Test for Dengue Virus, Japanese Encephalitis Virus, and Zika Virus Serology and Drug Screening. *Pathogens*, 13(1). doi:10.3390/pathogens13010008
- Halstead, S. B. (2014). Dengue Antibody-Dependent Enhancement: Knowns and Unknowns. *Microbiol Spectr*, 2(6). doi:10.1128/microbiolspec.AID-0022-2014
- Hamel, R., Surasombatpattana, P., Wichit, S., Dauve, A., Donato, C., Pompon, J., . . . Misse, D. (2019). Phylogenetic analysis revealed the co-circulation of four dengue virus serotypes in Southern Thailand. *PLoS One*, 14(8), e0221179. doi:10.1371/journal.pone.0221179
- Hamins-Puertolas, M., Buddhari, D., Salje, H., Cummings, D. A. T., Fernandez, S., Farmer, A., . . . Anderson, K. B. (2024). Household immunity and individual risk of infection with dengue virus in a prospective, longitudinal cohort study. *Nat Microbiol*, 9(1), 274-283. doi:10.1038/s41564-023-01543-3
- Hattakam, S., Elong Ngono, A., McCauley, M., Shresta, S., & Yamabhai, M. (2021). Repeated exposure to dengue virus elicits robust cross neutralizing antibodies against Zika virus in residents of Northeastern Thailand. *Scientific Reports*, 11(1), 1-10.
- Hou, B., Chen, H., Gao, N., & An, J. (2022). Cross-Reactive Immunity among Five Medically Important Mosquito-Borne Flaviviruses Related to Human Diseases. *Viruses*, 14(6). doi:10.3390/v14061213
- Huang, A. T., Takahashi, S., Salje, H., Wang, L., Garcia-Carreras, B., Anderson, K., . . . Cummings, D. A. T. (2022). Assessing the role of multiple mechanisms increasing the age of dengue cases in Thailand. *Proc Natl Acad Sci U S A*, 119(20), e2115790119. doi:10.1073/pnas.2115790119
- Ito, M., Takasaki, T., Yamada, K., Nerome, R., Tajima, S., & Kurane, I. (2004). Development and evaluation of fluorogenic TaqMan reverse transcriptase PCR assays for detection of dengue virus types 1 to 4. *J Clin Microbiol*, 42(12), 5935-5937. doi:10.1128/JCM.42.12.5935-5937.2004
- Jain, S., Vimal, N., Angmo, N., Sengupta, M., & Thangaraj, S. (2023). Dengue Vaccination: Towards a New Dawn of Curbing Dengue Infection. *Immunol Invest*, 52(8), 1096-1149. doi:10.1080/08820139.2023.2280698

- Jiravejchakul, N., Chan-In, W., Thuncharoen, W., Thailand, D., Sunnak, W., Charoensawan, V., . . . Matangkasombut, P. (2025). Cytokine and chemokine kinetics in natural human dengue infection as predictors of disease outcome. *Sci Rep*, 15(1), 15612. doi:10.1038/s41598-025-99628-y
- Lim, J. K., Chanthavanich, P., Limkittikul, K., Lee, J. S., Sirivichayakul, C., Lee, K. S., . . . Hattasingh, W. (2021). Clinical and epidemiologic characteristics associated with dengue fever in 2011-2016 in Bang Phae district, Ratchaburi province, Thailand. *PLoS Negl Trop Dis*, 15(6), e0009513. doi:10.1371/journal.pntd.0009513
- Limothai, U., Tachaboon, S., Dinhuzen, J., Singh, J., Leewongworasingh, A., Watanaboonyongcharoen, P., . . . Srisawat, N. (2024). Dengue virus transmission risk in blood donation: Evidence from Thailand. *J Med Virol*, 96(6), e29689. doi:10.1002/jmv.29689
- Maksikharin, A., Prommalikit, O., & Thisyakorn, U. (2023). The Surveillance of Acute Encephalitis Syndrome in Thailand, 2003-2019: A Perspective for Prevention and Control. *WHO South East Asia J Public Health*, 12(1), 45-50. doi:10.4103/WHO-SEAPPH.WHO-SEAPPH\_92\_22
- Monath, T. P. (2007). Dengue and yellow fever--challenges for the development and use of vaccines. *N Engl J Med*, 357(22), 2222-2225. doi:10.1056/NEJMp0707161
- Montoya, M., Collins, M., Dejnirattisai, W., Katzelnick, L. C., Puerta-Guardo, H., Jadi, R., . . . Harris, E. (2018). Longitudinal Analysis of Antibody Cross-neutralization Following Zika Virus and Dengue Virus Infection in Asia and the Americas. *J Infect Dis*, 218(4), 536-545. doi:10.1093/infdis/jiy164
- Perez, L. J., Yamaguchi, J., Weiss, S., Carlos, C., Meyer, T. V., Rodgers, M. A., . . . Berg, M. G. (2025). Climate, inter-serotype competition and arboviral interactions shape dengue dynamics in Thailand. *Commun Biol*, 8(1), 601. doi:10.1038/s42003-025-07999-9
- Pierson, T. C., & Diamond, M. S. (2020). The continued threat of emerging flaviviruses. *Nature microbiology*, 5(6), 796-812. doi:10.1038/s41564-020-0714-0
- Ribeiro Dos Santos, G., Buddhari, D., Iamsirithaworn, S., Khampaen, D., Ponlawat, A., Fansiri, T., . . . Anderson, K. B. (2022). Individual, Household, and Community Drivers of Dengue Virus Infection Risk in Kamphaeng Phet Province, Thailand. *J Infect Dis*, 226(8), 1348-1356. doi:10.1093/infdis/jiac177
- Rivera, L., Biswal, S., Saez-Llorens, X., Reynales, H., Lopez-Medina, E., Borja-Tabora, C., . . . Borkowski, A. (2022). Three-year Efficacy and Safety of Takeda's Dengue Vaccine Candidate (TAK-003). *Clin Infect Dis*, 75(1), 107-117. doi:10.1093/cid/ciab864

- Ruchusatsawat, K., Wongjaroen, P., Posanacharoen, A., Rodriguez-Barraquer, I., Sangkitporn, S., Cummings, D. A. T., & Salje, H. (2019). Long-term circulation of Zika virus in Thailand: an observational study. *Lancet Infect Dis*, 19(4), 439-446. doi:10.1016/S1473-3099(18)30718-7
- Saba Villarroel, P. M., Hamel, R., Gumpangseth, N., Yainoy, S., Koomhin, P., Misse, D., & Wichit, S. (2024). Global seroprevalence of Zika virus in asymptomatic individuals: A systematic review. *PLoS Negl Trop Dis*, 18(4), e0011842. doi:10.1371/journal.pntd.0011842
- Sridhar, S., Luedtke, A., Langevin, E., Zhu, M., Bonaparte, M., Machabert, T., . . . DiazGranados, C. A. (2018). Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy. *N Engl J Med*, 379(4), 327-340. doi:10.1056/NEJMoa1800820
- Sudjaritruk, T., Kaewpoowat, Q., Prasarakkee, C., Sarachai, S., Taurel, A. F., Sricharoen, N., . . . Yoksan, S. (2022). Seroepidemiological study of Japanese encephalitis virus in Chiang Mai: Immunity and susceptibility 28 years after introduction of a vaccination programme. *PLoS Negl Trop Dis*, 16(8), e0010674. doi:10.1371/journal.pntd.0010674
- Tully, D., & Griffiths, C. L. (2021). Dengvaxia: the world's first vaccine for prevention of secondary dengue. *Ther Adv Vaccines Immunother*, 9, 25151355211015839. doi:10.1177/25151355211015839
- Wang, L., Huang, A. T., Katzelnick, L. C., Lefrancq, N., Escoto, A. C., Duret, L., . . . Salje, H. (2024). Antigenic distance between primary and secondary dengue infections correlates with disease risk. *Sci Transl Med*, 16(744), eadk3259. doi:10.1126/scitranslmed.adk3259
- Wongsawat, J., Suttha, P., Chanama, S., Srisopa, S., Yonchoho, N., & Limpanadusadee, P. (2021). Serological Differences after Acute Zika Virus Infections between Children and Adults: Implication for Use of a Serological Test. *Am J Trop Med Hyg*, 105(4), 931-935. doi:10.4269/ajtmh.21-0134
- Yamanaka, A., Matsuda, M., Okabayashi, T., Pitaksajakul, P., Ramasoota, P., Saito, K., . . . Suzuki, R. (2021). Seroprevalence of Flavivirus Neutralizing Antibodies in Thailand by High-Throughput Neutralization Assay: Endemic Circulation of Zika Virus before 2012. *mSphere*, 6(4), e0033921. doi:10.1128/mSphere.00339-21

## CHAPTER V

### APPLICATION OF PHAGE DISPLAY TECHNOLOGY FOR RESEARCH ON DENGUE VIRUS

#### 5.1 Abstract

Dengue virus (DENV) poses a significant global health threat and is responsible for a wide spectrum of diseases ranging from mild dengue fever to severe dengue hemorrhagic fever and dengue shock syndrome. Till date, there are critical challenges in dengue research for the development of broadly neutralizing antibodies and vaccines effective across all four serotypes (DENV-1 to DENV-4). This study explores the application of phage display technology, utilizing a consensus envelope domain III (cEDIII) as a target, for advancing DENV research. The envelope (E) protein of DENV, particularly domain III of the envelope protein (EDIII), plays a significant role in virus-host interaction and is a key target for neutralizing antibodies. Hence, cEDIII representing conserved elements across all serotypes, can serve as an effective immunogen or screening bait to discover neutralizing antibodies using scFv phage display libraries. We successfully isolated four distinct IgG antibodies that exhibited cross-reactivity and demonstrated the ability to neutralize all four dengue virus serotypes (DENV-1 through DENV-4) to varying degrees. This approach holds promise for developing broadly neutralizing antibodies, novel antiviral drugs, and improved diagnostic tools. Furthermore, phage display can be employed for epitope mapping and vaccine development, contributing to a deeper understanding of DENV pathogenesis and immunity. Altogether, this research highlights the versatility of phage display technology in targeting cEDIII for comprehensive DENV research and the development of effective countermeasures.

#### 5.2 Introduction

Dengue virus (DENV) causing dengue fever refers to a group of four genetically and antigenically related viruses, DENV 1-4, that are known as serotypes are further classified into genotypes based on the lack of cross-neutralization by antibodies generated against individual strains (Kyle & Harris, 2008). Infection by any of the four



serotypes can result in a range of clinical manifestations for which the timing or sequence of infections can be an important determinant of disease severity and course.

Until now, there are no vaccines against DENV that can provide long durable protection against all four DENV serotypes and can be given to people of all ages irrespective their immune status against DENV which has been already described in previous chapters as well. Therefore, the search for serological biomarkers through monoclonal antibodies (mAbs) that identify unique viral regions can assist in the differential detection, whereas the development of recombinant antibodies with a neutralizing potential can lead to the establishment of efficacious treatments.

Phage display is a powerful tool to generate antibodies and peptides. The main principle of this technique is the presentation of libraries of molecules on the surface of a bacteriophage (phage), allowing the identification of a wide range of biomolecules, including peptides, antibodies, and other proteins (Clackson, Hoogenboom, Griffiths, & Winter, 1991). This technology is attractive because of the ability to rapidly select a molecule with the desired phenotype from a vast and diverse library repertoire. Antibody phage display is possible to generate antibodies against low immunogenic targets, avoid animal immunization, and generate non-immunogenic human antibodies for therapy (Marks et al., 1991).

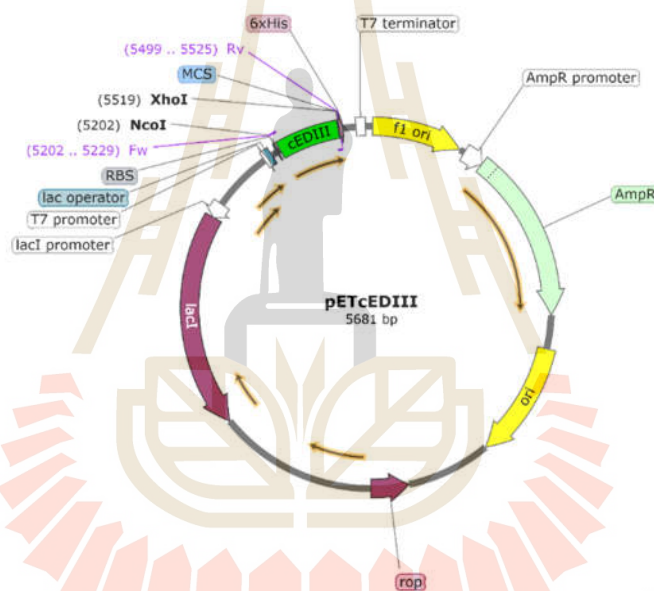
Among the structural proteins of DENV, the envelope protein (E protein) plays critical roles in virus attachment by binding to host cell receptors, fusion and then entry to host cells and is also the most immunogenic (Modis et al., 2004). Hence, from the perspective of vaccine design or neutralizing antibody, the envelope protein (E protein) of DENV is considered as an important antigen and is used even as a reagent for diagnostic purposes. E protein is a large and cysteine-rich protein that consists of three structurally distinct domains (I, II, and III). Domain I forms the structural domain, domain II links structural and binding domain and domain III is the major binding domain of the virus (Modis, Ogata, Clements, & Harrison, 2003). Domain III (EDIII) contains type specific neutralization sites unlike domain I and II, hence induces a type specific neutralizing response with a low potential for inducing cross-reactive antibodies and thereby potentially avoiding Antibody Dependent Enhancement. A consensus dengue virus envelope protein domain III (cEDIII) was also developed in *E. coli* host system that could produce recombinant proteins inducing cross-neutralizing antibodies and memory immunity against all 4 serotypes of DENV (H. W. Chen et al., 2013; H. J. Huang et al., 2022; Leng et al., 2009). Therefore, cEDIII represents as an attractive target

for the development of neutralizing DENV antibodies using phage display technology. In this study, we use cEDIII to produce recombinant monoclonal antibodies against DENV using phage display technology and further characterize its properties.

## 5.3 Material and Methods

### 5.3.1 cEDIII production

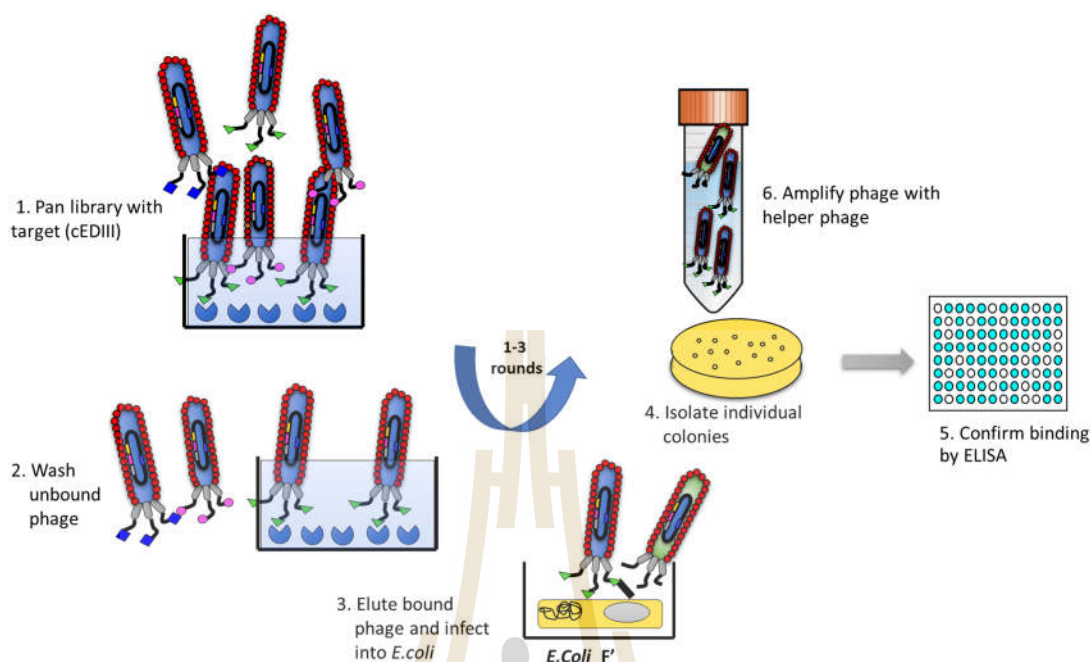
The target protein, consensus envelope domain III (cEDIII) was performed as described previously (Leng et al., 2009). Briefly, the cEDIII gene was cloned into the pET-21d (+) vector and expressed in *Escherichia coli*. After isopropylthiogalactoside (IPTG) induction, the recombinant cEDIII was purified with immobilized metal affinity chromatography (IMAC) columns.



**Figure 5.1** Cloning Consensus Envelope Domain III (cEDIII) in pET21d vector for expression in *E. coli*

### 5.3.2 Affinity selection

Selection of phage particles displaying specific scFvs were performed on immuno tubes (Nunc, Denmark) by bio-panning from a human phage display scFv library Yamo I (naive to dengue) (Potjamas Pansri, Nanthanit Jaruseranee, Kuntalee Rangnoi, Peter Kristensen, & Montarop Yamabhai, 2009) and Yamo II (dengue immune library constructed from people having neutralizing antibodies against dengue; unpublished data) produced in our laboratory using varied concentrations of cEDIII for different rounds of panning as previously described (Potjamas Pansri et al., 2009).



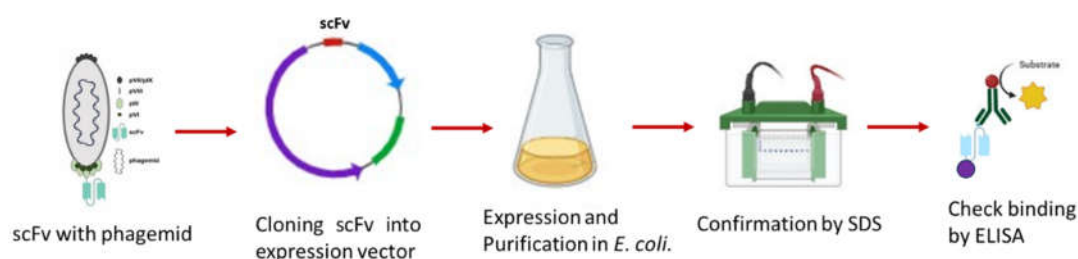
**Figure 5.2** Representation of a biopanning process using Phage display antibody library

### 5.3.3 Affinity determination by ELISA

The 96-well plate (F96 maxisorp, Nunc plate; Cat No. 442404) was coated with cEDIII protein in coating buffer and left overnight at 4°C. Non-specific binding was prevented by incubating the wells with 3% MPBS at room temperature for 2 hours. Following this, the wells were washed three times with PBS. Subsequently, 150  $\mu\text{L}$  of supernatant obtained from phage rescue of selected single colonies was added to each well, along with 50  $\mu\text{L}$  of 3% MPBS. The mixture was incubated at room temperature for another 2 hours to allow binding. After incubation, the wells were washed three times with PBS-T, followed by two washes with PBS. To detect bound phages, the secondary antibody HisProbe-HRP (ThermoFisher, Cat No. 15165) was applied. Then, 200  $\mu\text{L}$  of ABTS substrate (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; ABTS® BioChemica, A1088, 0010) in citric acid was added to each well. The plates were incubated at room temperature for 1 hour, with absorbance readings taken every 15 minutes. Absorbance was measured at 405 nm in an ELISA plate reader (Sunrise Microplate Reader). After initial screening, binding phage clones are again amplified in 5ml of culture media and then checked again by ELISA in same way.

#### 5.3.4 scFv expression and purification

The plasmid DNA of the binding clones were sequenced and full length scFvs were selected for expression. Selected scFvs were cloned into pET21d+ vector for plasmid extraction and then transformed into expression host *E. coli* (T7 SHuffle B cell). The transformed bacterial cells were cultured on LB agar plates supplemented with 100 µg/ml ampicillin and incubated overnight at 30 °C. Individual colonies were then selected to initiate overnight cultures. These cultures were diluted 1:100 into 200 ml of fresh LB medium containing ampicillin and incubated at 30 °C with shaking until the OD<sub>600</sub> reached between 0.8 and 0.9. IPTG was subsequently added to a final concentration of 0.4–0.8 mM to induce protein expression, and the cultures were further incubated at 25 °C with shaking for 20 hours. Cells were harvested by centrifugation at 4000 g for 30 minutes, and the supernatant was discarded. The resulting cell pellet was resuspended in binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.4) containing 1 mg/ml lysozyme and incubated on ice for 30 minutes. Cell lysis was performed by sonicating the suspension for 10 minutes (pulse and break 30 seconds each) on ice. The cell lysate was centrifuged at 10000 g for 30 minutes, 4 °C. Cleared supernatant was moved to the gravity flow chromatography containing 1 ml of the immobilized metal affinity chromatography (IMAC) resin (HisPur Ni-NTA RESIN, Gibthai) and incubated for 2 hours with rotating at 4°C. Then the column was placed on a stand and the flow was poured out by gravity and washed 3 times with washing buffer (20 mM of Tris-HCl, 500 mM of NaCl, 60 mM Imidazole, pH 7.4) and eluted with 5 column volumes of elution buffer (20 mM of Tris-HCl, 500 mM of NaCl, 250 mM Imidazole, pH 7.4). Every fraction was collected for SDS-PAGE analysis. After that elutes containing scFv were pooled and exchanged in PBS and concentration was measured by Nanodrop. The binding of scFvs to cEDIII and all serotypes of DENV were performed by ELISA.



**Figure 5.3** Representative steps for scFv expression, purification and binding analysis

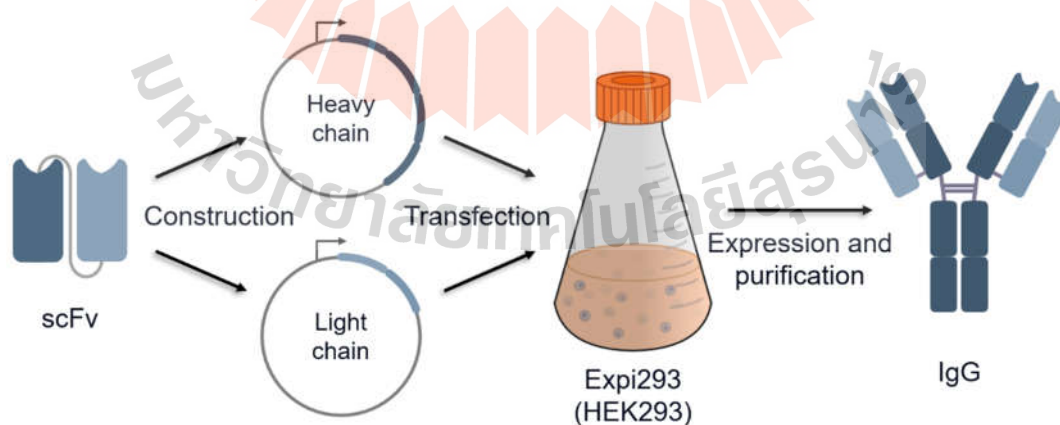
### 5.3.5 Expression of recombinant full-length anti-human monoclonal IgGs antibody and purification

Immunoglobulin G (IgG) is the most widely used format for therapeutic applications. Converting single-chain variable fragment (scFv) antibodies into full-length IgG can preserve or even enhance their antigen-binding capabilities. Therefore, selected scFv clones exhibiting strong neutralizing activity were reformatted into IgG. The phagemid vectors carrying the scFv genes encoding the VH and VL regions were amplified using PCR. These PCR products were then inserted into antibody expression plasmids (pTT28) containing the constant regions of the heavy and light chains, respectively. The resulting plasmids were co-transfected into Expi293 cells using the Expi293™ Expression System using serum free media, leading to the secretion of full-length recombinant IgG into the cell culture supernatant. Following transfection, the culture was centrifuged to collect the IgG-containing supernatant. Human IgG was subsequently purified using a HiTrap MabSelect Prisma protein A column (Cytiva). The column was first equilibrated and washed with a binding buffer composed of 20 mM sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ) and 0.15 M NaCl at pH 7.2. The antibody-containing supernatant, mixed with binding buffer, was loaded onto the column. After washing with 10 ml of binding buffer, the IgG was eluted using 0.1 M citric acid at pH 3.0. Eluted fractions were immediately neutralized with Tris-HCl buffer (pH 9.0), then buffer-exchanged to PBS. Purified IgG samples were stored at 4°C until further use.



**Table 5.1** Primers for construction of IgG vectors

| Primer    | Clones | Sequence                                |
|-----------|--------|-----------------------------------------|
| IgG1-HC-F | 1D1    | CTGTGCGCTAGCGAGGTGCAGCTGGTGCAG          |
|           | YE11   | 5'-GCCGCTAGCCAGGTGCAGCTGGTGCAGTC-3'     |
|           | YG1    | 5'-GCCGCTAGCCAGGTGCAGCTGGTGCAGTC-3'     |
|           | YH1    | 5'-CTGTGCGCTAGCGAGGTGCAGCTGCAGGAG-3'    |
| IgG1-HC-R | 1D1    | 5'-ACCGCTAGCTGAGGAGACGGTGACCAGGG-3'     |
|           | YE11   | 5'-ACCGCTAGCTGAGGAGACGGTGACCAGGG-3'     |
|           | YG1    | 5'-GCACAGGCTAGCTGAAGAGACGGTGACCATTG-3'  |
|           | YH1    | 5'-ACCGCTAGCTGAGGAGACGGTGACCAGGG-3'     |
| IgG1-LC-F | 1D1    | 5'-CTGTGCGCTAGCTCCTATGTGCTGACTCAGC-3'   |
|           | YE11   | 5'-CTGTGCGCTAGCTCCTATGTGCTGACTCAGC-3'   |
|           | YG1    | 5'-GGTGCTAGCTCTTCTGAGCTGACTCAGGACCC-3'  |
|           | YH1    | 5'-CTGTGCGCTAGCCACGTTATACTGACTCAACCG-3' |
| IgG1-LC-R | 1D1    | 5'-GCACAGCGTACGTAAAACGGTGAGCTGGGTC-3'   |
|           | YE11   | 5'-GCACGTACGTAGGACGGTCAGCTTGGTC-3'      |
|           | YG1    | 5'-GCACGTACGTAGGACGGTCAGCTTGGTC-3'      |
|           | YH1    | 5'-GCACGTACGTAGGACGGTCAGCTTGGTC-3'      |

**Figure 5.4** Representative figure for IgG expression

### 5.3.6 Affinity determination by ELISA

To assess the binding of human monoclonal antibodies (mAbs) from the expressed clones to DENV1-4, ELISA was conducted using cEDIII and whole virus coated onto plates in coating buffer (0.1 M  $\text{NaHCO}_3$ ) and incubated overnight at 4°C. The plates were then blocked with 3% skimmed milk in PBS for 2 hours. Following washing, antibody samples diluted in PBS were added and incubated for 1 hour. After another washing step with wash buffer, Peroxidase AffiniPure™ F(ab')<sub>2</sub> Fragment Goat Anti-Human IgG (H+L) was added as the secondary antibody. The binding reaction was visualized using ABTS substrate, and absorbance was measured at 405 nm using an ELISA plate reader.

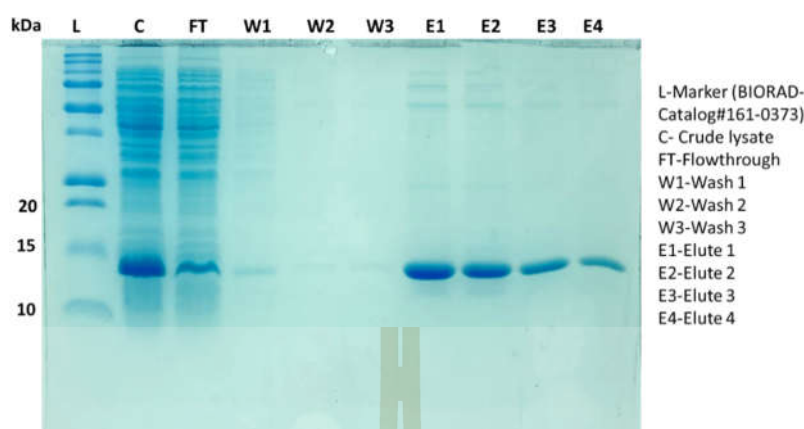
### 5.3.7 Neutralization test using Microneutralization Assay (MNT)

The Vero cells were cultured and seeded in 96 well plate and incubated overnight to get 80-100% confluency. The recombinant antibodies were serially diluted (2.5 mg/ml) in two-fold dilution and mixed with virus at a 1:1 ratio and incubated at 37°C. The resulting mixture was added to Vero cells seeded in 96-well plates. Following a 1-hour adsorption period, the plates were incubated at 37°C for 5 days. Cells were then fixed with methanol (Wako Pure Chemical Industries) for 10 minutes at room temperature and stained with 0.1% crystal violet (Wako Pure Chemical Industries) for 15 minutes. After washing off the excess stain, 1% SDS was used to dissolve the cells. Absorbance was measured at a wavelength of 595 nm. Then percentage neutralization was calculated for each of the antibodies against DENV1-4 (Haga et al., 2023). Titration of the viruses was performed same as described in Chapter IV.

## 5.4 Results

### 5.4.1 Expression and Purification of target protein

Target protein (cEDIII) was expressed in bacterial expression system. SDS-PAGE was performed to visualize and confirm the expression of the protein and expected band size (~15KD) was observed (Figure 5.5).



**Figure 5.5** SDS-PAGE (16%) for consensus envelope domain III (cEDIII) after purification

#### 5.4.2 Affinity selection of cEDIII and Immunogenetic analysis

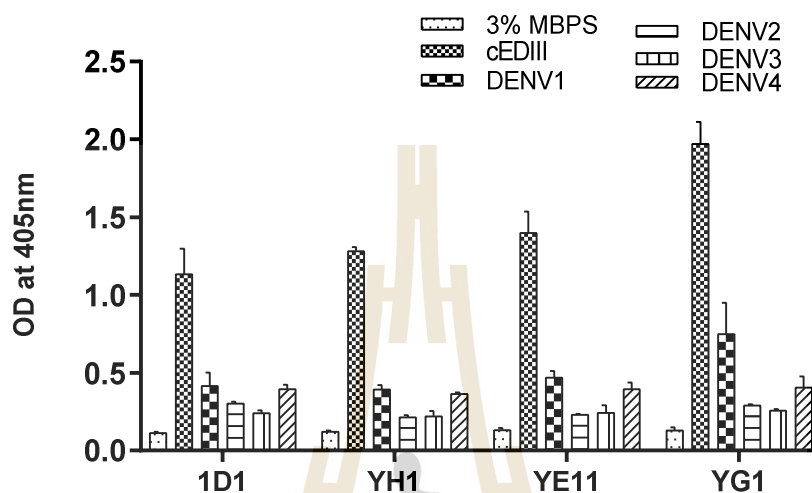
Biopanning was performed against cEDIII to get binding clones. More than 100 of clones were selected randomly for ELISA to check the binding. The binding clones are further grown in 5ml media for confirmation and checked again by ELISA. In the end, 8 clones from Yamo I and 7 clones from Yamo II showing high binding against cEDIII were selected and sent for sequencing. After sequencing, from Yamo I, 6 clones had same sequence. So, total of 3 clones (YE11, YG1 and YH1) were selected for further process. In case of Yamo II, only 1 clone (1D1) was selected for further process because rest had stop codons.

We further analyzed the sequences of 4 mAbs using the IMGT tool ([https://www.imgt.org/IMGT\\_vquest/input](https://www.imgt.org/IMGT_vquest/input)) to identify their closest VH and VL germline genes. The results showed that these antibodies originated from different B-cell lineages (Table 5.2).

**Table 5.2** Analysis of V-gene germline family

| YAMO Library                     |                 |          |            |          |                         |                 |          |             |             |                 |
|----------------------------------|-----------------|----------|------------|----------|-------------------------|-----------------|----------|-------------|-------------|-----------------|
| Clones                           | V-Gene germline | Identity | CDR1       | CDR2     | CDR3                    | V-Gene germline | Identity | CDR1        | CDR2        | CDR3            |
| VH                               |                 |          |            |          |                         | VL              |          |             |             |                 |
| Y5_1F10,1A1,1E11,1G5,1E12 (YE11) | IGHV1-24*01     | 85.76%   | GATVTELS   | FDHEQDEI | VTGDSFDY                | IGLV1-47*01     | 98.60%   | SSNIGSNY    | RNN         | AAWDDSLSGVV     |
| YG1                              | IGHV1-69*01     | 69.10%   | SGDFSSSA   | IIPMSGRT | AGGKQEARTIPQ<br>IYNWFDP | IGLV3-19*01     | 93.91%   | SLRNY<br>Y  | GDN         | NSRDNSGDI<br>LL |
| YH1                              | IGHV4-39*01     | 85.22%   | GGILRDSRYN | IHASGGT  | GRQGIFTGMIDS            | IGLV4-69*01     | 79.93%   | SGRGL<br>NG | INSDG<br>TF | QPWDNGN<br>VV   |
| Dengue Library                   |                 |          |            |          |                         |                 |          |             |             |                 |
| Clones                           | V-Gene germline | Identity | CDR1       | CDR2     | CDR3                    | V-Gene germline | Identity | CDR1        | CDR2        | CDR3            |
| VH                               |                 |          |            |          |                         | VL              |          |             |             |                 |
| 1D1                              | IGHV1-69*01     | 99.31%   | GGTFSSYA   | IPIFGTA  | ARDSGNADA<br>EYFQH      | IGLV3-21*02     | 94.62%   | NIGTKI      | DDS         | QVWDSSSD<br>HFV |

These scFvs were expressed and checked by ELISA (Figure 5.6) for their binding ability. All four of them bound with cEDIII and also little bit with DENV1-4. Hence, they were further expressed as full length IgGs.

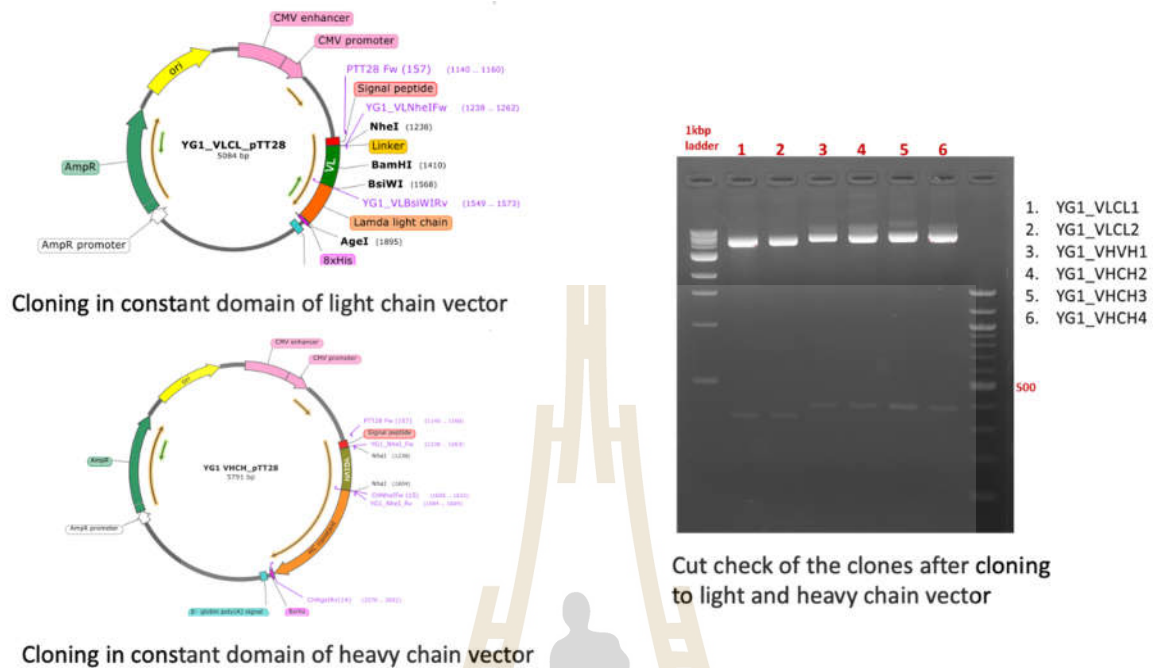


**Figure 5.6** ELISA showing binding of the scFv clones with cEDIII and DENV1-4

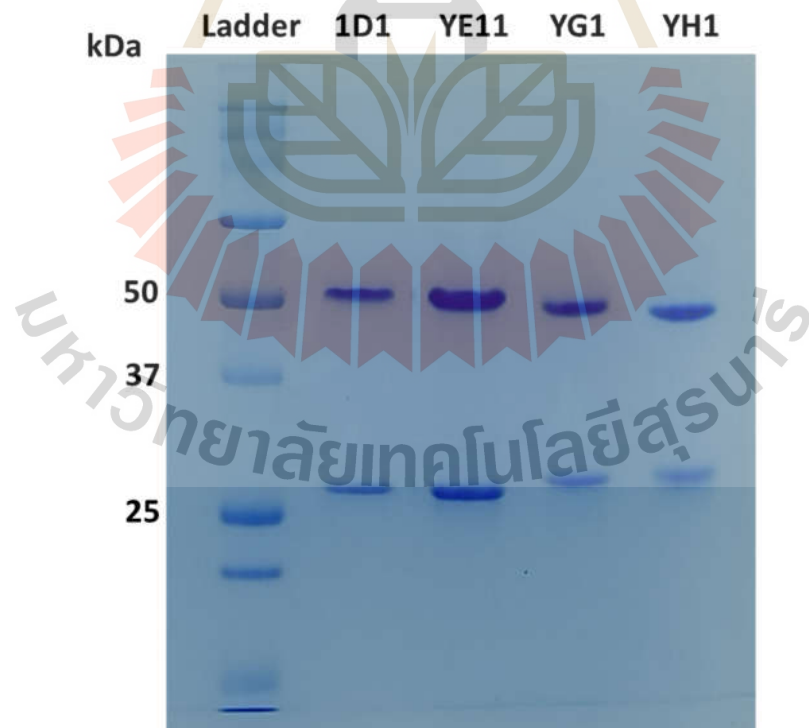
#### 5.4.3 Expression of full-length anti-human monoclonal IgGs antibody and purification

As IgG is the most accepted format for the therapeutic purpose, conversion of scFv antibody fragments into IgG can result in similar or even improved antigen binding. The VH and VL regions of scFv are separately amplified and cloned to plasmid containing constant domain of heavy and light chains respectively. Then transfection was performed to get full length IgG. Four different IgGs were expressed and purified. The expression of IgGs was confirmed by running SDS-PAGE. Heavy chain was observed in ~50kDa size and light chain was observed around 25kDa (Figure 5.8).





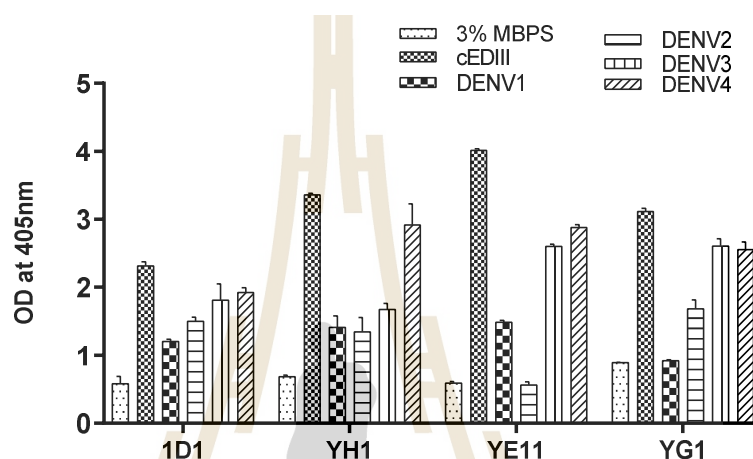
**Figure 5.7** Representative figure showing cloning of scFv VH and VL to heavy chain and light chain vectors respectively



**Figure 5.8** SDS-PAGE of the expressed recombinant monoclonal IgGs

#### 5.4.4 Check binding of IgGs by ELISA

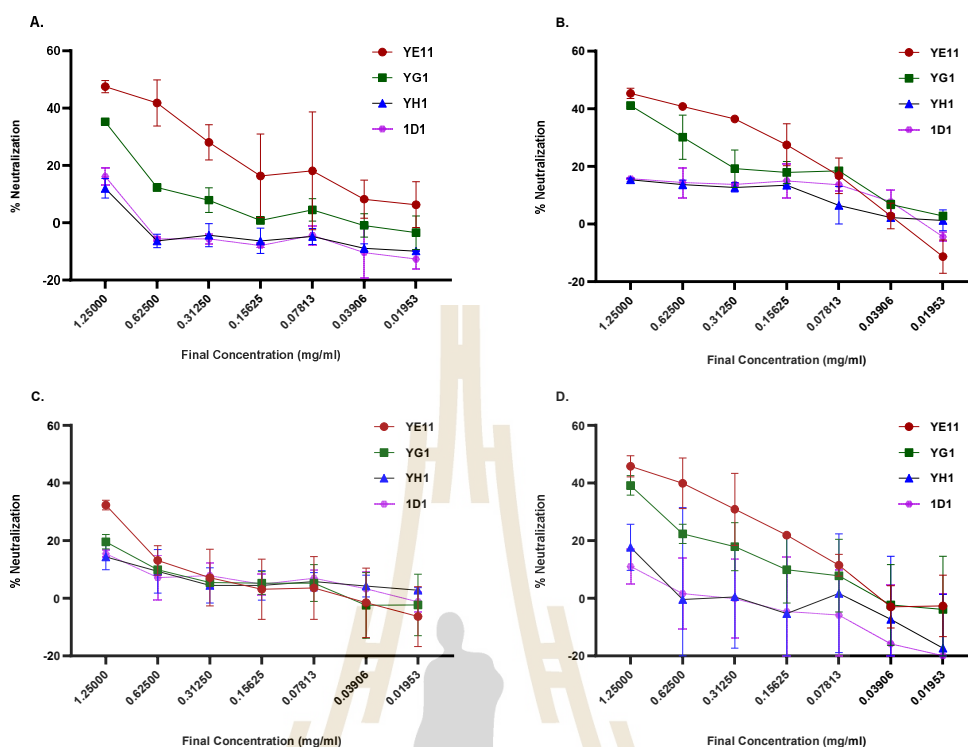
Four different IgGs have been successfully expressed in Expi293 medium and ELISA of the clones was performed against cEDIII and DENV1-4. The clones were shown to successfully bind with cEDIII and DENV serotypes with one clone having less specificity (Figure 5.9).



**Figure 5.9** ELISA showing binding of the recombinant monoclonal antibodies with cEDIII and DENV1-4

#### 5.4.5 Neutralization activity check by using MNT

These recombinant IgGs were checked for their neutralization ability using microneutralization assay against DENV1-4. The strains 01-44 (DENV1), 00St22A (DENV2), TL18 (DENV3) and TVP360 (DENV) were used. Sadly, the neutralization ability of all of the four antibodies was less than 50% even with the highest concentration 1.25mg/ml. The results shown in the graph are the mean of two independent experiments performed in duplicate. The mAb YE11 was shown to have highest neutralization activity against all 4 serotypes. YE11 showed 47.5%, 45.3%, 32.3% and 45.8% neutralization against DENV1, 2, 3 and 4 respectively. Another clone YG1 also showed 35.2%, 41.1%, 19.6% and 39.1% neutralization against DENV1, 2, 3 and 4 respectively. The remaining clones YH1 and 1D1 were very poorly neutralizing and showed less than 20% neutralization against all four serotypes (Figure 5.10).



**Figure 5.10** Percentage neutralization activity of recombinant antibodies.

A: Neutralization against DENV1, B: Neutralization against DENV2,  
C: Neutralization against DENV3 and D: Neutralization against DENV4

## 5.5 Discussion

Dengue fever is a serious concern because of its cross reactivity with its own serotypes together with other flavivirus. Also, there is no definitive treatment for dengue and only symptomatic treatment.

In the absence of a vaccine that can protect irrespective of the age group and immune status of a person, a therapeutic neutralizing antibody that could protect against all serotypes could be a major breakthrough against dengue. One effective method for comprehending the mechanism of DENV and ZIKV antiviral immunity, diagnostics, and treatments is the study of mAbs generated from individuals infected with these viruses (Dejnirattisai et al., 2015; Priyamvada et al., 2016). Hence, in this study we employed phage display technology from which we can get humanized monoclonal recombinant antibodies. We used two different kinds of scFv antibody libraries: one which has been constructed from dengue naive individuals (P. Pansri, N. Jaruseranee, K. Rangnoi, P. Kristensen, & M. Yamabhai, 2009) and another from people with neutralizing antibodies against dengue (unpublished data from our lab). We were

successful to get four monoclonal antibodies that bind to our target cEDIII. But sadly, these antibodies could not neutralize the viruses by 50%.

Even though, these antibodies could not neutralize the virus, we were able to show that phage display technology could be used to isolate the antibodies against dengue by using any desired target. Probable reason that the antibody might not have been able to neutralize could be because the target that we used was synthetic one and not directly from the virus. The focus on envelope domain III, while successful in identifying cross-reactive antibodies, may have limited the discovery of antibodies targeting other functionally important epitopes. Future research should consider broader epitope coverage to identify antibodies with complementary mechanisms of action. Additionally, the target directly from the virus which induces cross-reactive neutralizing antibody response could be used.

Also, further studies on these antibodies can be performed. The binding sites of these antibodies to the virus also greatly affect its neutralizing ability. Hence further studies on the structural properties of the antibody could also help us to increase its binding affinity and in turn probably the neutralization ability as well

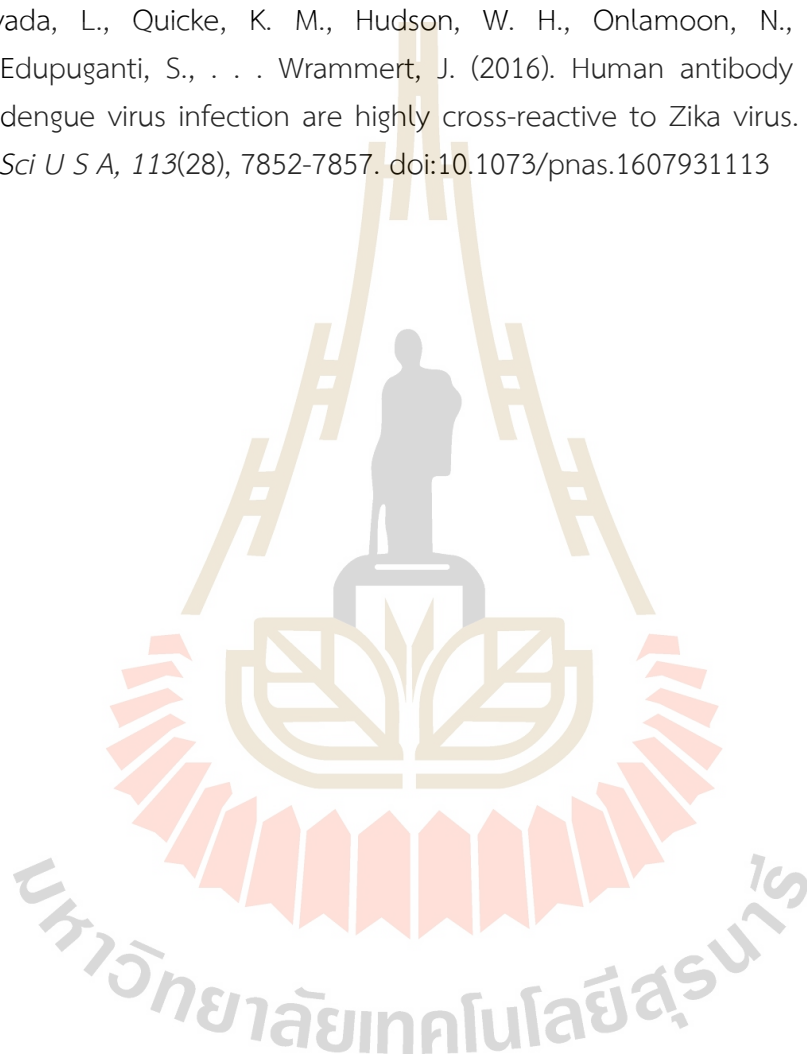
In conclusion, phage display technology could be used as an essential tool for research on dengue virus. The antibodies isolated from scFv antibody libraries were cross-reactive and were able to neutralize all four serotypes of DENV to some extent. Further works can be done on the antibodies to increase its neutralization ability so that it could be used as a therapeutic antibody in the future that could protect against severe dengue. Also, this study opens up the avenue to work against other viruses as well.

## 5.6 References

- Chen, H. W., Liu, S. J., Li, Y. S., Liu, H. H., Tsai, J. P., Chiang, C. Y., . . . Pan, C. H. (2013). A consensus envelope protein domain III can induce neutralizing antibody responses against serotype 2 of dengue virus in non-human primates. *Arch Virol*, 158(7), 1523-1531. doi:10.1007/s00705-013-1639-1
- Clackson, T., Hoogenboom, H. R., Griffiths, A. D., & Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature*, 352(6336), 624-628. doi:10.1038/352624a0
- Dejnirattisai, W., Wongwiwat, W., Supasa, S., Zhang, X., Dai, X., Rouvinski, A., . . . Screaton, G. R. (2015). A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat Immunol*, 16(2), 170-177. doi:10.1038/ni.3058
- Haga, K., Chen, Z. N., Himeno, M., Majima, R., & Moi, M. L. (2023). Utility of an In-Vitro Micro-Neutralizing Test in Comparison to a Plaque Reduction Neutralization Test for Dengue Virus, Japanese Encephalitis Virus, and Zika Virus Serology and Drug Screening. *Pathogens*, 13(1). doi:10.3390/pathogens13010008
- Huang, H. J., Yang, M., Chen, H. W., Wang, S., Chang, C. P., Ho, T. S., . . . Wan, S. W. (2022). A novel chimeric dengue vaccine candidate composed of consensus envelope protein domain III fused to C-terminal-modified NS1 protein. *Vaccine*, 40(15), 2299-2310. doi:10.1016/j.vaccine.2022.02.070
- Kyle, J. L., & Harris, E. (2008). Global spread and persistence of dengue. *Annu Rev Microbiol*, 62, 71-92. doi:10.1146/annurev.micro.62.081307.163005
- Leng, C. H., Liu, S. J., Tsai, J. P., Li, Y. S., Chen, M. Y., Liu, H. H., . . . Chen, H. W. (2009). A novel dengue vaccine candidate that induces cross-neutralizing antibodies and memory immunity. *Microbes Infect*, 11(2), 288-295. doi:10.1016/j.micinf.2008.12.004
- Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D., & Winter, G. (1991). By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J Mol Biol*, 222(3), 581-597. doi:10.1016/0022-2836(91)90498-u
- Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc Natl Acad Sci U S A*, 100(12), 6986-6991. doi:10.1073/pnas.0832193100
- Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature*, 427(6972), 313-319. doi:10.1038/nature02165



- Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., & Yamabhai, M. (2009). A compact phage display human SCFV library for selection of antibodies to a wide variety of antigens. *BMC biotechnology*, 9, 6. doi:10.1186/1472-6750-9-6
- Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., & Yamabhai, M. (2009). A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol*, 9, 6. doi:10.1186/1472-6750-9-6
- Priyamvada, L., Quicke, K. M., Hudson, W. H., Onlamoon, N., Sewatanon, J., Edupuganti, S., . . . Wrammert, J. (2016). Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. *Proc Natl Acad Sci U S A*, 113(28), 7852-7857. doi:10.1073/pnas.1607931113



## CHAPTER VI

### IMPEDIMETRIC IMMUNOSENSOR FOR DETECTION OF ANTI-DENGUE ANTIBODIES IN PLASMA

#### 6.1 Abstract

Dengue is one of the most rapidly spreading mosquito-borne viral disease, posing a significant global public health challenge in tropical and sub-tropical regions of the world. Hence early and accurate diagnosis is vital for effective triage and outbreak control. This study reports the development of a label-free impedimetric immunosensor for the rapid and sensitive detection of anti-dengue antibodies in human plasma using consensus envelope domain III (cEDIII) as a target antigen. The sensor utilizes a gold electrode modified with a self-assembled monolayer (SAM) of cEDIII antigen. Binding of anti-dengue antibodies from patient plasma to the immobilized antigen alters the interfacial properties of the electrode, leading to a measurable change in impedance. This change in impedance is directly proportional to the concentration of anti-dengue antibodies in the sample. The sensor's performance was evaluated first with monoclonal recombinant antibody that binds to cEDIII and then it was tested on patient samples. Results demonstrate significant analytical differences between these samples showing the potential of this technology in dengue diagnostics. The proposed impedimetric immunosensor offers a promising point-of-care diagnostic tool for dengue infection, providing rapid results without the need for complex labeling procedures or specialized equipment. This technology has the potential to significantly improve dengue surveillance and patient management, especially in resource-limited settings.

#### 6.2 Introduction

Laboratory diagnosis of DENV involves virus isolation or detection, antigen detection or antibody detection using different tools and techniques such as PCR, ELISA, PRNT, etc. Each of these techniques having its own advantages and disadvantages, a new approach involving biosensing is now emerging. This technique offers numerous benefits, like- early detection, cheap cost, ease in handling, no need

for a trained expert, highly stable, on-site detection, high specificity, and high sensitivity. The WHO emphasizes that diagnostic tools, including biosensors, must meet the “ASSURED” (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users) criteria. These criteria thus ensures that diagnostics are effective and accessible, predominantly in low-resource settings. Biosensor methods being developed recently can enhance dengue confirmation by enabling the rapid, sensitive, and specific detection of dengue virus antigens or viral RNA (Pimenta, Santos, Thomasini, & Ferreira, 2018; Siew, Pang, Loh, & Tan, 2021).

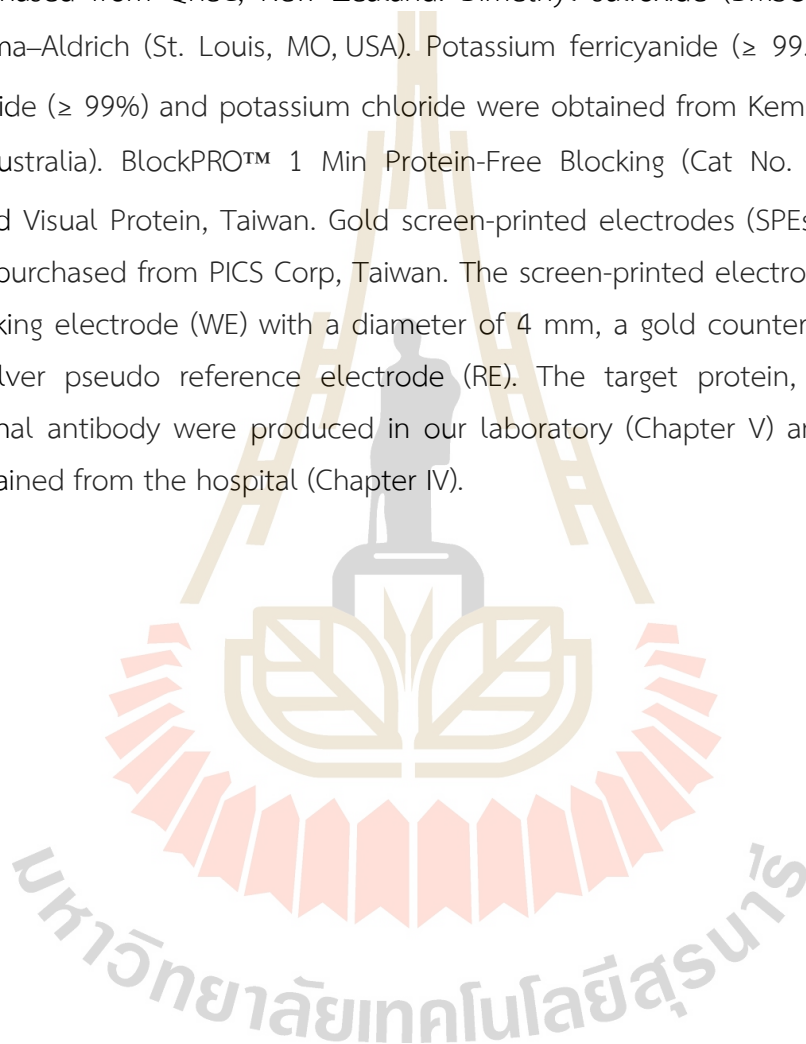
Biosensor refers to an innovative and powerful analytical gadget that integrates a bio-sensing component and has an extensive use. They are specifically designed to diagnose the biological analytes via transforming a biological item. These are simple to use providing one-step detection and fast analysis (Naresh & Lee, 2021). Among various kinds of biosensors, electrochemical biosensors integrate biological recognition elements (e.g., enzymes, antibodies, DNA) with electrochemical transducers to detect analytes in body fluids, enabling rapid, sensitive, and portable diagnostics. They offer simple instrumentation and operation, low cost, and rapid response times. Impedimetric sensors, in particular, measures the changes in electrical impedance at the electrode during biomolecular binding (Lisdat & Schäfer, 2008). The advantages of impedimetric sensors include low cost, sensitivity, miniaturization, and absence of reagents (Ahmed, Rushworth, Hirst, & Millner, 2014).

In this study, consensus envelope domain III (cEDIII) was used as the biorecognition element for the differentiation of dengue positive and negative samples. Firstly, the binding conditions were optimized using the recombinant monoclonal antibody that binds to cEDIII and having neutralizing ability against dengue (~40%) which has been described in detail in Chapter V has been used. After that positive and negative sample which had been already tested by ELISA and PCR (described in detail in Chapter IV) was tested for impedimetric sensing. The preliminary results show high promise for the development of portable device used for dengue diagnostics using cEDIII as the target.

## 6.3 Material and Methods

### 6.3.1 Chemicals, target and samples

Dithiobis-succinimidyl propionate (DSP; Cat No. D2473) and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Cat no. T1656) were purchased from TCI chemicals (Saitama, Japan). Hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 35%; Cat No.H8089-3-1000) was purchased from QReC, New Zealand. Dimethyl sulfoxide (DMSO) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Potassium ferricyanide ( $\geq 99.0\%$ ), potassium ferrocyanide ( $\geq 99\%$ ) and potassium chloride were obtained from Kemaus (New South Wales, Australia). BlockPRO™ 1 Min Protein-Free Blocking (Cat No. BM01-500) was purchased Visual Protein, Taiwan. Gold screen-printed electrodes (SPEs) model GMTE-200 was purchased from PICS Corp, Taiwan. The screen-printed electrode consists of a gold working electrode (WE) with a diameter of 4 mm, a gold counter electrode (CE), and a silver pseudo reference electrode (RE). The target protein, cEDIII and the monoclonal antibody were produced in our laboratory (Chapter V) and the samples were obtained from the hospital (Chapter IV).



### 6.3.2 Immunosensor fabrication

The gold screen-printed electrodes were pre-cleaned with deionized (DI) water, followed by treatment with piranha solution ( $\text{H}_2\text{SO}_4\text{:H}_2\text{O}_2$ ; 7:3 v/v) for 5 minutes and subsequent rinsing with DI water. To form the DSP self-assembled monolayer (SAM), a 2 mg/mL DSP solution in DMSO was treated with 5 mM TCEP and incubated at 37 °C for 15 minutes to facilitate reduce disulfide bonds (-S-S-) in DSP into free thiol groups (-SH). The electrodes were incubated in the DSP solution for 1 hour at room temperature to allow SAM formation. Subsequently, the unbound DSP was removed by rinsing with DMSO, followed by deionized water. The DSP-modified gold electrodes were immediately used for the covalent immobilization of cEDIII. Covalent attachment was achieved by incubating the working electrode with 3  $\mu\text{L}$  of a 10  $\mu\text{g/mL}$  cEDIII in PBS (pH 7.4) for 1 hr at room temperature. The target protein was covalently bound via a reaction of its amino groups and the succinimidyl groups on DSP linker. The resulting cEDIII/DSP/Au electrode was thoroughly washed with PBS and incubated with BlockPRO buffer for 5 min to block and minimize non-specific adsorption.

### 6.3.3 Cyclic Voltammetry (CV) and Electrochemical Impedance

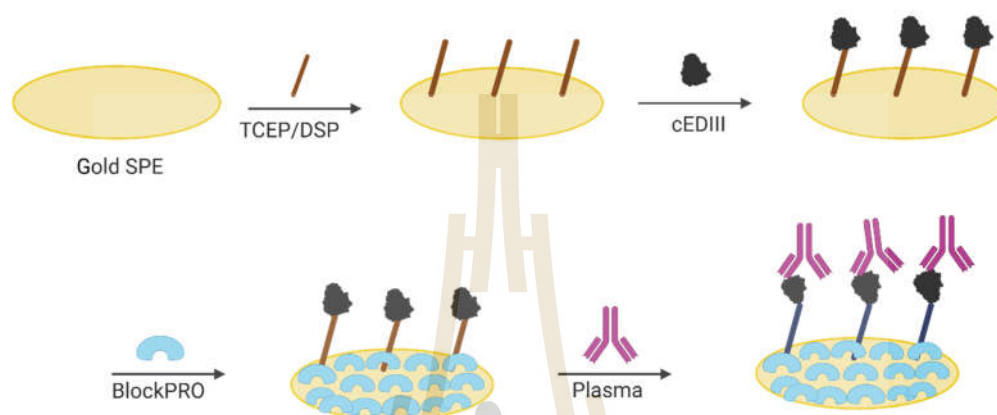
#### Spectroscopy (EIS) Measurements

Electrochemical analyses, including cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), were conducted using a PalmSens potentiostat/galvanostat controlled by PSTrace software (PalmSens, Houten, The Netherlands). CV was carried out by sweeping the potential of the working electrode from 0.4 V to -0.2 V at a scan rate of 0.10 V/s in 60  $\mu\text{L}$  of 10 mM  $[\text{Fe}(\text{CN})_6]^{3-}$  in 0.1 M KCl. For EIS, measurements were performed in 60  $\mu\text{L}$  of the electrolyte containing 5.0 mM equimolar  $[\text{Fe}(\text{CN})_6]^{3-}/^{4-}$  and 0.1 M KCl. A constant DC potential of 0.12 V was applied with a 5 mV AC amplitude over a frequency range of 100 kHz to 0.1 Hz. The resulting impedance spectra were fitted to a Randles equivalent circuit using PSTrace software. All measurements were conducted at room temperature.

For the estimation of anti-dengue antibodies in plasma, a dengue-negative plasma sample was diluted 1:100 with PBS before analysis. A 3  $\mu\text{L}$  aliquot of the diluted plasma was applied to the working electrode surface and incubated at either 37 °C or room temperature for 1 hr. Following incubation, the electrode was rinsed with DI/PBS to remove unbound components and subsequently subjected to CV and



EIS measurements to evaluate the electrochemical response. After that same procedure was followed for positive dengue sample.



**Figure 6.1** Schematic representation of cEDIII based dengue biosensor (created using Biorender)

## 6.4 Results and Discussion

### 6.4.1 Production of target protein

Target protein (cEDIII) was expressed in bacterial expression system and has been described in detail in Chapter V.

### 6.4.2 Sample details

The samples were obtained from the hospital. Clinically defined as positive and negative sample (Chapter IV) was used for this preliminary experiment.

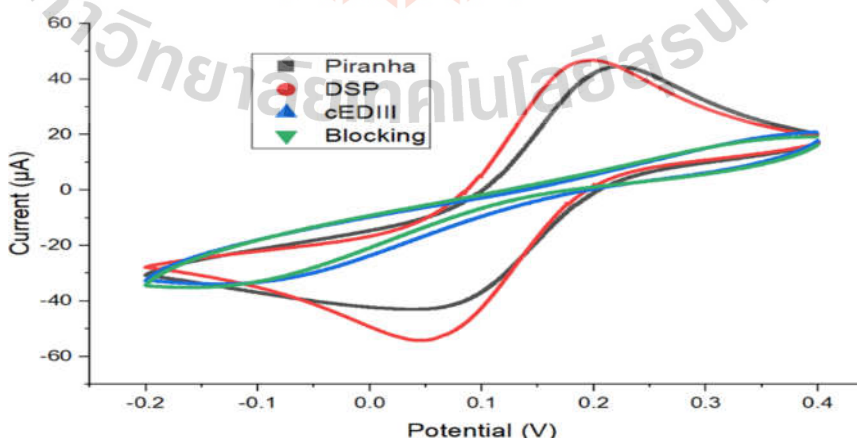
**Table 6.1** ELISA results for the samples

| ELISA Tests                     | Sub ID  | OD values | Interpretation |
|---------------------------------|---------|-----------|----------------|
| Serion IgG ELISA Detetction kit | SH/24/A | 3.176     | Positive       |
| cEDIII Inhouse ELISA            | SH/24/A | 3.981     | Positive       |
| Serion IgG ELISA Detetction kit | SH/07/A | 0.451     | Negative       |
| cEDIII Inhouse ELISA            | SH/07/A | 0.893     | Negative       |

#### 6.4.3 Electrochemical characterization of the immunosensor

The overall immunosensor fabrication and detection process in this study is illustrated in Figure 6.1. The immunosensor was constructed on a gold surface, antigen cEDIII was immobilized using DSP in the presence of TCEP.

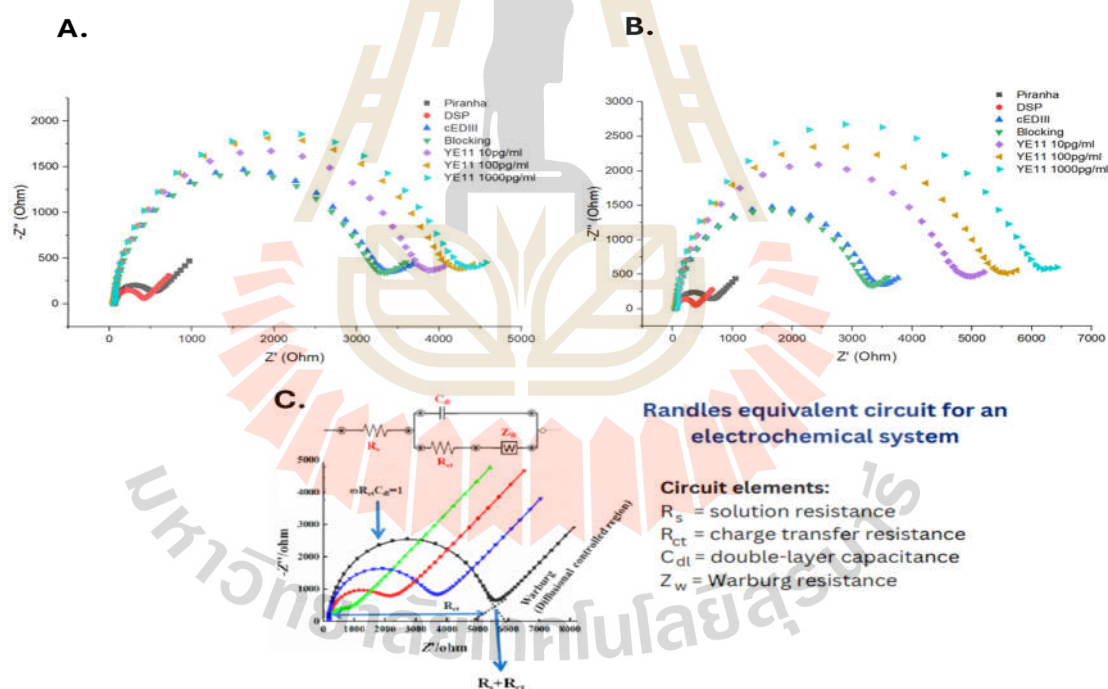
Cyclic voltammetry was employed to monitor the stepwise electrode modification by analyzing the redox behavior of the  $[\text{Fe}(\text{CN})_6]^{3-}/^{4-}$  couple, which serves as an electrochemical probe for assessing surface properties and electron transfer kinetics. After modification with DSP, a slight increase in both oxidation and reduction peak currents was observed (red curve), likely due to the introduction of hydrophilic functional groups from the DSP crosslinker. These groups enhanced the wettability of the electrode surface, thereby improving the accessibility of the redox probe to the electrode interface. Subsequent covalent immobilization of cEDIII (envelope domain III) led to a pronounced decrease in peak currents (blue curve), indicating successful protein attachment. This significant reduction is attributed to the steric hindrance and insulating nature of the protein layer, which impedes the diffusion of redox species to the electrode surface.



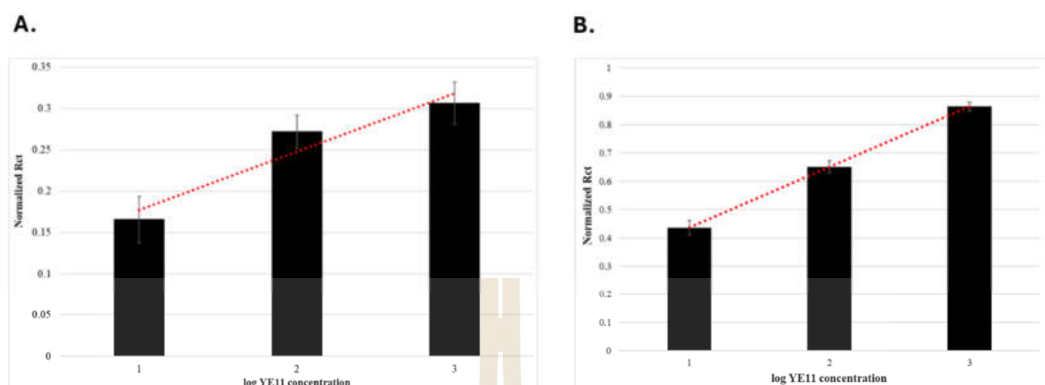
**Figure 6.2** Cyclic voltammograms of the immunosensor (SPE+cEDIII) measured after each stage of electrode modification.

Electrochemical impedance spectroscopy (EIS) was performed to characterize the functionalization of the immunosensor and to monitor the detection of anti-dengue antibodies in plasma samples. The impedance data were presented as Nyquist plots (Fig. 6.4), which depict the relationship between the real ( $Z'$ ) and imaginary ( $Z''$ ) components of impedance over a range of frequencies following each modification step. The experimental data were fitted to a Randles equivalent circuit to extract the charge transfer resistance ( $R_{ct}$ ), which reflects changes in interfacial electron transfer properties at the gold electrode surface. Variations in  $R_{ct}$  provided insight into the success of each surface modification and the binding events associated with antibody detection.

The optimizations were performed with YE11 antibody, which binds with cEDIII (Chapter V) and we could see good binding at both 37°C and in room temperature (Figure 6.2). After that the assay was performed with patient samples.

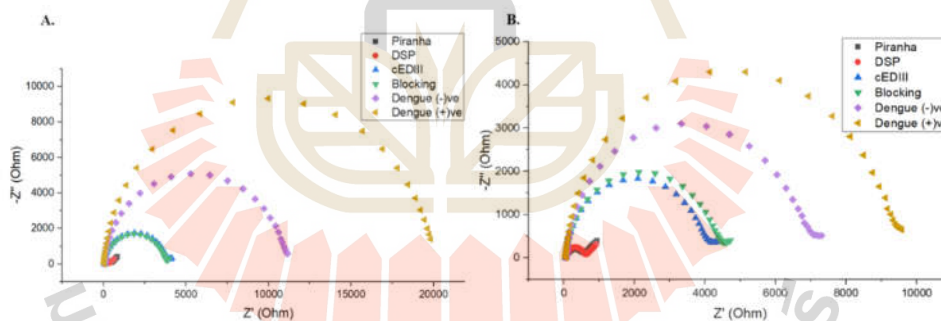


**Figure 6.3** EIS Nyquist diagram obtained in 10.0 mM  $K_3Fe(CN)_6/K_4Fe(CN)_6$  solution containing 0.10 M KCl showing effect of incubation temperature on binding between cEDIII (10 $\mu$ g/ml) and YE11 recombinant monoclonal antibody at different concentrations (10, 100 and 1000 pg/ml) and temperature. Amplitude = 5 mV. Frequency range: 105 to 0.5 Hz. [cEDIII]=10.0 $\mu$ g/mL. A: Room temperature; B: 37°C (C) Randles circuit used to model and fit the electrochemical impedance spectra (Nyquist plots (Magar, Hassan, & Mulchandani, 2021).



**Figure 6.4** Bar graph showing the comparison of YE11 (10, 100 and 1000 pg/mL) against delta charge transfer resistance value ( $R_{ct}$ ). A: Room temperature; B: 37°C (replicate = 3)

The results obtained indicate an increased electron transfer barrier to the redox couple after each modification step. Increasing  $R_{ct}$  values can also be qualitatively observed in the form of larger semicircles in the Nyquist plot after each modification step.

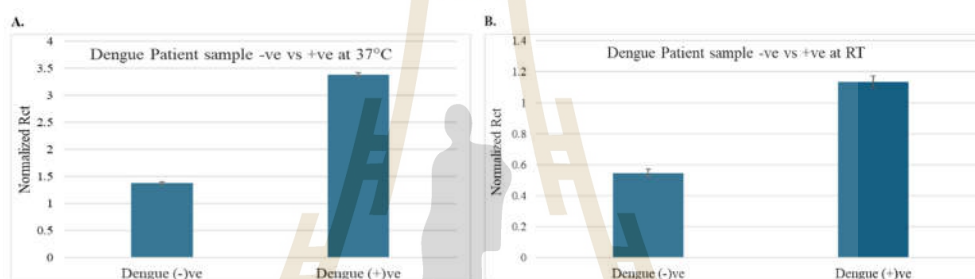


**Figure 6.5** EIS Nyquist diagram obtained in 10.0 mM  $K_3Fe(CN)_6/K_4Fe(CN)_6$  solution containing 0.10 M KCl for: cEDIII/plasma/SPE at the following negative and positive serum dilution: 1:100. Amplitude = 5 mV. Frequency range:  $10^5$  to 0.5 Hz. [cEDIII]=10.0ug/mL. A- incubation at 37°C and B- incubation at room temperature

#### 6.4.4 Impedance responses of the immunosensor toward anti-dengue antibodies detection

In these preliminary experiments performed so far, we optimized the immunosensor by using the recombinant monoclonal antibody using different concentrations of DSP and varying the incubation time and temperature.

Finally, the plasma samples were used in the experiment with 2mg/ml DSP and incubation at 37°C and room temperature. We could see significant differences in the analytical response between the positive and negative samples (Figure 6.4).



**Figure 6.6** Comparative analysis of the difference in ohm for negative and positive sample. A- incubation at 37°C and B- incubation at room temperature (replicate =3)

In conclusion, we were able to have immunosensor that could detect anti-dengue antibodies in the plasma. The developed immunosensor was able to differentiate positive and negative sample of dengue. Both positive and negative plasma samples exhibited responses due to the binding between cEDIII and anti-dengue antibodies, leading to an increased charge transfer resistance (Rct) at the gold screen-printed electrode. However, the signal obtained from the positive serum sample was significantly higher than that from the negative sample, as indicated by the normalized Rct values relative to the blocking step.



## 6.6 References

- Ahmed, A., Rushworth, J. V., Hirst, N. A., & Millner, P. A. (2014). Biosensors for Whole-Cell Bacterial Detection. *Clinical Microbiology Reviews*, 27(3), 631-646.  
doi:doi:10.1128/cmr.00120-13
- Lisdat, F., & Schäfer, D. (2008). The use of electrochemical impedance spectroscopy for biosensing. *Analytical and Bioanalytical Chemistry*, 391(5), 1555-1567.  
doi:10.1007/s00216-008-1970-7
- Magar, H. S., Hassan, R. Y. A., & Mulchandani, A. (2021). Electrochemical Impedance Spectroscopy (EIS): Principles, Construction, and Biosensing Applications. *Sensors (Basel)*, 21(19). doi:10.3390/s21196578
- Naresh, V., & Lee, N. (2021). A Review on Biosensors and Recent Development of Nanostructured Materials-Enabled Biosensors. *Sensors (Basel)*, 21(4).  
doi:10.3390/s21041109
- Pimenta, T. C., Santos, C. D. C., Thomasini, R. L., & Ferreira, L. F. (2018). Impedimetric immunosensor for dengue diagnosis using graphite screen-printed electrodes coated with poly(4-aminophenylacetic acid). *Biomed Microdevices*, 20(3), 78.  
doi:10.1007/s10544-018-0324-2
- Siew, Q. Y., Pang, E. L., Loh, H. S., & Tan, M. T. T. (2021). Highly sensitive and specific graphene/TiO(2) impedimetric immunosensor based on plant-derived tetravalent envelope glycoprotein domain III (EDIII) probe antigen for dengue diagnosis. *Biosens Bioelectron*, 176, 112895. doi:10.1016/j.bios.2020.112895

## CHAPTER VII

### CONCLUSION AND FUTURE DIRECTIONS

In summary, this multidisciplinary investigation provides compelling evidence for the complex immunological landscape of flavivirus infections in endemic regions. The documented cross-reactivity patterns, technological innovations, and diagnostic challenges revealed by this study contribute significantly to our understanding of dengue virus pathogenesis and immunity. The findings emphasize that effective dengue control strategies must account for the broader flavivirus ecosystem, incorporating comprehensive diagnostic approaches, enhanced surveillance systems, and innovative therapeutic strategies. The successful integration of molecular epidemiology, immunological characterization, and technological innovation demonstrated in this study provides a valuable framework for future research and public health interventions. As dengue continues to expand its geographic distribution and severity patterns evolve, the insights gained from this comprehensive analysis will be instrumental in developing more effective strategies for disease prevention, diagnosis, and treatment. Ultimately, this research underscores the critical importance of adopting a holistic approach to flavivirus research that considers the complex interplay between viral evolution, host immunity, and cross-reactive responses. These findings emphasize the urgent need for improved surveillance, diagnostics, and therapeutics, along with a deeper understanding of flavivirus cross-reactivity to refine vaccine development and public health strategies against DENV.

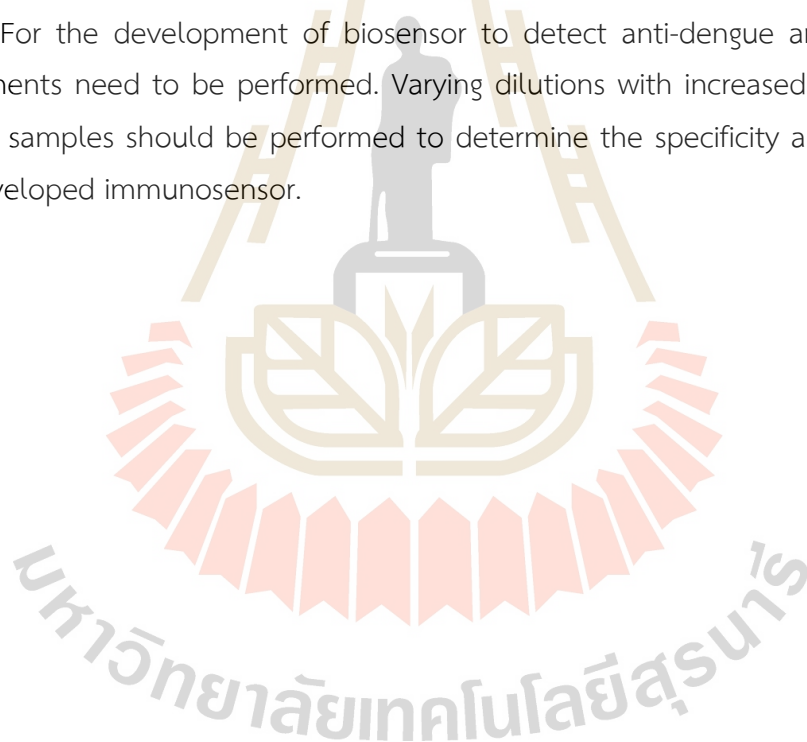
#### **Future Directions:**

The patient samples should be further analyzed to find the exact neutralization titers against other viruses as well. In the performed experiments only 1:80 dilution was performed for other viruses. Since, less neutralizing antibodies were observed; we can start with lesser dilution to find the neutralization titer. Also, the primary and secondary infection can be differentiated using the neutralization titer. Even now we can see that secondary infection is more prevalent, with the help of

neutralization titer we will have more accurate analysis for differentiation of primary and secondary infection. Additionally, presence of enhancing antibodies could be checked in the samples against DENV1-4 and other flaviviruses. Also sequencing of these samples in future will provide more details about the phylogenetic relationship and we can even find the genotypes of these circulating viruses.

Regarding the application of phage development technology, we were able to get antibodies which are not highly neutralizing. However, the use of this technique opens several avenues for future research and technological advancement. Antibody engineering approaches, including affinity maturation, half-life extension through Fc modifications, and the development of bispecific antibodies, represent opportunities for optimization.

For the development of biosensor to detect anti-dengue antibodies, more experiments need to be performed. Varying dilutions with increased number of the plasma samples should be performed to determine the specificity and sensitivity of the developed immunosensor.



## APPENDIX

### List of publications:

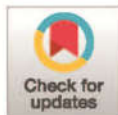
1. **Prajapati S**, Elong Ngono A, Mc Cauley M, Timis J, Shrestha S, Bastola A, et al. (2024) Genomic sequencing and neutralizing serological profiles during acute dengue infection: A 2017 cohort study in Nepal. PLOS Glob Public Health 4(11): e0002966. <https://doi.org/10.1371/journal.pgph.0002966>
2. **Prajapati S**, Napit R, Bastola A, Rauniyar R, Shrestha S, Lamsal M, et al. (2020) Molecular phylogeny and distribution of dengue virus serotypes circulating in Nepal in 2017. PLoS ONE 15(7): e0234929. <https://doi.org/10.1371/journal.pone.0234929>
3. Rauniyar R, **Prajapati S**, Manandhar B, Bastola A, Chalise BS, Shrestha S, et al. (2023), “Dengue virus infection during window period of consecutive outbreaks in Nepal and assessment of clinical parameters”; Scientific Reports; <https://doi.org/10.1038/s41598-023-35928-5>
4. Rai TM, Shrestha S, **Prajapati S**, Bastola A, Parajuli N, Ghimire PG, et al. (2023) “*Leishmania donovani* persistence and circulation causing cutaneous leishmaniasis in unusual-foci of Nepal”; Scientific Reports; <https://doi.org/10.1038/s41598-023-37458-6>
5. Bastola A, Shrestha M, Lamsal M, Shrestha S, **Prajapati S**, Adhikari A, et al. (2019) “A case of high altitude cutaneous leishmaniasis in a non-endemic region in Nepal”; Parasitology International; <https://doi.org/10.1016/j.parint.2019.101991>

## RESEARCH ARTICLE

## Genomic sequencing and neutralizing serological profiles during acute dengue infection: A 2017 cohort study in Nepal

Sabita Prajapati<sup>1,2\*</sup>, Annie Elong Ngono<sup>3\*</sup>, Melanie Mc Cauley<sup>3\*</sup>, Julia Timis<sup>3</sup>, Srijan Shrestha<sup>1</sup>, Anup Bastola<sup>4</sup>, Shrawan Kumar Mandal<sup>4</sup>, Sanjay Ray Yadav<sup>5</sup>, Rajindra Nait<sup>1</sup>, Meng Ling Moi<sup>6</sup>, Montarop Yamabhai<sup>6</sup>, October M. Sessions<sup>7,8,9\*</sup>, Sujan Shrestha<sup>3,10\*</sup>, Krishna Das Manandhar<sup>1\*</sup>

**1** Infectious and Viral Disease Research Laboratory, Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal, **2** Molecular Biotechnology Laboratory, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand, **3** Center for Vaccine Innovation, La Jolla Institute for Immunology, La Jolla, California, United States of America, **4** Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu, Nepal, **5** Department of Haematology and Biochemistry, Chitwan Medical College and Teaching Hospital, Chitwan, Nepal, **6** School of International Health, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, **7** Saw Swee Hock School of Public Health, National University of Singapore, Singapore, **8** Department of Pharmacy, National University of Singapore, Singapore, **9** Program in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore, **10** Division of Host-Microbe Systems and Therapeutics, Department of Pediatrics, University of California San Diego School of Medicine, La Jolla, California, United States of America



## OPEN ACCESS

**Citation:** Prajapati S, Elong Ngono A, Mc Cauley M, Timis J, Shrestha S, Bastola A, et al. (2024) Genomic sequencing and neutralizing serological profiles during acute dengue infection: A 2017 cohort study in Nepal. PLOS Glob Public Health 4(11): e0002966. <https://doi.org/10.1371/journal.pgph.0002966>

**Editor:** Sukanta Chowdhury, ICDDR B: International Centre for Diarrhoeal Disease Research Bangladesh, BANGLADESH

**Received:** October 22, 2023

**Accepted:** September 30, 2024

**Published:** November 13, 2024

**Copyright:** © 2024 Prajapati et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All data underlying the findings are reported in the submitted paper and also seen in the NCBI database (PP152366 and PP152367).

**Funding:** This study was supported by U01 AI151810 (KDM), a Tullie and Rickey Families SPARK Award for Innovations in Immunology at the La Jolla Institute for Immunology (AEN), and an e-Asia grant N10A650706 and N10A660577.

\* These authors contributed equally to this work.

\* Current address: Emerging Infectious Disease Branch, Walter Reed Army Institute of Research, Silver Spring, Maryland, United States of America

\* [krishna.manandhar@gmail.com](mailto:krishna.manandhar@gmail.com) (KDM); [October.sessions@nus.edu.sg](mailto:October.sessions@nus.edu.sg) (OMS); [sujan@lji.org](mailto:sujan@lji.org) (SS)

## Abstract

Dengue virus (DENV) is a mosquito-borne flavivirus that poses a threat to nearly 50% of the global population. DENV has been endemic in Nepal since 2006; however, little is known about how DENV is evolving or the prevalence of anti-DENV immunity within the Nepalese population. To begin to address these gaps, we performed a serologic and genetic study of 49 patients from across Nepal who presented at central hospitals during the 2017 dengue season with suspected DENV infection. Of the 49 subjects assessed, 21 (43%) were positive for DENV NS1 antigen; of these, 5 were also anti-DENV IgM<sup>+</sup> IgG<sup>+</sup>; 7 were DENV IgM<sup>+</sup> IgG<sup>-</sup>, 2 were IgM<sup>-</sup> IgG<sup>+</sup>, and 7 were IgM<sup>-</sup> IgG<sup>-</sup> by specific ELISAs. Seven of the 21 NS1 positive sera were RNA positive by RT-PCR (six DENV2, one DENV3), suggesting that DENV2 was the dominant serotype in our cohort. Whole-genome sequencing of two DENV2 isolates showed similarity with strains circulating in Singapore in 2016, and the envelope genes were also similar to strains circulating in India in 2017. DENV-neutralizing antibodies (nAbs) were present in 31 of 47 sera tested (66%); among these, 20, 24, 26, and 12 sera contained nAbs against DENV1, 2, 3, and 4 serotypes, respectively. Additionally, 27 (58%) samples had nAbs against multiple serotypes (2 or more). Serology analysis suggested that 12 (26%) and 19 (40%) of the 47 subjects were experiencing primary and secondary DENV infections, respectively. Collectively, our results provide evidence for current and/or past exposure to multiple DENV serotypes in our cohort. These data suggest that expanded local surveillance



(MY, in collaboration with SS). OMS was supported by an MOE-Tier1 award (Reimagine Research FY2020). SS was supported by R01 AI163188, R01 AI153500, and e-Asia grant G-202012-67125. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

of circulating DENV genotypes and population immunity will be important to effectively manage and mitigate future dengue outbreaks in Nepal.

## Introduction

Dengue virus (DENV) is a positive-sense single-stranded RNA virus of the *Flavivirus* genus, which also includes Japanese encephalitis, Zika, yellow fever, and West Nile viruses [1, 2], all of which are predominantly transmitted by infected *Aedes* spp. mosquitoes [3, 4]. The four antigenically distinct serotypes of DENV (DENV1–4) are responsible for about 400 million reported DENV infections per year [3, 4], which may be asymptomatic or have symptoms ranging from self-limiting dengue fever to severe disease characterized by hemorrhagic fever, shock and, in some cases, death [1].

Infection with DENV confers long-term protection against homologous serotypes but only limited cross-protection against heterologous serotypes; indeed, in some cases, secondary infection with a different serotype can elicit severe dengue in an antibody (Ab)-dependent manner [5–7]. At present, there are no DENV vaccines that provide durable protection against all four DENV serotypes and can be administered to people of all ages who are DENV-naïve or -immune. The first approved dengue vaccine (Dengvaxia) can only be administered to individuals with prior natural DENV infection [8], because it increases the risk of severe dengue in DENV-naïve individuals who are then exposed naturally after vaccination. A new vaccine, QDenga (Takeda) has been recently approved in Indonesia, Thailand, Argentina, Brazil, and several European countries for DENV-naïve and -immune individuals, but it provides robust long-term protection against only selected DENV serotypes [9–11]. Finally, a phase 3 trial of the tetravalent vaccine Butantan-DV (Instituto Butantan/NIAD) demonstrated good protection against DENV1, but was only moderately effective against DENV2 [12]. These data highlight the challenges in developing a DENV vaccine that induces protective immunity against all four serotypes regardless of prior natural exposure. Moreover, the potential dangers of vaccination mean that data on currently circulating DENV serotypes and population immune status must be available to enable governments to make informed decisions in selecting the optimal vaccine for distribution in any given year [13].

In Nepal, the first DENV infection was detected in 2004 in a Japanese traveler, from whom DENV2 was isolated [14]; by 2006, all 4 DENV serotypes had been documented in Nepal [15]. Since then, sporadic cases have been reported during the annual dengue season (September to December), interrupted by major outbreaks roughly every 3 years beginning in 2010 [16]. Notably, each successive outbreak has been accompanied by an increase in case numbers, geographic spread, morbidity, and mortality [17–19]. In 2017, a total of 2111 dengue cases were reported from 28 of Nepal's 77 districts whereas in 2019, 17,992 cases, including 6 deaths, were reported from 68 of the 77 districts [16, 20, 21], representing a 140-fold increase in incidence in just 2 years [16]. This trend continued in the 2022 outbreak, with 54,784 reported cases and 88 deaths involving all districts [17]. Based on these data, Nepal is on a trajectory to experience another DENV outbreak in 2025.

To date, very little data have been collected on DENV virology and immunology in the Nepalese population. In part, this is because Nepal is a low-income country with a limited scientific infrastructure. For example, the full genomes of only a few circulating DENV isolates have been sequenced since the first reported infection in Nepal in 2006 [22]. The present study was conducted as part of a long-term process of enabling local scientists/clinicians to

effectively surveil the circulation of, and population exposure to, DENV serotypes in Nepal. To this end, we describe here serological and genomic analyses of sera from 49 residents of districts across Nepal who presented with suspected dengue at hospitals in Kathmandu and Chitwan, two geographically and climatically distinct districts. All sera were analyzed for DENV-NS1 antigen, DENV-specific anti-IgM/IgG, and neutralizing antibody (nAb) response against all four DENV serotypes. A subset of NS1 positive sera were further serotyped by RT-PCR and subjected to whole-genome sequencing. The knowledge gained from this study has already facilitated the expansion of DENV genomic surveillance studies by local scientists/clinicians with the goal of mitigating the dengue outbreak predicted to occur in 2025.

## Materials and methods

### Ethics statement

Ethical approval was obtained from the Nepal Health Research Council (Reg. no. 378/2016). Patients were provided with a verbal explanation about the study, its potential significance, the tests to be performed on their samples, including genetic testing, and potential risks associated with drawing blood. The adult patient (or parent/guardian for patients under 18 years of age) then provided written informed consent to be enrolled in the study.

### Sample collection

The study cohort consisted of 49 febrile patients who presented with suspected dengue at Sukraraj Tropical and Infectious Disease Hospital in Kathmandu and at Chitwan Medical College and Teaching Hospital in Chitwan during the 2017 dengue outbreak (September 2017 through January 2018). A diagnosis of suspected dengue was made by attending physicians. Venous blood samples (5 mL) were collected into EDTA tubes, and demographic and clinicopathological information was recorded. All steps were performed by the Nepal-based authors and teams, with advice from the authors based in the US, Japan, and Thailand.

### Enzyme-linked immunosorbent assays

Plasma was isolated from the blood samples and stored at  $-80^{\circ}\text{C}$  until analyzed. Levels of DENV NS1 antigen, anti-DENV IgM, and anti-DENV IgG were quantified using commercial ELISA kits (InBios: DNS1-R, DDMS-1 and DDGS-R respectively). Assay procedures, calculation of immune status ratios, and classification as seronegative or seropositive were all performed according to the manufacturer's instructions. ELISAs were performed at the Infectious and Viral Disease Research Laboratory, Central Department of Biotechnology, Tribhuvan University, Nepal in 2018.

### DENV serotyping by RT-PCR

Viral RNA was isolated from plasma samples using a QIAamp Viral RNA Mini kit (Qiagen, 52906). DENV1–4 serotypes were identified by multiplex RT-PCR as described previously [23–26] using US Centers for Disease Control and Prevention real-time RT-PCR assay kits (KK0128). The analysis was performed at the La Jolla Institute for Immunology in 2018.

### Whole-genome DENV sequencing

All procedures were conducted according to the kit manufacturer's recommendations. Illumina libraries were constructed from total RNA using the NEB Next Ultra Directional RNA Library Prep Kit (New England Biolabs, E7760) per the manufacturer's instructions. Libraries of 400–600 nucleotides were obtained using Mag-Bind RxnPure Plus beads (Omega Bio-Tek,



M1386-01), purified with the MinElute PCR Purification Kit (Qiagen, 28004), and quantified using a Bioanalyzer High-Sensitivity DNA Assay (Agilent Technologies, 5067–4626). Targeted DENV genome enrichment was achieved using custom-designed biotinylated 120-mer xGen Lockdown baits (Integrated DNA Technologies) with complementarity to DENV1–4 serotypes, as previously described [27], all per manufacturer's instructions. Genome assembly was performed using the VIPR4 pipeline (<https://github.com/nf-core/vipr/>). Whole-genome sequencing was performed and data were analyzed at the National University of Singapore in 2019.

### Phylogenetic analysis

Multiple sequence alignment of DENV2 full-genome sequences from the present study ( $n = 2$ ) and the National Center for Biotechnology Information (NCBI) ( $n = 1777$ ) was carried out using a fast Fourier transformation method in MAFFT v7.490. An approximately maximum likelihood phylogenetic tree was generated using a generalized time-reversible model of nucleotide evolution in FastTree v2.1.11, which uses SH-like local supports with 1000 resamples to estimate and validate the reliability of each split in the tree. The branch containing the 2 sequences from the 2017 Nepal outbreak and 29 complete genome sequences from the NCBI were selected, and a more robust maximum likelihood phylogenetic tree created using RAXML v8.2.11 and the GTR GAMMA model with 1000 bootstrap replications. Trees were visualized using FigTree v1.4.4.

### Flow cytometry-based DENV1–4 neutralization assay

Neutralization assays were performed as previously described [28], using a U937 DC-SIGN cell-based flow cytometry assay. Briefly, plasma samples were serially diluted 5-fold starting at 1:40, and incubated with pre-titrated DENV1–4 serotypes (WHO reference standards) for 1 h at 37°C. U937 DC-SIGN cells were then incubated with the plasma/virus mixtures for 2 h at 37°C, washed, and incubated again for 16 h at 37°C. As controls, cells were incubated with virus in the absence of serum to obtain the baseline infection rate. The cells were then surface stained with anti-CD209-PE Ab (DC-SIGN) and intracellularly stained with anti-FITC-labeled 4G2 Ab (pan-flaviviral envelope [E] protein), and analyzed using a FACSCanto LSR cytometer and FlowJo V9 software (BD Biosciences).

Serum was considered positive for neutralizing activity if DENV infection was reduced by  $\geq 90\%$  at a serum dilution of  $>1:40$  (cutoff =  $NT_{90} > 40$ ).  $NT_{90}$  values were used to classify primary infection (first infection with a single serotype) or secondary infection (prior infections with other serotypes), as previously described [29]. In brief, primary infection was defined as (i) an  $NT_{90} \geq 40$  against a single DENV serotype and  $< 40$  against all other serotypes, or (ii) a response against a single DENV serotype, with an  $NT_{90}$  4-fold higher than against the other serotypes. Secondary infection was defined as (i) an  $NT_{90} \geq 40$  against at least two DENV serotypes, and/or (ii) a response with an  $NT_{90} \leq 4$ -fold higher than the next highest response [29]. This assay and analysis were performed at the La Jolla Institute for Immunology in 2018 and 2019.

## Results

### Patient demographics and disease association with platelet count

The study cohort consisted of 49 patients who presented with suspected dengue at two major hospitals in Kathmandu and Chitwan, Nepal during the 2017 dengue season (September 2017 to January 2018) [30]. The majority of the subjects were male (71%, 35/49) and were aged 20

Table 1. Characteristics of study participants.

| Age range (years) | Male (N = 35) | Female (N = 14) | Total (N = 49) |
|-------------------|---------------|-----------------|----------------|
| 13–19             | 8             | 3               | 11             |
| 20–59             | 24            | 7               | 31             |
| 60–81             | 3             | 4               | 7              |

Abbreviation: N, number.

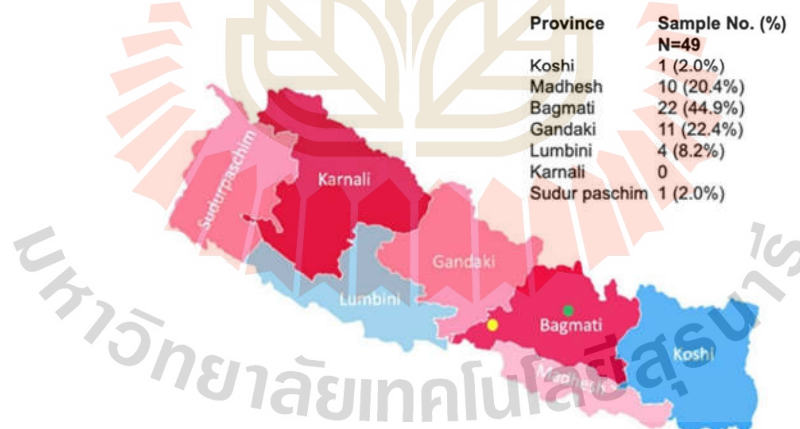
<https://doi.org/10.1371/journal.pgph.0002966.t001>

to 59 years (63%, 31/49), with a median age of 34 years (range 13–81) (Table 1). Although the 49 subjects presented to the two major hospitals in Kathmandu and Chitwan, they originated from 6 of Nepal's 7 provinces (Fig 1).

All subjects were symptomatic at the time of sample collection, although the time between symptom onset and blood collection was not recorded. Thrombocytopenia is a potential indicator of dengue disease severity [31], and platelet counts of  $41\text{--}100 \times 10^3/\mu\text{L}$ ,  $21\text{--}40 \times 10^3/\mu\text{L}$ , and  $\leq 20 \times 10^3/\mu\text{L}$  are considered to reflect low, moderate, and high risk, respectively, of bleeding associated with severe dengue (normal platelet counts:  $100\text{--}450 \times 10^3/\mu\text{L}$ ) [32]. Most samples (31/48) had normal platelet counts, with 13, 2, and 2 samples having counts within the low-, moderate-, and high-risk categories, respectively (Table 2). These results are consistent with the majority of our study cohort having mild dengue disease.

### DENV serostatus and serotypes

NS1 antigen was detected in 43% (21/49) of samples, anti-DENV IgM Ab in 29% (14/49) and anti-DENV IgG Ab in 43% (21/49). Thus, 47% (23/49) of our cohort were considered to have active dengue at the time of sample collection based on the presence of  $\geq 1$  of the following 5 criteria: NS1<sup>+</sup> (14%, n = 7), NS1<sup>+</sup>IgM<sup>+</sup> (14%, n = 7), NS1<sup>+</sup>IgG<sup>+</sup> (4%, n = 2), NS1<sup>+</sup>IgM<sup>+</sup>IgG<sup>+</sup>



**Fig 1. Geographic distribution of the home residences of the study subjects.** Patients originated from across Nepal and were seen at hospitals in Kathmandu (green dot) and Chitwan (yellow dot). White borders demarcate the seven provinces in Nepal. Abbreviation: No, number. Image modified from Wikipedia ([https://en.m.wikipedia.org/wiki/File:Provinces\\_of\\_Nepal.png](https://en.m.wikipedia.org/wiki/File:Provinces_of_Nepal.png)).

<https://doi.org/10.1371/journal.pgph.0002966.g001>

Table 2. Platelet counts and DENV serology, serotyping, neutralization titers, and infection status.

| Patient ID | Location  | Platelets<br>( $\times 10^3/\mu\text{L}$ ) <sup>a</sup> | DENV<br>NS1 | Anti-DENV |     | DENV Serotype <sup>c</sup> | NT90 titer <sup>d</sup> |        |         |       | DENV infection status |
|------------|-----------|---------------------------------------------------------|-------------|-----------|-----|----------------------------|-------------------------|--------|---------|-------|-----------------------|
|            |           |                                                         |             | IgM       | IgG |                            | DENV1                   | DENV2  | DENV3   | DENV4 |                       |
| 1          | Kathmandu | 101                                                     | +           | +         | +   | —                          | <40                     | 1708   | 1735    | <40   | Sec                   |
| 2          |           | 128                                                     | +           | +         | —   | —                          | 42                      | 1010   | 99      | 89    | Prim                  |
| 3          |           | 94                                                      | +           | +         | —   | DENV2                      | <40                     | 82     | <40     | <40   | Prim                  |
| 4          |           | 20                                                      | —           | +         | +   | NE                         | 4070                    | 51,382 | 140,960 | <40   | Sec                   |
| 5          |           | 80                                                      | —           | Equ       | +   | NE                         | 120,158                 | 46,411 | 158,838 | <40   | Sec                   |
| 6          |           | 76                                                      | +           | +         | —   | DENV2                      | 245                     | 16,286 | 23,817  | <40   | Sec                   |
| 7          |           | 196                                                     | +           | —         | Equ | DENV3                      | <40                     | <40    | <40     | <40   | —                     |
| 8          |           | 111                                                     | +           | +         | +   | —                          | <40                     | 2523   | <40     | <40   | Prim                  |
| 9          |           | 53                                                      | +           | —         | —   | DENV2                      | <40                     | <40    | <40     | <40   | —                     |
| 10         |           | 210                                                     | +           | —         | —   | —                          | <40                     | <40    | <40     | 125   | Prim                  |
| 11         |           | 143                                                     | —           | —         | +   | NE                         | <40                     | 68     | 54      | 52    | Sec                   |
| 12         |           | 66                                                      | +           | +         | —   | —                          | 56                      | 67     | 721     | <40   | Prim                  |
| 13         |           | 61                                                      | +           | +         | —   | —                          | <40                     | <40    | <40     | <40   | —                     |
| 14         |           | 76                                                      | —           | Equ       | +   | NE                         | 16,887                  | 19,645 | 9985    | 352   | Sec                   |
| 15         |           | 69                                                      | +           | +         | +   | —                          | 50,072                  | 18,730 | 14,242  | 722   | Sec                   |
| 16         |           | 136                                                     | +           | —         | +   | DENV2                      | 202                     | 12,665 | 226     | 200   | Sec                   |
| 17         |           | 365                                                     | —           | +         | +   | NE                         | 57                      | 238    | 1251    | <40   | Prim                  |
| 18         |           | 244                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 19         |           | 51                                                      | +           | —         | —   | —                          | <40                     | <40    | <40     | <40   | —                     |
| 20         |           | 91                                                      | +           | —         | +   | —                          | 14,584                  | 7496   | <40     | 914   | Sec                   |
| 21         |           | 97                                                      | +           | +         | —   | —                          | 45,639                  | 384    | <40     | 87    | Prim                  |
| 22         | Chitwan   | 21                                                      | +           | +         | +   | —                          | NA                      | NA     | NA      | NA    | NA                    |
| 23         |           | 50                                                      | +           | +         | +   | DENV2                      | NA                      | NA     | NA      | NA    | NA                    |
| 24         |           | 159                                                     | +           | —         | —   | DENV2                      | <40                     | <40    | <40     | <40   | —                     |
| 25         |           | 124                                                     | —           | —         | +   | NE                         | 94                      | <40    | 60      | 102   | Sec                   |
| 26         |           | 95                                                      | +           | +         | —   | —                          | <40                     | 3190   | 108     | 118   | Prim                  |
| 27         |           | 165                                                     | +           | —         | —   | —                          | <40                     | 74     | 308     | <40   | Prim                  |
| 28         |           | 206                                                     | —           | —         | +   | NE                         | <40                     | 58     | 75      | <40   | Sec                   |
| 29         |           | 194                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 30         |           | 121                                                     | —           | —         | —   | NE                         | 49                      | <40    | 55      | <40   | Sec                   |
| 31         |           | 113                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 32         |           | 231                                                     | —           | —         | +   | NE                         | <40                     | 45     | 73      | <40   | Sec                   |
| 33         |           | 189                                                     | +           | —         | —   | —                          | <40                     | <40    | <40     | <40   | —                     |
| 34         |           | N/D                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 35         |           | 106                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 36         |           | 189                                                     | —           | —         | —   | NE                         | 65                      | <40    | 44      | <40   | Sec                   |
| 37         |           | 357                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 38         |           | 120                                                     | —           | —         | +   | NE                         | <40                     | <40    | 68      | <40   | Prim                  |
| 39         |           | 135                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 40         |           | 100                                                     | —           | —         | —   | NE                         | 60                      | <40    | 53      | <40   | Sec                   |
| 41         |           | 30                                                      | —           | —         | +   | NE                         | <40                     | 118    | 77      | 143   | Sec                   |
| 42         |           | 245                                                     | —           | —         | —   | NE                         | 46                      | <40    | 49      | <40   | Sec                   |
| 43         |           | 130                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 44         |           | 107                                                     | —           | —         | +   | NE                         | 356                     | 128    | 81      | <40   | Sec                   |
| 45         |           | 108                                                     | —           | —         | +   | NE                         | 137                     | 46     | 84      | <40   | Sec                   |
| 46         |           | 205                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 47         |           | 140                                                     | —           | —         | +   | NE                         | 1245                    | 318    | 152     | <40   | Prim                  |
| 48         |           | 176                                                     | —           | —         | —   | NE                         | <40                     | <40    | <40     | <40   | —                     |
| 49         |           | 15                                                      | —           | +         | +   | NE                         | 89                      | 193    | 940     | 74    | Prim                  |

Abbreviations: Equ, equivocal (inconclusive after 2 tests); NA, not available (insufficient plasma); NE, not evaluated (only NS1+ samples were evaluated by RT-PCR);

NT<sub>90</sub>, 90% neutralization titer; Prim, primary; Sec, secondary.

<sup>a</sup>Platelet counts ( $\times 10^3/\mu\text{L}$ ) and dengue risk: low, 41–100; moderate, 21–40; high, <20 (normal, 100–450  $\times 10^3/\mu\text{L}$ ).

<sup>b</sup> By ELISA

<sup>c</sup> By serotype-specific RT-PCR

<sup>d</sup> Lowest dilution (1:40) was the designated cutoff value for positivity.

<https://doi.org/10.1371/journal.pgph.0002966.t002>



(10%,  $n = 5$ ) and IgM<sup>+</sup> IgG<sup>+</sup> (4%,  $n = 2$ ). Evidence of previous dengue infections were observed in subjects with IgG alone (24%,  $n = 12$ ) (Table 2). Samples from 29% (14/49) of subjects showed no evidence of current or prior infection (NS1<sup>-</sup>, IgM<sup>-</sup>, and IgG<sup>-</sup>). The 21 NS1 positive sera were further analyzed for DENV serotype by RT-PCR, 7 (33%) were found to be positive for DENV RNA, with 6 samples serotyped as DENV2 and 1 as DENV3 (Table 2). Thus, despite the small number of RT-PCR RNA-positive samples, DENV2 appears to have been the dominant circulating serotype in our 49-subject cohort at the time of the 2017 outbreak.

### Neutralizing Ab response against DENV1–4 serotypes

Of the 49 samples collected, 47 were evaluated in cell-based neutralizing assays (Fig 2A). The mean NT<sub>50</sub> titers for DENV1, DENV2, and DENV3 were similar, whereas the mean NT<sub>50</sub> titer for DENV4 was much lower overall, and significantly lower when compared with DENV2 (Fig 2A). Anti-DENV4 nAbs were undetectable in 35 samples (74%) (Fig 2A). Strikingly, 3 of the 7 samples positive for DENV RNA by RT-PCR were negative for nAbs against any of the DENV serotypes (Table 2). By evaluating anti-DENV nAb activity, it is possible to infer whether DENV infection is primary or secondary for both single- and multiple-serotype infections [33]. Using this approach, 26% (12/47) and 40% (19/47) of samples were from patients experiencing primary and secondary infections, respectively (Table 2). The remaining 34% (16/47) of samples had NT<sub>50</sub> values <40 for all 4 serotypes; of these, 10 were NS1<sup>-</sup> IgM<sup>-</sup> IgG<sup>-</sup> and an additional 4 were IgM<sup>-</sup> IgG<sup>-</sup> (Table 2). Thus, our study cohort consisted of patients who experienced both primary and secondary DENV exposure during the 2017 outbreak, with a higher prevalence of secondary infections.

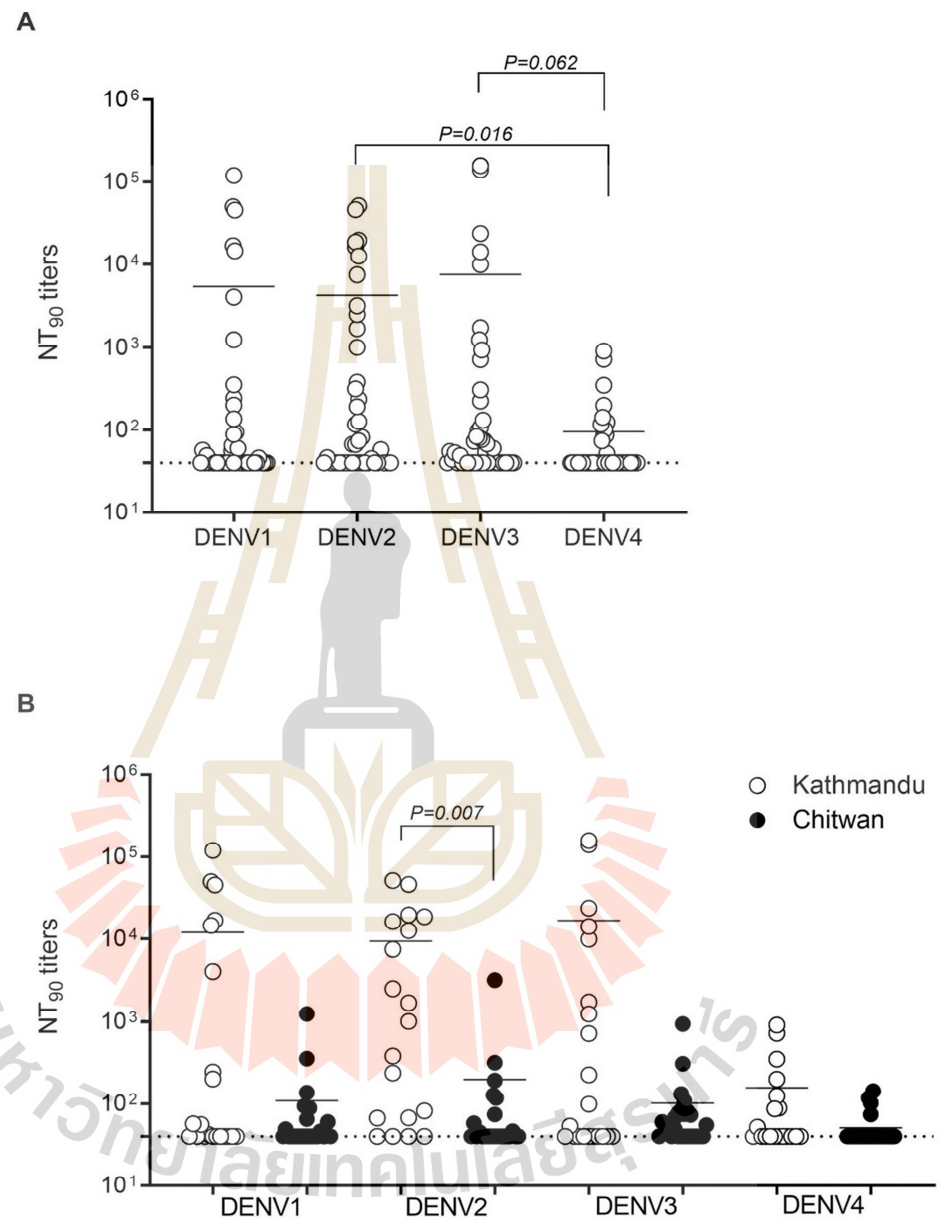
### Phylogenetic and nucleotide sequence analysis of DENV isolates

Full-genome sequences were obtained from the 7 serum samples shown to be positive for DENV RNA by RT-PCR (Table 2). Of the 7 genomes, 2 complete sequences and 5 incomplete sequences were obtained. Whole-genome phylogenetic analysis of the complete DENV2 genomes (PP152366 and PP152367, NCBI database) indicated that they belong to the Cosmopolitan Genotype, with the closest relatives being DENV2 sequences from Singapore in 2016 and 2014 (MW512465 and MW512428, respectively) (Fig 3). Because of the scarcity of full-genome sequences from Nepal ( $n = 2$ ), we also analyzed the phylogeny of the E gene of PP152366 and PP152367 and E gene sequences from global DENV2 isolates. Close relationships were detected between PP152366 and PP152367 and E genes from 12 additional DENV2 sequences isolated in 2017 from Nepal [34] and 4 from India in 2017 (S1 Fig). Thus, the 2017 DENV2 strains from Nepal and India likely originated from Singapore.

### Discussion

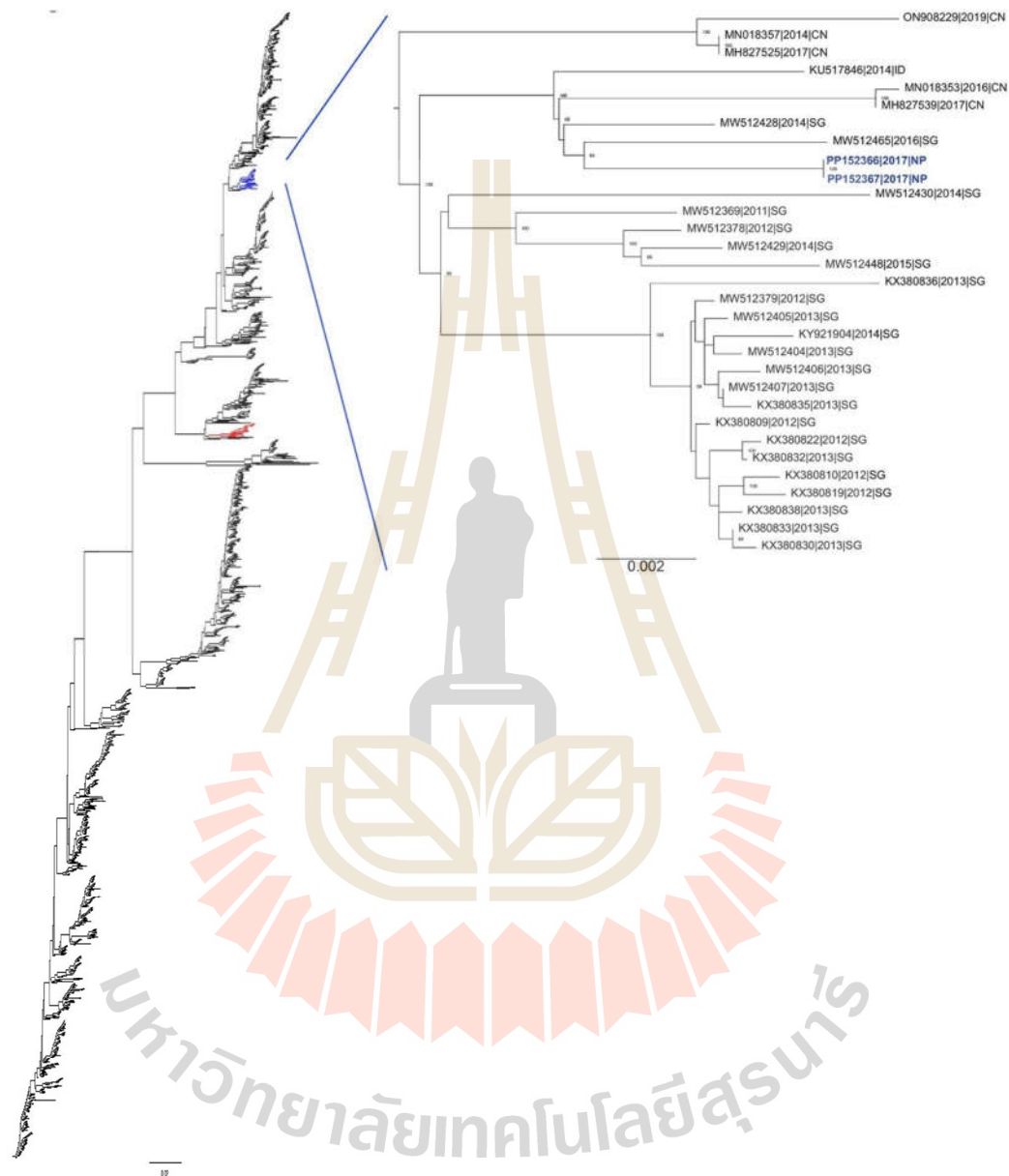
Over the past decade, Nepal has experienced increasingly severe outbreaks of dengue in approximately 3-year cycles, and cases were reported from all 77 districts during the 2019 outbreak [25, 35, 36]. Despite this public health burden, little was known about the DENV genotypes and serotypes circulating during each outbreak, or of the DENV serological status of the population. This was in large part because the physical infrastructure and knowledge base needed to collect and analyze such data in a systematic manner were lacking, and this study was designed to begin this process with local scientists/clinicians. The overall goal is to amass sufficient data to enable local and central governments to plan for future dengue outbreaks and, eventually, the selection and distribution of DENV vaccines.

We report here the analysis of samples collected during the 2017 dengue season from 49 patients presenting at the main hospitals in Kathmandu and Chitwan in central and southern



**Fig 2. Distribution of nAb titers in samples collected during the 2017 outbreak.** (A and B) NT<sub>90</sub> titers were determined according to DENV1–4 reference serotypes (A) and sample collection site (B). Mean values for  $n = 47$  ( $n = 21$  Kathmandu,  $n = 26$  Chitwan) with circles representing individual samples. Dashed lines represent the cutoff value for nAb positivity (NT<sub>90</sub> = 40). Mean values were compared using the non-parametric Kruskal–Wallis test.

<https://doi.org/10.1371/journal.pgph.0002966.g002>



**Fig 3. Phylogenetic analysis of full-genome sequences of the Nepal 2017 DENV2 isolates generated here and other DENV2 isolates.** Phylogenetic tree comparing the DENV2 isolates from this study (blue, PP152366[2017]NP and PP152367[2017]NP), with other DENV2 full genome sequences present in NCBI. Strains are labeled by GenBank ID, as well as year and country of isolation (CN, China; SG, Singapore; NP, Nepal).

<https://doi.org/10.1371/journal.pgph.0002966.g003>



In conclusion, despite its limited size, our study not only sheds light on the prevalence of DENV serotypes and nAbs in a Nepalese cohort during the 2017 dengue season, but also marks a significant advance in establishing, for the first time, the scientific infrastructure to perform genomic and immunologic surveillance of DENV in Nepal. Our analysis demonstrates that a DENV2 genome closely related to strains from Singapore in 2016 was most likely the dominant serotype circulating in Nepal in 2017. The shift in dominant DENV serotypes between outbreaks and the prevalence of both primary and secondary DENV infections underscore the need for sustained surveillance of DENV at virologic and immunologic levels. As noted earlier, the clinical outcome of DENV vaccination will be influenced by the vaccine efficacy profile, the DENV strains circulating at the time of vaccination, and the subjects' exposure prior to and after vaccination. Thus, our results represent a springboard for further studies to understand DENV virology and immunology across Nepal and thereby inform the selection of one or more DENV vaccine candidates.

### Study limitations

The small cohort size limits the interpretative value of our results. Additionally, we were unable to obtain complete clinical data or information on patient outcomes for all participants, which limited our ability to make correlations with specific serotypes or other biomarkers. Analysis of samples collected at a single time point during the acute phase of infection, rather than paired acute/convalescent samples, allowed us to infer, but not establish, the subjects' DENV infection status [50].

### Supporting information

**S1 Fig. Phylogenetic analysis of E gene sequences from the 2017 DENV2 genomes from Nepal obtained in the present study shows a close relationship with other 2017 DENV2 isolates from Nepal and India.** Phylogenetic tree comparing E gene sequences of the two 2017 DENV2 strains from Nepal in the current study (blue font, PP152366[2017]NP and PP152367[2017]NP) with other E gene sequences in NCBI. Strains are labeled by GenBank ID, followed by the year and country of isolation (CN, China; ID, Indonesia; IN, India; NP, Nepal; PG, Papua New Guinea; SG, Singapore; TW, Taiwan). (TIF)

### Acknowledgments

We thank the Flow Cytometry Core (Cheryl Kim) at the La Jolla Institute for Immunology for their assistance with experiments, and Dr. Aunji Pradhan from Tribhuvan University for her constructive comments on the manuscript. We are grateful to the clinical staff at Sukraraj Tropical and Infectious Disease Hospital and at Chitwan Medical College and Teaching Hospital, and to the study participants and their families. We would also like to thank Karius Inc. (Redwood City, CA, USA) for its training on regulatory compliance for the source documents and Dr. David Wang (Washington University in St. Louis, NIH grant U01 AI151810) for helping us to build scientific infrastructure at Tribhuvan University and the Prebys Foundation award (to SS).

### Author Contributions

**Conceptualization:** Annie Elong Ngono, Sujan Shrestha, Krishna Das Manandhar.

**Data curation:** Sabita Prajapati, Annie Elong Ngono, Melanie Mc Cauley.

**Formal analysis:** Sabita Prajapati, Annie Elong Ngono, Julia Timis, Rajindra Napit, October M. Sessions, Sujan Shrestha, Krishna Das Manandhar.

**Funding acquisition:** Annie Elong Ngono, Montarop Yamabhai, Sujan Shrestha, Krishna Das Manandhar.

**Investigation:** Sabita Prajapati, Annie Elong Ngono, Melanie Mc Cauley, Julia Timis, Srijan Shrestha, Anup Bastola, Sanjay Ray Yadav, Rajindra Napit.

**Methodology:** Sabita Prajapati, Annie Elong Ngono, Melanie Mc Cauley, Julia Timis, Srijan Shrestha, Anup Bastola, Shrawan Kumar Mandal, Sanjay Ray Yadav, Rajindra Napit, October M. Sessions.

**Project administration:** Sabita Prajapati, Annie Elong Ngono, Anup Bastola, Sujan Shrestha, Krishna Das Manandhar.

**Resources:** Annie Elong Ngono, Melanie Mc Cauley, Anup Bastola, Shrawan Kumar Mandal, Sanjay Ray Yadav, Sujan Shrestha.

**Software:** Sabita Prajapati, Annie Elong Ngono, October M. Sessions.

**Supervision:** Annie Elong Ngono, Sujan Shrestha, Krishna Das Manandhar.

**Validation:** Sabita Prajapati, Annie Elong Ngono, Sujan Shrestha, Krishna Das Manandhar.

**Visualization:** Sabita Prajapati, Annie Elong Ngono, Sujan Shrestha, Krishna Das Manandhar.

**Writing – original draft:** Sabita Prajapati, Annie Elong Ngono, Melanie Mc Cauley, Julia Timis, Sujan Shrestha.

**Writing – review & editing:** Meng Ling Moi, Montarop Yamabhai, October M. Sessions, Sujan Shrestha, Krishna Das Manandhar.

## References

1. Guzman MG, Harris E. Dengue. *Lancet*. 2015; 385(9966):453–65. Epub 2014/09/19. [https://doi.org/10.1016/S0140-6736\(14\)60572-9](https://doi.org/10.1016/S0140-6736(14)60572-9) PMID: 25230594.
2. Pierson TC, Diamond MS. The continued threat of emerging flaviviruses. *Nat Microbiol*. 2020; 5(6):796–812. Epub 2020/05/06. <https://doi.org/10.1038/s41564-020-0714-0> PMID: 32367055.
3. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. *Nature*. 2013; 496(7446):504–7. <https://doi.org/10.1038/nature12060> PMID: 23563266.
4. WHO M. Dengue and severe dengue. Geneva. 2019.
5. Katzelnick LC, Bos S, Harris E. Protective and enhancing interactions among dengue viruses 1–4 and Zika virus. *Curr Opin Virol*. 2020; 43:59–70. Epub 2020/09/27. <https://doi.org/10.1016/j.coviro.2020.08.006> PMID: 32979816.
6. Halstead SB. Dengue Antibody-Dependent Enhancement: Knowns and Unknowns. *Microbiol Spectr*. 2014; 2(6). <https://doi.org/10.1128/microbiolspec.AID-0022-2014> PMID: 26104444.
7. Ngono AE, Shrestha S. Immune Response to Dengue and Zika. *Annu Rev Immunol*. 2018; 36:279–308. Epub 2018/01/19. <https://doi.org/10.1146/annurev-immunol-012617-053142> PMID: 29345964.
8. Sridhar S, Luedtke A, Langevin E, Zhu M, Bonaparte M, Machabert T, et al. Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy. *N Engl J Med*. 2018; 379(4):327–40. Epub 2018/06/14. <https://doi.org/10.1056/NEJMoa1800820> PMID: 29697841.
9. Aguiar M, Stollenwerk N. The Impact of Serotype Cross-Protection on Vaccine Trials: DENVax as a Case Study. *Vaccines (Basel)*. 2020; 8(4). Epub 2020/11/18. <https://doi.org/10.3390/vaccines8040674> PMID: 33198303.
10. Patel SS, Rauscher M, Kudela M, Pang H. Clinical Safety Experience of TAK-003 for Dengue Fever: A New Tetravalent Live Attenuated Vaccine Candidate. *Clin Infect Dis*. 2023; 76(3):e1350–e9. Epub 2022/06/01. <https://doi.org/10.1093/cid/ciac418> PMID: 35639602.



11. Thomas SJ. Is new dengue vaccine efficacy data a relief or cause for concern? *NPJ Vaccines*. 2023; 8(1):55. Epub 2023/04/16. <https://doi.org/10.1038/s41541-023-00658-2> PMID: 37061527 support for a wide variety of dengue research initiatives. He has/is supporting dengue countermeasure development efforts as a consultant, advisory board member, or blinded case adjudication committee member for GlaxoSmithKline, Sanofi, Takeda, and/or Merck and was/is compensated for his time. S.J.T. has a patent related to dengue vaccines and all rights were assigned to the U.S. government.
12. Kallas EG, Cintra MAT, Moreira JA, Patino EG, Braga PE, Tenorio JCV, et al. Live, Attenuated, Tetravalent Butantan-Dengue Vaccine in Children and Adults. *N Engl J Med*. 2024; 390(5):397–408. Epub 2024/01/31. <https://doi.org/10.1056/NEJMoa2301790> PMID: 38294972.
13. OhAinle M, Balmaseda A, Macalalad AR, Tellez Y, Zody MC, Saborio S, et al. Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. *Sci Transl Med*. 2011; 3(114):114ra28. Epub 2011/12/23. <https://doi.org/10.1126/scitranslmed.3003084> PMID: 22190239.
14. Takasaki T, Kotaki A, Nishimura K, Sato Y, Tokuda A, Lim CK, et al. Dengue virus type 2 isolated from an imported dengue patient in Japan: first isolation of dengue virus from Nepal. *J Travel Med*. 2008; 15(1):46–9. <https://doi.org/10.1111/j.1708-8305.2007.00165.x> PMID: 18217869.
15. Malla S, Thakur GD, Shrestha SK, Banjerree MK, Thapa LB, Gongal G, et al. Identification of all dengue serotypes in Nepal. *Emerg Infect Dis*. 2008; 14(10):1669–70. Epub 2008/10/02. <https://doi.org/10.3201/eid1410.080432> PMID: 18826846.
16. Rijal KR, Adhikari B, Ghimire B, Dhungel B, Pyakurel UR, Shah P, et al. Epidemiology of dengue virus infections in Nepal, 2006–2019. *Infect Dis Poverty*. 2021; 10(1):52. Epub 2021/04/17. <https://doi.org/10.1186/s40249-021-00637-0> PMID: 33858508.
17. EDCD MoHaP, Services DoH, Division EaDC. Situation update of Dengue 2022 (As of 31th Dec,2022). 2022.
18. Rimal S, Shrestha S, Pandey K, Nguyen TV, Bhandari P, Shah Y, et al. Co-Circulation of Dengue Virus Serotypes 1, 2, and 3 during the 2022 Dengue Outbreak in Nepal: A Cross-Sectional Study. *Viruses*. 2023; 15(2). Epub 2023/03/01. <https://doi.org/10.3390/v15020507> PMID: 36651721.
19. Organization WH. Dengue-Nepal. 2022.
20. Poudyal P, Sharma K, Dumre SP, Bastola A, Chalise BS, Shrestha B, et al. Molecular study of 2019 dengue fever outbreaks in Nepal. *Trans R Soc Trop Med Hyg*. 2021; 115(6):619–26. Epub 2020/09/29. <https://doi.org/10.1093/trstmh/traa096> PMID: 32987406.
21. Adhikari N, Subedi D. The alarming outbreaks of dengue in Nepal. *Trop Med Health*. 2020; 48:5. Epub 2020/02/15. <https://doi.org/10.1186/s41182-020-0194-1> PMID: 32055230.
22. Manandhar KD, McCauley M, Gupta BP, Kurmi R, Adhikari A, Nguyen AV, et al. Whole Genome Sequencing of Dengue Virus Serotype 2 from Two Clinical Isolates and Serological Profile of Dengue in the 2015–2016 Nepal Outbreak. *Am J Trop Med Hyg*. 2021; 104(1):115–20. Epub 2020/10/20. <https://doi.org/10.4269/ajtmh.20-0163> PMID: 33073748.
23. (FDA) USFDA. 510(k) substantial equivalence determination decision summary: CDC DENV-1-4 real-time RT-PCR assay. 2012. [https://www.accessdata.fda.gov/cdrh\\_docs/reviews/k113336.pdf](https://www.accessdata.fda.gov/cdrh_docs/reviews/k113336.pdf).
24. Waggoner JJ, Abeynayake J, Sahoo MK, Gresh L, Tellez Y, Gonzalez K, et al. Single-reaction, multiplex, real-time rt-PCR for the detection, quantitation, and serotyping of dengue viruses. *PLoS Negl Trop Dis*. 2013; 7(4):e2116. Epub 2013/05/03. <https://doi.org/10.1371/journal.pntd.0002116> PMID: 23638191.
25. Prajapati S, Napit R, Bastola A, Rauniyar R, Shrestha S, Lamsal M, et al. Molecular phylogeny and distribution of dengue virus serotypes circulating in Nepal in 2017. *PLoS One*. 2020; 15(7):e0234929. Epub 2020/07/08. <https://doi.org/10.1371/journal.pone.0234929> PMID: 32634137 USA. This does not alter our adherence to PLOS ONE policies on sharing data and materials. Please check these statements carefully and let us know if they are correct and have your approval. If they are incorrect, please edit them and provide us with updated, accurate, versions.
26. Santiago GA, Vergne E, Quiles Y, Cosme J, Vazquez J, Medina JF, et al. Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus. *PLoS Negl Trop Dis*. 2013; 7(7):e2311. <https://doi.org/10.1371/journal.pntd.0002311> PMID: 23875046.
27. Kamaraj US, Tan JH, Xin Mei O, Pan L, Chawla T, Uehara A, et al. Application of a targeted-enrichment methodology for full-genome sequencing of Dengue 1–4, Chikungunya and Zika viruses directly from patient samples. *PLoS Negl Trop Dis*. 2019; 13(4):e0007184. Epub 2019/04/26. <https://doi.org/10.1371/journal.pntd.0007184> PMID: 31022183.
28. de Alwis R, de Silva AM. Measuring antibody neutralization of dengue virus (DENV) using a flow cytometry-based technique. *Methods Mol Biol*. 2014; 1138:27–39. [https://doi.org/10.1007/978-1-4939-0348-1\\_3](https://doi.org/10.1007/978-1-4939-0348-1_3) PMID: 24696329.

29. Hattakam S, Elong Ngono A, McCauley M, Shrestha S, Yamabhai M. Repeated exposure to dengue virus elicits robust cross neutralizing antibodies against Zika virus in residents of Northeastern Thailand. *Sci Rep*. 2021; 11(1):9634. Epub 2021/05/07. <https://doi.org/10.1038/s41598-021-88933-x> PMID: 33953258.
30. Nepal Go. Annual Report, Department of Health Services, Ministry of Health & Population. 2012/2013 and 2013/2014.
31. Jayashree K, Manasa GC, Pallavi P, Manjunath GV. Evaluation of platelets as predictive parameters in dengue Fever. *Indian J Hematol Blood Transfus*. 2011; 27(3):127–30. Epub 2012/09/04. <https://doi.org/10.1007/s12288-011-0075-1> PMID: 22942561.
32. Ojha A, Nandi D, Betra H, Singhal R, Annarapu GK, Bhattacharyya S, et al. Platelet activation determines the severity of thrombocytopenia in dengue infection. *Sci Rep*. 2017; 7:41697. Epub 2017/02/01. <https://doi.org/10.1038/srep41697> PMID: 28139770.
33. Nguyen THT, Clapham HE, Phung KL, Nguyen TK, TT DI, Nguyen THQ, et al. Methods to discriminate primary from secondary dengue during acute symptomatic infection. *BMC Infect Dis*. 2018; 18(1):375. Epub 2018/08/09. <https://doi.org/10.1186/s12879-018-3274-7> PMID: 30086716.
34. Ngwe Tun MM, Pandey K, Nabeshima T, Kyaw AK, Adhikari M, Raini SK, et al. An Outbreak of Dengue Virus Serotype 2 Cosmopolitan Genotype in Nepal, 2017. *Viruses*. 2021; 13(8). Epub 2021/08/29. <https://doi.org/10.3390/v13081444> PMID: 34452310.
35. Gupta BP, Tuladhar R, Kurmi R, Manandhar KD. Dengue periodic outbreaks and epidemiological trends in Nepal. *Ann Clin Microbiol Antimicrob*. 2018; 17(1):6. Epub 2018/02/24. <https://doi.org/10.1186/s12941-018-0258-9> PMID: 29471819.
36. Khetan RP, Stein DA, Chaudhary SK, Rauniyar R, Upadhyay BP, Gupta UP, et al. Profile of the 2016 dengue outbreak in Nepal. *BMC Res Notes*. 2018; 11(1):423. Epub 2018/07/05. <https://doi.org/10.1186/s13104-018-3514-3> PMID: 29970132.
37. Rauniyar R, Prajapati S, Manandhar B, Bastola A, Chalise BS, Shrestha S, et al. Dengue virus infection during window period of consecutive outbreaks in Nepal and assessment of clinical parameters. *Sci Rep*. 2023; 13(1):9262. Epub 2023/06/08. <https://doi.org/10.1038/s41598-023-35928-5> PMID: 37286625.
38. Gupta BP, Haselbeck A, Kim JH, Marks F, Saluja T. The Dengue virus in Nepal: gaps in diagnosis and surveillance. *Ann Clin Microbiol Antimicrob*. 2018; 17(1):32. Epub 2018/07/17. <https://doi.org/10.1186/s12941-018-0284-7> PMID: 30008269.
39. Gupta BP, Lamsal M, Chaulagain S, Rauniyar R, Malla R, Shrestha S, et al. Emergence of dengue in Nepal. *Virusdisease*. 2018; 29(2):129–33. Epub 2018/06/19. <https://doi.org/10.1007/s13337-018-0439-3> PMID: 29911144.
40. (WHO) WHO. Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses 2007.
41. Pandey BD, Nabeshima T, Pandey K, Rajendra SP, Shah Y, Adhikari BR, et al. First isolation of dengue virus from the 2010 epidemic in Nepal. *Trop Med Health*. 2013; 41(3):103–11. <https://doi.org/10.2149/tmh.2012-17> PMID: 24155651.
42. Singh S, Gupta BP, Manakkadan A, Das Manandhar K, Sreekumar E. Phylogenetic study reveals co-circulation of Asian II and Cosmopolitan genotypes of Dengue virus serotype 2 in Nepal during 2013. *Infect Genet Evol*. 2015; 34:402–9. Epub 2015/07/15. <https://doi.org/10.1016/j.meegid.2015.07.006> PMID: 26160542.
43. Katzelnick LC, Montoya M, Gresh L, Balmaseda A, Harris E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proc Natl Acad Sci U S A*. 2016; 113(3):728–33. Epub 2016/01/06. <https://doi.org/10.1073/pnas.1522136113> PMID: 26729879.
44. Bos S, Graber AL, Cardona-Ospina JA, Duarte EM, Zambrana JV, Ruiz Salinas JA, et al. Protection against symptomatic dengue infection by neutralizing antibodies varies by infection history and infecting serotype. *Nature Communications*. 2024; 15(1):382. <https://doi.org/10.1038/s41467-023-44330-8> PMID: 38195666.
45. Yenamandra SP, Koo C, Chiang S, Lim HSJ, Yeo ZY, Ng LC, et al. Evolution, heterogeneity and global dispersal of cosmopolitan genotype of Dengue virus type 2. *Sci Rep*. 2021; 11(1):13496. Epub 2021/07/01. <https://doi.org/10.1038/s41598-021-92783-y> PMID: 34188091.
46. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. 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*. 2002; 40(2):376–81. Epub 2002/02/05. <https://doi.org/10.1128/JCM.40.02.376-381.2002> PMID: 11825945.

47. Huhtamo E, Hasu E, Uzategui NY, Erra E, Nikkari S, Kantele A, et al. Early diagnosis of dengue in travelers: comparison of a novel real-time RT-PCR, NS1 antigen detection and serology. *J Clin Virol*. 2010; 47(1):49–53. Epub 2009/12/08. <https://doi.org/10.1016/j.jcv.2009.11.001> PMID: [19963435](#).
48. Singh MP, Majumdar M, Singh G, Goyal K, Preet K, Sarwal A, et al. NS1 antigen as an early diagnostic marker in dengue: report from India. *Diagn Microbiol Infect Dis*. 2010; 68(1):50–4. Epub 2010/08/24. <https://doi.org/10.1016/j.diagmicrobio.2010.04.004> PMID: [20727470](#).
49. Alm E, Lindegren G, Falk KI, Lagerqvist N. One-step real-time RT-PCR assays for serotyping dengue virus in clinical samples. *BMC Infect Dis*. 2015; 15:493. <https://doi.org/10.1186/s12879-015-1226-z> PMID: [26527283](#).
50. Collins MH, McGowan E, Jodi R, Young E, Lopez CA, Baric RS, et al. Lack of Durable Cross-Neutralizing Antibodies Against Zika Virus from Dengue Virus Infection. *Emerg Infect Dis*. 2017; 23(5):773–81. Epub 2017/04/19. <https://doi.org/10.3201/eid2305.161630> PMID: [28418292](#).





## BIOGRAPHY

The author, Miss Sabita Prajapati, was born on 11 June 1993 in Nepal. She graduated from Tri-Chandra Multiple Campus, Tribhuvan University with a Bachelor's degree in science, microbiology (2011-2014). Later she completed her Master's degree in sciences in Biotechnology from Central Department of Biotechnology, Tribhuvan University (2015-2018). During this period, she also worked as a short-term research scholar at La Jolla Institute for Immunology, USA. Now, she is doing her Ph.D. at the School of Biotechnology, Suranaree University of Technology, since 2021. She has also worked as a visiting scholar in The University of Tokyo for a period of 3 months. She has research experiences in infectious diseases like dengue, leishmania for couple of years with hands on skill on phage display technology, antibody engineering, cell culture, protein expression, molecular cloning, and other immuno-molecular tools and techniques. During her academic journey she has 5 research publications so far. Her research career goal is to be able to do something good leading to benefit to mankind.

