MINING ENVELOPE DOMAIN III OF DENGUE VIRUS FOR RECOMBINANT TETRAVALENT DNA VACCINE CANDIDATE FROM NEPALESE SAMPLES

Master in Science Thesis

2021 AD

Submitted to

Central Department of Biotechnology,

Tribhuvan University,

Kirtipur, Kathmandu, Nepal

By

Machchhendra Thapa

BT 409/073

Central Department of Biotechnology, TU.

Supervisor:

Prof. Dr. Krishna Das Manandhar Ph.D.

Head of Department

Central Department of Biotechnology,

TU, Kirtipur

TU Registration No: 5-3-28-228-2016

Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Date: Dec , 2021

RECOMMENDATION

This is to certify that the research work entiled “**MINING ENVELOPE DOMAIN III OF DENGUE VIRUS FOR RECOMBINANT TETRAVALENT DNA VACCINE CANDIDATE FROM NEPALESE SAMPLES”** has been carried out by **Mr. Machchhendra Thapa** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

……………………………….

(Supervisor)

Prof. Dr. Krishna Das Manandhar

Head of Department

Central Department of Biotechnology

Tribhuvan University,

Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Date: Dec , 2021

Certificate of Evaluation

This is to certify that this entitled "**MINING ENVELOPE DOMAIN III OF DENGUE VIRUS FOR RECOMBINANT TETRAVALENT DNA VACCINE CANDIDATE FROM NEPALESE SAMPLES"** presented to evaluation committed by **Mr. Machchhendra Thapa** is found satisfactory for the partial fulfillment of Master of Science and Technology.

……………………………………

Prof. Krishna Das Manandhar, Ph.D
Head of Department
(Supervisor)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

…………………………..

(Internal Examiner)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

…………………………………………..

(External Examiner)

Acknowledgment

This research title entitled **"MINING ENVELOPE DOMAIN III OF DENGUE VIRUS FOR RECOMBINANT TETRAVALENT DNA VACCINE CANDIDATE FROM NEPALESE SAMPLES"** was done for the partial fulfillment of Master of Science in Biotechnology under the course code BT 621 in Central Department of Biotechnology. It is one of the esteemed institute for research in the field of science and technology and provide student researchers with lab facilities and chemicals for which I am very grateful.

I would like to show my veneration to **Prof. Dr. Krishna Das Manandhar**, Head of Department and my thesis supervisor for his precious supervision, constant support, and valuable guidance that was pivotal for the thesis work. He has been very kind and generous with his valuable time to guide me through this study. He has empowered me to excel in the research through his guidance and encouragement.

I would also like to thank my senior **Ms. Sabita Prajapati** for guidance and support in critical step of the thesis work and enabling me to tackle the problem faced during the thesis. And particularly I would like to thank my friend **Ms. Chetana Khanal** for her help and support during tough times which has encouraged me to achieve the objectives and enabled me to advance throughout this study.

Also I would like to thank my all my mates **Roji Raut, Tinmaya Rai, Bandana Thakur, Sishir Gautam** and senior brothers and sisters including **Srijan Shrestha, Tika Bahadur Buda, Ramanuj Rauniyar, Mahesh Lamsal** for immense support during the time of work. Ms. Tinmaya Rai, Mr. Sishir Gautam and Mr. Ramanuj Rauniyar provided helping hands during sample collection. I am thankful to the entire **CDBT faculty, senior, juniors and my friends from 8th batch of CDBT.**

My sincere thanks goes to **Parmananda Bhandari**, Senior lab technician, Sukraraj Tropical and Infectious Disease Hospital and **Dr. Sanjay Ray Yadav**, Chitwan Medical College and Teaching Hospital, **Anil Yadav**, Hetauda Hospital and **Ramesh Gautam**, Bijaypur hospital whose enermous help during sample collection have enabled us to collect large numbers of samples from vast geographical regions.

I am in debt to **all the patients** who cooperated with us and signed the consent and participated in the study.

My sincere thanks goes to **Chinese Academy of Sciences (CAS)** for providing me with MSc Thesis Research Grant.

Finally I would like to show my deepest love and respect to **my Family** for their constant support and motivation to perform well during this thesis work.

Glossary Acronyms

ADE: Antibody-dependent enhancement

ALT: Alanine transaminase

ANOVA: Analysis of Variance

APC: Allophycocyanin

AST: Aspartate transaminase

BP: Blood pressure

BSL: Biosafety level

CCL4: CC-chemokine ligand 4

CD: Cluster of Differentiation

CD4: Cluster of differentiation 4, T helper cell surface glycoprotein

CD8: Cluster of differentiation 8, T cell co-receptor transmembrane glycoprotein

CDBT: Central Department of Biotechnology

CDC: Central for disease control

cDNA: Complimentary DNA

CFR: Case-fatality rate

CMC: Chitwan Medical College and Teaching Hospital, Chitwan

COMBI: Communication for behavioural impact

CRF: Case report form

Da: Dalton

DALYs: Disability Adjusted Life Years

DDT: Dichlorodiphenyltrichloroethane

DEET: Diethyl-meta-toluamide

DEN: Dengue

DENCO: Dengue and Control study (multi-country study)

DENRA: Dengue Recombinant Antigen

DENV: Dengue virus

DF: Dengue fever

DHF: Dengue haemorrhagic fever

DNA: Deoxyribonucleic acid

DSS: Dengue shock syndrome

DT: Tablet for direct application

E/M: Envelop/membrane antigen

EC: Emulsifiable concentrate

EIP: Extrinsic Incubation Period

ELISA: Enzyme-linked immunosorbent assay

FBC: Full blood count

Fc: Receptor fragment, crystallisable region, a cell receptor

FDA: Food and Drug Administration

FITC: Fluorescein isothiocyanate

FRhL: Fetal rhesus lung cells

GIS: Geographical Information System

GOARN: Global Outbreak Alert and Response Network

GPS: Global positioning system

GR: Granule

HCV: Hepatitis C Virus

HEPES: 4-2-hydroxyethyl-1-piperazineethanesulfonic acid

HI: Haemagglutination-inhibition

HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome

HLA-DR: Human leucocyte antigen- DR isotype

HSC: Human Specimen Control

ICU: Intensive care unit

IEC: Information, education, communication

IFNγ: Interferon-γ

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IIC:	Intrinsic Incubation Period
IL-10:	Interleukin-10
IL-2:	Interleukin-2
INF:	Interferon
IPCS:	International Programme on Chemical Safety
IR3535:	3-[N-acetyl-N-butyl]-aminopropionic acid ethyl ester
ISR:	Immune Status Ratio
ITM:	Insecticide treated material
IV:	Intravenous
JEV:	Japanese encephalitis virus
LAV:	Live attenuated vaccine
MAC-ELISA:	IgM antibody-capture enzyme-linked immunosorbent assay
MCMC:	Markov Chain Monte Carlo
MHC:	Major Histocompatibility Complex
MIA:	Microsphere-based immunoassays
MoE:	Ministry of Education
MoH:	Ministry of Health
NAAT:	Nucleic acid amplification test
NASBA:	Nucleic acid sequence based amplification
NCA:	Normal Cell Antigen
NDEN:	Non-dengue
NGO:	Nongovernmental organization
NHRC:	Nepal Health Research Council
NK:	Natural killer
NKT:	Natural Killer T cells
NS:	Non-structural protein
NS1:	Non-structural proteins1
NSAID:	Non-steroidal anti-inflammatory drugs
OD:	Optical Density

ORF:	Open reading frame
ORS:	Oral rehydration solution
PAHO:	Pan American Health Organization
PBMCs:	Peripheral blood mononuclear cells
PCR:	Polymerase Chain Reaction
PDVI:	Pediatric Dengue Vaccine Initiative
PE:	Phycoerythrin
PerCP:	Peridinin Chlorophyll Protein
pH:	Measure of the acidity or basicity of a solution
prM:	a region of the dengue genome
PRNT:	Plaque reduction and neutralization test
RNA:	Ribose nucleic acid
RT-PCR:	Reverse transcription-polymerase chain reaction
SNP:	Single Nucleotide Polymorphism
STIDH:	Sukraraj Tropical and Infectious disease Hospital
T cells:	A group of lymphocytes important for cell-mediated immunity
TBEV:	Tick-borne encephalitis virus
TDR:	Special Programme for Research and Training in Tropical Diseases
TMB:	Trimethyl Benzidine
TNF alfa:	Tumor necrosis factor alfa
TNF:	Tumour necrosis factor
UCMS:	Universal College of Medical Science
WBC:	White blood cells
WHO:	World Health Organization
WNV:	West Nile virus
WP:	Wettable powder
YF:	Yellow fever
YFV:	Yellow Fever Virus

Contents

Acknowledgment iv
Glossary Acronyms v
List of Figures xiii
List of tables xv
Abstract 1
CHAPTER I 2
INTRODUCTION 2
1.1 Background of Dengue Virus and Dengue Fever 2
1.2 History of Dengue Infection 2
1.3 Dengue in Global Scenario 3
1.4. Dengue prevalence in Nepal 4
1.5 Virus taxonomy 6
1.5.1 Taxonomy of Dengue Virus 6
1.6. Dengue Virus Morphology 7
1.7. Genomic organization of Dengue virus 7
1.7.1. Dengue virus serotype 9
1.8. Vectors and Mode of Transmission 10
1.8.1. Vectors of Dengue 10
1.8.2. Life cycle of Dengue vector 11
1.8.3. Mode of transmission of Dengue 12
1.9 Virus Replication 13
1.10 Dengue symptoms and clinical manifestations 14
1.11 Classification of Dengue ailment 16
1.12 Diagnosis of Dengue virus 18
1.13 Host Immune Response 20
1.14 Secondary Dengue Infections 22
1.15 Dengue Prevention and Control 24
1.15.1. Methods of Vector Control 24
1.16Theoretical/Conceptual Framework 27
1.17 Hypothesis/ Research Questions 28
1.18. OBJECTIVES: 29
1.18.1 General Objective: 29

1.18.2 Specific Objective: 29
1.19 Problem statement / Rationale 30
CHAPTER II 31
LITERATURE REVIEW 31
2.1 Dengue Virus 31
2.2. Dengue virus serotypes and their genotypes 32
2.3 Epidemiology of Dengue in Nepal 33
2.4 Mode of transmission of dengue 35
2.3 Clinical manifestations of dengue 36
2.4 Laboratory diagnosis of dengue 37
2.4.1 Serological / Immunological Diagnosis Approach 39
2.4.2 Enzyme Linked Immunosorbent Assay (ELISA) 40
2.5. Molecular identification/ Nucleic acid detection 43
2.6. Vaccine Development status 44
2.7 Cell Cultures of Dengue 47
2.8 Animal model for dengue research 48
CHAPTER III 49
MATERIALS AND METHOD 49
3.1 Ethical Approval 49
3.2 Site Selection 49
3.3 Sample Collection and Storage 50
3.3.1 Sample Size 50
3.3.2 Sample Collection and Storage 51
3.4 Sample Inclusion and Exclusion criteria 51
3.5. Validation of dengue cases 51
3.6 Immunological and serological Assay 52
3.6.1 Enzyme-linked Immuno-sorbent Assay (ELISA) 52
3.6.2. InBios NS1Ag ELISA: 52
3.6.3 InBios IgM ELISA 53
3.6.4 InBios IgG ELISA 54
3.6.5 Statistical Analysis: 54
3.7 Molecular Assay 55
3.7.1 Viral RNA Isolation by QIAamp® viral RNA Mini kit 55
3.7.2 Serotyping by Reverse Transcriptase Real-Time Polymerase Chain Reaction 56

3.7.3 Viral RNA Isolation by TRIzol 58
3.7.4 cDNA preparation 58
3.7.5 Nested RT-PCR and Primer Designing 59
3.7.6 PCR amplification of Domain III region of all four serotype with overlapping region 62
3.7.7 Sequencing of the construct 63
3.7.8 *Insilico* Analysis 63
3.8 Dengue virus propagation 63
3.9 Animal immunization 64
3.10 Inhouse ELISA for serotype specific IgG detection 65
3.10.1 ELISA Plates and reagents 65
3.10.2 Antigen 65
3.10.3 Human sera: 65
3.10.4 Enzyme conjugated secondary antibody: 65
3.10.5 Substrate: 65
3.10.6 Stop solution: 65
3.10.7 Test controls: 65
3.10.8 ELISA procedure: 66
CHAPTER IV 67
RESULTS 67
4.1 Study Population 67
4.2 Gender and Age based Susceptibility 68
4.3. Dengue detection by RDT kit 68
4.4 Dengue detection by ELISA 68
4.4.1 Dengue antigen detection by NS1 capture ELISA 69
4.4.2 Anti- DENV IgM detection by IgM Capture ELISA 69
4.4.3 Anti- DENV IgG detection by IgG Capture ELISA 70
4.5 DENV infection and serotypes as detected by Real-time RT-PCR 71
4.5.1 Nested PCR for amplification of envelope Domain III 73
4.5.2 Amplification of envelope Domain III with overlapping region. 73
4.5.3 Sequence Analalysis 74
4.5.4 *Insilico* Analysis of the construct. 76
4.6 Cell culture for the virus propagation 78
4.7 Animal Immunization 78

4.8 Detection of serotype specific antibody by In-House ELISA....80
CHAPTER V....86
DISCUSSION....86
CHAPTER VI....89
CONCLUSION....89
LIMITATIONS OF THE STUDY....90
RECOMMENDATIONS/ FUTURE PERSPECTIVES....90
References....91
Appendices....100
Appendix 1.1 Ethical Approval for sample collection.....100
Appendix 1.2 Consent form for sample collection....101
Appendix 1.3 Case Report Form (CRF) Form....103
Appendix 2.1 Sample Details regarding site, year of collection and RDT test results....104
Appendix 2.2. Presentation of Dengue NS1 capture ELISA along with mean OD values at 450nm, ISR and sero-status.....113
Appendix 2.3. Presentation of Dengue IgM capture ELISA with mean OD for DENRA and NCA, ISR ratio and sero status.....115
Appendix 2.4.Presentation of IgG ELISA the mean OD for DENRA and NCA, ISR and serostatus.....118
Appendix 2.5. Cycle threshold (CT) values for Real time PCR positive samples....121
Appendix 3.2 Sequence used in Phylogeny of D1DIII....122
Appendix: 3.3 Sequence used for Phylogeny of D2DIII....122
Appendix 4.1.....123
4.1.1 Mean of Non endemic control (NEC), endemic control (EC) and cut off values for DENV-1 and DENV-2.....123
4.1.2 Mean of Non endemic control (NEC), endemic control (EC) and cut off values for DENV-3 and DENV-4.....124
4.1.3 Mean of Samples and status of presence of different antibody for dengue in samples....124
4.1.4. Comparison of In-House ELISA results with In-Bios kit ELISA.....126

List of Figures

Fig: 1. 1 Countries or areas at risk for dengue outbreak. 4

Fig: 1. 2 Prevalence of Dengue Cases in Nepal 2006 to 2019..5

Fig: 1. 3 Structure of the Dengue virion..7

Fig: 1. 4. Schematic diagram of genomic organization of Dengue virus..............................8

Fig: 1. 5. Representation of pre-dicted RNA elements at the 3'UTR of the DENV genome 9

Fig: 1. 6. Mosquitoes vector of Dengue. 11

Fig: 1. 7. (1) *Aedes aegypti* life cycle (2) Dengue transmission cycle................................12

Fig: 1. 8. Dengue virus replication..14

Fig: 1. 9. Manifestation of dengue virus infection 16

Fig: 1. 10. Schematic representation of dengue infection..16

Fig: 1. 11. Classification of dengue according to severity...17

Fig: 1. 12. Immune response to dengue infection. 19

Fig: 1. 13. Process for the diagnosis of Dengue (CDC). 20

Fig: 1. 14. Model of antibody-dependent enhancement of dengue infection 23

Fig: 1. 15. Multiple theories of dengue immune pathogenesis..23

Fig 2. 1. Year-wise cases of dengue cases in Nepal from 2013/2014–2018/2019. 34

Fig 2. 2. Dengue confirmed Provinces in the year 2019 35

Fig 2. 3. Replication of dengue virus 36

Fig 2. 4. Course of Dengue illness. 37

Fig 2. 5. Dengue diagnosis...39

Fig 2. 6. Different diagnosis techniques and days when tests become positive 40

Fig 2. 7. Overview of steps in different types of ELISA. 42

Fig 2. 8. Crystal structure of dengue envelope protein 46

Fig 3. 1. Hospitals where samples were collected 49

Fig 3. 2 Major steps used in ELISA 66

Fig 4. 1. Sample Distribution Sites.. 67

Fig 4. 2. Population distribution among different age group 68

Fig 4. 3. Scatter plot of Immune Status Ratio (ISR) of NS1, IgM and IgG..........................71

Fig 4. 4. Serotyping by Real time PCR. 72
Fig 4. 5. Gel images under UV Transilluminator. 73
Fig 4. 6. Amplification of envelope Domain III...74
Fig 4. 7. Choromatogram of D1DIII with overlapping region..74
Fig 4. 8: Sequence of D1DIII with overlapping region..74
Fig 4. 9. Chromatogram of D2DIII with overlapping region..75
Fig 4. 10. Sequence of D2DIII with overlapping region..75
Fig 4. 11. Phylogenetic Analysis of D1DIII. 75
Fig 4. 12. Phylogenetic Analysis of D2DIII...75
Fig 4. 13 Amino acid sequence of recombinant tetravalent construct.. 76
Fig 4. 14. 2D structure of the construct..77
Fig 4. 15. Secondary structure prediction of the construct. 77
Fig 4. 16. Cell culture of DENV3 in C6/36 cell line. 78
Fig 4. 17. Mouse used in animal immunization. 79
Fig 4. 18. Comparative study of Mean of (NEC), (EC), Samples 81
Fig 4. 19. Comparative study of Mean of (NEC), (EC), Positive samples 83
Fig 4. 20. Scatter diagram showing the OD values of samples...83
Fig 4. 21. Comparison of Mean OD for positive and negative samples.............................84

List of tables

Table 1. 1. Vector control measures for dengue vector 24

Table 2. 1. Summary of operating characteristics and dengue diagnosis methods. 38

Table 2. 2. Current status of Dengue vaccine 47

Table 3. 1 Master-mix preparation for multiplex PCR (DENV1-4) 57

Table 3. 2. Master-mix preparation for HSC reaction...56

Table 3. 3. Program setting for PCR 57

Table 3. 4. Components used for cDNA Synthesis 59

Table 3. 5. Reaction Protocol for cDNA synthesis..59

Table 3. 6. Primers used for dengue virus confirmation and serotype specific PCR 60

Table 3. 7. Master-mix preparation for PCR 61

Table 3. 8. PCR Program for Envelope region of Dengue 61

Table 3. 9. Primers Details for the Fusion PCR..62

Table 3. 10. PCR Protocol for Fusion PCR 62

Table 4. 1. Province-wise sample distribution and districts involved. 67

Table 4. 2. Epitope prediction of the constructed sequence. 76

Table 4. 3. NS1 ELISA from mouse serum. 79

Table 4. 4. IgM ELISA from mouse serum 79

Table 4. 5. IgG ELISA from Mouse Serum. 80

Table 4. 6. OD of ELISA at 450nm using different antigens of dengue virus 81

Table 4. 7. Comparison of Mean of negative samples with positive control 82

Table 4. 8. Comparative analysis of antibody titer for positive, endemic and non-endemic samples 82

Abstract

Dengue is caused by a single stranded positive sense RNA flavivirus, Dengue virus (DENV), which is transmitted predominantly by female mosquito vectors *Aedes aegypti, Aedes albopictus.* It leads to disease in human from mild dengue fever (DF) to a life threatening, severe Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS). All dengue viruses (serotypes 1-4) can infect human. Once infected the patients develops immunity against all serotype for limited time period. Risk of secondary infection increases as immunity against other serotype decreases. There is higher risk of development of severe dengue during secondary infection due to Antibody Dependent Enhancement (ADE). ADE results in enhanced virus entry and greater virus replication which lead to severe dengue. It also one of the hurdles in the vaccine development and acceptance.

Early diagnosis can help patients to get necessary treatment in time and effective preventive measures should be taken to reduce the dengue cases and mortality due to dengue fever. So there is an urgent need for a dengue vaccine that induces long-lasting, simultaneous protection to all four serotypes of dengue while avoiding the immune enhancement of viral infection. For the dengue vaccine development the Envelope region of dengue virus has been widely studied. Envelope region contains three domains, among Domains I, II and III of envelope region, Domain III remain choice of interest due to its reduced risk of Antibody Dependent Enhancement (ADE) in dengue infection. Envelope Domain III (EDIII) of the dengue envelope protein has been implicated in receptor binding, and is also the target of specific neutralizing antibodies thus EDIII has emerged as a promising region for a vaccine candidate. Considering EDIII as candidate instead of whole envelope might address the solution to existing problems of dengue vaccine in use and it might eliminate the risk of ADE.

In the present study, we aim to identify all four serotypes and amplify serotype specific EDIII region. Further we aim to fuse EDIII region of different dengue virus serotypes and construct recombinant bivalent construct and tetravalent construct. Then we aim to recombine the construct into a mammalian expression vector to make a single recombinant tetravalent ED III dengue vaccine construct for its subsequent use as a novel vaccine candidate because it is exposed to the surface and thus becomes the primary target for antibody-mediated neutralization.

Keywords: Dengue, Envelope Domain III, Antibody Dependent Enhancement, Recombinant DNA, Vaccine

CHAPTER I

INTRODUCTION

1.1 Background of Dengue Virus and Dengue Fever

Dengue is caused by Dengue virus (DENV), a single stranded positive sense RNA flavivirus, which is transmitted predominantly by female mosquito vectors *Aedes aegypti, Aedes albopictus* (Guzman et al., 2010) and cause a wide range of disease in human from a self -limited Dengue Fever (DF) to a life-threatening syndrome called Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS). The term 'dengue virus' refers to a group of four genetically and antigenically related viruses commonly known as serotypes (DENV-1, DENV-2,DENV-3 and DENV-4)(Kyle & Harris, 2008). All four dengue virus serotypes cause the same clinical manifestations and show similar patterns of systemic dissemination, with tropism principally for monocytes, macrophages and dendritic cells (WHO, 2009).

Approximately 40% of the world's population is at the risk for transmission of one or more of the four dengue virus (DENV) serotypes, and there is continuous spread of mosquito vectors that transmit these viruses to different parts of the world, thus dengue fever is now becoming a global epidemic (Yung et al., 2015). The infection of Dengue virus causes a wide spectrum of clinical manifestations ranging from normal dengue fever to fatal hemorrhagic diseases. Dengue fever is clinically characterized by acute febrile illness with chills, body pain, headache, retro-ocular pain and arthralgia followed by nausea, vomiting and rash (Halstead, 2008). A severe form of dengue fever known as dengue hemorrhagic fever (DHF) can cause severe bleeding, and Dengue Shock Syndrome (DSS) with sudden drop in the blood pressure (shock) and lead to death(Seema & Jain, 2005).

1.2 History of Dengue Infection

The earliest record of probable dengue fever case is in a Chinese medical encyclopedia from the Jin Dynasty (265–420 AD) which referred as "water poison" associated with flying insects (Gubler, 1997). Although there was descriptions of epidemics in the 17th century, but earliest probable reports of dengue epidemics are from 1779 and 1780, when an epidemic swept across Asia, Africa and North America. Since then epidemic of dengue were infrequent till 1940 (Gubler, 1997). In 1906, vector for dengue transmission by the Aedes mosquitoes was confirmed, and in 1907 dengue was the second disease (after yellow fever) that was known to be caused by a virus (Henchal & Putnak, 1990). The first confirmed case report dates from 1789 and is by Benjamin Rush, who coined the term "break-bone fever" because of the symptoms of myalgia and arthralgia (Guzman et al., 2010; Wei & Li, 2017).

In 1943, Dengue virus was first isolated in Japan by Ren Kimura and Susumu Hotta. They were studying blood samples of patients collected during the dengue epidemic in Nagasaki, Japan in 1943. During World War II in 1942 to 1944, an epidemic of dengue fever involving at least 200,000 cases had occurred in Japanese port cities such as Nagasaki, Kobe, and Osaka. (Hotta, 1952) The infections originated from traveler returning from the tropics, in particular Southeast Asia and the Pacific islands. Then Albert B. Sabin and Walter Schlesinger, Japanese and American investigators independently isolated DENV-1 from Hawaiian in pacific during World War II and shortly thereafter, DENV-2 from Papua New Guinean samples were isolated. DEN-3 and DEN-4 were subsequently isolated in the 1950s during epidemics occurred in the Philippines and Thailand (Mackenzie et.al, 2004).

Only in the 20th century, the viral etiology and the transmission by mosquitoes were deciphered. The socioeconomic impact of World War II played critical role in increased spread of dengue globally. Nowadays, about 40% of the world's population are at risk as they live in areas of high risk of dengue transmission (Yung et al., 2015). Dengue have spread to more than 100 countries in Asia now, and the origins of DENV have been the subject of speculation for decades. Phylogenetic relationships to other flaviviruses provide little insight because the closest relatives to DENV occur in several continents. It was hypothesized that endemic DENV evolved from sylvatic strains in Africa or Asia that utilize nonhuman primate hosts and gallery forest-dwelling Aedes vectors (not the endemic/epidemic vectors *Ades*. *aegypti* or *Ades*. *albopictus*) (Gubler, 1997). The sylvatic cycle is presumed to be ancestral because efficient inter human transmission is thought to require a minimum human population size of 10,000–1 million, which did not exist until about 4000 years ago when urban civilizations arose (Weaver & Vasilakis, 2009).

1.3 Dengue in Global Scenario

Although DENVs achieved distribution throughout the tropics in the 18th and 19th centuries, during the 20th and 21st centuries, globalization enabled their accelerated spread and the introduction of multiple viral serotypes into susceptible areas, resulting in most tropical regions becoming hyper endemic to dengue. This rapid spread began with a pandemic of dengue in South-East Asia in the 1950s that was associated with regional economic and urban growth after World War II (Mackenzie John, S, 2004). Epidemic activity dramatically accelerated in the 1970s and 1980s, leading to a global geographical expansion of viruses and mosquito vectors, and the consequent widespread DENV transmission across the tropics and subtropical areas. This geographical expansion of dengue resulted in increased frequency and magnitude of epidemics and increased frequency of severe disease (Kyle & Harris, 2008). The principal driving force of this 20th century pandemic were global trends, such as human population growth, urbanization,

modern transportation, global trade and the absence of effective mosquito control in endemic countries. This development led to more-frequent epidemics followed by clinically silent or undetected transmission during inter-epidemic periods. Large cities tend to be hyper endemic, with co-circulation of all four serotypes. When dengue is persistent in limited areas for long time, epidemics might occur due to decrease of herd immunity to one of the four serotypes or when a new epidemic strain of virus emerges or is introduced (Endy et al., 2002).

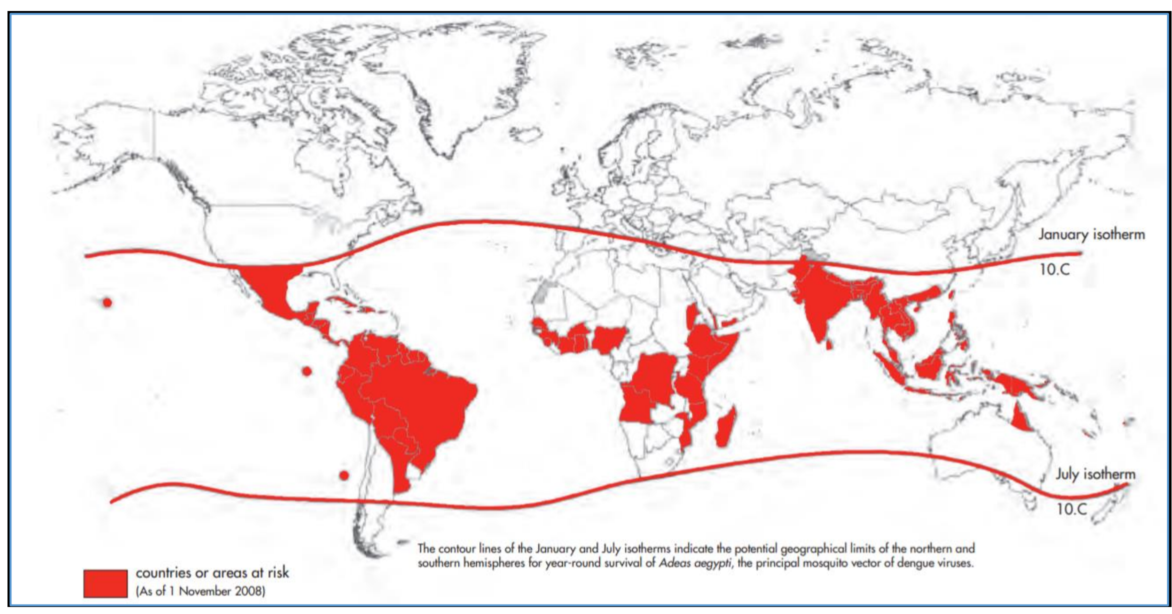


Fig: 1. 1 Countries or areas at risk for dengue outbreak (WHO, 2009).

Although not documented, an increase or change in vector competence of the mosquito population might also influence epidemic transmission. The World Health Organization (WHO) estimates that more than 50 million dengue virus infections and 20,000 dengue disease-related deaths occur annually worldwide (Organji et al., 2017). A recent disease distribution model using a boosted regression tree framework estimated there to be 390 million dengue disease infections in 2010, of which 96 million are clinically apparent (Mota et al., 2017). The frequency of reported cases of dengue to WHO has increased 8 fold in the time difference of 10 years i.e.; 505,430 cases in 2000, to over 2.4 million in 2010, and 5.2 million in 2019. (WHO, 2020)

1.4. Dengue prevalence in Nepal

The first case of dengue virus in Nepal was observed in 2004 (Pandey et al., 2004; Takasaki et al., 2008) and since then there is rapid spread of dengue fever across the country within the short period of time. More than 1500 cases of dengue infection were reported from different districts in year 2016 (EDCD, 2016). All 4 serotype has been seen in Nepal (Malla et al., 2008b). Although there is presence of all serotype, mostly the serotype 2 was seen in the year 2004, serotype 1 in 2010, serotype 2 in the year 2013 (Gupta et al., 2015b),

serotype 1 in 2016 (Gupta, et al., 2018) (Gupta et al., 2015a). The shift of the viral disease from the subtropical Terai belt to the temperate hill region of Nepal is concern to scientific community as the disease could easily spread all over the world in the future, irrespective of climate(Gupta et al., 2018).

A trend of increasing dengue outbreak in every three years interval in Nepal. Although the first confirmed dengue case was reported in 2004 from Japanese traveler, the outbreak of dengue then occurred in 2006 with a remarkable number of 32 confirmed cases (Malla et al., 2008b). No any confirmed cases observed in almost three consecutive years from 2007 to 2009 and reoccurred again during a massive outbreak in 2010 (Pandey et al., 2013). Cases of dengue continued to be reported in the subsequent year 2011 and 2012 and two major outbreaks were witnessed in 2013 and 2016, clear 3 year cyclic outbreak demonstrated by massive in 2010, 2013 and 2016 (Gupta et al., 2018). Incidence of dengue cases in 2017 was found to be five more than previous year. Although lesser case were reported in 2018 than 2017 there was massive rise in confirmed cases in 2019 with 17992 confirmed cases (Rijal et al., 2021). In Nepal, dengue has been spreading in more districts year by year with 68 districts with confirmed cases in 2019 (Rijal et al., 2021).

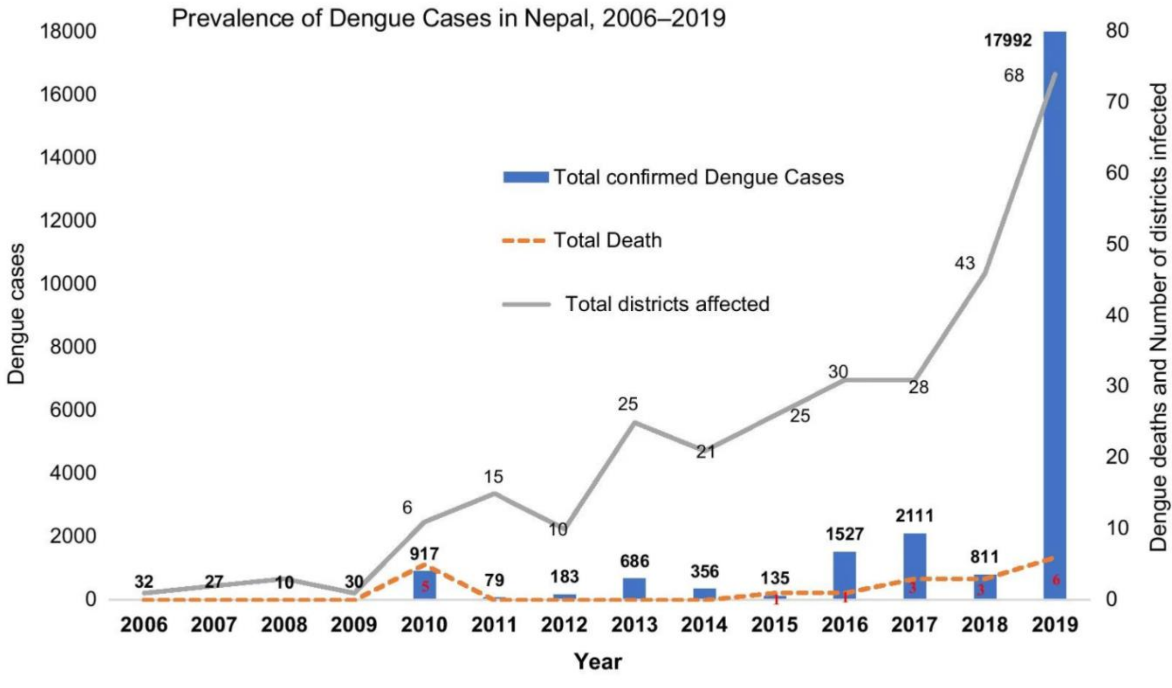


Fig: 1. 2 Prevalence of Dengue Cases in Nepal 2006 to 2019 (Rijal et al., 2021).

1.5 Virus taxonomy

Family:	Flaviviridae
Genus:	*Flavivirius*
Species:	DENV-1, 2, 3 &4
Nucleic acid type:	Single stranded RNA
Sense:	Positive sense RNA genome
Nucleotide:	10.7 kb

1.5.1 Taxonomy of Dengue Virus

There are three known genera in the family, Flaviviridae (formerly known as group B *arboviruses*) namely *Flavivirus*, *Pestivirus* and *Hepacivirus*. The DENV belongs to the group B Arbovirus, family Flaviviridae and genus *Flavirus*. The genus *Flavivirus* consists of 55 identified virus species. The word *Flavi* is a derivation from the Latin word "flavus" meaning "yellow" and the type species of the genus is the yellow fever virus. The flaviviruses are thus named due to the jaundice observed in yellow fever patients. Many flaviviruses are important human pathogens, most notably the dengue viruses, yellow fever virus, Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV) (Murugesan & Manoharan, 2020). The flaviviruses are predominantly transmitted by mosquitoes and ticks, whereas vector are unknown in some flaviviruses. (Katzelnick et al., 2017). Phylogenetic inference from molecular data showed agreement with the antigenic complex classification while comparing with available sequence data. In addition, it revealed there is clear clustering of the *Flavivirus* genus into non vector and vector-borne virus clusters, with the latter splitting into mosquito borne and tick-borne virus clusters (Ramos-Castañeda et al., 2017). The dengue virus was basically divided into four groups called serotypes based on antigenic properties. Subsequent evidence from molecular data acknowledged this classification and also provided a clearer understanding of the phylogeny of the four serotypes: among the dengue viruses, DENV-4 diverged first from the common ancestor, followed by DENV-2, and finally DENV-1 and DENV-3 (Huntington et al., 2016).

There are four distinct serotypes and all of them can cause Dengue fever/Dengue hemorrhagic fever. All four serotypes have a similar natural history, and share humans as a primary host and *Aedes* mosquitoes as a primary mosquito vector. The genomic material of the DENV is contained in a positive-sense, single-stranded RNA molecule approximately 10.7kb in length. It consists of a single open reading frame that encodes a poly protein of between 3387 and 3392 amino acids (Gubler, 1997).

1.6. Dengue Virus Morphology

The dengue virion, like those of other flaviviruses, is spherical and 40-50 nm in diameter. It is comprised of a nucleocapsid about 30 nm in diameter that is enclosed in a lipid envelope which consists of a lipid bilayer in which Envelope, E, and Membrane, M, proteins are embedded as E and M part. The nucleocapsid contains the viral capsid and RNA genome(Modis et al., 2004). The lipid-containing envelope consists of a lipid bilayer, an envelope protein between 51,000 and 59,000 Da that mediates attachment, fusion, and penetration, and a small non-glycosylated internal matrix protein of approximately 8,500 Da. The envelope protein is glycosylated in most flaviviruses and is exposed on the virion surface(Perera & Kuhn, 2008).

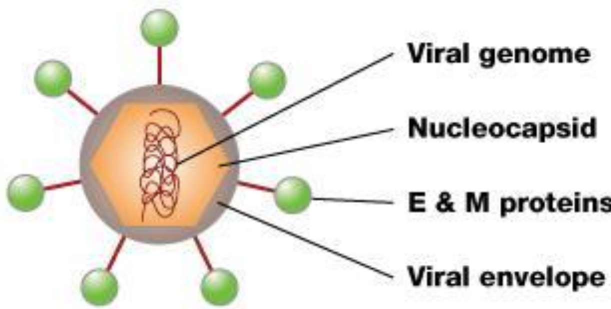


Fig: 1. 3 Structure of the Dengue virion. (E: enveloped protein and M: Membrane protein, the virus contains RNA as genome and different proteins as structural and non-structural proteins. There are 180 identical copies of the envelope (E) protein attached to the surface of the viral membrane by a short trans-membrane segment (Guzman et al., 2010).

1.7. Genomic organization of Dengue virus

The genomic organization of the dengue virus, and by extension all flaviviruses, is relatively simple compared to other arboviral families such as the *Togaviridae* formerly known as group A, *arboviruses*, *Bunyaviridae* or *Rhabdoviridae*. The DENV genome consists of a single-stranded, positive sense RNA molecule roughly 10.7 kb in size. It contains a single translated open reading frame (ORF) that encodes a precursor polypeptide of around 3390 amino acids which is processed catalytically into ten viral proteins (Perera & Kuhn, 2008). There is no evidence of alternative or overlapping reading frames that are translated and there is also no hyper-variable region in the DENV genome like those reported in the Hepatitis C Virus (HCV) genome.

The virus has a genome of about 10700 bases that encodes an Open Reading Frame (ORF). The DENV ORF is flanked at its 5’ terminus by an untranslated region (UTR) of 95-101 nucleotides long which consists of two RNA domains having role in its genome synthesis. The 3’ UTRs of DENV are about 500 nucleotides long and consists of 3 domains. . The 5’ UTR of the genome has a type I cap (m7GpppAmp) and 3’ UTR lacks polyadenylation at 3’ terminus. Lack of poly-adenylation distinguishes the flavivirus from other positive strand

RNA viruses (Dwivedi et al., 2017). The dengue virus ORFs are 10,688; 10,173;10,170 and 10,158 nucleotides in length that encode a poly-protein precursor of 3396,3391,3390 and 3386 amino acid in DENV-1,-2,-3 and-4 viruses, respectively. The translated polyprotein is cleaved co- and post-translationally by viral and host proteases into ten viral proteins: three structural proteins (C, capsid; prM/M, precursor of membrane; E, envelope) encoded at the 5' end of the ORF, and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) encoded at the 3' end (Gebhard et al., 2011). Of the three structural proteins, the E protein is the most studied as virus envelope. It is glycosylated at two sites (Asn-67 and Asn-153) and is responsible for viron attachment to receptors of susceptible host cells and fusion of the virus envelope with the target cell membrane. The E glycoprotein also contains the main neutralization epitopes recognized by neutralizing antibodies. Such epitopes are also found to a lesser extent on the M glycoprotein. In addition to the E glycoprotein, only one other viral protein, NS1 has been associated with a role in protective immunity. NS3 is a protease and a helicase, whereas NS5 is the RNA polymerase in charge of viral RNA replication. (Rice et al., 2005).

.

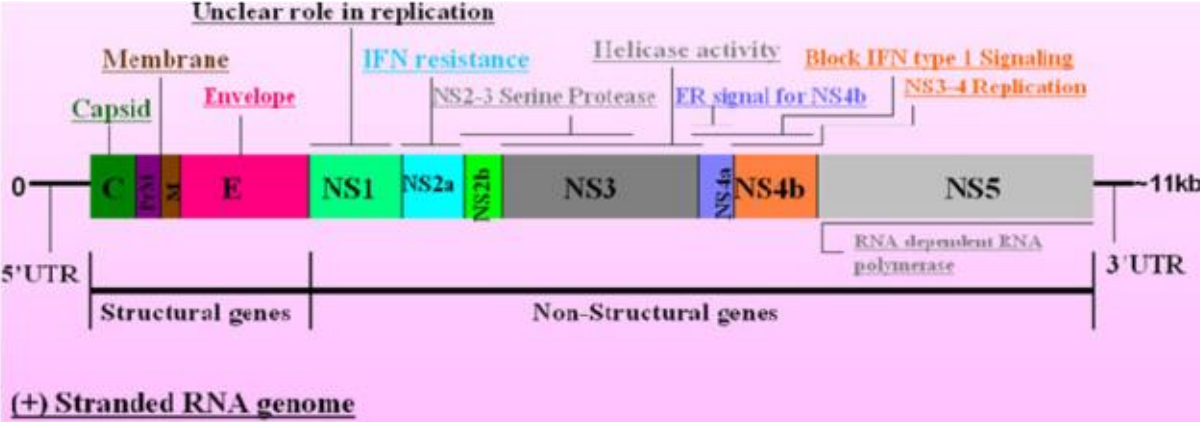


Fig: 1. 4. Schematic diagram of genomic organization of Dengue virus (C: Capsid protein; PrM & M: Membrane associated protein; E: Enveloped protein; NS1-5: Non-Structural protein 1-5)(Faheem et al., 2011)

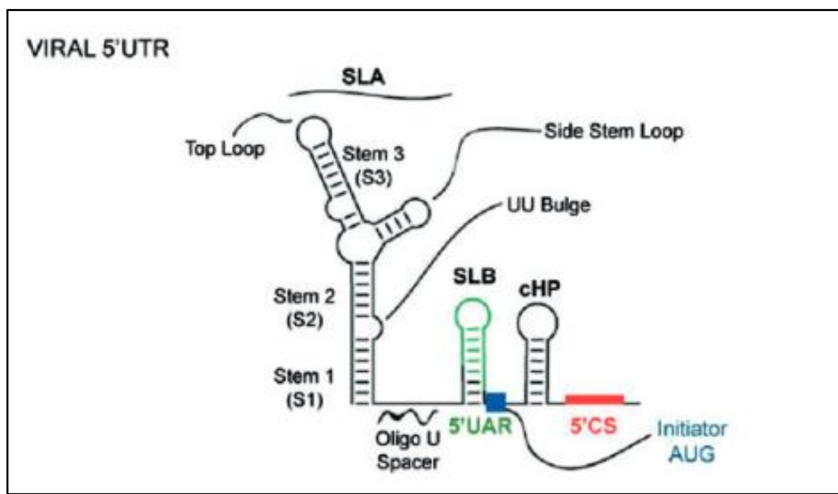


Fig: 1.5. Predicted secondary structure of 5' terminal region of viral genome (Dwivedi et al., 2017). Structural elements that are located at the 5'end are shown as stem loop A (SLA), stem loop B (SLB), oligo (U) track spacer, 5'UAR (Upstream AUG region), translation initiator AUG, capsid region hairpin (cHP) and the 5'CS element (Ng et al., 2017).

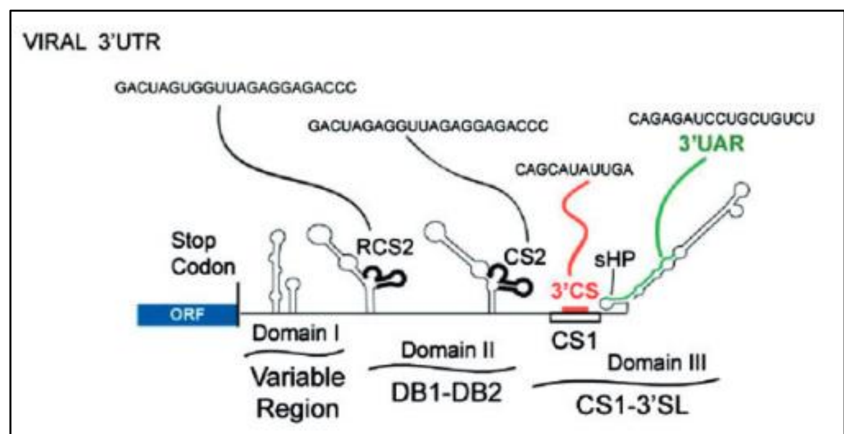


Fig: 1. 5. Representation of pre-dicted RNA elements at the 3'UTR of the DENV genome (Dwivedi et al., 2017).

The predicted secondary structures of the three defined domains are shown: DI comprising variable region (VR), DII comprising DB (Domain Binding) structures (DB1 and DB2) and DIII consisting conserved sequence CS1 and 3'SL(stem loop). In addition, the location and sequence of each of the conserved elements corresponding to RCS2 (repeated conserved sequence), CS2, 3'CS and 3'UAR (upstream AUG region) are also indicated (Ng et al., 2017).

1.7.1. Dengue virus serotype

The term 'dengue virus' refers to a group of four genetically and antigenically related viruses that are known as serotypes (DENV1, DENV2, DENV3 & DENV4)(C. F. Yung et al., 2015). 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

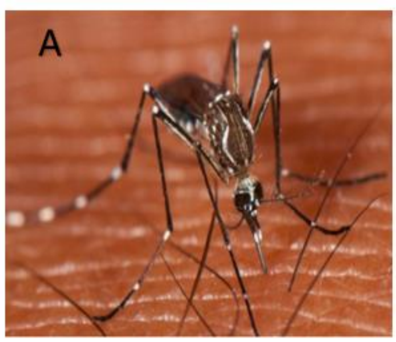
severity and course. The fifth serotype DENV-5 has been discovered in 2013 from the patient suffering from Dengue in 2007. There has been report of only one case of DENV-5 which indicates probability of low transmission rate but there is probability of fresh outbreak of Dengue (Mustafa et al., 2015). Dengue illness can evolve into three phases: the acute febrile phase observed in most of the patients, the critical phase and the recovery (convalescent) phases (Henchal & Putnak, 1990).

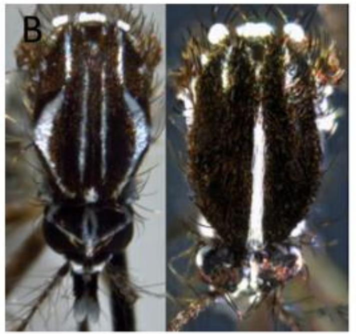
1.8. Vectors and Mode of Transmission

Dengue transmission is vector mediated and is transmitted from person to person through the bites of infected female mosquitoes. The etiological agent for dengue is believed to have sylvatic/enzootic transmission cycles involving nonhuman primate hosts and vector species living in forests. The virus was transmitted to humans when human and mosquito came into contact and continuous human-mosquito cycles was maintained in around human population centers (Gubler, 1997).

1.8.1. Vectors of Dengue

Many species from the genus *Aedes* of the family *Culicidae* are known to transmit DENV, but the principal vector is *Aedes aegypti* which is also the vector of the yellow fever virus (YFV), Chikungunia virus, Zika virus. The first person to suggested *Aedes aegypti* as a carrier of dengue fever was an Australian naturalist Thomas Lane Bancroft in 1906 based on epidemiological grounds, which was confirmed in 1916 by John Burton Cleland (Piovezan et al., 2019). *Aedes aegypti* is known to be a day-biting mosquito that prefers to breed in clean water contained in domestic and peri-domestic water containers. Due to its adaptation to human habitats and its desiccation-resistant eggs, *Aedes aegypti* has enabled it to flourish in urban centers. The secondary vector for dengue is *Aedes albopictus* which is also known as the Asian tiger mosquito. Its role as dengue vector in semi-tropical regions was first identified in Taiwan in 1917 (Seltenrich, 2016). *Aedes albopictus* serves as the primary vector for dengue in countries where *Aedes aegypti* is absent and as a maintenance vector in rural areas where both species coexist (Saito et al., 2016). In the Pacific islands *Aedes polynesiensis* has been suggested as the primary dengue vector whereas *Aedes scutellaris* was identified as the 'jungle' vector for dengue (Bharati & Saha, 2018).





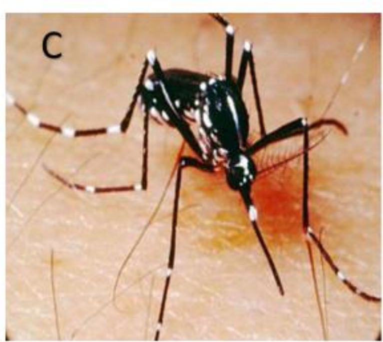


Fig: 1. 6. Mosquitoes vector of Dengue. (A) *Aedes aegypti,* (B) Thorax region of *Aedes aegypti* (left), *Aedes albopictus* (right), (C) *Aedes albopictus.*

Aedes aegypti and *Aedes albopictus* have both been shown to be anthropophilic, i.e. prefer to feed on humans and are widely distributed in both urban and semi-urban areas in the tropics and subtropics. Both species have also been demonstrated to pose high vector competence for the dengue virus (Akter et al., 2017).

1.8.2. Life cycle of Dengue vector

The larvae of the principal vector *Aedes agypti* under naturally changing temperature are capable of developing into adults in conditions lower than 10°C, whereas those of *Aedes albopictus* can survive even lower temperatures (Akter et al., 2017). Consequently the two species can be found between latitudes 35°N and 35°S, approximately corresponding to a winter isotherm of 10°C (Kumar & Sharma, 2016). A dengue outbreak reported in Buenos Aires, Argentina (34°36'S) in early 2009 is very close to this isotherm and is the furthest south dengue has spread. Dengue is the most common arboviral (arthropod-transmitted) disease and it also ranks as the most important mosquito-borne viral disease in the world. Around 2.5 billion people living in tropical and sub-tropical regions are at risk of dengue infection, which equates to about two-fifths of humanity (Kumar & Sharma, 2016). There is an estimated 50-100 million infections occurring globally every year, with 500,000 cases requiring hospitalization and causing 24,000 deaths (Hernández & Mendoza, 2016). Female *Aedes aegypti* commonly lays eggs on the inner walls of artificial containers. When the containers filled with water, mosquito larvae hatch from the eggs. After developing through four larval stages, the larvae metamorphose into pupas. Like the larval stage, the pupal stage is also aquatic. After two days, a fully developed adult mosquito forms and breaks through the skin of the pupa. It takes around 7-10 days for mosquito to become adults from eggs. The adult mosquito can fly and has a terrestrial habitat. The dengue virus is spread through a human-to-mosquito-to-human cycle of transmission (Yung et al., 2015).

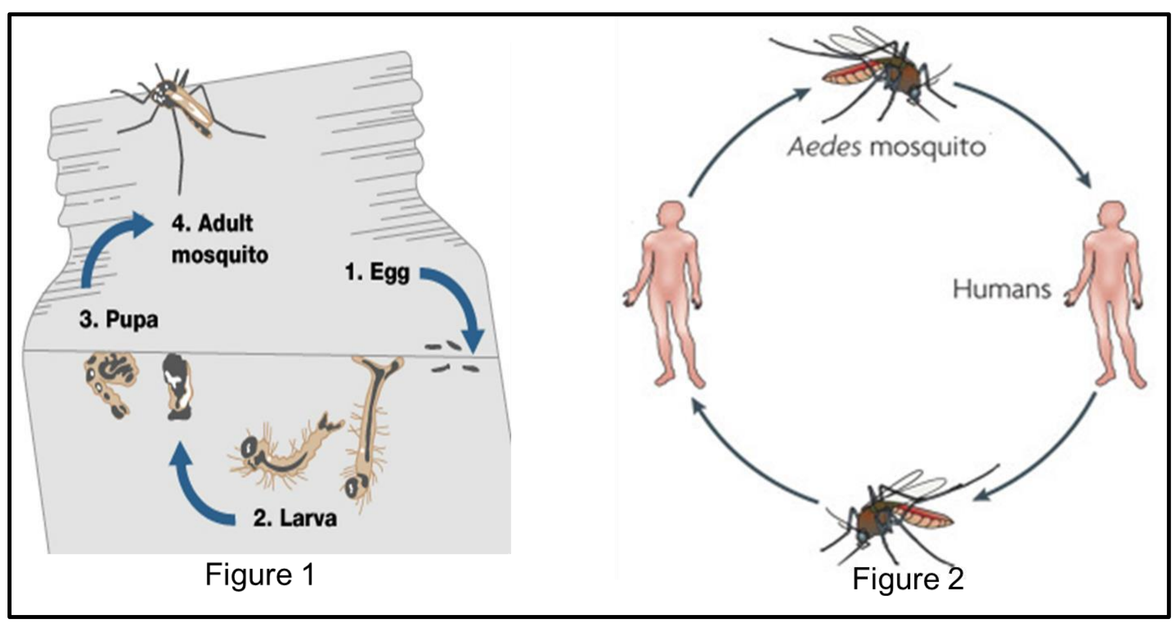


Fig: 1. 7. (1) *Aedes aegypti* life cycle (Guzman et al., 2010b) (2) Dengue transmission cycle

1.8.3. Mode of transmission of Dengue

Humans are the main amplifying host of the virus. Dengue virus circulating in the blood of viraemic humans is ingested by female mosquitoes during feeding. The extrinsic incubation period (EIP) is the viral incubation period between the time when a mosquito takes a viremic blood meal and the time when that mosquito becomes infectious. The intrinsic incubation period (IIP) is the time between a human being infected and the onset of symptoms due to the infection. The virus then infects the mosquito mid-gut and subsequently spreads systemically over a period of 6-15 days. After this extrinsic incubation period, the virus can be transmitted to other humans during subsequent probing or feeding. The extrinsic incubation period is influenced in part by environmental conditions, especially ambient temperature. The EIP is higher in lower temperature and lower in higher temperature. The IIP is usually 3-10 days long. Thereafter the mosquito remains infective for the rest of its life (Chan & Johansson, 2012).

Aedes aegypti is one of the most efficient vectors for arboviruses because it is highly anthropophilic, frequently bites several times before completing oogenesis, and thrives near humans. Vertical transmission of dengue virus has been demonstrated in the laboratory but rarely in the field. The significance of vertical transmission for maintenance of the virus is not well understood. Several factors can influence the dynamics of virus transmission - including environmental and climate factors, host-pathogen interactions and population immunological factors (WHO, 2009).

The dengue virus enters via the skin while an infected mosquito is taking a blood meal. During the acute phase of illness, the virus is present in the blood and its clearance from this compartment generally coincides with effervescence. Humoral and cellular immune responses are considered to contribute to virus clearance via the generation of neutralizing antibodies and the activation of CD4+ and CD8+ T lymphocytes. In addition, innate host defense may limit infection by the virus. After infection, serotype-specific and cross-reactive antibodies and CD4+ and CD8+ T cells remain measurable for years. It can proceed in two ways, primary infection leads to the direct entry of DENV into immune cells like Macrophages, Monocytes and Dendritic cells. This pathway can further activate DENV specific T cells, cause cytolysis, cytokines production, complement activation and finally leading to plasma leakage. In case of secondary infection, antibody based enhancement occurs which leads to cytokines production and complement activation, high levels of cytokines and complement activation can damage vascular endothelial cells resulting in plasma leakage (Bilal Waqar, 2016)

1.9 Virus Replication

The dengue virus attaches to the surface of a host cell and enters the cell by a process called endocytosis. Once deep inside the cell, the virus fuses with the endosomal membrane and gets released into the cytoplasm. The virus particle comes apart, releasing the viral genome. The viral RNA (vRNA) is translated into a single polypeptide that is cut into ten proteins, and the viral genome is replicated.The newly synthesized viral RNA is enclosed in the C proteins, forming a nucleocapid. The nucleocapsid enters the rough ER and is enveloped in the ER membrane and surrounded by the M and E proteins. Virus assembly occurs on the surface of the endoplasmic reticulum (ER) when the structural proteins and newly synthesized RNA bud out from the ER. This step adds the viral envelope and protective outer layer. The immature viruses travel through the Golgi apparatus complex, where the viruses mature and convert into their infectious form. The mature viruses are then released from the cell and can go on to infect other cells (Mukhopadhyay et al., 2005).

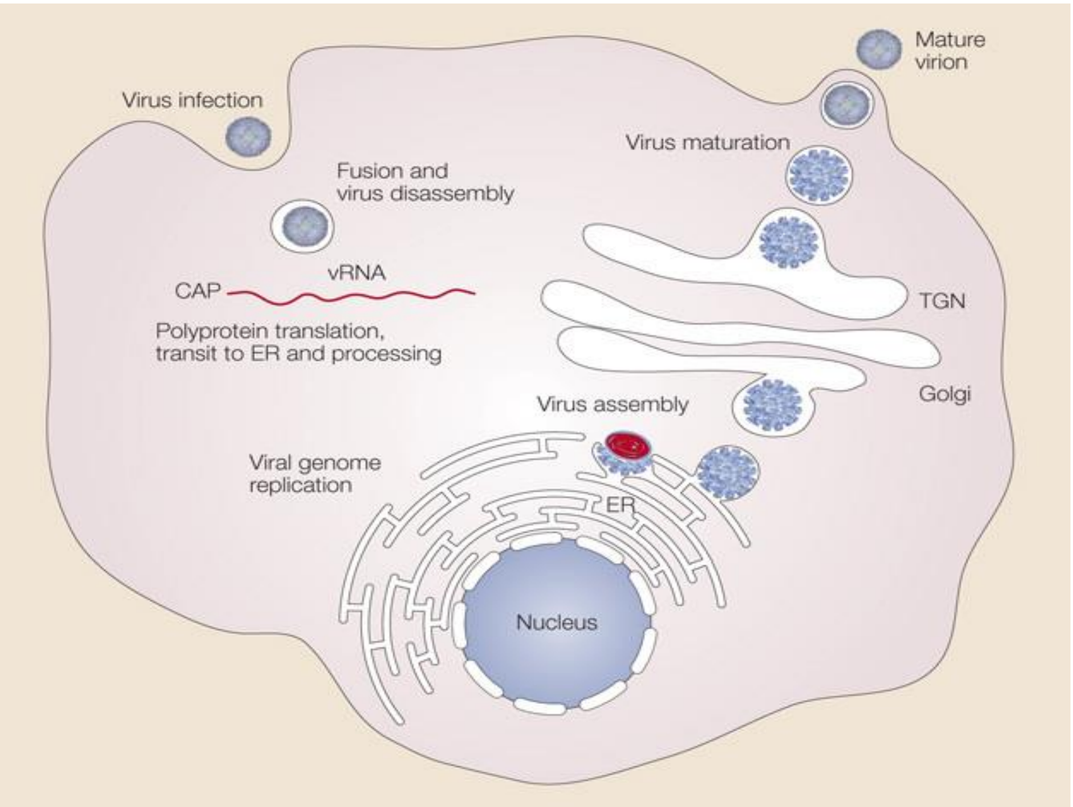


Fig: 1. 8. Dengue virus replication (Mukhopadhyay et al., 2005)

1.10 Dengue symptoms and clinical manifestations

Infection with any of the four serotypes (DENV1–4) of dengue virus (DENV) can produce a broad spectrum of symptoms, ranging from asymptomatic infection to a severe life threatening illness. Dengue is a dynamic illness, despite its short duration (about 1 week in nearly 90% of cases). Its clinical expression can change as the days go by and can also worsen quickly (WHO, 2009).

The assessment of warning signs is designed to permit the early identification of patients with more-severe disease manifestations who require supportive therapy. Dengue illness can also be divided into three separate phases: the acute (febrile) phase, the critical (plasma leakage) phase and the convalescent or reabsorption phase. The 2009 classification replaced the previous 1997 WHO system that addressed and underscored the two pathological phenomena associated with the disease: plasma leakage and abnormal haemostasis. Under this classification, patients were designated as having either dengue fever-a nonspecific febrile illness and the most common manifestation of DENV infection-or dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS)-a combination of plasma leakage and coagulopathy, sometimes accompanied by bleeding that can lead to a rapid fall in blood pressure and consequently to circulatory shock and organ impairment (WHO Press, 1997).

Fever occurs during the acute febrile stage and is generally the first clinical manifestation of illness with a variable intensity. It is associated with headache and vomiting, as well as body pains (Siddiqi et al., 2019). In children, fever is frequently the only clinical manifestation or is associated with rash and/or unspecific digestive symptoms. Redness can be overserved in pharynx but other signs and symptoms of the respiratory system are not frequent or clinically significant. Slight abdominal pain and more frequent diarrhea occurs in patients who are <2 years of age and in adults. In general, compared with children, adolescents and adults show a ‘flu-like syndrome’ including headache and body pains with more prominent digestive symptoms than respiratory symptoms (WHO, 2009). During the febrile stage, leukocyte counts are usually decreased. Petechiae (small spots on the skin caused by broken capillaries) or ecchymosis (large subcutaneous bleeding spots) can be present, with or without thrombocytopenia. After 2–5 days, these symptoms can be followed by rapid clinical deterioration (CDC, 2021). Most patients with dengue recover after defervescence; however, the clinical state of some patients worsens when the fever drops. Thus, the period during which the fever subsides indicates the beginning of the critical phase.

The critical phase coincides with the leakage of plasma that can lead to shock, which is characterized by coldness in the teguments, weak pulse, delayed capillary filling, tachycardia, oliguria and hypotension. Shock is caused by low blood volume (hypovolaemia) (Rajapakse, 2011). At the beginning, not all clinical signs of shock are observed. At this stage, patients usually have a flushed face, a warm trunk, cold and clammy extremities, diaphoresis (sweating), slow venous filling, restlessness, irritability, pain in the upper and middle abdomen and decreased urinary output. In addition, patients might also exhibit signs of impaired haemostasis, including scattered petechiae on the forehead and extremities, spontaneous ecchymoses, easy bruising and bleeding at venipuncture sites, and circumoral and peripheral cyanosis (blue skin discolouration) (Prajapati, Napit, Bastola, Rauniyar, Shrestha, Lamsal, Adhikari, Bhandari, Ray Yadav, et al., 2020).

Gastrointestinal bleeding occurs in <10% of patients and usually follows a period of uncorrected hypotensive shock. Patients with shock also experience rapid and potentially laboured breathing, a weak pulse and have a rapid heartbeat that sounds ‘thready’. Finally, their livers are usually firm, tender and can become enlarged to 4–6 cm below the costal margin, the haematocrit level is increased and the platelets reach their lowest count (Rajapakse, 2011). In those who recover, this critical phase lasts for 24–36 hours and is followed by a rapid convalescence. Convalescence can involve complications, such as encephalopathy, bradycardia, ventricular extarsystoles and, rarely, myocarditis and encephalitis. Disease severity and the manifestation of clinical symptoms are known to be

influenced by the environmental factors, the serotype/genotype of dengue virus, the immune response and genetic background of host (Santos et al., 2017).

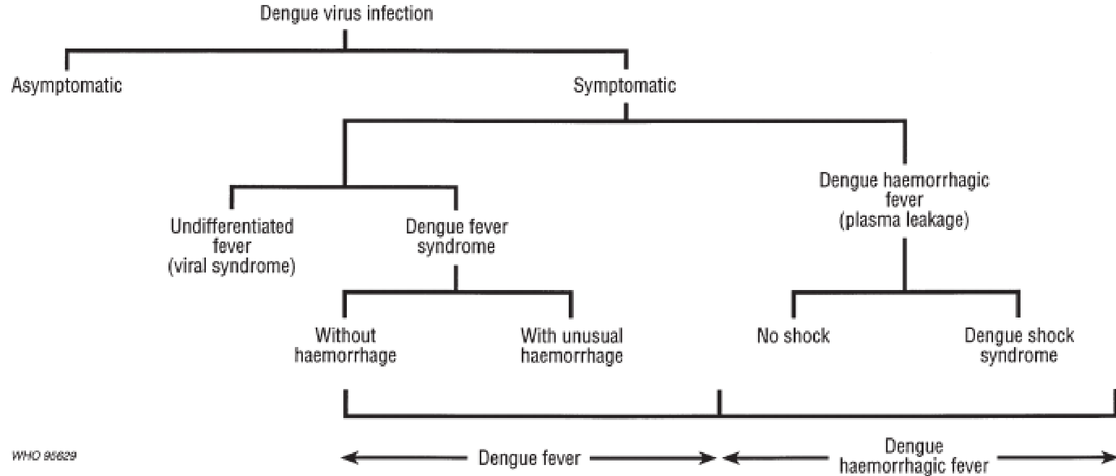


Fig: 1. 9. Manifestation of dengue virus infection (Siddiqi et al., 2019)

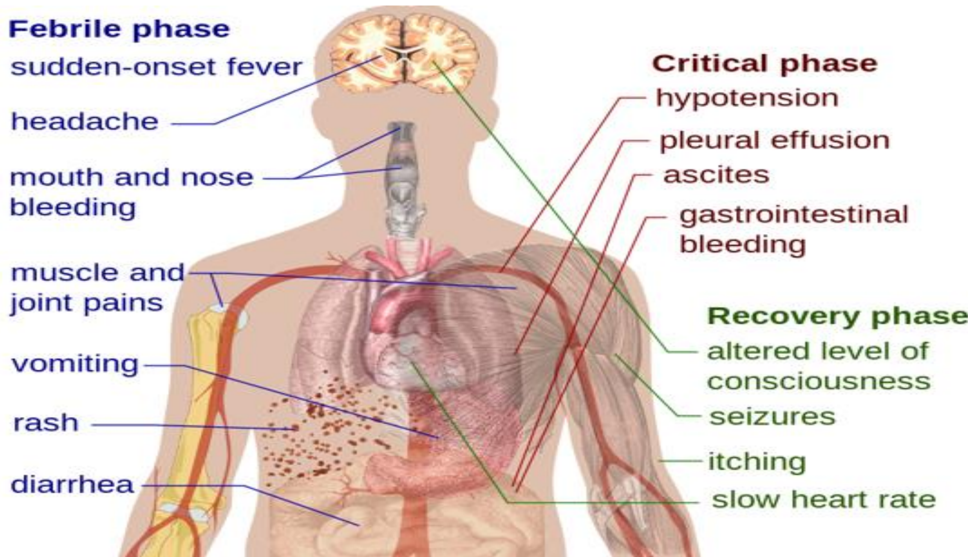


Fig: 1. 10. Schematic representation of dengue infection (WHO, 2009)

1.11 Classification of Dengue ailment

Dengue illness is clinically classified as either dengue with or without warning signs or severe dengue (WHO, 2009) . This classification was launched by the WHO in 2009 for the purpose of improving clinical management.

i) **Dengue fever without warning signs**: The subjects who have fever and any two of the symptoms of nausea, vomiting, rash, aches and pains, leucopenia and positive tourniquet test are said to have dengue without warning signs.

ii) **Dengue fever with warning signs:** The warning signs of the dengue fever includes abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation (ascites, pleural effusion), mucosal bleeding, lethargy, liver enlargement >2cm, laboratory increase in hematocrit concurrent with rapid decrease in platelet counts.

iii) **Severe dengue:** Severe dengue is defined having at least one of the following criteria: shock, fluid accumulation with respiratory distress and severe organ impairment (WHO, 2009).

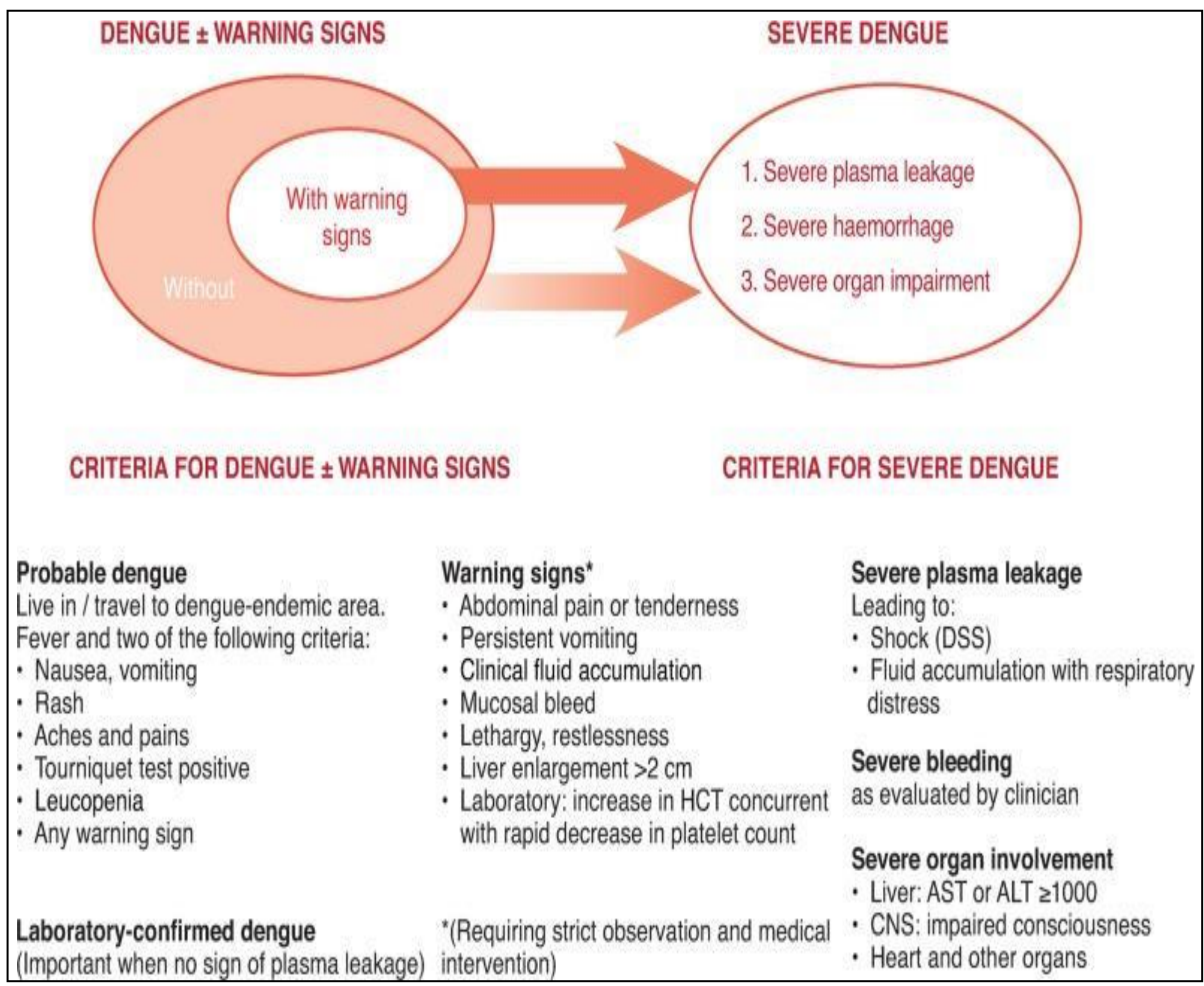


Fig: 1. 11. Classification of dengue according to severity (Hadinegoro, 2012)

1.12 Diagnosis of Dengue virus

Progression of symptoms in some occurs over a short period and accurate disease diagnosis is imperative to assess treatment success, and predict patient prognosis. The most common diagnosis technique is the enzyme linked immunosorbent assay (ELISA)(Vázquez et al., 2003). Other detection method includes molecular diagnosis approaches like Polymerase Chain Reaction (PCR)(Calvo et al., 2016). In Nepal, ELISA is the foremost detection technique of Dengue.

The positive results for one of the following methods will confirm the case by laboratory criterion:

1) Isolation and identification of dengue virus (DENV) in blood sample (serum, plasma), organ fragments collected *post mortem* (liver, spleen, kidney, heart, lung, nervous system.
2) Detection of viral nucleic acid (RNA) using the reverse transcription-polymerase chain reaction (RT-PCR) technique.
3) Detection of non-structural 1 (NS1) protein (antigen) in serum and/or plasma sample.
4) Detection of IgM antibodies specific for dengue in a single serum sample.
5) Increase of four times or more in the IgG antibodies titers; hemagglutination inhibiting antibodies, and/or neutralizing antibodies in paired serum samples, collected during the acute phase of illness and convalescence, for one or more DENV antigens.
6) Demonstration of viral antigen in necropsied tissues by immunohistochemistry. (WHO, 2009) (Guzmán & Kourí, 2004a).

Dengue has a wide spectrum of clinical presentations, often with unpredictable clinical evolution and outcome. While most patients recover following a self-limiting non-severe clinical course, a small proportion progress to severe disease, mostly characterized by plasma leakage with or without haemorrhage. Intravenous rehydration is the therapy of choice; this intervention can reduce the case fatality rate to less than 1% of severe cases. The group progressing from non-severe to severe disease is difficult to define, but this is an important concern since appropriate treatment may prevent these patients from developing more severe clinical conditions (WHO, 2009). DENV RNA and NS1 are detectable during the first week of illness. Anti-DENV IgM is detectable starting approximately 5 days after illness onset. Although most cases only have detectable IgM anti-DENV for 14–20 days after illness onset, in some cases it may be detectable for up to 90 days. Detection of anti-DENV IgG is neither sensitive nor specific in identifying patients with dengue (Kuhn et al., 2002).

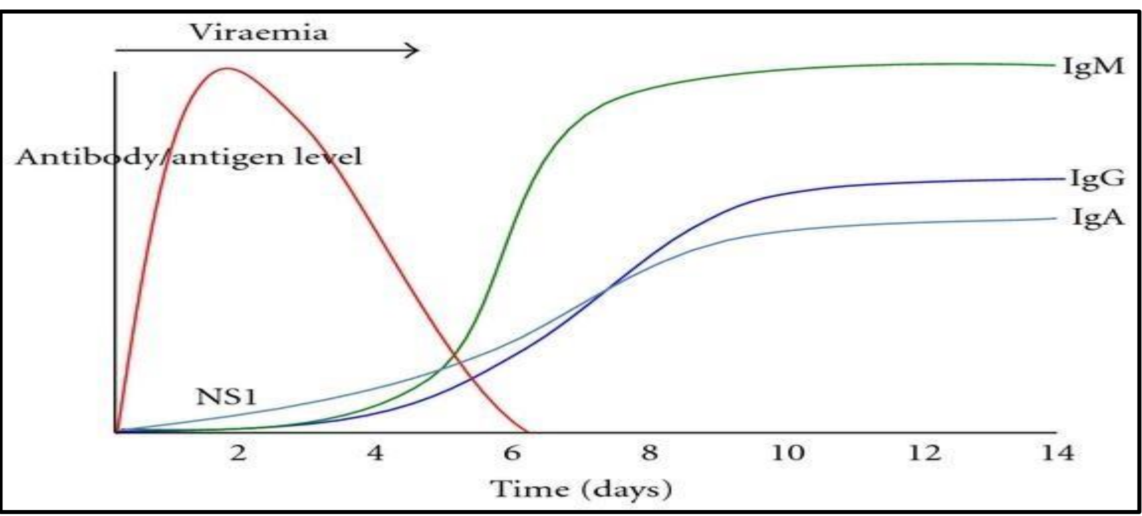


Fig: 1. 12. Immune response to dengue infection (Guzman et al., 2010).

An infected person experiences the acute symptoms of dengue when there is a high level of the virus in the bloodstream. As the immune response fights the dengue infection, the person's B cells begin producing IgM and IgG antibodies that are released in the blood and lymph fluid, where they recognize and neutralize the dengue virus and viral molecules such as the dengue NS1 protein. The immune response eliminates the virus, leading to recovery (Guzman et al., 2010).

Dengue can be diagnosed by isolation of the virus, by serological tests, or by molecular methods. Diagnosis of acute (on-going) or recent dengue infection can be established by testing serum samples during the first 5 days of symptoms and/or early convalescent phase (more than 5 days of symptoms). Acute infection with dengue virus is confirmed when the virus is isolated from serum or autopsy tissue specimens, or the specific dengue virus genome is identified by reverse transcription-polymerase chain reaction (RT–PCR) from serum or plasma, cerebrospinal fluid, or autopsy tissue specimens during an acute febrile illness. Methods such as one-step, real-time RT–PCR or nested RT–PCR are now widely used to detect dengue viral genes in acute-phase serum samples (CDC, 2021). Several formats of ELISA are designed for detecting DENV antibodies. Classical indirect ELISA and immunoglobulin antibody capture ELISA are the 2 most common formats. Indirect ELISA uses the viral antigens to coat the microtiter plates. Following serial incubation with patient serum and enzyme-conjugated anti-human immunoglobulin, the chromogen substrate is added during the final step for colour development. The colour is then read using a spectrophotometer. Recently, NS1 serotype-specific IgG ELISA was also found to be a reliable method for differentiating primary and secondary virus infections (K.-J. Huang et al., 2005).

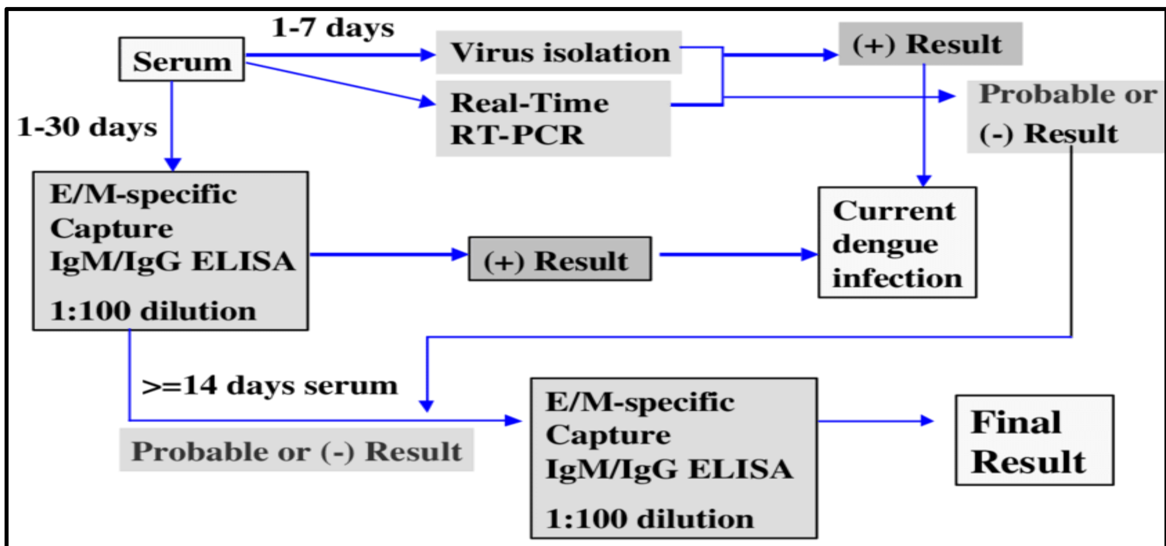


Fig: 1. 13. Process for the diagnosis of Dengue (CDC).

Different types of capture ELISA like IgM, IgG and NS1 are performed for the early diagnosis and confirmation of primary and secondary infection. PCR is performed for the confirmation of the viral infection and serotyping is done for the confirmation of the prevalent serotype in a year and in a certain locality (K.-J. Huang et al., 2005).

1.13 Host Immune Response

When a human body is invaded by any pathogen body defends it by its immune system which is made up of two parts. First part, called the innate immune system, provides the body with immediate and general protection from any invading pathogen. The innate immune response rapidly recognizes and responds to pathogens, but it does not provide a person with long-term immunity against an invading pathogen. The second part of the immune system, the adaptive immune system, produces cells that specifically and efficiently target the pathogen and infected cells (King et al., 2021). The cells produced by the adaptive immune system include antibody-secreting B cells and cytotoxic T cells. The antibodies (called immunoglobulin, or Ig) secreted by the B cells specifically recognize and bind to foreign molecules. The cytotoxic T cells kill cells that are infected with pathogens. The adaptive immune system takes longer to respond to an invading pathogen than the innate immune response, but it provides a person with long-term immunity against a pathogen (Stephens, 2009).

When an infected mosquito feeds on a person, it injects the dengue virus into the bloodstream. The virus infects nearby skin cells called keratinocytes, the most common cell type in the skin. The dengue virus also infects and replicates inside a specialized immune cell located in the skin, a type of dendritic cell called a Langerhans cell. Langerhans cells detect invading pathogens and display molecules from the pathogens, called antigens, on their surface. The Langerhans cells then travel to the lymph nodes and

alert the immune system to trigger the immune response because a pathogen is in the body (King et al., 2021). Lymph nodes are small organs located throughout the body connected by vessels that form a network called the lymphatic system. The lymph nodes are stations in the body for immune cells that help fight against infections (Marcial-Juárez et al., 2017).

The infected Langerhans cells display dengue viral antigens on their surface, which activates the innate immune response by alerting two types of white blood cells, called monocytes and macrophages, to fight the virus. Normally, monocytes and macrophages ingest and destroy pathogens, but instead of destroying the dengue virus, both types of white blood cells are targeted and infected by the virus (King et al., 2021). The dengue virus tricks the immune system to get around its defenses and infect more cells. As the infected monocytes and macrophages travel through the lymphatic system, the dengue virus spreads throughout the body. During its journey, the dengue virus infects more cells, including those in the lymph nodes and bone marrow, macrophages in both the spleen and liver, and monocytes in the blood. The spread and increase of the virus results in viremia, a condition in which there is a high level of dengue virus in the bloodstream (Imad et al., 2020).

Although the dengue virus has tricked the immune system to infect cells and spread throughout the body, the immune system has additional defenses to fight the virus. The infected cells produce and release small proteins called interferons that are part of a large group of proteins called cytokines (Imad et al., 2020). Interferons have the ability to interfere with viral replication, and they activate both the innate and adaptive immune system defenses. They help the immune system recognize dengue-infected cells and help protect uninfected cells from infection.

As the adaptive immune response starts fighting the dengue infection, B cells produce antibodies called IgM and IgG that are released in the blood and lymph fluid, where they specifically recognize and neutralize the dengue viral particles. In another adaptive immune response, cytotoxic T cells, or killer T cells, recognize and kill the cells that are infected with the dengue virus. The innate immune response activates the complement system, a response that helps the antibodies and white blood cells remove the virus. Together, the innate and adaptive immune responses neutralize the dengue infection, and the patient recovers from dengue fever (King et al., 2021).

1.14 Secondary Dengue Infections

After recovering from a first dengue infection, a person is protected from infection with the remaining three dengue serotypes for two to three months. Unfortunately, it is not long-term protection, and after that short period, a person can be infected with any of the remaining three dengue serotypes (Wei & Li, 2017).

In the 1960s, Dr. Scott Halstead and his colleagues were studying the dengue virus in Thailand. They noticed that people who had been exposed to dengue a second time had an increased risk of severe dengue compared with those who had not been previously exposed (Halstead, 2008).

Normally after an infection with a pathogen, the body "remembers" the infection for a long time because cells — called memory B cells and memory T cells — remain in the body. Because they remember the first infection, these memory cells can react rapidly to provide an adaptive response when an infection strikes a second time. Memory cells can remain in a person's body for many years, even an entire lifetime. Halstead proposed a phenomenon called "antibody-dependent enhancement of infection" to explain these observations (Wei & Li, 2017). There are four different types of dengue viruses (serotypes), but the memory cells only provide immunity from reinfection with the dengue serotype that caused the first infection. When a person is infected with a second dengue serotype, Halstead proposed that antibodies from the first infection actually help spread the dengue viral infection and increase viremia, the amount of virus in the bloodstream (Endy et al., 2002). This phenomenon can also happen in children who received antibodies against dengue from their mothers while in the womb. Surprisingly, instead of destroying the virus, the existing antibodies and the antibodies newly produced by the memory B cells can actually help the virus infect host cells more efficiently.

Ironically, the consequence of antibody-dependent enhancement is that the body's immune system response actually makes the clinical symptoms of dengue worse and raises the risk of severe dengue illnesses (K.-J. Huang et al., 2005) . Antibody (Ab)-dependent enhancement of infection occurs when preexisting antibodies present in the body from a primary (first) dengue virus (DENV) infection bind to an infecting DENV particle during a subsequent infection with a different dengue serotype. The antibodies from the primary infection cannot neutralize the virus. Instead, the Ab–virus complex attaches to receptors called Fcγ receptors (FcγR) on circulating monocytes (Whitehead et al., 2007a). The antibodies help the virus infect monocytes more efficiently. The outcome is an increase in the overall replication of the virus and a higher risk of severe dengue.

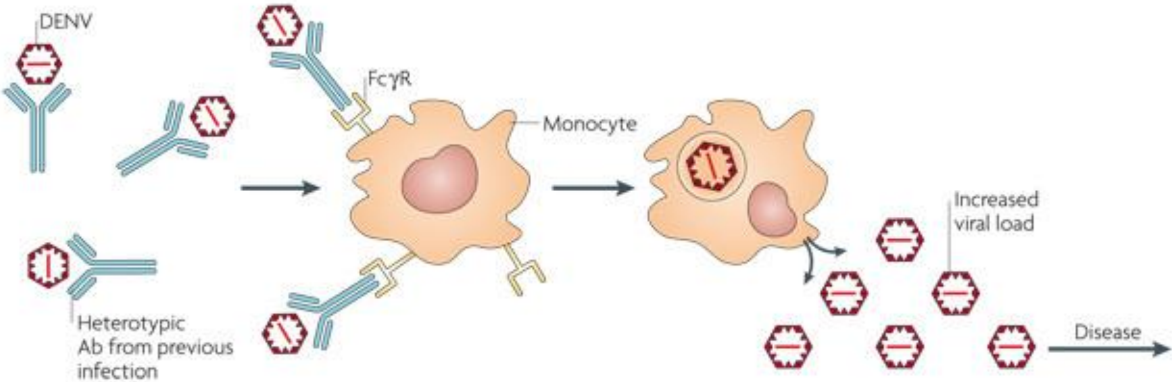


Fig: 1. 14. Model of antibody-dependent enhancement of dengue infection (Whitehead et al., 2007a)

Studies also showed that during a second infection with dengue, the cytotoxic T cells produced by the immune system provide only partial immunity against the new dengue serotype. The cytotoxic T cells do not effectively clear the virus from the body, and they release excess quantities of molecules called cytokines. In normal quantities, cytokines help the immune response; however, in high quantities, cytokines can produce serious inflammation and tissue damage such as leakage from the capillaries, possibly contributing to the development of severe dengue diseases (St. John, 2013). Certain chronic diseases — including asthma, sickle cell anemia, and diabetes mellitus — can increase a person's risk of developing a severe form of the disease. Variations in human leukocyte antigen alleles (a group of genes involved in immune system function) may also increase that risk.

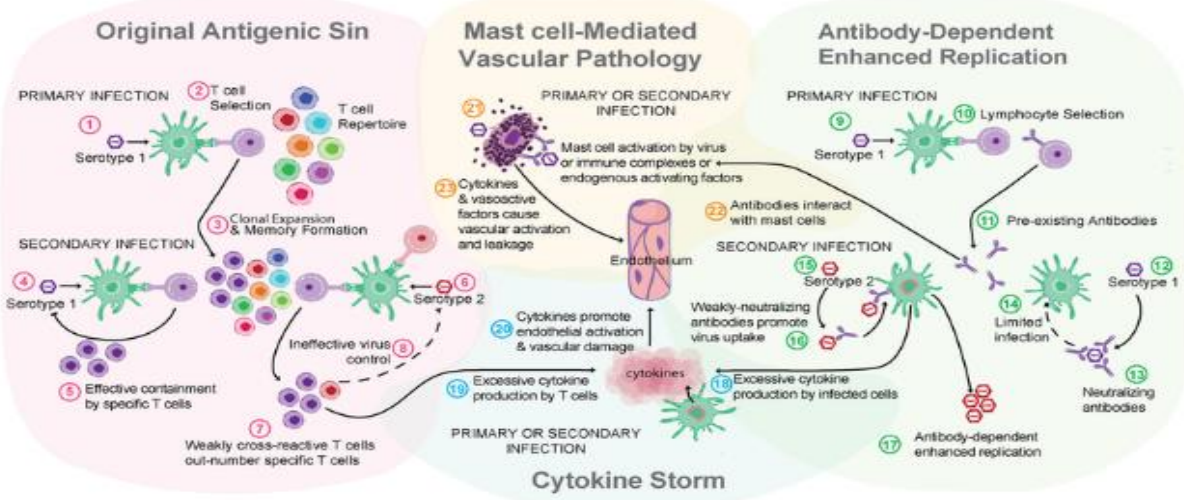


Fig: 1. 15. Multiple theories of dengue immune pathogenesis (St John, 2005)

1.15 Dengue Prevention and Control

In the continued absence of safe vaccines and specific treatment, effective vector control by either though fogging that kills adult mosquitoes, application of larvicides that target the aquatic stage of mosquitoes, or source reduction that reduces their breeding habitat is currently the most practical method available for reducing the incidence of dengue disease (WHO, 2021). Dengue preventive measures can be grouped into different catagories:

1.15.1. Methods of Vector Control

Aedes vector control should target all Aedes mosquito life stages (egg, larva, pupa and adult). *Aedes aegypti* uses a wide range of confined larval habitats, both man-made and natural. It may not be feasible or cost-effective to attempt to control the immature stages in all such habitats. Some man-made container habitats produce large numbers of adult mosquitoes, whereas others are less productive. Control activities should target the habitats that are most productive and hence epidemiologically more important rather than all types of container, especially in a resource constraints settings. Various vector control measures for dengue are Anti-Larval Measures, Environmental management, Chemical Control- Larvicides, Biological larvicides , Insect growth regulators (EDCD, 2019).

Table 1. 1. Vector control measures for dengue vector

Larvae	**Adult**	**Personal Protection**
Environmental	Chemical	Mosquito net
Chemical	Genetic	Screening
Biological		Repellants
Insect Growth Regulators		Protective Clothing

Environmental Control

Environmental management includes prevention or minimization of vector propagation and human contact with the vector-pathogen by destroying, altering, removing or recycling non-essential containers that provide larval habitats. It includes good piped water supply, frequent emptying and cleaning of water storage containers, cleaning gutters, proper disposal of discarded containers and tyres. Mosquito-proofing of water-storage containers, solid waste management, street cleansing which removes discarded water bearing containers such as plastic cups, bottles, bottle caps, plastic package covers, etc and regular cleaning of drains helps to reduce larval propagation(EDCD, 2019).

Change in human habitation or behavior such as use of mosquito screening nets in windows, doors, bed nets for sleeping during day time. Collection, recycle and disposal of unused or damaged drain water storage tank, drums, flower vase with water, potted plants with saucers can aid in dengue control. Also protection from mosquito bites to dengue patient during first week of illness is also important measures to prevent mosquito from getting dengue virus and transmitting to other humans (WHO, 2021).

Chemical Control

Chemical control should be considered as complementary to environmental management and except in emergencies it should be restricted to containers that cannot otherwise be eliminated or managed (WHO, 2009). It is difficult and expensive to apply chemical larvicides on a long term basis. Larvicides may be impractical to apply in hard-to-reach natural sites such as leaf axils and tree holes, which are common habitats of *Aedes albopictus*, or in deep wells. The difficulty of accessing indoor larval habitats of *Aedes aegypti* (e.g. water storage containers, plant vases, saucers) to apply larvicides is a major limitation in many urban contexts. As *Aedes aegypti* often deposit eggs in water-storage containers, the larvicides should have low toxicity to other species and should not significantly change the taste, odor or color of the water. Commonly used chemicals are Temephos (1 mg/liter), Methoprene (1 mg/liter), Pyriproxyfen (0.01 mg*/liter). Other chemical methods include fogging for adult mosquito control (EDCD, 2019).

Biological Control

Biological control methods are mostly effective against the immature stages of vector mosquitoes in the larval habitat. It is based on the introduction of organisms that prey upon, parasitize, compete with or otherwise reduce populations of the target species. Against Aedes vectors of dengue, only certain species of larvivorous fish- *Gambusia affinis*, *Poecilia reticulata* and predatory copepods (small freshwater crustaceans) have proved effective (EDCD, 2019).

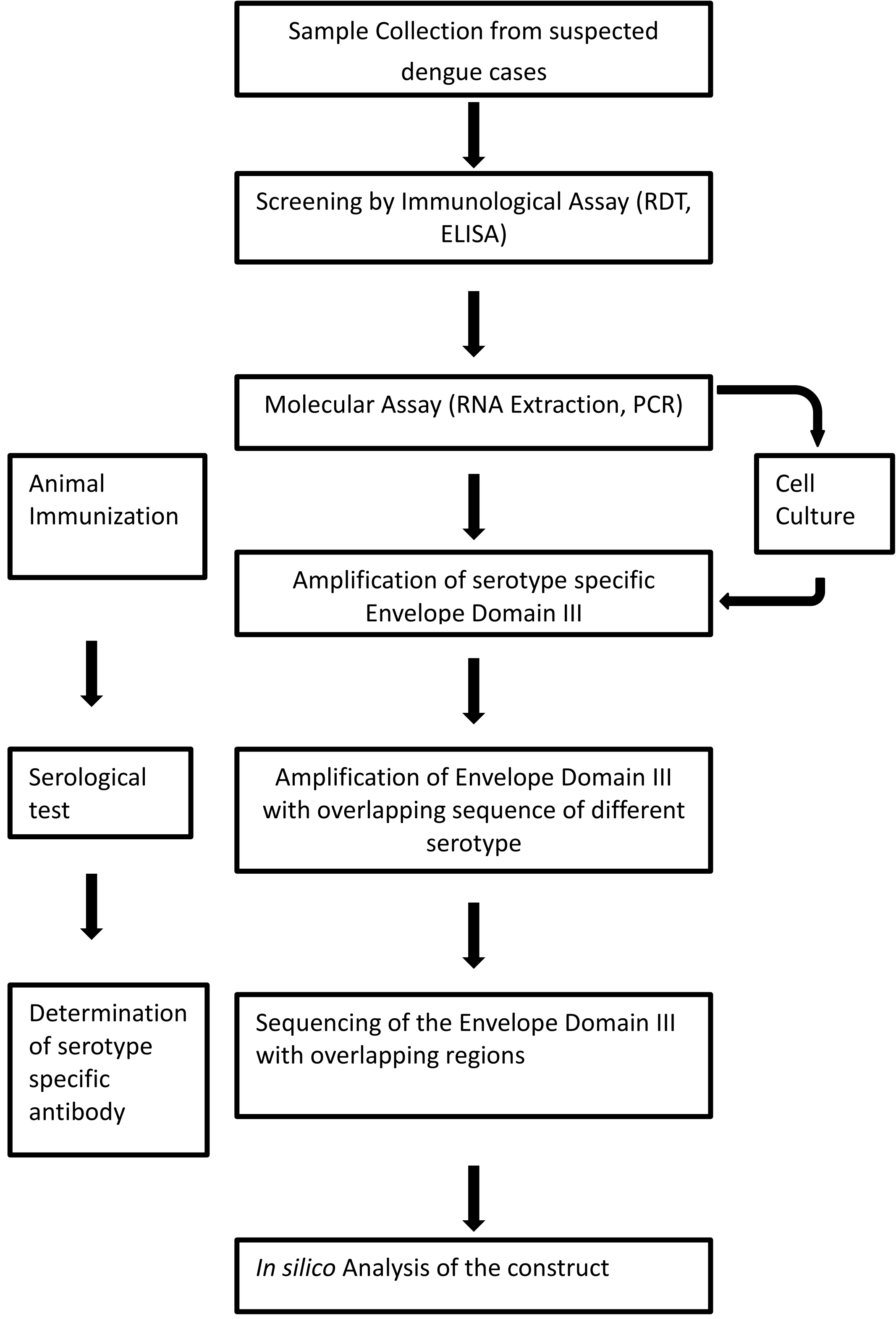
Insect growth regulators (IGR) or growth hormones are highly toxic to insect larvae or pupae interfering with development into adults. However, it is costly and limited availability. Some genetic control methods are available for adult mosquito control such as sterile male technique, chromosomal translocations, gene replacement, release of *Wolbachia spp* infected *Aedes* vector are some of understudy technique worldwide for dengue control (WHO, 2021). Use of microorganism such as *Wolbachia* for dengue control have been applied in countries like Singapore, Austrilia, etc. It’s main aim is to increase number of the sterile mosquitoes in the wild.

Vaccination against Dengue

Vaccination plays one of the integral part in dengue prevention and control strategy. The first dengue vaccine, Dengvaxia® (CYD-TDV) developed by Sanofi Pasteur was licensed in December 2015 and has now been approved by regulatory authorities in around 20 countries. The vaccine is targeted for persons living in endemic areas, ranging from 9-45 years of age, who have had at least 1 documented dengue virus infection previously (WHO, 2021).

The ideal DENV vaccine would be free from significant reactogenicity and it would induce the level of protection afforded by infection with any of the four serotypes of dengue viruses. It would provide lifelong protection. As many regions with endemic DENV are in developing countries with limited health resources, the vaccine must be economical with minimal or no repeat immunization (Whitehead et al., 2007b).

1.16Theoretical/Conceptual Framework



1.17 Hypothesis/ Research Questions

Is Envelope Domain III region of Dengue virus good candidate for vaccine development?

Will the use of Envelope Domain III region eliminate the risk of Antibody Dependent Enhancement (ADE) in Dengue Vaccine?

Will recombinant envelope Domain III vaccine candidate provide protection against all four serotypes of Dengue?

Null Hypothesis:

Envelope Domain III is not the suitable candidate for safe vaccine production.

Tetravalent DNA vaccine construct of dengue will not provide immunity against all four serotypes.

Alternative hypothesis

Envelope Domain III is the suitable candidate for safe vaccine production.

Tetravalent DNA vaccine construct of dengue will provide immunity against all four serotypes.

1.18. OBJECTIVES:

1.18.1 General Objective:

- Mining Envelope Domain III of Dengue Virus for Recombinant Tetravalent DNA Vaccine Candidate from Nepalese Samples

1.18.2 Specific Objective:

- Virus detection in the collected samples.
- Serotyping of virus.
- Propagation of dengue virus in cell lines
- Construction of recombinant envelope domain III of all serotypes.
- Sequencing of recombinant envelope domain III.
- *In silico* Analysis of the recombinant construct
- Animal Immunization and serological test.
- Determination of serotype specific IgG antibody.

1.19 Problem statement / Rationale

Dengue disease is becoming a global epidemic, with nearly 40% of the world's population at risk for transmission of one or poly serotypes among the four dengue viruses (DENV). Since 1970s, endemic Dengue has spread from 9 nations to over 100, moved into urban areas, and explosive outbreaks of disease have become increasingly common. Four different serotypes of DENV (DENV 1-4) have been identified as causes of dengue infection since first recognized in 1943, later accompanied by the fifth serotype (DENV-5) discovered in 2013.

Dengue is endemic in Terai of Nepal and recently suffered a huge outbreak with 17992 reported case and the number could be more considering the spread rate and spread area. Also the outbreaks have been showing some familiar pattern and there is a major outbreak in every 3 years which might lead to development of severe case in Nepal in upcoming year. In today's context, major focus of research on disease management is based on pathogen rather than host. Increasing host immunity through vaccination is crutial for long term dengue control. In Nepal there is no vaccination programme for dengue.

Many dengue infections lead to hospitalizations, which can overwhelm weak health care structures, in particular during times of outbreaks. Given the unpredictability of outbreaks, the increasing magnitude and frequency of such outbreaks, and the current lack of highly effective and sustainable vector control interventions, there is a clear indication for a dengue vaccine for endemic populations.

Also, there are not many safe vaccine available at this stage. The first dengue vaccine, Dengvaxia® (CYD-TDV) developed by Sanofi Pasteur was licensed in December 2015 and has now been approved by regulatory authorities in about 20 countries. In November 2017, the results of an additional analysis to retrospectively determine serostatus at the time of vaccination were released. The analysis showed that the subset of trial participants who were inferred to be seronegative at time of first vaccination had a higher risk of more severe dengue and hospitalizations from dengue compared to unvaccinated participants. As such, use of the vaccine is targeted for persons living in endemic areas, ranging from 9-45 years of age, who have had at least 1 documented dengue virus infection previously.

This research will also play pivotal role in development of the safe vaccine which will address the problems in current vaccine. Thus enabling us towards proper dengue prevention and control.

CHAPTER II
LITERATURE REVIEW

2.1 Dengue Virus

Dengue virus (DENV) is a mosquito-borne single stranded, positive sense encapsulated RNA virus, approximately 10.7 kb long. The genome of DENV codes for three structural proteins- a nucleocapsid or core (C), a membrane-associated (M), an enveloped (E) glycoprotein and seven non-structural (NS) proteins. Dengue fever caused by DENV is one of the most important arthropod-borne tropical viral infections with an estimation of 390 million infections annually (Bhatt et al., 2013). The disease is transmitted by mosquitoes, primarily *Aedes aegypti* and *Aedes albopictus*. Dengue is the most common and widespread arboviral infection in the tropical and subtropical regions with significant morbidity and mortality. The clinical picture ranges from a mild dengue fever (DF) to severe cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which might be fatal (Rajapakse, 2011). The primary infection with any single serotype usually causes fever but the severe cases are usually an outcome of a secondary infection with a heterologous serotype along with the presence of non-neutralizing antibodies. The later phenomenon is known as Antibody Dependent Enhancement (Wei & Li, 2017). The presence of four classical serotypes among the Dengue viruses that have no cross-protective immunity in infected patients and hence dramatically increases the complexity of this disease (Prajapati, et al., 2020).

Dengue virus (DENV) is a RNA virus transmitted by mosquitoes which causes dengue fever. Dengue virus (DENV) is a single stranded, positive sense encapsulated RNA virus, approximately 10.7 kb long(Sim & Hibberd, 2016). The genome codes for three structural proteins- a nucleocapsid or core (C), a membrane-associated (M), an enveloped (E) glycoprotein and seven non-structural (NS) proteins. Dengue fever caused by DENV is one of the most important arthropod-borne tropical viral infections with an estimation of 390 million infections annually. The disease is transmitted by mosquitoes, primarily *Aedes aegypti* and *Aedes albopictus*. Dengue is the most common and widespread arboviral infection in the tropical and subtropical regions with significant morbidity and mortality(Samsa et al., 2009). The clinical picture ranges from a mild dengue fever (DF) to severe cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which might be fatal.

2.2. Dengue virus serotypes and their genotypes

All four DENV serotypes are transmitted by *Aedes* mosquitoes and, in principle, cause the same clinical manifestations and show similar patterns of systemic dissemination, there are some biological differences between them (Bara et al., 2013). The associations between particular serotypes or genotypes and disease severity, epidemic potential and the efficiency of transmission have been described, but these associations could be influenced by factors other than intrinsic viral characteristics, such as host immunity, the ability of the mosquito vector to become infected and to transmit the virus to humans, and the conditions that support the displacement of one genotype by another (Halstead, 2008).

Each serotype of the dengue virus can be further classified into several genetic groups called *genotypes* (the term *subtype* is used interchange) based on sequence diversity. Initially it was defined a dengue genotype as a group of dengue viruses having no more than 6% sequence divergence within a 240-nucleotide region of the DENV-1 and DENV-2 E/NS1 junction. Since then, both the length and region of virus genome selected for sequencing varied greatly depending on research groups, ranging from the complete sequence of single genes to the complete genome of the DENV. Assignment of genotypes now relies on phylogenetic analysis rather than arbitrary cut-off values in sequence diversity (Flamand et al., 2017).

DENV-1 can be divided into five genotypes based on the complete E gene sequence as described (Dempsey et al., 2017). Earlier work also classified DENV-1 into five groups based on the 240- nucleotide E/NS1 junction sequences, but with some minor differences from the newer scheme, the DENV-1 genotypes, all have a wide area of distribution apart from genotype III (sylvatic) and genotype II which consists of Thai strains from the 1950s and 1960s (Singh et al., 2013).Viruses of genotype I and IV have recently been implicated as causing epidemics in the Pacific between 2000 and 2004 and genotype V viruses are frequently isolated during epidemics in the Americas (Kyle & Harris, 2008). However, it is still inconclusive whether any of these three DENV-1 genotypes can be consistently associated with causing more severe dengue.

DENV-2 is the most studied serotype among the dengue viruses. DENV-2 has been attributed to cause a more severe disease even during primary infections. At molecular genetic level, DENV-2 has been divided into 6 genotypes namely Cosmopolitan, Asian I, Asian II, American, American/Asian and Sylvatic (Gupta et al., 2015b). These genotypes have a 5% or more genetic divergence in their envelope as well as whole genome nucleotide sequences. The Asian I genotype comprises of strains isolated from Thailand and Malaysia while Asian II genotype consists of strains circulating in China, Philippines, Sri Lanka, Taiwan. The strains circulating in China, Thailand, Vietnam, Brazil, Venezuela

and the Caribbean belonged to American/Asian genotype while the isolates from Australia, Pacific Islands, South East Asia, and Indian sub-continent, Middle East, Africa and Mexico fall in Cosmopolitan genotype (Prajapati et al., 2020).The existence of six genotypes of DENV-2 based on the complete E gene sequence following earlier work. Sylvatic DENV-2 strains that are closely related have been isolated from several countries in West Africa and Malaysia, two locations that are far apart, leading to hypothesise that the DENV sylvatic ancestor arose in the Asian-Oceanic region before diverging into today's four DENV serotypes (Abello et al., 2016).

The current genotype classification for DENV-3 follows four genotypes based on prM/E sequences. These four genotypes are similar to the four groups described using a 195-nucleotide region at the 5' terminus of the E gene. Introduced to the Americas via Nicaragua in 1994, genotype III DENV-3 is now widely found in Central and Southern America and is considered as the most virulent of the four DENV-3 genotypes. It is worthy to note that genotype IV has never been associated with any DHF epidemics (Kumar & Sharma, 2016).

DENV-4 initially had been separated into two genotypes, I and II, based on the complete E gene sequence. A further two genotypes were subsequently described, with one found only in non-human primates in Malaysia and another, genotype III, found only in Bangkok, Thailand (Phommanivong et al., 2016). Genotype II DENV-4 is the most widespread of the four following an introduction to the Western hemisphere in 1981, possibly via the Pacific islands. Although DENV-4 is the least frequently sampled serotype, it is often associated with haemorrhagic fever during secondary infection (Shim, 2016).

Except for the sylvatic genotypes, genotype classification can often unveil the geographical origin of the dengue virus strains. This has enabled tracking the route of virus transmissions across distant time and place, and has served as the basis of molecular epidemiological studies that can determine whether dengue epidemics are caused by introduction of new viruses or the result of re-emergence of endemic strains. Introduction of new viruses inevitably leads to the question whether particular genotypes of DENV are associated with higher virulence or severe disease (Prajapati et al., 2020).

2.3 Epidemiology of Dengue in Nepal

Dengue is the global public health issue which has become also a major public health issue of Nepal. Dengue virus (DENV) is the causative agent for dengue fever and causes more than 400 million cases of dengue infection annually in globe and there is increasing trend of dengue cases in Nepal (Pandey et al., 2008). All four serotypes of dengue were found circulating in Nepalese population thus increasing chances of developing more severe dengue cases by secondary infection (Pun, 2011).

Nepal, a landlocked country, surrounded by India on three sides and China to the North is an endemic nation for many vector borne diseases, like Malaria, Kala-azar, Japanese encephalitis, lymphatic Filariasis and Dengue fever (Dhimal et al., 2015). The first report of dengue fever was in 2004 from a Japanese traveler in Nepal (Pandey et al., 2004) and it was reported to be serotype 2 with >99% similarity with viral strains from India (Takasaki et al., 2008). In 2006, dengue fever was seen in southern lowland districts' patients without any history of travel to endemic areas along with the presence of the primary dengue vector mosquito *A. aegypti* confirmed the establishment of local DENV transmission in Nepal. In addition, clinical and laboratory test results confirmed the circulation of all four DENV serotypes during the 2006 outbreak in Nepal (Malla et al., 2008a). A few intermittent cases were reported nationwide from 2007 to 2009 with 2, 8 and 16 cases per year respectively (Fernandez et al., 2013). Since then, annual sporadic clinical cases of dengue are being reported with major outbreaks in the years 2010, 2013 and 2016 with shift of serotype from Genotype V of DENV-1 in 2010 (Pandey et al., 2013) to Cosmopolitan Iva Asian II genotypes of DENV-2 in 2013 (Singh et al., 2015) and again DENV-1 in 2016 (Gupta et al., 2018). In 2017 outbreak DENV-1, 2 and3 serotypes were found to be co-circulating in Nepal. From the study in 2017 sample DENV-1 isolated from Nepalese population were found to be closely related to Indian isolates belonging to genotype V and DENV-2 detected was reported to be related to genotype cosmopolitan Iva which is closely related to Indonesian isolates.(Prajapati et al., 2020) With minimal cases in 2018, 2019 was reported with highest cases declaring dengue epidemic in Nepal.

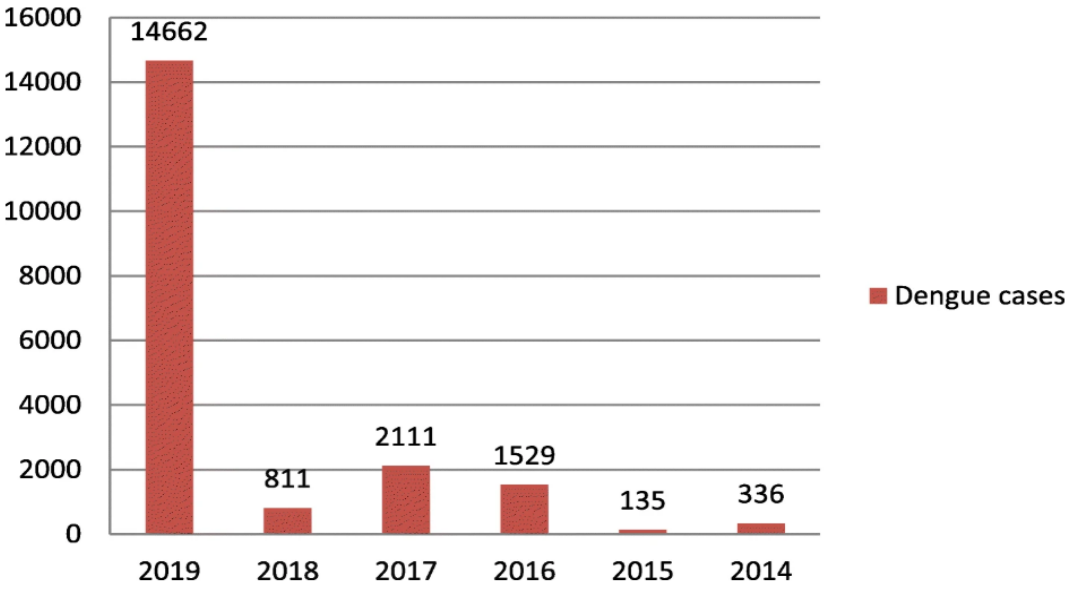


Fig 2. 1. Year-wise cases of dengue cases in Nepal from 2013/2014–2018/2019 (Adhikari & Subedi, 2020a). This data is based on dengue update by EDCD till Nov10 2019. Data might differ in later updates.

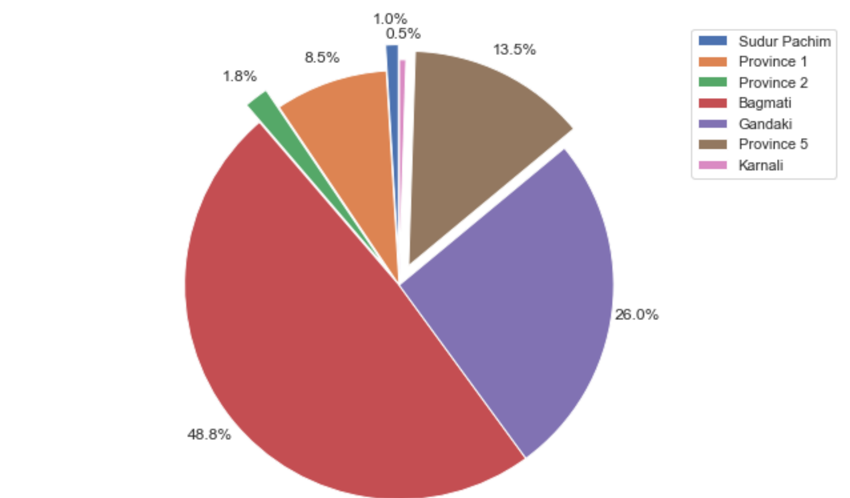


Fig 2. 2. Dengue confirmed Provinces in the year 2019 (Adhikari & Subedi, 2020b)

Nepal, a Himalayan country, surrounded by India on three sides and China to the North is an endemic nation for many vector borne diseases, like malaria, kala-azar, Japanese encephalitis, lymphatic filariasis and dengue fever (Dhimal et al., 2015). The first report of dengue fever was in 2004 from a Japanese traveler in Nepal (Pandey et al., 2004) and it was reported to be of serotype 2 with >99% similarity with viral strains from India (Takasaki et al., 2008). In 2006, dengue fever was seen in southern lowland districts' patients without any history of travel to endemic areas along with the presence of the primary dengue vector mosquito A. aegypti confirmed the establishment of local DENV transmission in Nepal. In addition, clinical and laboratory test results confirmed the circulation of all four DENV serotypes during the 2006 outbreak in Nepal (Malla et al., 2008). A few intermittent cases were reported nationwide from 2007 to 2009 with 2, 8 and 16 cases per year respectively (Fernandez et al., 2013). Since then annual sporadic clinical cases of dengue are being reported with major outbreaks in the years 2010, 2013 and 2016 with shift of serotype from Genotype V of DENV-1 in 2010 (Pandey et al., 2013) to Cosmopolitan Iva Asian II genotypes of DENV-2 in 2013 (Singh et al., 2015) and again DENV-1 in 2016. (Gupta et al., 2018).

2.4 Mode of transmission of dengue

The life cycle of dengue involves endocytosis via a cell surface receptor. The virus uncoats intracellularly. When the virus is carried into the cell and into lysozomes, the acidic environment causes the protein to disintegrate cause the virus membrane to fuse with lysozome. This releases the RNA into the cell and infection starts(Rodenhuis-Zybert et al., 2010). The DENV RNA genome is in the infected cell translated by the host ribosomes. The subsequently formed immature virions are assembled by budding of newly formed nucleocapsids into the lumen of the endoplasmic reticulum (ER), thereby acquiring a lipid

bilayer envelope with the structural proteins prM and E. The virions mature during transport through the acidic trans-Golgi network, where the prM proteins stabilize the E proteins to prevent conformational changes. Before release of the virions from the host cell, the maturation process is completed when prM is cleaved into a soluble pr peptide and virion-associated M by the cellular protease furin (Wilder-Smith et al., 2010). Outside the cell, the virus particles encounter a neutral pH, which promotes dissociation of the pr peptides from the virus particles and generates mature, infectious virions. At this point the cycle repeats itself.

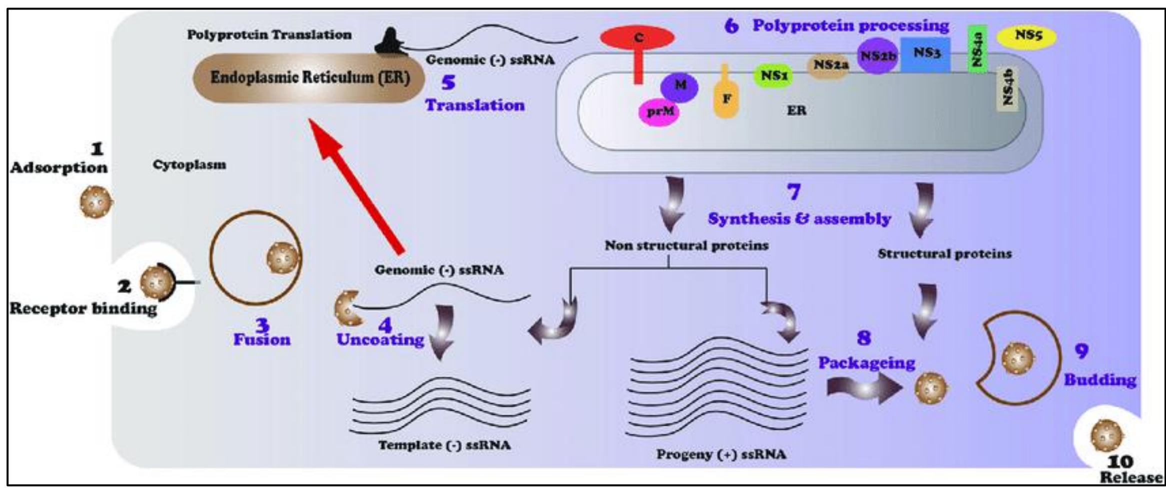


Fig 2. 3. Replication of dengue virus (Idrees & Ashfaq, 2012)

2.3 Clinical manifestations of dengue

Dengue viruses show same clinical manifestations and show similar patterns of systemic dissemination having tropism principally for monocytes, macrophages and dendritic cells (Durbin et al., 2008, Jessie et al., 2004). The pathophysiologic changes that occur during dengue fever are still not fully understood. Thrombocytopenia has always been one of the criteria by WHO guidelines as a potential indicator of clinical severity and a number of studies have documented platelet dysfunction in DENV infection (WHO, 2009). Although dengue is a non hepatotropic virus, liver injury due to dengue infection is common. Hepatic involvement can be characterized by manifestations of acute hepatitis, hepatomegaly, pain in the right hypochondrium, jaundice and raised aminotransferase levels (Gubler, 1997). Clinical studies documenting hepatic involvement in dengue infection are rare. Also the hematological parameters like hematocrit and hemoglobin should be monitored during dengue fever.

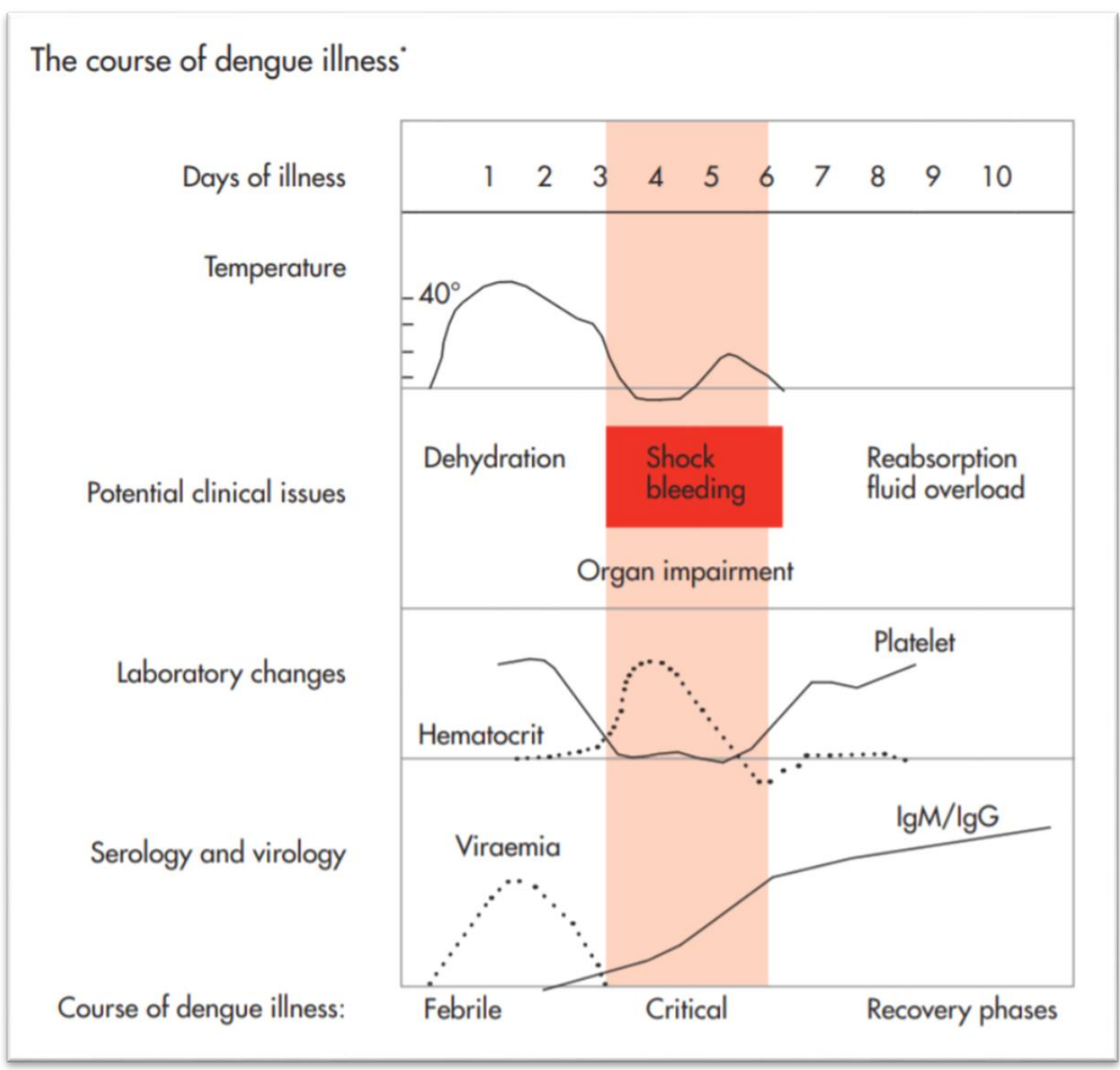


Fig 2. 4. Course of Dengue illness (WHO, 2009).

2.4 Laboratory diagnosis of dengue

Laboratory diagnosis methods for confirming dengue virus infection may involve detection of the virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques. After the onset of illness, the virus can be detected in serum, plasma, circulating blood cells and other tissues for 4–5 days. During the early stages of the disease, virus isolation, nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis (WHO, 2009).

Table 2. 1. Summary of operating characteristics and dengue diagnosis methods.

Diagnostic Methods	**Specimen**	**Specimen collection time**	**Facilities**
Viral isolation and serotype identification	Whole blood, serum, tissue	1-5 days	BSL-2 laboratory, molecular biology equipment
Nucleic acid detection	Tissue, whole blood, serum, plasma	1-6 days	BSL-2 laboratory, molecular biology equipment
Antigen detection	Serum	1-5 days	ELISA facilities
IgM ELISA	Serum, plasma, whole blood	After 5 days	ELISA facilities
IgG ELISA	Serum, plasma, whole blood	Acute sera, 1-5 days; Convalescent after 15 days	ELISA facilities

DENV viremia is detectable 24–48 hours before fever onset and continues for 5–6 days. During this period, infective virus, its specific RNA and the NS1 protein can be detected in patient blood, serum and plasma, and also in tissues from fatal cases (Gubler et al., 1997). Virological, molecular and serological methods are used to confirm DENV infection for epidemiological surveillance and clinical diagnosis. Anti-DENV IgM antibody detection is the most widely used test in routine practice. Anti-DENV IgM titres in sera from patients in the acute phase of disease are measured to serologically confirmed infection, whereas patients in convalescence are identified through IgM and IgG seroconversion by comparing antibody titres in paired acute and convalescent sera (Gubler et al., 1997). For patients who are suspected of having dengue, a presumptive diagnosis can be made by the detection of anti-IgM antibodies in samples collected at day 6 of acute symptoms. Commercial kits for IgM or IgG detection in Enzyme-Linked Immunosorbent Assay (ELISA) and less-sensitive rapid test formats are available (Hunsperger et al., 2009). Reverse transcription PCR (RT-PCR), real-time RT-PCR, DENV isolation in mosquito cell lines and by mosquito inoculation facilitate confirmation and identification of the agent virologically. Although virus isolation and identification is highly specific, it has a relatively low sensitivity and is resource-consuming and time-consuming.

By contrast, DENV RNA detection provides a rapid, sensitive and specific method for virological diagnostic confirmation. NS1 protein detection provides a window of opportunity for early aetiological diagnosis. The sensitivity and specificity of DENV NS1 detection depend on the infecting serotype, the timing of sample collection and the parity of DENV infection (primary versus secondary), as well as the format of the test (Hunsperger et al., 2009).

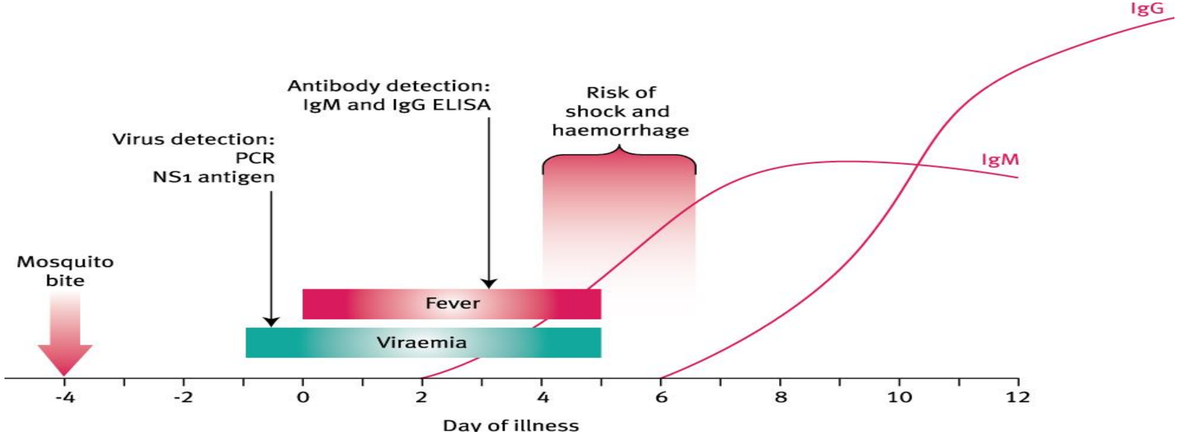


Fig 2. 5. Dengue diagnosis Viremia, Non-Structural 1 (NS1) antigen and antibodies change over time; thus, different diagnostic tests will be appropriate depending on the stage of infection. ELISA, Enzyme-Linked Immunosorbent Assay; RT, reverse transcription Adapted from REF. 153, Nature Publishing Group

The dengue virus detection can be done by immunological and molecular techniques. Immunological diagnosis detects specific antibodies to the virus and complements the viral diagnosis. The detection of antibodies can be made using Enzyme linked Immuno-Sorbent Assay (ELISA) and immune-chromatographic techniques; test for hemagglutination inhibition (HI), plaque reduction neutralization test (PRNT), etc (WHO, 2009). Molecular characterization of dengue virus can be done by using both Real time RT-PCR and conventional RT-PCR. The use of conventional RT-PCR in early diagnosis of suspected cases and to monitor viral circulation has proven to be a valuable diagnostic tool, with the advantage of not providing significant difference in sensitivity, in both primary and secondary cases, or even in the presence or absence of IgM in the serum sample tested (Arya et al., 2011).

2.4.1 Serological / Immunological Diagnosis Approach

Among different dengue diagnosis techniques ELISA is the widely used technique. Before day 5 of illness, during the febrile period, dengue infections may be diagnosed by virus isolation in cell culture, by detection of viral RNA by nucleic acid amplification tests (NAAT), or by detection of viral antigens by ELISA or rapid tests (Dussart et al., 2008). The isolation and identification of dengue viruses in cell cultures usually takes several days.

Nucleic acid detection assays with excellent performance characteristics may identify dengue viral RNA within 24–48 hours(Guzmán & Kourí, 2004b). However, these tests require expensive equipment and reagents. NS1 antigen detection kits now becoming commercially available can be used in laboratories with limited equipment and yield results within a few hours.

Fig 2. 6. Different diagnosis techniques and days when tests become positive.(Hang et al., 2009)

After day 5, dengue viruses and antigens disappear from the blood coincident with the appearance of specific antibodies. NS1 antigen may be detected in some patients for a few days after defervescence. Low levels of a detectable dengue IgM response or the absence of it in some secondary infections reduces the diagnostic accuracy of IgM ELISA tests. A four-fold or greater increase in antibody levels measured by IgG ELISA or by haemagglutination inhibition (HI) test in paired sera indicates an acute or recent flavivirus infection(Blacksell et al., 2012). However, waiting for the convalescent serum collected at the time of patient discharge is not very useful for diagnosis and clinical management and provides only a retrospective result(De Paula & Fonseca, 2004).

2.4.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. Other names, such as enzyme immunoassay (EIA), are also used to describe the same technology. In an ELISA, an antigen must be immobilized on a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by

assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction(Kuno et al., 1991).

Types of ELISA

Different types of ELISA are commonly used. They are described as follows:

1. Direct ELISA: It is the simplest configuration in which the antigen is bound by passive adsorption to the solid phase, washed to remove any unbound molecules and then directly incubated with a conjugated_antibody. Following the incubation period and additional washing, substrate is added to produce signal that is allowed to develop. After certain time, the substrate reaction is stopped and the resulting signal quantified(Aydin, 2015). It is commonly used for tittering conjugated secondary antibodies and very useful to estimate antigen cross-reactivity.

2. Indirect ELISA: In this system, initial antigen binding and washing steps are the same as the direct method. The main difference in this case is the use of unconjugated antibody to bind the immobilized antigen upon incubation at optimal temperature (usually 37°C). Following a washing step to remove unbound antibodies, the remaining antigen-bound antibodies are targeted by a conjugated secondary antibody that will generate the readout signal as described for direct ELISA. This system has been widely applied in diagnostics because it allows large number of samples to be screened with a single conjugated secondary antibody(Lin, 2015).

3. Sandwich ELISA: This assay requires a compatible antibody pair that recognize different antigenic targets (epitopes) on the same antigen. The first antibody, called capturing antibody, is coated on the plate and used to immobilize the antigen upon binding during incubation with the sample. Free antigen is removed by a washing step and then a detecting antibody is added to bind the captured antigen and enable subsequent detection(“Sandwich ELISA,” 2011).

Step	Direct	Indirect	Sandwich Direct	Sandwich Indirect
Coating (Adsorption to solid phase)	Antigen	Antigen	Capture Antibody	Capture Antibody
Blocking	Addition of blocking agent to prevent non-specific binding			
Wash	Separate bound / unbound analytes			
Analyte (Addition of testing sample)	Enzyme- or Fluorescence-Conjugated antibody	Unconjugated Antibody	Antigen sample	Antigen sample
Wash	Separate bound / unbound analytes			
Secondary Reagent	N/A	Enzyme- or Fluorescence-Conjugated antibody	Enzyme- or Fluorescence-Conjugated Detection Antibody	Biotin-conjugated or Unconjugated Detection Antibody
Wash	Separate bound / unbound analytes			
Additional Reagent	N/A	N/A	N/A	Enzyme- or Fluorescence-Conjugated Streptavidin or Secondary Antibody
Wash	Separate bound / unbound analytes			
Signal Development	Addition of substrate for enzyme-conjugated antibodies			
Stop Signal Development	For end-point reading of enzyme-based detection systems			
Signal Detection	Colorimetric, fluorescent or chemiluminescent detection			

Fig 2. 7. Overview of steps in different types of ELISA (AbCAM, 2016).

Development of In-House ELISA

The first step of ELISA is coating. It is a process where a suitably diluted antigen or antibody is incubated until adsorbed to the surface of the well. Coating helps to stabilize the antigen or antibody which is used to coat the ELISA multi-well plate, maximizing adsorption to the plate and optimizing interactions with the detection antibody. The dilution is done on the coating buffer which helps in adsorptive immobilization of proteins and antibodies on plastic surfaces or other binding surfaces. After coating, the plates are incubated at 4◦C overnight. Then the plates are blocked using blocking buffer. Blocking is necessary to prevent the non-specific binding of detection antibodies to the multi-well plate surface itself. The blocking buffers usually contain an unrelated protein or a protein derivative that does not react with any of the antibodies being used in the detection step(Wang & Sekaran, 2010). The plates are washed using phosphate-buffer saline which is a water based salt solution. It is preferred because it is non-toxic to most cells. The washing step helps to remove the unbound materials(Wasonga et al., 2015).

2.5. Molecular identification/ Nucleic acid detection

Reverse Transcriptase PCR and Real time PCR have been the method of choice for genome detection. Viral RNA can be extracted from serum, blood, plasma, tissues (including formalin-fixed specimens), blood collected on filter paper, and (more recently) saliva. Since the 1990s, several reverse transcriptase-polymerase chain reaction (RT-PCR) assays have been developed (Hunsperger et al., 2009). They offer better sensitivity compared to virus isolation with a much more rapid turnaround time.

Nucleic acid detection assays involve three basic steps: nucleic acid extraction and purification, amplification of the nucleic acid, and detection and characterization of the amplified product. Extraction and purification of viral RNA from the specimen can be done by traditional liquid phase separation methods (e.g. phenol, chloroform) but has been gradually replaced by silica based commercial kits (beads or columns) that are more reproducible and faster, especially since they can be automated using robotics systems. Many laboratories utilize a nested RT-PCR assay, using universal dengue primers targeting the C/prM region of the genome for an initial reverse transcription and amplification step, followed by a nested PCR amplification that is serotype-specific (Gupta et al., 2015b). A combination of the four serotype-specific oligonucleotide primers in a single reaction tube (one-step multiplex RT-PCR) is an interesting alternative to the nested RT-PCR (Poudyal et al., 2021).

The real-time RT-PCR assay is a one-step assay system used to quantitate viral RNA and using primer pairs and probes that are specific to each dengue serotype. The use of a fluorescent probe enables the detection of the reaction products in real time, in a specialized PCR machine, without the need for electrophoresis. Many real-time RT-PCR assays have been developed employing TaqMan or SYBR Green technologies (Gupta et al., 2015b).

2.7. Host cellular immune response

Cellular immune response does not involve antibodies, but rather involves the activation of phagocytes, antigen-specific T-lymphocytes and the release of various cytokines in response to an antigen. Cellular immunity protects the body through T-cell mediated immunity by activating antigen-specific cytotoxic T-cells, macrophage and natural killer cell action enabling the destruction of pathogens via recognition and secretion of cytotoxic granules and phagocytosis and stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive and innate immune responses (Janeway, 2000).

Cellular immunity plays a vital role during dengue infection. Dengue virus-specific T-cells recognize virus-infected cells and respond with a diverse set of effector functions,

including proliferation, target cell lysis and the production of a range of cytokines (Rothman, 2011). A broad array of cytokines is produced by dengue virus-specific T cells in response to the recognition of peptide–Major histocompatibility complex (MHC) on target cells. For most T cells studied, the pattern of cytokine production follows a T helper 1 (TH1) - or TH0-like profile. Thus, these T cells produce interferon-γ (IFNγ), tumour necrosis factor (TNF), interleukin-2 (IL-2) and CC-chemokine ligand 4 (CCL4; also known as MIP1β), whereas the production of TH2-type cytokines, such as IL-4, is less common (Rothman, 2011). In addition to increases in cytokine levels cellular markers for T-cell activation, CD69, CD38 and CCR7 have been shown to be increased in dengue infection (Mathew & Rothman, 2008).

2.6. Vaccine Development status

Because effective vector-control measures are not scalable or sustainable, community based approaches have led to mixed results, and promising novel strategies such as *Wolbachia* are still under development, a dengue vaccine would appear to be the best intervention (Wilder-Smith, 2019). Challenges and hurdles in the development of dengue vaccines. Several difficulties have hampered the development of a dengue vaccine. One challenge is the lack of an appropriate animal model and poor knowledge of correlates, both for protection and disease enhancement. But the biggest hurdle is the interaction among the four serotypes (Guzman et al., 2010). As a tetravalent immune response is desired, when a mixture of all four serotypes in a tetravalent live attenuated vaccine is given, each component would need to independently result in four different monotypic immune responses that are solid to each serotype (Murugesan & Manoharan, 2020). This has, unfortunately, proven to be difficult to achieve. Dengue vaccine development. Despite more than 30 years of efforts using various vaccine platforms including inactivated, DNA, and live vaccines, only live attenuated vaccines have entered phase 3 trials. Three live attenuated dengue vaccines are now in late stage development, with one candidate having completed phase 3 trials including long-term follow-up of 5 years: CYD-TDV by Sanofi Pasteur, Lyon, France, with the trade name of Dengvaxia. Dengvaxia have been approved by FDA in 2015 (Wilder-Smith, 2019).

CYD-TDV dengue vaccine

CYD-TDV, a tetravalent live attenuated vaccine with a yellow fever back-bone, is the first dengue vaccine to be licensed. Phase 3 trials revealed a vaccine efficacy that depended on age, and serotype but also showed a population level benefit. Interference manifested by asymmetric immunological responses to the mixtures of four dengue vaccine viruses was recognized as a possible reason for this varied vaccine performance (Katzelnick et al., 2017). Post hoc retrospective analyses of the long-term safety data using a novel nonstructural protein (NS1) antibody assay revealed an excess risk of severe dengue in

those who were seronegative at baseline, which means those who were dengue naive at the time of administration of the first dose (WHO, 2021). The reasons for the excess cases are not fully understood, but a plausible hypothesis is that Dengvaxia may trigger an immune response to dengue in seronegative persons that predisposes them to a higher risk of severe disease, analogous to what is seen in natural secondary dengue infections (Wilder-Smith, 2019).

Second generation dengue vaccines

Two live attenuated dengue vaccines are now in phase 3 trials. One such live attenuated dengue vaccine is being developed by Takeda: DENVax vaccine consists of an attenuated DENV-2 (DEN2-PDK-53), whereby three chimeric viruses containing the prM and envelope proteins of DENV-1, DENV-3, and DENV-4 are inserted into the DEN2-PDK-53 backbone (Wilder-Smith, 2019). The difference from Dengvaxia, therefore, is the presence of nonstructural (NS) proteins due to the DENV2 backbone. This vaccine has performed well in phase 1and phase 2 clinical trials, with high titers of neutralizing antibody to all four serotypes in nonhuman primates and humans, including cross-reactive T-cell-mediated responses that may be necessary for broad protection against dengue fever. The other tetravalent live attenuated dengue vaccine was developed by the U.S. National Institutes of Health (NIH) and is currently in a phase 3 trial in Brazil, but it was also licensed to Merck and various other vaccine manufacturers for further development outside Brazil (Wilder-Smith, 2019). This vaccine consists of three full-length dengue virus (DENV) serotypes attenuated by one or more deletions in the 3'untrans-lated region with DEN1, DEN2, and DEN4, while the fourth component is a chimeric virus in which the prM and E proteins of DENV-2 replace those of DENV-4 in the DEN4 back-ground. This vaccine performed well and was safe in phase 1 and phase 2 trials. A single dose induced tetravalent antibody and cellular T-cellresponses and resulted in 100% efficacy in a human challenge study. The capacity to elicit CD4+ cell responses closely mirrored those observed in a population associated with natural immunity (Murugesan & Manoharan, 2020).

The advantages of these second generation dengue vaccines are the inclusion of NS proteins of the dengue backbone and more convenient dosing, with reduced numbers of doses needed: While Dengvaxia is licensed for three doses 6 months apart, the Takeda vaccine is currently being considered for two doses 3 months apart and the NIH vaccine for a single dose. Whether these secondgeneration vaccines will provide balanced high protection against all four serotypes and thus overcome the serostatus dependent problem of CYD-TDV remains unknown and can be addressed only by the long-term results of the pending phase 3 trials (Wilder-Smith, 2019).

Envelope Domain III as vaccine candidate

There are numerous dengue vaccine candidates in the pre-clinical and clinical development (Durbin et al., 2013). However, the development of dengue tetravalent vaccine is the most challenging. A safe and effective tetravalent dengue vaccine that can simultaneously induce a high level, long-lasting immune response against all four serotypes is necessary. Domain III of envelope protein (EDIII) contains an immunoglobulin-like structure and is found to be involved in host cell development as it is exposed to binding*(Babu et al., 2008*). Domain III has single disulfide bond (SS6) in DIII between Cys 302 and Cys 333, which is very critical for antigenic structure presence. It has relatively stable conformation and useful as a virus-specific antigen. Domain III has the advantage of reducing the risk of ADE because of the absence of other non-neutralizing and cross-reactive Epitopes. It also plays a crucial role in dengue pathogenesis and immunity. In addition, it possesses multiple and serotype specific neutralizing epitopes making it a suitable candidate for vaccine development. Neutralizing antibodies produced against domain III may block the entry of the virus into the cell *(Khanam et.al.,2009).*

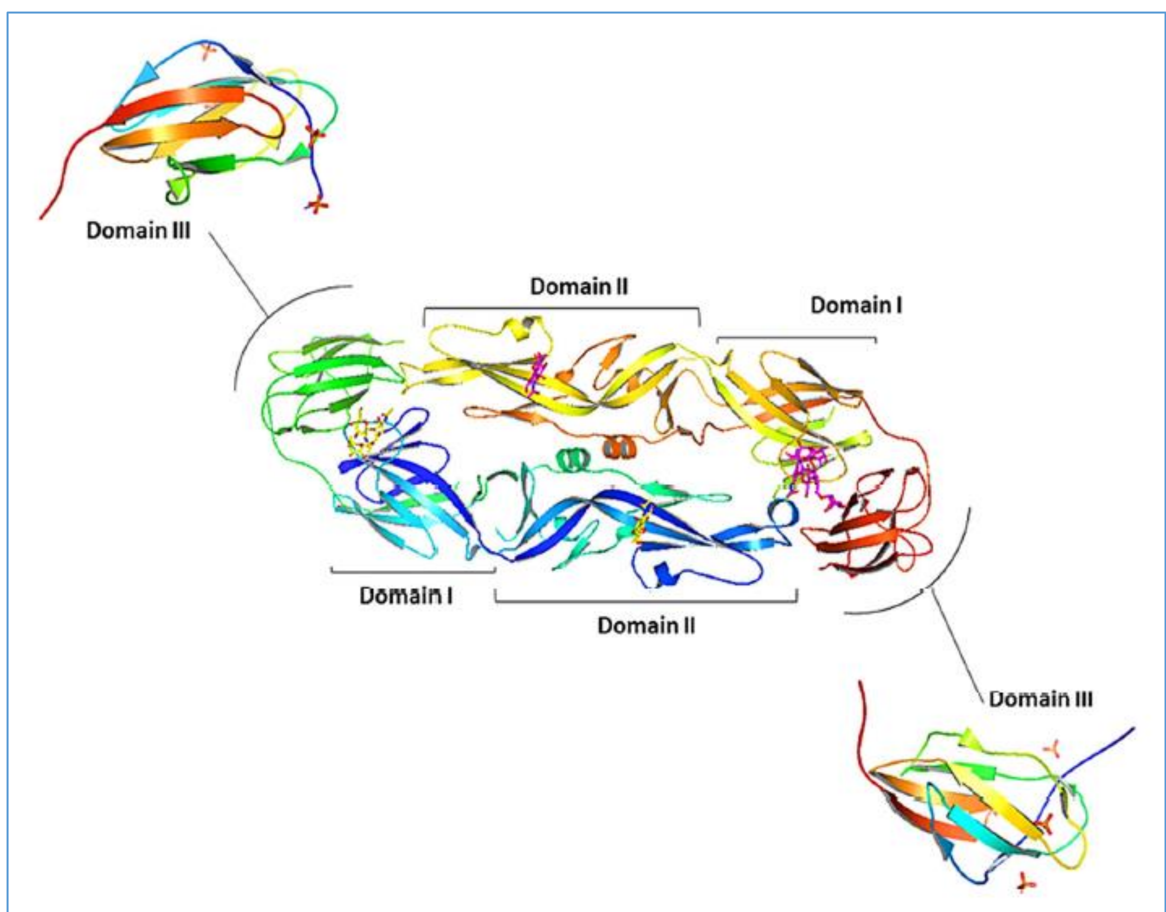


Fig 2. 8. Crystal structure of dengue envelope protein (Guzman et al., 2010)

Table 2. 2. Current status of Dengue vaccine (C. H. Huang et al., 2021)

Vaccine Type	**Name/ Strategy**	**Clinical Trial Phase**
Live-Attenuated chimera	**CYD-TDV**, Yellow fever YFV-17D vaccine virus backbone with prM/E genes of DENVs 1–4.	Licensed
	DENVax, DENV-2 PDK-53 attenuated vaccine with prM/E of other serotypes (DENV-2/1, −2/ 3, and −2/4 chimeras); contains whole dengue non-structural proteins.	Phase III
	TV003/TV005, attenuated by remove of 30 nucleotides from 3’ UTR of DENV-1, DENV-3, DENV-4, with full-length DENV1, 3, 4 genomes; and a chimeric rDEN4Δ30 backbone with prM and E genes of DENV-2.	Phase III
Inactivated virus	Tetravalent dengue purified inactivated vaccine (DPIV) with different adjuvants (e.g. aluminum hydroxide, AS01E or AS03B)	Phase I
	Tetravalent dengue purified inactivated vaccine (DPIV) with AS03B adjuvant.	Phase I/II
Subunit vaccine	**V180**, 80% of N-terminal truncated E protein (DEN-80E) which is produced by insect cells with or without adjuvants.	Phase I
DNA vaccine	**D1ME100**, recombinant plasmid vector encodes monovalent DENV-1 prM and E protein.	Phase I
	TVDV, tetravalent DNA vaccine with Vaxfectin adjuvant (Vical, Boulder, CO, USA)	Phase I
Heterologous prime boost	TLAV Prime/PIV boost and reverse order combination vaccination strategy.	Phase I

2.7 Cell Cultures of Dengue

Before the availability of molecular approaches, laboratory used direct C6/36 cell culture and *Toxorhynchites splendens* amplification followed by C6/36 cell culture for dengue virus isolations. The high sensitivity of the C6/36 clone of Aedes albopictus cells to dengue viruses has been well documented in recent years. These cells provide a simple and rapid method for dengue virus isolation and, together with the use of serotype-specific monoclonal antibodies for identification, provide the basis for an effective and economical method for dengue virus surveillance (Kuno et al., 1985).

2.8 Animal model for dengue research

DENV-susceptible mouse models have been developed by genetically modifying the immune system to maintain the infected state. However, this model has limitations related to the immune response, and it is not applicable to all DENV serotypes (Suzarte et al., 2015). At present, there are no experimental models that reflect the pathological characteristics and clinical symptoms in humans, in part because DENV only infects apes and people. The use of primate species as experimental models also has limitations, including the high cost and challenges of managing them, and such models are not appropriate in certain situations, since they present viremia without clinical symptoms (Zompi & Harris, 2012).

Rodent models are generally used as a first step for pre-clinical development of vaccines since they cost significantly less than other models, such as non-human primate (NHP) models. However, immune responses in mouse models can be underestimated since the DENV replication rate in mice is low. In addition, immunocompromised mouse models, one of the most frequently used rodent models, lack interferon (IFN)-α/β and -γ receptors; therefore, they cannot develop full immune responses (Zellweger et al., 2014). Currently available immunocompetent mice models include the BALB/c, A/J, and AG129 models. Although no humanized BALB/c mouse models are available, they are important models because they are widely used, provide adequate relevance to dengue infection, and enable evaluation of innate systems or vaccine/antiviral studies. The A/J mouse models are similar to the BALB/c models in that they are easily accessible and show better proximity to humans in terms of DENV infection and their feasibility for evaluating the innate response in vaccine/antiviral studies (Zompi & Harris, 2012). AG129 models are similar to BALB/c and A/J mice in terms of their relevance to DENV infection and their availability, and they provide superior information when evaluating innate immunity and in vaccine studies (Zellweger et al., 2014).

CHAPTER III

MATERIALS AND METHOD

3.1 Ethical Approval

The ethical approval for the molecular and immunological study of dengue has been taken from NHRC (Reg No: 121/2019). The samples were collected after taking written consent from the patients. Then the laboratory work has been performed in Central Department of Biotechnology, TU which is well equipped and in accordance with the ethical guidelines of NHRC. Also the universal safety guidelines were followed during collection, transportation, storage of samples and laboratory works. (Appendix 1.1; Appendix 1.2)

3.2 Site Selection

The sites for sample collection were mainly hospital based and five major hospitals from five different districts, where most of referral cases from eastern, central and western regions of Nepal were treated were included in this study. The samples were collected from Sukraraj Tropical and Infectious Disease Hospital (STIDH) - Kathmandu, Chitwan Medical College and Teaching Hospital (CMC)-Chitwan, Bijayapur Hospital (BH)-Dharan, Sunsari, Universal College of Medical Sciences and Teaching Hospital (UCMS) - Lumbini and Hetauda Hospital (HH)-Hetauda, Makwanpur**.** The samples collected in 2017 A.D, 2018 A.D and 2019 A.D were included in this study. The study was designed as a cross-section descriptive study.

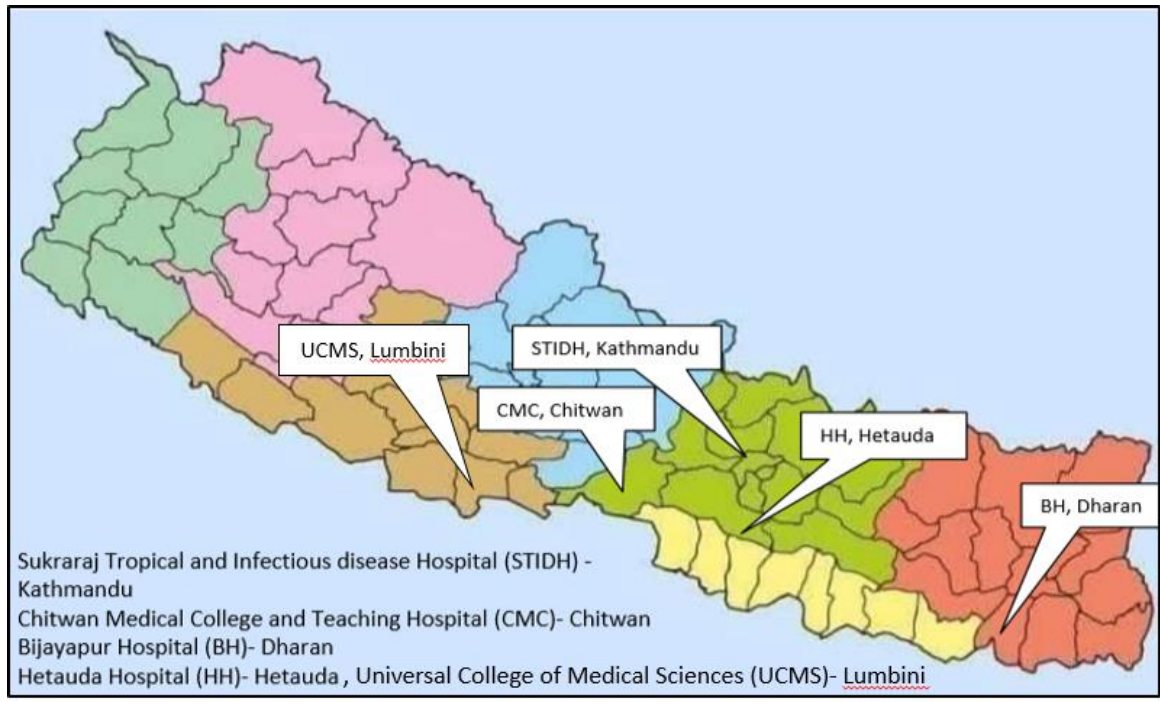


Fig 3. 1. Hospitals where samples were collected: STIDH, CMC, BH, UCMS and HH.

Patients with an acute febrile illness and showing the symptoms of dengue fever were enrolled in the study. WHO, 2009 guidelines were strictly followed for determination of dengue-case identification. Only those subjects were enrolled who had fever with two of the following symptoms: nausea, vomiting, rashes and aches and pains. The other recorded symptoms were abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, liver enlargement >2cm, severe bleeding, shock, organ failure or fluid accumulation with respiratory distress. Demographic information like age, sex and address, symptoms and diagnosis were recorded by attending physicians in the Study CRF (Case report form). (Appendix 1.3)

3.3 Sample Collection and Storage

3.3.1 Sample Size

The sample size for this study was calculated by using formula:

For unlimited population,

$$n = \frac{z^2 \times p(1-p)}{\varepsilon^2}$$

where, z is the z score at 95% confidence level

ε is the margin of error

p is the population proportion

n is the sample size

Assuming a population proportion of 0.5, and unlimited population size. Keeping z score at 95% confidence as 1.96 and error margin of 5% the sample size was calculated as

$$n = \frac{1.96^2 \times 0.5(1-0.5)}{0.05^2}$$

n = 384.16 ~ 385

Thus, a sample size of at least 385 samples were needed used in this study. The sample size of 400 samples were used in this study.

The reported dengue case in 2019 is around 18000 which is the highest reported case till date in Nepal and population of Nepal is more than 26 million so the population proportion of Dengue fever with whole population is low and sample size calculation considering these data will result in sample size of 1 sample which will not justify the purpose of the study. Thus sample size has been calculated assuming population proportion on 0.5 and unlimited population size.

3.3.2 Sample Collection and Storage

The whole blood samples from 400 suspected Dengue patients were collected and stored at -80°C until further processing. These samples used in the study are from three different years. There are 50 samples from 2017 were collected by team members from dengue project from different hospitals in Kathmandu, Chitwan, Lumbini. 30 samples from 2018 outbreak are also included and all of them were collected from Kathmandu. Remaining 320 samples were from 2019 outbreak which collected from Dharan, Chitwan and Kathmandu. In Kathmandu, samples were collected from Sukraraj Tropical and Infectious Disease Hospital (STIDH), Teku and Gorakhkali Polyclinic, Banasthali. In Chitwan, samples were collected from Chitwan Medical College and in Dharan samples were collected from Bijayapur Hospital. The samples in Kathmandu were collected and immediately transferred to Central Department of Biotechnology (CDBT), TU for further processing. In other collection sites, whole blood was collected and plasma/serum were separated at the collection site using their own lab facilities and serum samples were frozen and transported to CDBT and further processing was done there. To avoid rupture of red blood cells (RBC) during freezing the serum samples were preferred for long distance sample transport. Also leftover whole blood were transported in freezing condition for host genetic study. The samples were classified according to their severity as Dengue without Warning signs (Fever with two of the following symptoms: nausea, vomiting, rashes, muscles and joints pain), Dengue with Warning signs (abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy or liver enlargement >2cm) and Severe Dengue (severe bleeding, shock, organ failure or fluid accumulation with respiratory distress).

3.4 Sample Inclusion and Exclusion criteria

The sample collection criteria was according to WHO case definition 2009. The subjects must have fever with two of the other symptoms of nausea, vomiting, rash, aches and pains, leukopenia or positive tourniquet test. Samples were not collected from the patients who did not consent for our study. Also samples were not collected from children below 2 years, pregnant women and HIV infected patients.

3.5. Validation of dengue cases

Laboratory diagnosis methods for confirming dengue virus infection may involve detection of the virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques. In our study, the suspected dengue samples were confirmed as dengue by ELISA (NS1 antigen detection and anti-DENV IgM and IgG antibody detection) and Real time Reverse transcriptase PCR (viral nucleic acid detection). The samples which were either NS1+/ IgM+/ PCR+ were classified as confirmed dengue cases (WHO, 2009).

3.6 Immunological and serological Assay

Collected samples will be tested by Rapid Diagnostic Kit (RDT) at the collection site. RDT kit would detect presence of NS1 component, IgM antibody and IgG antibody. It takes short period of time for the detection and very useful for the mass screening of dengue. Those samples showing band in control, NS1 and/or IgM region were considered to be dengue positive. Samples showing bands in all three region namely NS1, IgM and IgG were also considered positive. Samples showing band in control but not in any of these 3 regions are considered negative. Also it is necessary to show band in control region for both positive and negative case. After RDT test the samples were subjected to In-Bios ELISA (NS1, IgM, IgG).

3.6.1 Enzyme-linked Immuno-sorbent Assay (ELISA)

ELISA was carried for detection of dengue antigen NS1, anti-dengue IgM and anti-denge IgG antibodies using the standard kit of InBios (InBios International, Inc., Seattle, WA, catalogue no. REF DNS1-R for NS1, REF DDGS-R for IgG and REF DDMS-1 for IgM).

3.6.2. InBios NS1Ag ELISA:

Preparation of Reagents:

Preparation of 1X wash buffer: The 10X wash buffer was diluted to 1X using high- grade deionized water. To prepare 1X buffer solution, 120 ml 10X wash buffer was mixed with 1080 ml of deionized water and mixed thoroughly such that the solution was uniform and no precipitate was present.

Preparation of conjugate solution: The conjugate solution was prepared by adding 120 µl of 100X conjugate for Dengue NS1 ELISA directly to the 12ml bottle of Conjugate diluent for Dengue NS1 (1 part: 100 parts). The bottle was then inverted several times to mix the solution.

Microtitration wells: The required number of coated wells required for the assay were selected and the remaining unused wells were repackaged immediately with the supplied dessicant and stored at 4°C until further use. The first column of wells were defined for controls, the controls were run in duplicates in the first two wells as negative control followed by positive control and cut-off control respectively.

Test Procedure:

Sample Diluent containing secondary antibody for Dengue NS1 ELISA (50 µl) was dispensed into each well and 50 µl of each undiluted sera (test sample) were added to the test wells and, positive and negative kit provided sera were also added in duplicate in their

respective wells. The plate was gently rocked from side to side for 5 times. Then the plate was covered and incubated at 37°C for 1 hour. After incubation the plate was washed with 300 µl wash buffer for 6 times using automated ELISA plate washer. The prepared conjugate solution was dispensed (100µL/well) into all wells followed by incubation at 37°C for 30 minutes. The plate was washed as above. Liquid TMB substrate at the rate of 100µL per well was added to all wells followed by incubation in the dark, at room temperature for 20 minutes. Finally 50 µl per well of Stop Solution was added to stop the reaction. The plate was then read at 450nm wavelength in ELISA Plate reader (Thermo Electron Corporation Original Multiskan EX) for OD values.

Interpretation of the test was done by calculation of Immune Status Ratio (ISR). ISR was calculated by rationalizing sample OD by mean of cut-off OD. If the value was less than 1 it was considered negative and vice versa. Quality control of the test was determined by ratio of mean OD of positive control (PC) divided by mean OD of negative control (NC), $R_{(PC/NC)}$. For the test to be valid $R_{(PC/NC)}$ should be greater than 8 and mean OD of cut-off should be greater than mean OD of Negative control.

3.6.3 InBios IgM ELISA

Preparation of Reagents:

Preparation of 1X wash buffer: The 10X wash buffer was diluted to 1X using high- grade deionized water. To prepare 1X buffer solution, 120 ml 10X wash buffer was mixed with 1080 ml of deionized water and mixed thoroughly such that the solution was uniform and no precipitate was present.

Test Procedure:

ELISA plate coated with goat anti-human IgM and blocked was taken and 50 µl of diluted negative control, positive control and test sera of patients were added to well. The plate was incubated at 37°C for 1 hour and washed with 300µl wash buffer. Then 50 µl of ready to use Dengue Recombinant Antibody, DENRA and Normal Control Antigen, NCA, were added to plates (DENRA in row A-D and NCA in row E-H) followed by 1 hour incubation at 37°C and washing by 300µl wash buffer for 6 times. 50 µl of ready to use enzyme conjugated horseradish peroxidase (HRP) was added to wells and incubated at 37°C for 1 hour followed by washing. Then 150 µl of En wash was added to each wells and incubated at room temperature for 5 minutes followed by washing. Substrate tetramethylbenzidine (TMB), 75µl was added to the plate and incubated at 20°C in a dark place for 10 minutes followed by addition of 50µl of stop solution. After 1 minute incubation absorbance at OD450nm was taken from ELISA plate reader.

Interpretation was done by calculating ISR ratio which was calculated as ratio of OD of DENRA by OD of NCA. If the value was ≤1.65 then it was considered negative, values ≥2.84 were considered positive and values in between were considered equivocal. For equivocal samples test should be repeated. For quality control ratio mean OD of DENRA by mean OD of NCA of positive control should be greater than 5.

3.6.4 InBios IgG ELISA

Preparation of Reagents:

Preparation of 1X wash buffer: The 10X wash buffer was diluted to 1X using high- grade deionized water. To prepare 1X buffer solution, 120 ml 10X wash buffer was mixed with 1080 ml of deionized water and mixed thoroughly such that the solution was uniform and no precipitate was present.

Test Procedure:

Plates coated with DENRA (row A-D) and NCA (row E-H) was taken and 50µl of diluted samples, positive and negative controls were added and it was incubated at 37°C for 1 hr then washed with 300 µl of wash buffer for 6 times. Then 50 µl of ready to use HRP conjugate for IgG was added to each well followed by 1 hr incubation at 37°C and washing.

Then 150 µl of En wash was added to each wells and incubated at room temperature for 5 minutes followed by washing. Substrate tetramethylbenzidine (TMB), 75µl was added to the plate and incubated at 20°C in a dark place for 10 minutes followed by addition of 50µl of stop solution. After 1 minute incubation absorbance at OD450nm was taken from ELISA plate reader.

Interpretation was done by calculating ISR ratio which was calculated as ratio of OD of DENRA by OD of NCA. If the value was ≤1.65 then it was considered negative, values ≥2.84 were considered positive and values in between were considered equivocal. For equivocal samples test should be repeated. For quality control ratio mean OD of DENRA by mean OD of NCA of positive control should be greater than 5.

3.6.5 Statistical Analysis:

Data obtained in this study were recorded in MS-Excel 2013 and statistical analyses were performed using Graph-pad Prism V 7.0 software. The descriptive statistics were reported using the Mean ± Standard deviation for descriptive parameters.

3.7 Molecular Assay

3.7.1 Viral RNA Isolation by QIAamp® viral RNA Mini kit

Viral RNA was extracted from 140 µL of serum for each sample using QIAamp® viral RNA Mini kit using spin protocol (Cat. No. 52904) according to the manufacturer's instructions. Also manual extraction was done using Trizol method for the comparision of the extracted RNA from the samples.

Preparation of reagents

Carrier RNA was added to Buffer AVL. Buffer AVE, 310 µL was added to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µL. The carrier RNA was dissolved thoroughly and divided into required sized aliquots and stored at -20°C. 130 ml of absolute ethanol was added to buffer AW1 and 160 ml to buffer AW2 as indicated in the bottle to make buffer AW1 and AW2 complete.

RNA isolation by using Spin protocol

For RNA isolation, 560 µL of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5ml microcentrifuge tube. Serum sample, 140 µL was added to the Buffer AVL–carrier RNA in the microcentrifuge tube and mixed by pulse-vortexing for 15 s. The mixture was incubated at room temperature (15–25°C) for 10 min. After the incubation the tubes were briefly centrifuged to remove the drops from inside the lid. 560 µL of ethanol was added to the sample and mixed by pulse-vortexing for 15s. After mixing, the tubes were centrifuged briefly to remove the drops from inside the lid. Now, 560 µL the solution from the solution from the tube was added to the QIAamp Mini column (in a 2-ml collection tube) without wetting the rim. The cap was closed and centrifuged at 6000 x *g* (8000 rpm) for 1 min. The QIAamp Mini column was placed into a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp Mini column was carefully opened and same step as mentioned before was repeated. QIAamp Mini column was carefully opened and 500 µL of Buffer AW1 was added to it and again centrifuged at 6000 x *g* (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2 ml provided collection tube, and the tube containing the filtrate was discarded. Again, similar steps with the Buffer AW2 was repeated and centrifuged at (20,000 x *g*; 14,000 rpm) for 3 min. After the removal of AW2 buffer, the QIAamp Mini column was placed in a 1.5 ml collection tube, opened and then 40 µL of Buffer AVE equilibrated to room temperature was added. It was then centrifuged at 6000 x g (8000 rpm) for 1 min and the viral RNA was collected in the collection tube. The extracted viral RNA was stored at -80°C until used.

3.7.2 Serotyping by Reverse Transcriptase Real-Time Polymerase Chain Reaction

Preparation of Reagents:

Primer and Probe Preparation: Primers and probes were rehydrated using 100µl of PCR grade water into each dried PCR primer or probe and allowed to dissolve at room temperature for 1 hour. Aliquots were made and stored at -20°C or lower. Positive control mix, Human Specimen Control (HSC) were also checked and stored at -20°C. All the reagents were thawed at 4°C and placed above cool pack during the reagent preparation process.

Test Procedure:

Dengue diagnosis kit provided by Center for Disease Control and Prevention (CDC), USA were used for identification of DENV serotypes keeping separate dengue controls (DENV 1 to 4) catered in the kit. A master mix at the rate of 20 µL per well were prepared [RNase-free water-3.7µL, 2Xs Buffer-12.5, Primer Forward and Reverse for DENV 1-4 (D1 F/R–D4F/R)-100 µM, Probe for DENV 1-4 (P1-P4) -10 µM, Superscript III Platinum one-step qRT-PCR system enzyme (Invitrogen Cat No. 11732-020)-0.5 µL] in two separate labeled Eppendorf tubes for DENV reaction and Human Specimen Control (HSC) reaction. The PCR plate was loaded to Real-Time PCR machine (BIORAD-CFX96 Touch™ Real-Time PCR Detection System) having program set of reverse transcription at 50°C for 15 min, inactivation at 95°C for 2 min followed by 45 PCR amplification cycles (melting temp. 95°C for 15 sec with ramp rate 4.4°C/s, and annealing and extension temp. 60°C for 1 min. with ramp rate 2.2°C/s), melting curve set for 1 cycle (melting temp. 95°C for 10 sec, and annealing and extension temp. 65°C for 1 min.; continuous acquisition mode at 97°C); cooling program set at 50°C for 20 min with ramp rate 2.2°C/s) and finally the reaction was left at 4°C. The results were interpreted as positive to DENV 1, 2, 3 and 4 if the amplification of probes; FAM (Blue), VIC (Green), Texas Red (Red) and Cy5 (Purple) curve were amplified respectively within CT value 37.

Table 3. 1. Master-mix preparation for HSC reaction.

Reagents	**Concentration**	**Vol (µl)/Rxn**
RNAse Free Water	-	5.5
2X Premix	-	12.5
Primer RP-F	100 µM	0.5
Primer RP-R	100 µM	0.5
Probe RP	10 µM	0.5
Superscript III RT/ Platinum Taq Mix		0.5
Total		20

Table 3. 2 Master-mix preparation for multiplex PCR (DENV1-4)

Reagents	**Concentration**	**Vol (µl)/Rxn**
RNAse Free Water	-	2.2
2X Premix	-	12.5
Primer D1-F	100 µM	0.5
Primer D1-R	100 µM	0.5
Primer D2-F	100 µM	0.25
Primer D2-R	100 µM	0.25
Primer D3-F	100 µM	0.5
Primer D3-R	100 µM	0.5
Primer D4-F	100 µM	0.25
Primer D4-R	100 µM	0.25
Probe DENV-1	10 µM	0.45
Probe DENV-2	10 µM	0.45
Probe DENV-3	10 µM	0.45
Probe DENV-4	10 µM	0.45
Superscript III RT/ Platinum Taq Mix		0.5
Total		20

Table 3. 1. Program setting for PCR

	Stage 1	Stage2	Stage 3			Stage 4
Process	cDNA preparation	Denaturation	Denaturation	Extension	Cycle Go to	Hold
Temperature	50°C	95°C	95°C	(Capture) 60°C	45 cycle	4°C
Time	30 min	2 min	15 sec	1min		∞

Result Interpretation

Amplication plot was observed and baseline was set manually to determine Ct values for the curve. Based on color of curve determined by probe and Ct value the serotype of the samples were determined.

3.7.3 Viral RNA Isolation by TRIzol

This protocol uses TRIzol (also known as TRI REAGENT) for the isolation of total RNA. Trizol is a mixture of guanidine thiocyanate and phenol, which effectively dissolves DNA, RNA and protein on homogenization or lysis of sample. After adding chloroform, centrifugation was performed and the mixture separates into 3 phases with the upper clear aqueous phase containing the RNA. The next steps in the extraction are washes and precipitation of the RNA. The RNA can then be stored for longer periods of time at -80°C.

Reagents: a. TRIzol b. Chloroform c. Isopropyl alcohol d. 75% Ethanol e. RNase-free water

PROCEDURE

1. Homogenization: 600µl of TRIzol was pipetted on Eppendorf tube and 200µl of serum sample was added, the cells were lysed and homogenized by vortexing for 15 seconds.

2. Phase separation: The homogenized samples were incubated for 5 minutes in ice. 200µl of chloroform was added. The tubes were shaken vigorously by hand for 15 seconds and incubated them at room temperature for 5 minutes. The samples were centrifuged for 5 minutes at 12,000 rpm. The aqueous phase was transferred to new Eppendorf tubes. (lower phenol-chloroform phase, an interphase, and upper aqueous phase.).

3. RNA Precipitation: The RNA was precipitated from the aqueous phase by mixing with 300µL of isopropyl alcohol. The mixture was centrifuged for 10 minutes at 10,000 rpm

4. RNA Wash: The supernatant was removed. The RNA pellet was washed with 1ml of 75% ethanol. The samples were inverted and mixed and centrifuged at 9,500 rpm for 10 minutes.

5. Re-dissolving RNA: The RNA pellet was dried and was dissolved in RNase-free water.

3.7.4 cDNA preparation

BIORAD-iScript^{TM} cDNA Synthesis Kit (catalog no. 1708890) was used for preparation of cDNA. This kit includes three tubes, which contain all the reagents required for successful reverse transcription. The iScript Reverse Transcriptase is RNase H+, which provides greater sensitivity than RNase H–enzymes in qPCR. iScript is a modified Moloney murine leukemia virus (MMLV) reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided pre-blended with RNase inhibitor.

Table 3. 2. Components used for cDNA Synthesis

Components	**Volume per reaction (µl)**
5X iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease - free water	5
RNA template (100 µg–1 µg total RNA) *	10
Total volume	20µL

Table 3. 3. Reaction Protocol for cDNA synthesis

	Stage 1	Stage2	Stage 3	Stage 4
Process	Priming	Reverse Transcription	RT Inactivation	Hold
Temperature	25°C	46°C	96°C	4°C
Time	5 min	20 min	1 min	∞

3.7.5 Nested RT-PCR and Primer Designing

Nested PCR of the cDNA was performed for envelope protein primers (Table 3.4) designed by targeting partial region of envelope and Solis Biodyne -5x FIREPol® Master Mix (Cat. No. 04-11-00125). Primers AA6EP_F (10 pm/µl) and AA7EP_R (10 pm/µl) were used for the first round PCR. The PCR conditions were set to initial denaturation at 95°C for 3 min followed by 35 cycles of the PCR with cycling conditions of denaturation 95°C, annealing 59°C and extension 72°C. The final extension was done at 72°C for 5 min and the reaction was kept at hold at 4°C. The PCR product was diluted in the ratio 1:10 and used as template for second round PCR using serotype-specific primers (D1DIIIF & D1DIIIR for DENV1, D2DIIIF & D2DIIIR for DENV2, D3DIIIF & D3DIIIR for DENV3 and D4DIIIF & D4DIIIR for DENV4). The PCR conditions were same as of the first round and only annealing temperature was different for different serotypes (63.7°C, 54.4°C, 55.8°C and 52.4°C for DENV1, DENV2, DENV3 and DENV4 respectively). Annealing temperature for the serotype specific primers were determined by gradient PCR ranging temperature from 3-5°C below the given melting temperature of primers. Agarose gel electrophoresis was run in 1.5% agarose and the bands were visualized under UV transilluminator.

Primer Designing:

Primers used for serotyping and fusion PCR were designed using bioinformatics tool such as National Center for Biotechnology and Informatics, NCBI genome database for genome sequence retrieval. Further Oligo calc. tool of North western.edu was used for oligonucleotides properties calculation such as melting temperature, tm, length, etc. Oligo analyser tools from Integrated DNA Technology, IDT, were used to check for the homodimer and heterodimer formation. Homodimer is checked with the same primer and it checks if the 5' end of primer is open or not. 5' end must have at least 5-7 bp free for binding to template. Similarly heterodimer is checked between the forward and reverse primer. It determines if the primer forms dimer during the PCR run. ΔG of the dimers are also checked. Lower the ΔG value lesser is the chance of dimer formation. Also mfold tool from unafold.org was used to check if the primer forms folding which would hamper the binding of primer to template. Further for easy approach Primer 3 tool of NCBI was also used in some primer development. It important to design the forward and reverse primer with similar base pairs and similar melting temperature which would make PCR easier.

Table 3. 4. Primers used for dengue virus confirmation and serotype specific PCR

Primer	**Sequence (5' to 3')**	**Ref. seq. (GenBank accession no.)**	**Expected size (bp)**
AA6EP_F	TGG CTG GTG CAC AGA CAA TGG TT	MF381049.1	600
AA7EP_R	GCT GTG TCA CCC AGA GTG GCC AT	KY849753.1	
D1DIIIF	TGG CAG AGA CCC AGC ATG GAA CC	JF297577	299
D1DIIIR	CCT TCG TGC TCC TCG GGC GG		
D2DIIIF	TCA TAC TCC ATG TGC ACA GGA	JQ993227	306
D2DIIIR	GCC GAT AGA ACT TCC TTT CTT		
D3DIIIF	ATG AGC TAT GCA ATG TGC TTG	JN697379	296
D3DIIIR	CCC TTC CTG TAC CAG TTG ATT TT		
D4DIIIF	ATT GAG AAT TAA GGG AAT GTC A	JQ915085	307
D4DIIIR	CCT GAA CCA ATG GAG TGT TAA		

Table 3. 5. Master-mix preparation for PCR

PCR1		**PCR2**	
Reagents	**Vol. per reaction**	**Reagents**	**Vol. per reaction**
Master mix (5x)	5µl	Master mix (5x)	5µl
AA6EP_F (10pM/ µl)	1µl	Serotype specific FP (10pM/ µl)	1µl
AA7EP_R (10pM/ µl)	1µl	Serotype specific RP(10pM/ µl)	1µl
Template cDNA	2.5µl	Diluted PCR 1 (1:10)	1µl
DNA grade water	15.5µl	DNA grade water	17µl
Total	**25µl**	**Total**	**25µl**

Table 3. 6. PCR Program for Envelope region of Dengue in PCR1 and Domain III for all 4 serotypes in PCR 2.

PCR Steps	**Temperature of all 4 Dengue serotype**				**Time**
	DENV1	**DENV2**	**DENV3**	**DENV4**	
PCR1					
Initial Denaturation	95°C	95°C	95°C	95°C	5 min
Denaturation	95°C	95°C	95°C	95°C	1 min
Annealing	59°C	59°C	59°C	59°C	45 sec
Extension	72°C	72°C	72°C	72°C	45 sec
35 cycles of Denaturation, Annealing and Extension					
Final extension	72°C	72°C	72°C	72°C	5 min
Hold	4°C	4°C	4°C	4°C	
PCR2					
Initial Denaturation	95°C	95°C	95°C	95°C	5 min
Denaturation	95°C	95°C	95°C	95°C	1 min
Annealing	**63.7°C**	**54.4°C**	**55.8°C**	52.4°C	45 sec
Extension	72°C	72°C	72°C	72°C	45 sec
Cycles	35 cycles for all serotypes				
Final extension	72°C	72°C	72°C	72°C	5 min
Hold	4°C	4°C	4°C	4°C	

3.7.6 PCR amplification of Domain III region of all four serotype with overlapping region

Domain III region of envelope of all four serotype were amplified using conventional PCR. This PCR product was then used as template for constructing Domain III with overlapping region of other serotypes which will be used for fusion PCR. The primers for the fusion PCR was designed by adding 16 to 18bp sequence from other serotype in 5' region of existing serotype specific reverse primers. The reaction mixture were prepared as for PCR2 in nested PCR. Then the PCR was done two step PCR technique. For first 5 cycles, annealing was done at 2°C lower than annealing temperature of later 30 cycles. Forward primer for DENV1 serotype would contain site for restriction digestion.

Table 3. 7. Primers Details for the Fusion PCR

Primer Name	**Sequence (5' to 3')**
D1DIII F-cloning	AAC TCT GCC AGG ATC CTG GCA GAG ACC CAG CAT GGA ACC
D1DIII R-cloning	TCC TGT GCA CAT GGA GTA TGA CCT TCG TGC TCC TCG GGC GG
D2DIII F-cloning	TCA TAC TCC ATG TGC ACA GGA
D2DIII R-cloning	CAA GCA CAT TGC ATA GCT CAT GCC GAT AGA ACT TCC TTT CTT
D3DIII F-cloning	ATG AGC TAT GCA ATG TGC TTG
D3DIII R-cloning	TGA CAT TCC CTT AAT TCT CAA TCC CTT CCT GTA CCA GTT GAT TTT
D4DIII F-cloning	ATT GAG AAT TAA GGG AAT GTC A
D4DIII R-cloning	CAT CAG TGG TGA ATT CCC TGA ACC AAT GGA GTG TTA A

Table 3. 8. PCR Protocol for Fusion PCR

PCR Steps	DEN1	DEN2	DEN3	DEN4	Time
Intital Denaturation	95°C	95°C	95°C	95°C	3 min
Denaturation	95°C	95°C	95°C	95°C	30 sec
Annealing	63.6°C	53.8°C	54.7°C	50.4°C	30 sec
Extenstion	72°C	72°C	72°C	72°C	30 sec
For 5 cycles					
Denaturation	95°C	95°C	95°C	95°C	30 sec
Annealing	65.6°C	55.8°C	56.7°C	52.4°C	30 sec
Extenstion	72°C	72°C	72°C	72°C	30 sec
For 30 cycles					
Final Extension	72°C	72°C	72°C	72°C	5 min
Hold	4°C	4°C	4°C	4°C	∞

3.7.7 Sequencing of the construct

After PCR was performed with primers having overlapping region between multiple serotype the PCR product with homology between the Domain III of different serotype were produced which are then subjected to sequencing for construction verification and phylogenetic analysis. Only Domain III of DENV 1 with overlaps of DENv2 and Domain III of DENV2 with overlaps of DENV3 were sequenced.

For sequencing PCR product and primers were sent to National Academy of Science and Technology, NAST. Data generated were analyzed by using Bio Edit Software and generated sequence was subjected to BLAST (Basic Local Alignment Search Tool) analysis from NCBI for finding similarity between the sequences and Phylogenetic analysis was done using MUSCLE (Multiple Sequence Comparison by Log- Expectation) by EMBL-EBI (European Bioinformatics Institute).

3.7.8 *Insilico* Analysis

Insilico analysis of the probable construct was done by using bioinformatics tools such as BLAST from NCBI for sequence alignment, Expasy for the translation of DNA sequence into amino acid sequences. Further the aminoacid sequence were subjected to epitopes prediction tools, IEDB analysis resource. Also seconday structure, binding region and other structural prediction was done using predict protein online tool. The *insilico* analysis of recombinant tetravalent construct having DomainIII from all four serotypes fuzed into one single construct was done as the candidate for vaccine development.

3.8 Dengue virus propagation

Virus propagation was diagnostic tool before molecular assay but it had various other uses in neutralization assays, antibody production, kit development as well as virus multiplication. Virus propagation for dengue was done in C6/36 cell lines for selected samples and virus isolation will be performed for molecular assays. Option of cell culture in vero cell line was also considered. For C6/36 insect cell line serum was diluted by 100 fold and infected with day1 C6/36 cell culture. RPMI (Roswell Park Memorial Institute) 1640 was used as culture media.

Media Preparation

First the incomplete media (RPMI) was taken and Fetal Bovine Serum (FBS) was thawed, HEPES buffer and antibiotics were taken. For preparation of 50ml complete media, 5ml FBS (10%), 1.25ml HEPES (25mM), antibiotics 600µl (1.2%) and remaining RPMI was added to make final volume 50ml. Then the complete media was sterilized by using syrinze filter of 0.2 micron. The media was used for cell revival and passaging.

Culture Procedure

T-flask of 25ml was taken and labelled with cell type, passage number and date. Then 5ml of complete media was added into the flask and distributed evenly. Simultaneously, the cell line was thawed in water bath set at 37°C till the small size ice remained in the tube and it was transferred quickly into 15ml flacon tube containing complete media. The cells were then mixed gently by pipetting and centrifuged at 1200 rpm for 5 minutes. Then the supernatant was discarded a pellet was resuspended on 2ml fresh media. This was done to minimize the toxic effect of DMSO (Dimethyl sulphoxide) used for preservation. The resuspended cells were transferred to flask slowly and gently mixed and incubated at 37°C. Brief introduction of CO_2 (carbondixide) had positive effect on cell growth. Passaging and media feeding was done in alternate days.

Virus Propagation

The serum sample was diluted 100 fold and 200µl of diluted sample was added after 1 day of culture and changes in cell were observed. This was done to propagate the virus for the further analysis.

3.9 Animal immunization

Three female albino mice of 7 weeks old which were reproduced in Central Department by another project running in CDBT was used in this study. The mouse were levelled as control (mouse C) and dilution 1:10 (mouse A) and 1:30 (mouse B). BALB/c mice are generally used in immunological study but due to its lack of availability and easy access of albino mice, the albino mice were used here.

Mouse Innoculation

For Mouse A, 40µl of sample, 160µl of PBS (Phosphate Buffer Saline) and 200µl of Freud's complete adjuvant was mixed properly using T-shaped canulla. Similarly for mouse B, 15µl serum, 210 µl PBS and200µl of Freud's complete adjuvant were mixed. For control 400µl of PBS was used.

The inoculum was injected by using 1ml syringe in peritoneal cavity of mouse was changes were observed. Blood samples from tail vein were taken on 14 and 28 days which was subjected to ELISA test.

3.10 Inhouse ELISA for serotype specific IgG detection

3.10.1 ELISA Plates and reagents

The flat bottom, high binding, polystyrene, 96 well ELISA plates (costar) were used for this study. Reagents like coating buffer, blocking buffer, wash buffer, serum diluent were prepared in lab and its composition is provided in appendix

3.10.2 Antigen

The antigen used DENV1-4 were provided by Microbix, Canada. The antigen contains virus particles cultured in Vero cells and purified from tissue culture supernatants by precipitation, ultracentrifugation and chromatography.

3.10.3 Human sera:

Total of 50 suspected human sera samples of dengue were collected from different hospitals of Nepal were taken and diluted in serum diluent in 1:100 fold. This would act as primary antibody in the test.

3.10.4 Enzyme conjugated secondary antibody:

Antibody present in the serum was detected with the help of enzyme conjugated secondary antibody. In this study, Goat anti-human IgG Horse radish peroxidase(HRP)conjugated secondary antibody was used.

3.10.5 Substrate:

3,3’,5,5’-Tetramethylbenzidine or TMB substrate was used. It is a white crystal powder that forms a pale blue-green liquid in solution with ethyl acetate. TMB can act as a hydrogen donor for the reduction of hydrogen peroxide to water by peroxidase enzymes such as horseradish peroxidase. TMB should be kept out of direct sunlight as it is photosensitive.

3.10.6 Stop solution:

The reaction between TMB and HRP can be halted by using stop solution. The stop solution used in this assay is1N sulfuric acid. Using sulfuric acid turns TMB yellow.

3.10.7 Test controls:

For the test controls, the positive and negative control samples were obtained from the In-Bios ELISA kit. Other controls Blank, Primary Antibody absent and Secondary antibody absent were also taken. Non endemic control (NEC) were taken for dengue unaffected areas and endemic control (EC) were taken from healthy people of dengue affected areas. Cut off values were calculated from both NEC and EC considering them as negative controls for in-house ELISA.

3.10.8 ELISA procedure:

The antigen was diluted in carbonate-bicarbonate buffer (pH 9.6) with different concentration for different antigens. Then four different ELISA micro titer plates were coated with antigen (DENV1-4) diluted in coating buffer (100 µl/well). Plates were then incubated overnight at 4◦C and then washed with wash buffer (1X PBS+0.05% Tween-20) 5 times using automated ELISA plate washer and then blotted dry. The unbound sites were blocked with 1% casein in phosphate buffer solution and again incubated at room temperature for 1 hour. After incubation the plates were again washed and dried as before. Then 50µl of primary antibody diluted in serum diluents (0.1% casein in 1X PBS) in the ratio 1:100 were added to the respective wells and incubated at 37◦C for one hour. The plates should be covered by using paraffin tape. After incubation the plates were washed and dried as before. 25µl of secondary antibody (Goat anti-human HRP diluted in blocking buffer in the ratio 1:5000) were added to all the wells and again incubated at 37◦C for an hour by covering the plates as before. The plates were washed and dried as before. To all the plates 100µl of TMB substrate were added and incubated in dark for 7 minutes. After that 50µl of stop solution (1N sulfuric acid) was added to each wells and incubated for 1 minute. Then optical density reading was taken at 450nm using ELISA plate reader. The absorbance was recorded.

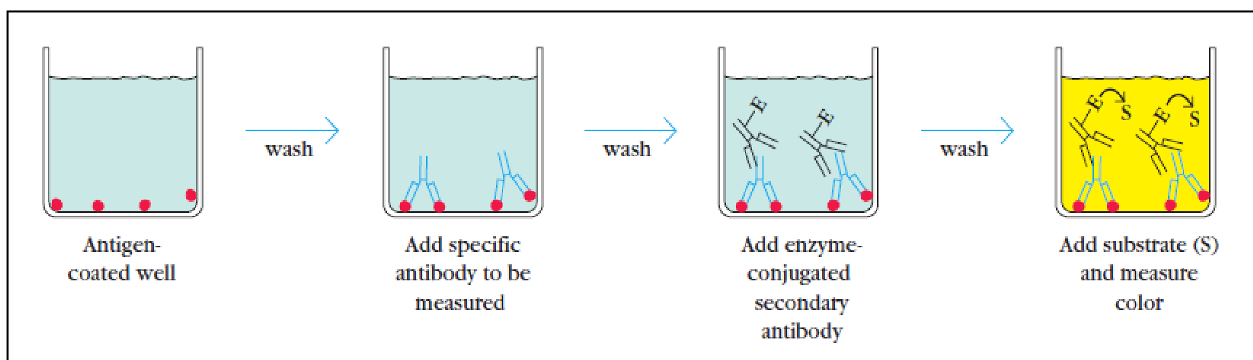


Fig 3. 2 Major steps used in ELISA.(Konstantinou, 2017)

Determination of Cut-off value:

Cut-off value was determined by running negative control (Non-endemic control and Endemic control) in every set of experiments. The mean and standard deviation of negative controls were calculated. Three times of the SD added to the mean value was taken as cut-off point.

Cut-off OD = Mean OD of negative control + 3 * SD

CHAPTER IV

RESULTS

4.1 Study Population

A total of 400 samples from suspected cases were collected from above mentioned hospitals. Among 400 samples, majority of samples collected from Sukraraj Tropical and Infectious Disease Hospital, Chitwan Medical College (CMC), Hetauda Hospita (HH), Makawanpur and Bijayapur Hospital (BH), Dharan were from Kathmandu, Chitwan, Hetauda and Dharan. The distribution of the study samples included 30 districts from all other Provinces except Province-6. The samples from three different years were included in the study where 50 samples were collected during 2017 outbreak, 30 samples were collected in 2018 outbreak and 320 samples were collected during 2019 outbreak. The details of the study population are presented in Appendix 2.1.

Fig 4. 1. Sample Distribution Sites. The samples were from 30 districts of Nepal including all the provinces except province-6.

Table 4. 1. Province-wise sample distribution and districts involved.

Province Name	No. of samples	Districts Included
Province 1	198	Jhapa, Udayapur, Morang, Sunsari, Ilam, Terathum
Province 2	17	Sarlahi, Rautahat, Bara, Parsa
Bagmati Province	149	Kathmandu, Chitwan, Nuwakot, Dhading, Makwanpur, Bhaktapur, Ramechap
Gandaki Province	9	Tanahun, Syangja, Gorkha, Kaski, Nawalpur
Lumbini Province	25	Rupandehi, Nawalparasi, Kapilvastu, Bardiya, Palpa, Dang
Karnali Province	0	
Sudurpashchim Province	2	Kailali, Kanchanpur

Province 1 had highest number of samples (198) which were mainly collected Sunsari during Dharan dengue outbreak in 2019. Province 3 was second highest (149) with most of the cases from Kathmandu, Chitwan and Hetauda. Samples from Lumbini Province (25), Province 2 (17) and Sudurpashchim (2) were also included in the study. Samples from 30 districts from 6 different proviences were included in this study.

4.2 Gender and Age based Susceptibility

The study consisted of 59% males (n=236) and 41% (n=164) females with the ratio of male: female as 3:2. The age group ranged from 3 years to 90 years of age with mean age of 31.915 years. The frequency of age group of 30 years to 40 years were highest in male whereas the age group of 20 years to 30 years were highest in female population. The details of age and gender of 400 suspected dengue case are defined in appendix 2.1.

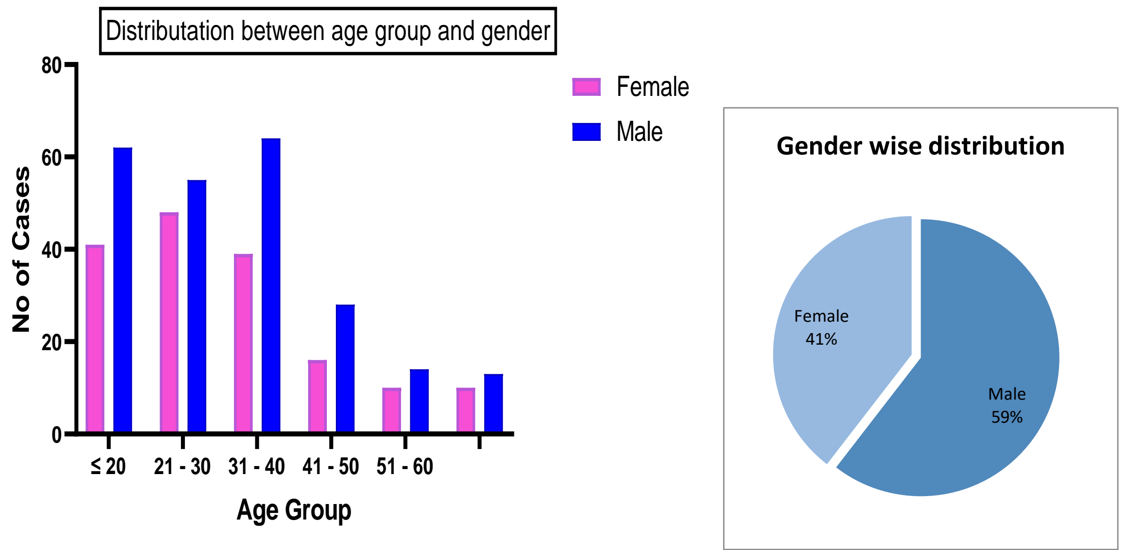


Fig 4. 2. Population distribution among different age group for male and female of suspected cases (n=400). Graph on the left indicates distribution of age group and chart on right indicates gender distribution

4.3. Dengue detection by RDT kit

All 400 samples were subjected to rapid diagnosis test at the site of collection which resulted in 318 positive cases and 82 negative cases. (Appendix 2.1) RDT was used as first selection criteria for the dengue case. Since this study required only 4 samples, one samples from each all four serotypes. And for further works in this study positive cases with fever for 2-7 days were prioritized for ELISA and fever for 2-5 days were included for Molecular Assay.

4.4 Dengue detection by ELISA

Among 312 RDT positive samples, 100 RDT positive samples with onset of fever from 2 days ago to 7 days (dengue diagnosis period by ELISA) were subjected to ELISA which

detected presence of NS1 antigen, IgM antibody and IgG antibody in selected 100 RDT positive cases.

4.4.1 Dengue antigen detection by NS1 capture ELISA

InBios NS1 ELISA of selected samples was performed and absorbance at OD450 were recorded for samples and controls. The ISR of the samples were calculated ratio of mean OD and mean cutoff.

Quality control for NS1 ELISA

Controls	Mean OD
Negative Control	**0.0535**
Positive Control	**1.9135**
Cut off control	**0.109**

Discrimination Capacity:

$R_{(PC/NC)}$	35.7664

The quality control for NS1 ELISA was valid as the positive control OD, negative control OD, and cut off OD were in range provided by the kit. The ISR ratio for all the samples were above 1.1 so all 100 samples were NS1 positive. The highest ISR ratio for NS1 was 36.61 in sample (Nep-44) and lowest ISR ratio was 1.74 in sample (Nep-12) with mean ISR ratio of 22.68. The detail presentation of Dengue NS1 capture ELISA along with mean OD values at 450nm, ISR and sero-status is presented in appendix 2.2.

4.4.2 Anti- DENV IgM detection by IgM Capture ELISA

InBios IgM ELISA of selected samples was performed and absorbance at OD450 were recorded for samples and controls. The ISR of the samples were calculated ratio of mean OD of DENRA and mean OD of NCA.

Quality control for IgM ELISA

Controls	Mean DENRA	Mean NCA	IgM ISR
Negative Control	0.07	0.05	1.40
Positive Control	1.307	0.0485	26.95

The quality control for IgM ELISA was valid as the ISR of positive control was above 8 as provided by the kit. The ISR ratio for all the samples that were above 2.84 were positive which constituted 56 samples out of 100. The number of samples having ISR ratio less than 1.65 were considered negative which constituted 27 samples and remaining 17

samples were in equivocal whose ISR was between 1.65 and 2.84. The highest ISR ratio for IgM was 19.51 in sample (DHR/BH_03) and lowest ISR ratio was 0.38 in sample (Nep-40) with mean ISR ratio of 5.00. The detail presentation of Dengue IgM ELISA along with mean OD values at 450nm, ISR and sero-status is presented in appendix 2.3.

4.4.3 Anti- DENV IgG detection by IgG Capture ELISA

InBios IgG ELISA of selected samples was performed and absorbance at OD450 were recorded for samples and controls. The ISR of the samples were calculated ratio of mean OD of DENRA and mean OD of NCA.

Quality control for IgG ELISA

Controls	Mean DENRA	Mean NCA	ISR
Negative Control	0.1825	0.1655	1.10
Positive Control	1.6645	0.08	20.81

The quality control for IgG ELISA was valid as the ISR of positive control was above 8 as provided by the kit. The ISR ratio for all the samples that were above 2.84 were positive which constituted 42 samples out of 100. The number of samples having ISR ratio less than 1.65 were considered negative which constituted 43 samples and remaining 15 samples were in equivocal whose ISR was between 1.65 and 2.84. The highest ISR ratio for IgG was 25.83 in sample (KTM/STH_27) and lowest ISR ratio was 0.63 in sample (Nep-2) with mean ISR ratio of 3.69. The detail presentation of Dengue IgG ELISA along with mean OD values at 450nm, ISR and sero-status is presented in appendix 2.4.

NS1 antigen was observed in all samples but IgM and IgG were observed in limited samples only. 56 cases had developed IgM antibody against dengue and only 42 cases have developed IgG antibody against dengue. And only 29 cases had both IgM and IgG antibodies. The samples selected were most probable for NS1 antigen test as the antibodies production require certain interval of time. As expected fewer samples were positive for IgG than IgM. The scatter plot of the samples with NS1, IgM and IgG ELISA results are shown in figure below.

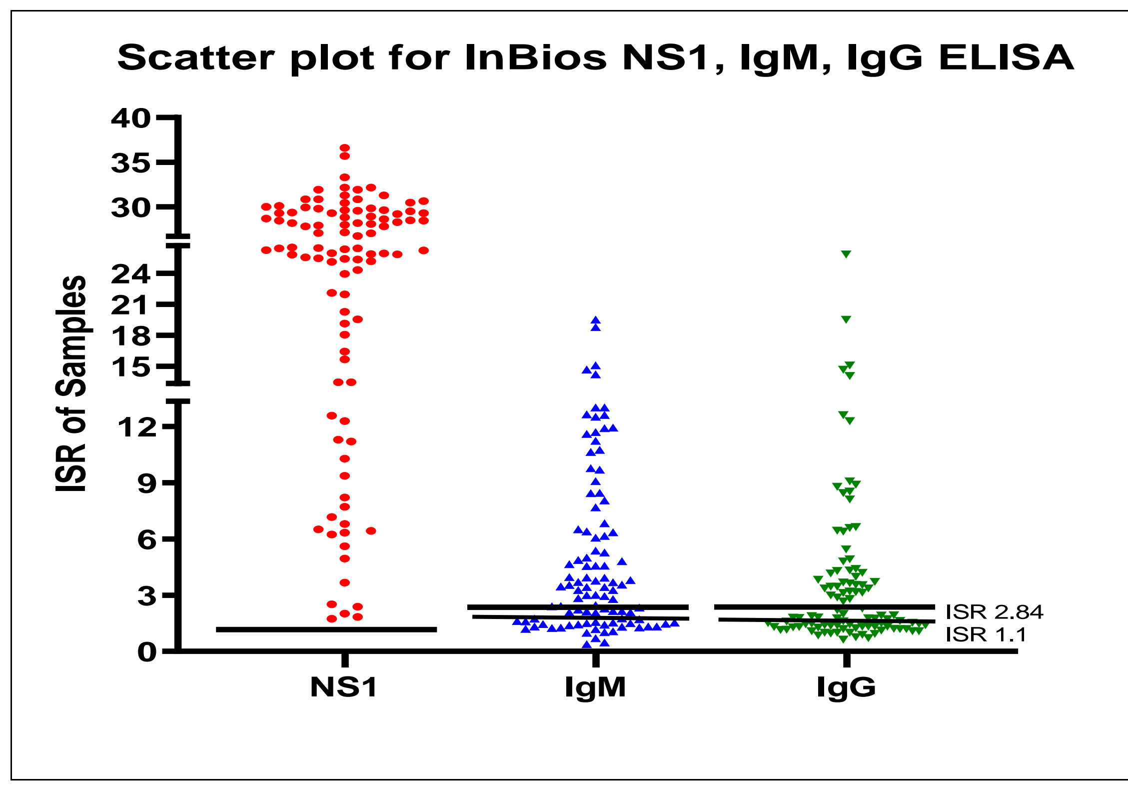


Fig 4. 3. Scatter plot of Immune Status Ratio (ISR) of NS1 antigen, IgM and IgG antibodies of the samples (n=100). The line represents ISR for the test. The samples above the ISR lines were considered positive for the test. Equivocal and negative samples lied below ISR line. Equivocal range is between ISR ratio 1.65 and 2.84.

4.5 DENV infection and serotypes as detected by Real-time RT-PCR

Among 56 NS1 positive samples, 30 selected samples based on clinical parameters such as fever days of 2-5 days (dengue detection period by PCR), body pain, digestive problems and platelets count less than 200000 platelets per ml of blood. All 30 samples were positive to Real-Time PCR performed by using Center for Disease Control (CDC), USA serotyping kit following procedure as described in methodology 3.7.2. Out of 30 samples 27 samples were serotype 2 (all for 2018 and 2019 samples), 2 samples were serotype 1 and only one sample was serotype 3 (both samples were from 2017 outbreak).

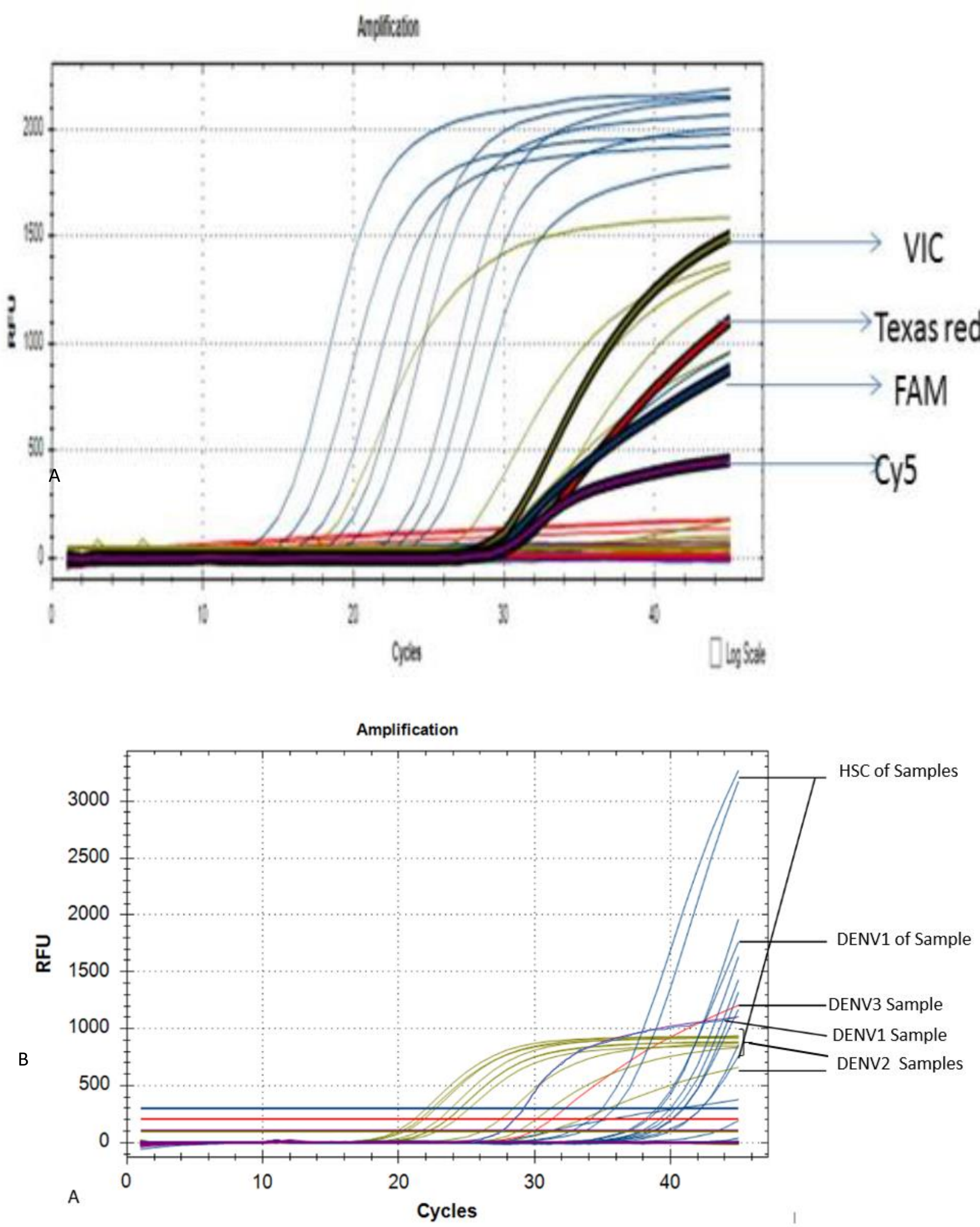


Fig 4. 4. Serotyping by Real time PCR. FAM fluorophore blue in colour is specific for DENV1, also for HSC . VIC fluorophore green in colour is specific for DENV2, Texas Red fluorophore is specific for DENV3. (A) Graph showing the amplification of positive control and (B) Graph showing the amplification of samples

Among 30 PCR positive samples, the Ct values of DENV3 (Nep-7) was 23.33. The Ct values for two DENV1 samples Nep-33 and Nep-43 were 26.23 and 20 respectively. The highest Ct values among 27 DENV2 positive sample was 33.68 for Nep-41 and lowest Ct value was 20.32 for DHR/BH_14 sample. The details of Ct values for all positive samples are detailed in appendix 2.5.

4.5.1 Nested PCR for amplification of envelope Domain III

Only three serotypes among four were obtained through serotyping by CDC Real Time kit. DENV4 was not found in this study so only three serotypes were further studied. There was only one DENV3 but multiple of DENV2 samples. However there were many options for DENV2 and two options for DENV1. The samples with lowest Ct value were selected for serotype 1 and serotype 2.

Sample Nep 43 for DENV1, DHR/BH_14 for DENV2 and Nep7 for DENV3 were further used for amplification of serotype specific envelope Domain III. Nested PCR was done individually for all 3 serotypes. Nested PCR for envelope DomainIII of DENV1 amplified amplicon of 299bp. Similarly, amplicon of 306bp was amplified for envelope DomainIII of DENV2 and amplicon of 296bp was amplified for envelope Domain III of DENV3.

Fig 4. 5. Gel images under UV Transilluminator of the second round nested PCR of envelope Domain IIIregion that 299 bp amplicon corresponds to DENV1, 306 bp amplicon corresponds to DENV2 and 296 bp amplicon corresponds to size of DENV3. 100bp DNA ladder was used to determine size around 300bp representing all serotypes.

4.5.2 Amplification of envelope Domain III with overlapping region.

Amplified serotype specific Domain III of DENV1 and DENV2 serotype was overlapped with 21 bp sequence from DENV2 in DENV1 and DENV3 in DENV2. PCR was done using amplified envelope Domain III of DENV1 as template for amplicon of envelope DoaminIII of DENV1 with overlapping sequence from DENV2. Similarly, envelope Domain III of DENV2 with overlapping sequence from DENV3 was amplified using amplified Domian III of DENV2. This was done to fuse the envelope Domain III of two serotype into recombinant bivalent construct. Due to lack of availability of DENV4 serotype only envelope Domain III of DENV1 with overlaps (D1DIII) from DENV2 of size 320bp and DENV2 with overlaps from DENV 3 (D2DIII)of size 326 bp were amplified.

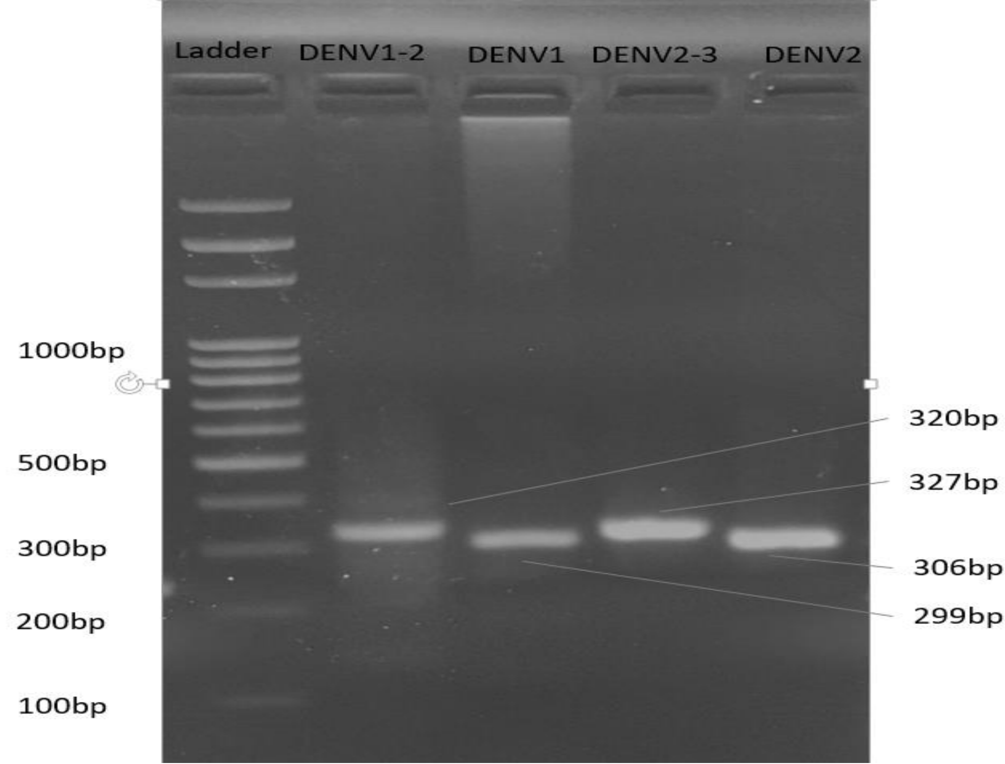


Fig 4. 6. Amplification of envelope Domain III with overlapping from different serotypes.

4.5.3 Sequence Analalysis

Sequence obtained from NAST were analysed by using BioEdit software and sequence were generated by comparing the forward and reverse sequence (bidirectional sequencing) for both D1DIII and D2DIII sequences. The sequence and chromatogram of D1DIII and D2DIII are detailed below.

Sequencing Data Analysis

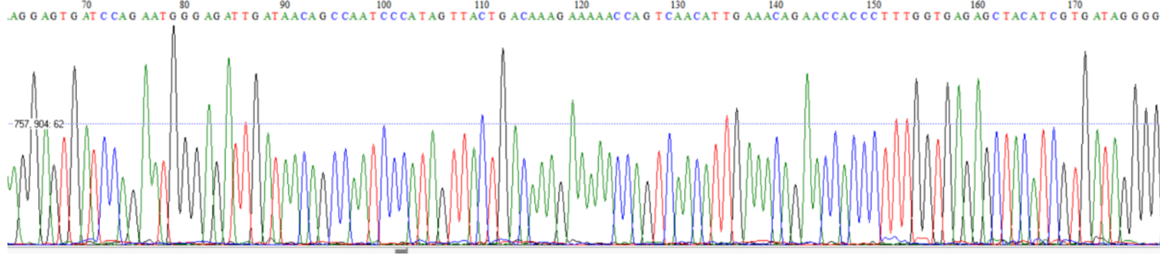


Fig 4. 7. Choromatogram of D1DIII with overlapping region.

>D1DIIIClo_Nep43_2017 sequence obtained from BioEdit.

GGCAGGATAAATATGAGGACAGATGCACCATGCAAGATTCCAATTTCAACCCAAGATGAGAAA
GGAGTGATCCAGAATGGGAGATTGATAACAGCCAATCCCATAGTTACTGACAAAGAAAAACCA
GTCAACATTGAAACAGAACCACCCTTTGGTGAGAGCTACATCGTGATAGGGGCGGGTGAAAAA
GCTTTGAAACTAAGCTGGTTCAAGAAAGGAAGCAGCATAGGGAAAATGTTCGAAGCTACCGCC
CGAGGAGCACGAAGGTCATACTCCATGTGCACAGGAAAC

Fig 4. 8: Sequence of D1DIII with overlapping region.

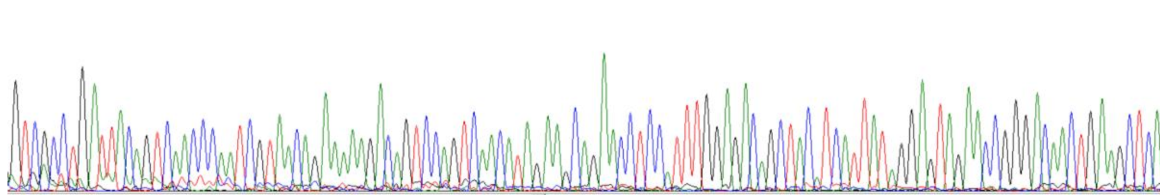


Fig 4. 9. Chromatogram of D2DIII with overlapping region.

>D2DIIICl_Nepal_DHR/BH_14_2019 sequence obtained from BioEdit.

GTAGCGGCTGGCTTGCATGTGCTTGTCGTCGACTCCATGTGCACGGAGGGAAGGCTCTTTTGTC
GATCCCTTTTGAGATAATGGATTTGGAAAAAASACATGTCTTAGGTCGCCTGATTACAGTCAACC
CAATCGTAACAGAAAAAGACAGTCCAGTCAACATAGAAGCAGAACCTCCATTTGGAGACAGCTA
CATCATTATAGGAGTAGAACCGGGACAACTGAAGCTCAGCTGGTTTAAGAAAGGAAGTTCTATC
GGCATGAGCTATGCAATGTGCTTG

Fig 4. 10. Sequence of D2DIII with overlapping region.

Further sequences were analyzed in BLAST analysis which generated maximum percentage identity of 98 percent for D1DIII and 97 percent for D2DIII. Further the phylogenetic tree for both D1DIII and D2DIII were subjected to multiple sequence alignment. The Phylogenetic analysis of D1DIII showed D1DIII was closer to Indian 2009 DENV1 strain whereas, D2DIII was somewhat closer to 2019 DENV2 from China.

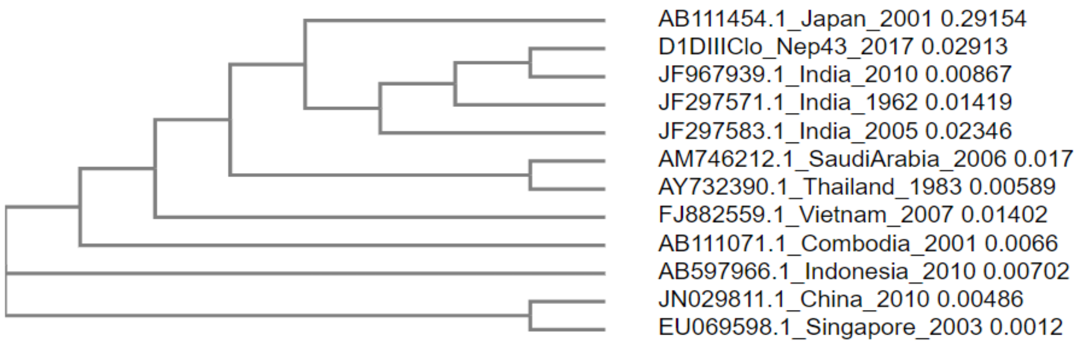


Fig 4. 11. Phylogenetic Analysis of D1DIII.

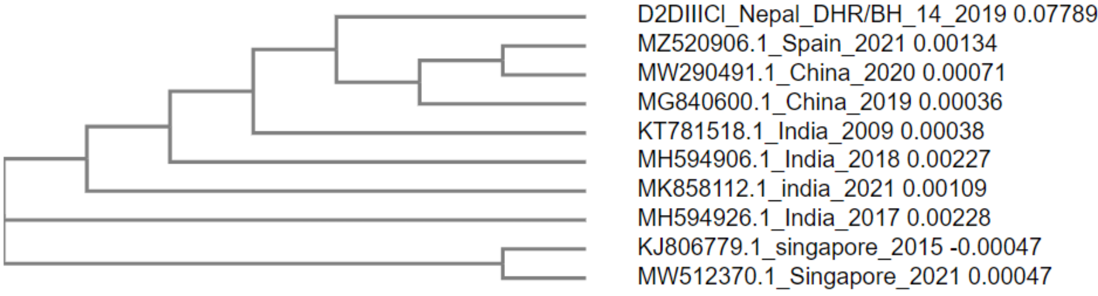


Fig 4. 12. Phylogenetic Analysis of D2DIII

4.5.4 *Insilico* Analysis of the construct.

The DNA sequence for D1DIII and D2DIII retrived from BioEdit were translated using Expasy tool. In case, of DENV3 and DENV4 the sequence used for primer desigining were used to retrieve aminoacid sequence of the recombinant tetravalent construct. The translated sequence was analysed for epitope prediction using iedb tool (Immune Epitope Database and Analysis Resource and protein prediction was done by using predict protein tool. Analysis showed the potein consists of 400 aminoacid with 11 epitopes present in the protein. And protein was classified as mixed protein.

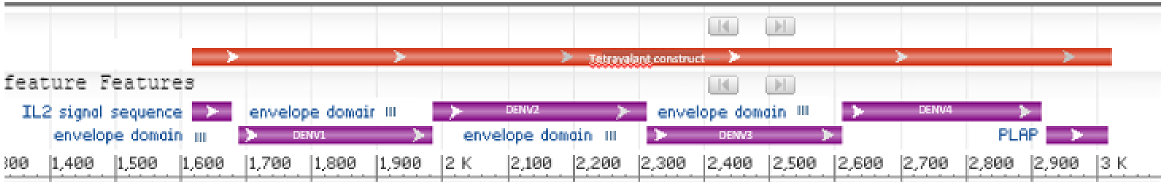


Fig 4. 13. Graphical view of the tetravalent construct.

Synthetic Peptides construct

MYRMQLLSCIALSLALVTNSARILGRINMRTDAPCKIPISTQDEKGVIQNGRLITANPIVTDKEKPVNIE
TEPPFGESYIVIGAGEKALKLSWFKKGSSIGKMFEATARGARRSYSMCTGNSGWLACACRRLHVHG
GKALLSIPFEIMDLEKHVLGRLITVNPIVTEKDSPVNIEAEPPFGDSYIIIGVEPGQLKLSWFKKGSSIGM
SYAMCLMSYAMCLNTFVKKEVSETQHTILIKVEYKGEDAPCKIPFSTEDGQGKAHNGRLITANPVVT
KKEKPVNIEAEPPFESIVISISDKALKINWKGLRIKGMSYTMCSGKFSIDKEMAETQHGTTVKVKYEGA
PCKVPIEIRDVNKEKVVGRIISSTPFAEYTNSVTNIELEPPFGDSYIVIGVGDSALTLHWEREFTTDAAH
PGRSVVPALLPLLAGTLLLETATAP

Fig 4. 14 Amino acid sequence of recombinant tetravalent construct. Amino Acid sequence of the construct. Here different color indicate sequence obtained from different serotype. Blue from D1DIII, green D2DIII, red D3DIII and purple D4DIIII.

Table 4. 2. Epitope prediction of the constructed sequence.

No.	Start	End	Peptide	
1	5	20	QLLSCIALSLALVTNS	16
2	43	49	DEKGVIQ	7
3	89	99	LKLSWFKKGSS	11
4	123	135	GWLACACRRLHVH	13
5	194	233	LKLSWFKKGSSIGMSYAMCLMSYAMCLNTFVKKEVSETQH	40
6	257	262	GQGKAH	6
7	286	294	PPFESIVIS	9
8	317	335	CSGKFSIDKEMAETQHGTT	19
9	353	357	DVNKE	5
10	404	416	EREFTTDAAHPGR	13
11	434	434	T	1

From *In silico* analysis 11 different epitopes were predicted in the construct which facilitates binding of complementary antibodies. Peptides number varies from 1 to 40 in these predicted epitopes.

2D structure prediction of the synthetic peptide.

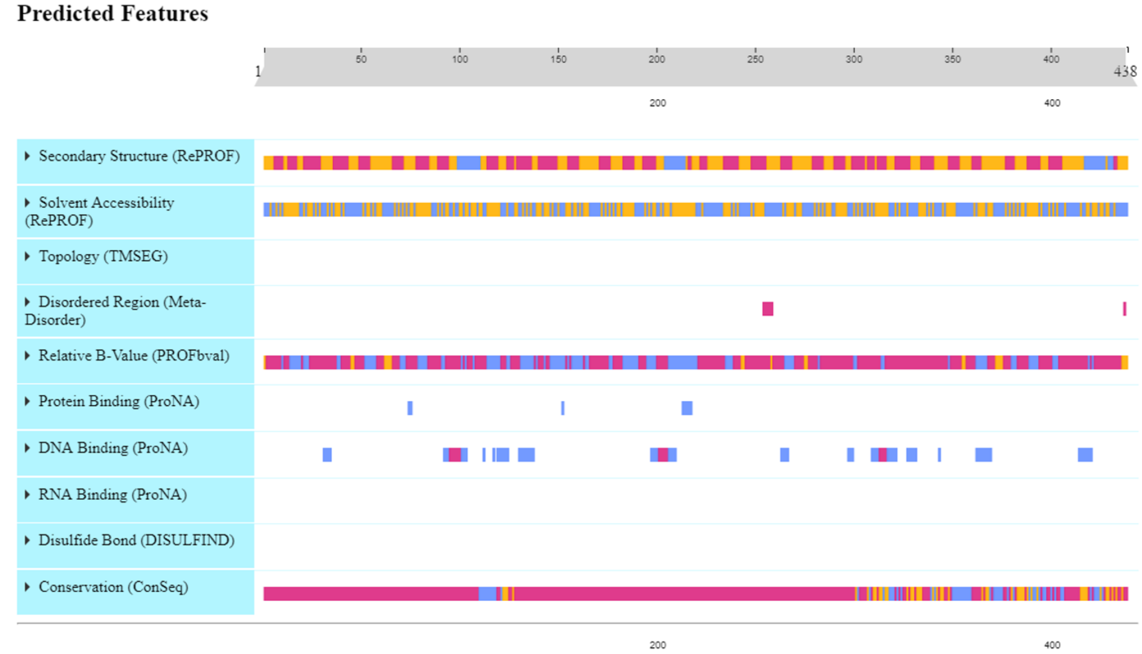


Fig 4. 15. 2D structure of the construct.

In secondary structure prediction, location of alpha helix represented by blue color, beta sheet represented by pink color and other components represented by yellow color can be observed. Also solvent accessibility region which are accessible can be observed in blue. Relative B- value representing rigid and flexible region denoted by different colors, Protein binding region, DNA binding region and conserved sequences are also ovserved in the structure prediction.

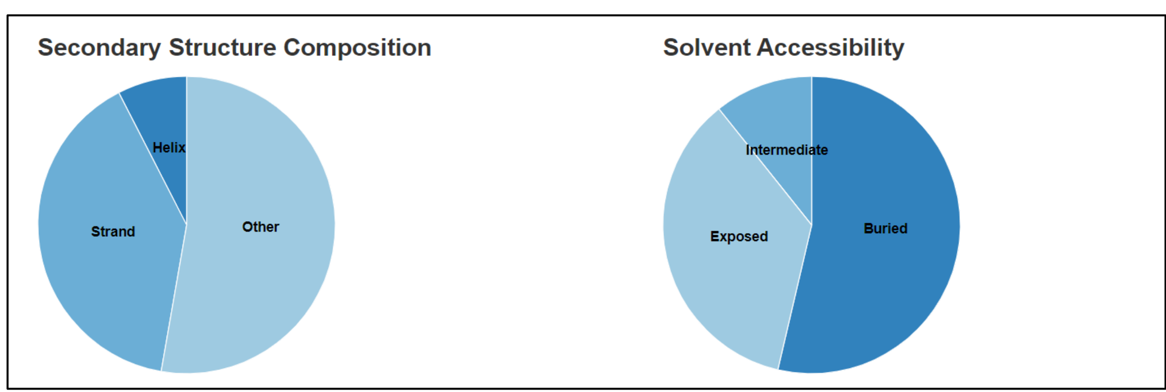


Fig 4. 16. Secondary structure prediction of the construct.

According to secondary structure prediction, this protein can be classified as mixed protein because the presence of other structure is more than alpha helix and beta sheet/strand. Similarly the protein had lesser exposed region for solvent accessibility.

4.6 Cell culture for the virus propagation

Due to lack of enough amount of DENV3 and DENV1 samples the cell culture for these samples were done in C6/36 cell lines. After cell was fully grown the virus infection was done by inoculating 200µl of diluted serum. A very little change was seen in day 1 but there was drastic death of cell in day 3 as seen in fig 4.9. The pellet obtained after harvesting cells in day 3 were used for molecular assay.

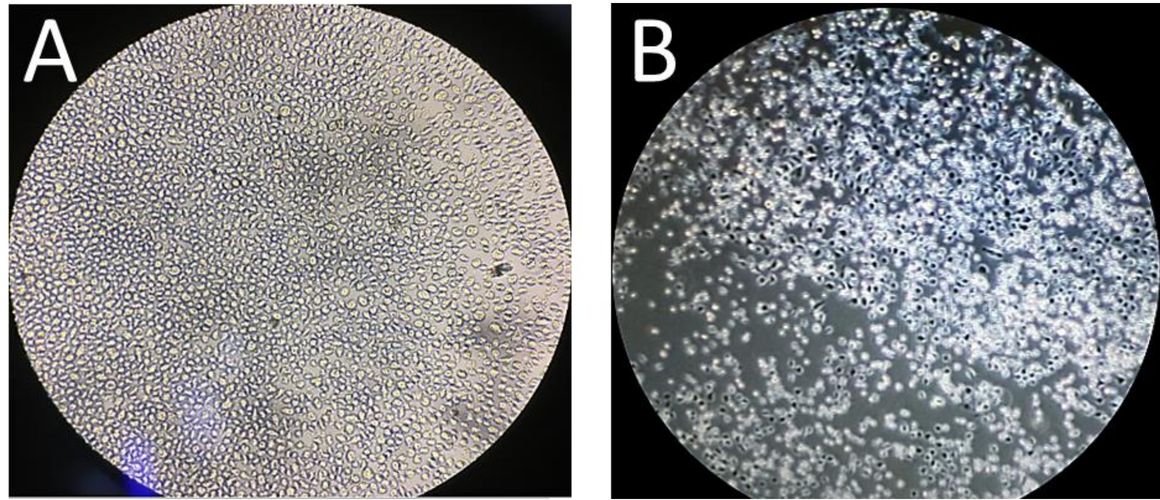


Fig 4. 17. Cell culture of DENV3 in C6/36 cell line. A is a day old C6/36 cell line, B is inoculate cell line after 3 days.

4.7 Animal Immunization

All three mouse were kept separately after inoculation and singns and symptoms were observed daily. No any physical sign and symptoms of Dengue were shown by the mouse. All the mouse seemed healthy as their activities such eating, walking, etc were normal. The blood samples were taken from tail vein at day 14 and day 28 for immunological assay.

Fig 4. 18. Mouse used in animal immunization. After inoculation they were separated and kept in vessel with rice husk.

Immunological Assay

The whole blood from mouse tail vein was drawn using 1ml syringe and serum was separated in Eppendorf tube. Due less volume of sample more test were not carried out.

Mouse A (1:10 serum dilution) was weakly positive in day 14 samples whereas Mouse B (1:30 serum dilution) and Mouse C (Control) were negative for NS1 ELISA. All samples were negative in day 28. Similarly, for IgM ELISA mouse A showed equivocal status whereas mouse B and mouse C were negative in day 14 and all samples were negative in day 28. For IgG ELISA, at day 14 mouse A was equivocal and mouse B and mouse C were negative. At day 28 mouse A was positive, mouse B was equivocal and mouse C was negative as shown in table below.

Table 4. 3. NS1 ELISA from mouse serum.

	In-Bios NS1	
Sample ID	**Mean OD**	**ISR**
Negative Control	**0.0535**	0.49083
Positive Control	**1.9135**	17.555
Cut off control	**0.109**	

	14 days			28 days		
Sample	Mean OD	ISR	Status	Mean OD	ISR	Status
Mouse A	0.11	1.01	Positive	0.0813	0.75	Negative
Mouse B	0.091	0.83	Negative	0.0734	0.67	Negative
Mouse C	0.062	0.57	Negative	0.0681	0.62	Negative

Table 4. 4. IgM ELISA from mouse serum

Control	Mean DENRA	Mean NCA	IgM ISR					
Negative Control	0.07	0.05	1.40					
Positive Control	1.307	0.0485	26.95		Mean DENRA	Mean NCA	IgM ISR	
Sample		14 days		Status		28 days		
MouseA	0.161	0.073	2.20	Equivocal	0.071	0.077	0.92	Negative
MouseB	0.091	0.062	1.46	Negative	0.057	0.056	1.01	Negative
MouseC	0.059	0.055	1.07	Negative	0.091	0.061	1.43	Negative

Table 4. 5. IgG ELISA from Mouse Serum.

	IgG							
Control	Mean DENRA	Mean NCA	IgG ISR					
Negative Control	0.1825	0.1655	1.10					
Positive Control	1.6645	0.08	20.81		Mean DENRA	Mean NCA	IgG ISR	
Sample		14 days		Status		28 days		
MouseA	0.173	0.093	1.86	Equivocal	0.184	0.062	2.97	Positive
MouseB	0.082	0.062	1.32	Negative	0.157	0.076	2.07	Equivocal
MouseC	0.069	0.065	1.06	Negative	0.095	0.072	1.32	Negative

4.8 Detection of serotype specific antibody by In-House ELISA

Once infected the infected person is believed to have protection against all type of serotype for certain period of time. To determine the presence of serotypic specific antibody dengue suspected serum samples were taken and In-House ELISA for the four antigens of dengue virus was performed. The OD values for all the controls and the samples is shown in the appendix 4.1.

The OD readings at 450nm of all the variables are given in **Table 4.8.** The mean OD of samples is considerably higher than the cut-off value for DENV-2. In case of DENV-1, DENV-3 and DENV-4 the mean OD is relatively lower than cut-off value. Similarly, for Non-endemic control (NEC) the mean OD was found near to cut-off

value and for Endemic control(EC), the mean OD was found to be considerably lower than cut-off value.

Table 4. 6. OD of ELISA at 450nm using different antigens of dengue virus

Antigens	NEC (n=5) (Mean ± SD)	EC (n=10) (Mean ± SD)	Cut-off value OD	Samples(n=50) (Mean ± SD)	Sample Status
DENV 1	1.1834±0.0436	1.0982±0.0812	1.3279	1.1876 ±0.5292	+ve=18 -ve= 32
DENV 2	1.7107±0.0629	1.2816± 0.0444	1.6572	1.9575± 0.5638	+ve=36 -ve= 14
DENV 3	1.7703±0.0799	1.6824 ± 0.0276	1.8877	1.4275± 0.4527	+ve= 7 -ve= 43
DENV 4	1.3049±0.1054	1.2719± 0.0561	1.5306	1.1141± 0.3484	+ve= 5 -ve= 45

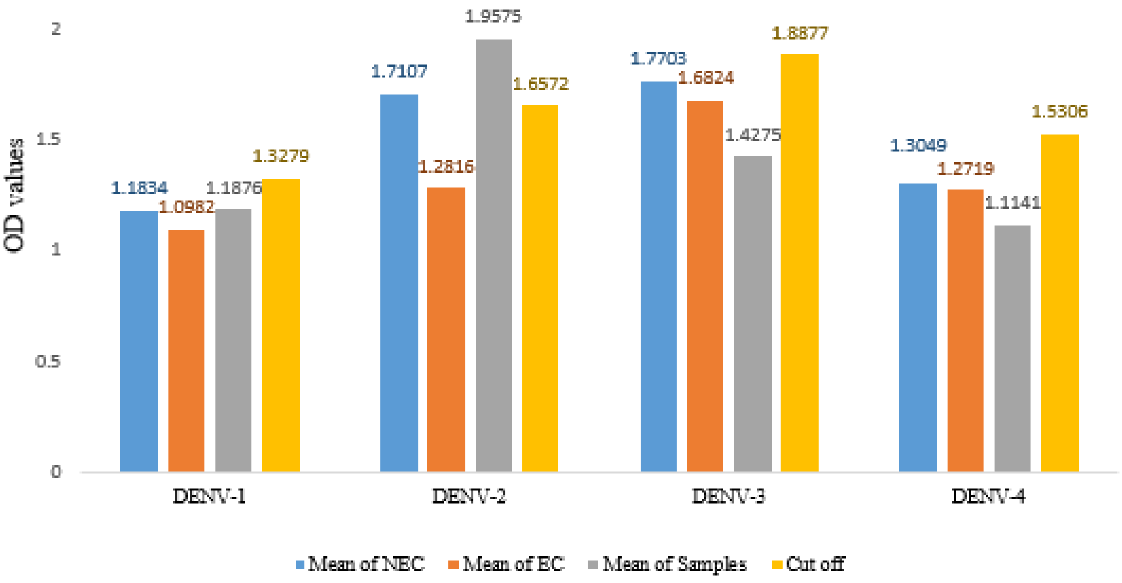


Fig 4. 19. Comparative study of Mean of Non-endemic control (NEC), Endemic control (EC), Samples and cut off OD value for different antigens of Dengue.

The mean of negative samples was found to be lower than the OD value of positive control. The comparative analysis is shown in **Table 4.9**. The mean OD for DENV-1 was to be 1.59 times lesser than that of positive control. For DENV-2 it was found to be 2.03 times, for DENV-3 it was 1.47 times and for DENV-4 it was 1.5 times lower than positive control.

Table 4. 7. Comparison of Mean of negative samples with positive control

Antigen	Mean of Negative samples	Positive control	Cut off value
DENV-1	0.8571	1.365	1.3279
DENV-2	1.2548	2.543	1.6572
DENV-3	1.3148	1.9315	1.8877
DENV-4	1.0551	1.5795	1.5306

The mean of non-endemic control and endemic control was found to be significantly lower than cut-off value but for non-endemic control, the antibody titer for DENV-2 was found to be higher than cut-off value. The mean value for positive samples was found to be significantly higher than cut-off value. Also there is significant difference between the antibody titer of non-epidemic control with epidemic control.

Table 4. 8. Comparative analysis of antibody titer for positive, endemic and non-endemic samples

Antigen	Cut-off value	Mean of NEC	Mean of EC	Mean of positive samples
DENV-1	1.3279	1.1834	1.0982	1.7891
DENV-2	1.6572	1.7107	1.2816	2.2586
DENV-3	1.8877	1.7703	1.6824	2.1198
DENV-4	1.5306	1.3049	1.2719	1.6449

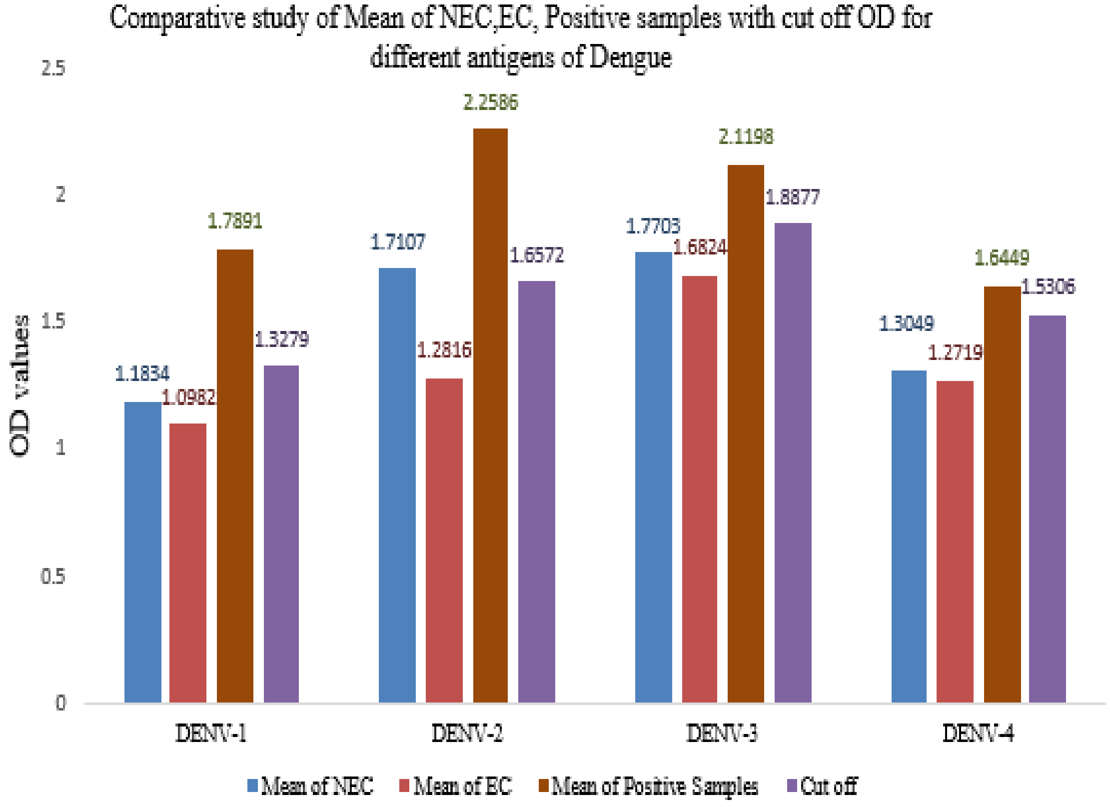


Fig 4. 20. Comparative study of Mean of non- endemic control (NEC), endemic (EC), Positive samples with cut off OD for different antigens of Dengue.

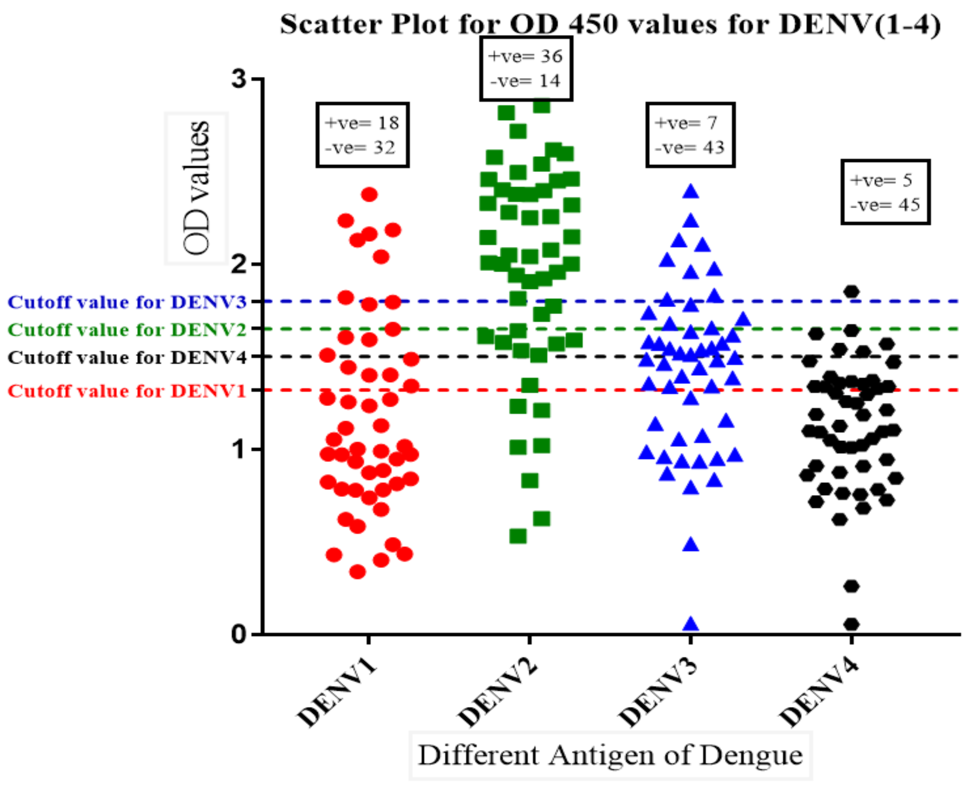


Fig 4. 21. Scatter diagram showing the OD values of samples for different antigens of dengue

The above scatter diagram shows the OD values which represents the antibody titer for the antigens of dengue virus. Each dot represents the mean of each sample. The samples that lie above the cut-off value are positive samples. Out of 50 samples, the total no. of positive samples was 18,36,7 and 5 for DENV-1, DENV-2, DENV-3 and DENV-4 respectively. The antibody titer for DENV-2 was found to be highest and for DENV-4 it was lowest.

The comparison of In-House ELISA positive samples and negative samples were done. The statistical analysis was also performed by unpaired t-test and One-way ANOVA. The p-value was found to be <0.0001 and P=0.0001. P<0.05 means there was significant difference between the mean of the positive and negative samples for different antigens of dengue.

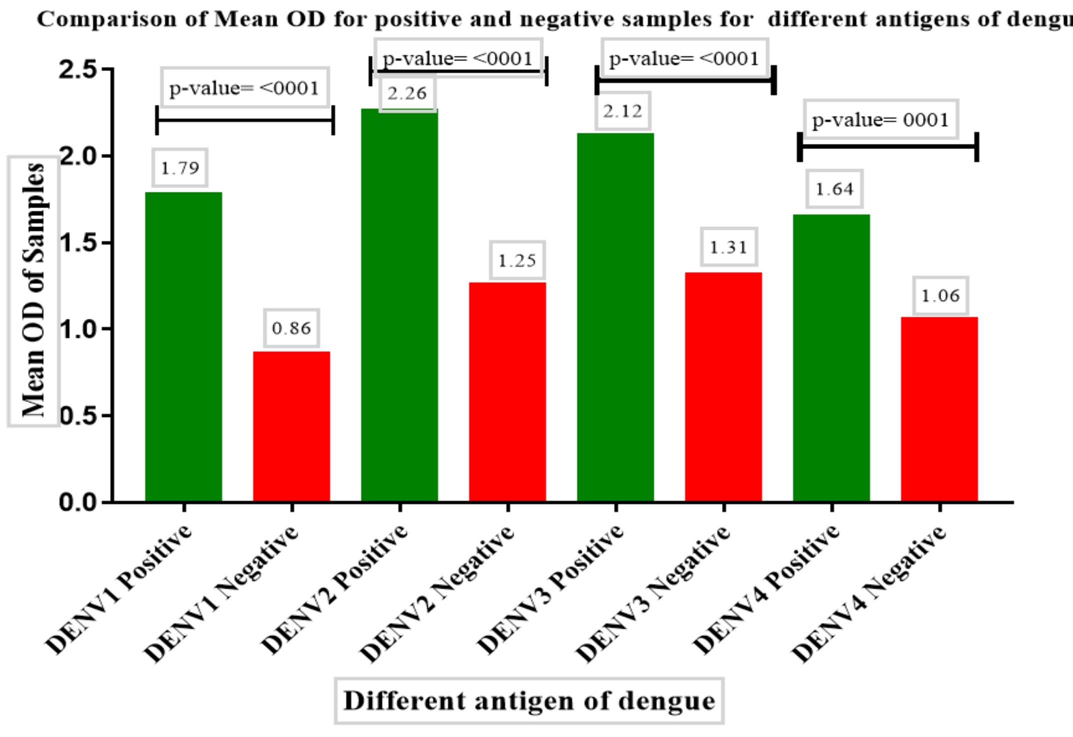


Fig 4. 22. Comparison of Mean OD for positive and negative samples for different antigens of dengue.

The comparison with the secondary data from In-Bios kit also shows that the kit is relatively sensitive for the detection of antibodies. Most of the samples which are positive in In-Bios Kit has been shown to have higher antibody titer for all antigens. The samples which are negative for the kit have also lower antibody titer. The details of In House ELISA are presented in appendix 4.1.

CHAPTER V

DISCUSSION

Several vector-borne tropical infectious diseases like malaria, lymphatic filariasis, visceral leishmaniasis, dengue fever and Japanese encephalitis have been found in Nepal (Dhimal et al., 2015). Dengue is one of them which was reported in 2004 for the first time in Japanese traveller followed by annual reports of indigenous circulation from the year 2006 (Malla et al., 2008). Dengue has infected 68 out of 77 districts of the country in 2019 in Nepal (Rijal et al., 2021) spreading rapidly all over the country from the East to the West encompassing even the temperate hilly regions (Khetan et al., 2018). The endemicity of dengue continued to spread towards highland hilly regions and has been found in Khotang and Panchthar districts which is really a serious concern as the disease could be extended pandemic to Nepal (Prajapati et al., 2020). The major reasons behind are rapid urbanization, quick transportation facility and better adaptation of *Aedes spp* mosquito to the comparatively cold environment. However, gender-wise infection verified 1.5 folds higher male patients than female accordance with our previous report (Gupta et al., 2018). This could be attributed to the two facts; one the men are outdoor field workers to be exposed to the vector and the next, men have preferentially quick access to the healthcare system than females do.

The infection of dengue is not well understood due to antibody-dependent enhancement (ADE), original T cell antigenic sin and viral virulence (Elong Ngono & Shresta, 2018), although viral serotype shift and genetic drift play major roles. The CDC DENV-1–4 RT-PCR Assay has been developed as an in-vitro diagnostic platform and approved by the US Food and Drug Administration (FDA) for detection of dengue in patients with signs or symptoms of mild or severe dengue. The primers and probes of this test have been designed to detect currently circulating strains of DENV-1–4 from around the world at comparable sensitivity. Using sequencing as a positive indicator, the RT-PCR Assay had a 97.92% positive agreement indicating that the CDC DENV-1–4 RT-PCR Assay provided a reliable diagnostic platform capable for confirming dengue in suspected cases (Santiago et al., 2013). The 2017 serotypes circulating in Nepalese population are DENV1, 2 and 3 and the report of dengue serotype 3 is for the first time after 11 years. Samples of 2018 outbreak and 2019 outbreak were DENV2 serotype in our study. And several study have shown DENV2 as major serotype circulating in Nepal from 2017 to 2019 (Poudyal et al., 2021).

Dengue disease is becoming a global epidemic, with nearly 40% of the world's population at risk for transmission of one or more of the four dengue virus serotypes(Bhatt, et al., 2013). It is one of the major public health problems. In the context of Nepal, the dengue epidemic is rising each year. Physical methods to control has not been as effective and

there is urgent need of safe vaccine for effective control (EDCD, 2019). There is only one vaccine Dengvaxia produced by Sanofi Pasteur Inc. and approved by FDA till now which also provide protection to previously infected person (WHO, 2021). So there is urgent need for vaccine development for prevention and control for the disease.

During vaccine development various factors such as prevalence of disease for vaccination in target population need to be considered (Lahariya, 2016). Prevalence study of dengue in our study will be advantageous in our vaccine development process. The vaccine developed should be safe for all and should not have any side effects. The main problems associated with current vaccine is due to presence of other non-neutralizing and cross-reactive epitopes which would lead to ADE in patients (Guzman et al., 2010). Use of Domain III as vaccine candidate has advantage of reducing risk of ADE because of absence of non-neutralizing and cross- reactive epitopes and also plays crucial role in dengue pathogenesis and immunity (Suzarte et al., 2015). *Insilico* analysis of recombinant tetravalent construct showed presence of epitopes suitable for producing antigenicity and immunogenicity.

Although EDIII presents only a fraction the Envelope protein, the absence of other epitopes which elicit non-neutralizing, cross-reactive antibodies could have advantages in reducing risk for developing ADE. In this context, the EDIII of the E protein has emerged as a very promising vaccine candidate in recent years (Guzman et al., 2010). It is very important presenting antigens from all serotypes in a manner to elicit immune responses in equal proportion. Whenever a dengue vaccine does not provide protection against all four serotypes, a vaccinated individual will be susceptible to development of a more severe disease (DHF/DSS) if infected by untargeted serotypes (Suzarte et al., 2015). One approach that has been suggested to minimize the possibility of antibody-dependent infection enhancement occurring after vaccination is to design a tetravalent subunit dengue vaccine (Wilder-Smith et al., 2010). This type of vaccine contains epitopes inducing high levels of specific neutralizing antibody to all four serotypes, but contains a minimum of dengue complex cross-reactive epitopes which are presumably responsible for the induction of enhancing antibodies. To achieve equal expression of antigens from all four serotypes, it should design the EDIII antigens of the four serotypes of virus as an individual protein (Wilder-Smith, 2019). However, the detailed mechanism of dengue infection and also vaccine provided protection are not clearly explained, up to now. In addition, for designing a dengue vaccine which is protective against all four serotypes, without any potential risk of disease severity enhancement, the molecular mechanism of dengue pathogenesis must be considered (Screaton et al., 2015). It is expected that in the coming years more data on dengue pathogenesis and protection mechanisms will be available.

During development, vaccine challenge to observe the immune response of wild serotype are done in animal models before human trails (Sekhar & Kang, 2020). Usually dengue virus are revived in cell culture and revived cultures are inoculated in animal models to observe the immune response of virus. Due to lack of proper animal model it has been difficult to study immunological changes and use animal model vaccine development. Several study have tried to use different animal model for study but there is still lack of proper animal model for dengue (Zompi & Harris, 2012). In this study albino mouse with 10 folds serum dilution showed dengue infection in serology by no any physical sign and symptoms were observed. Further study in mass number is necessary to draw any conclusion since this was just preliminary test with 3 mice.

Finally, we intended to check cross-reactivity of the antibody between the samples as cross-reactivity of samples has found to enhance the dengue infection (Dejnirattisai et al., 2016). In our study, we have found out cross-reactivity in some samples which will enable us to alert the Nepalese population in case of future Dengue outbreak and prevent infected people from development in to severe dengue cases.

CHAPTER VI

CONCLUSION

Dengue is one of the neglected tropical disease vector borne disease in Nepal though it is frequently occurring seasonal disease in Nepal. The vaccination could provide a promising approach for controlling dengue virus infections but the approved vaccine can only be provide to certain age group of seropositive people thus providing immunity to limited people. Dengue vaccine must be tetravalent that will induce a balanced immune response against all four serotypes and provide long-term protection. Despite other vaccine types, recombinant DNA vaccines are attractive candidates for effective tetravalent dengue vaccine due to its ability of generating antigen-specific immune responses, stability, ease of preparation in large scale with high purity, simplicity of delivery, and safety.

In the present study, we have constructed recombinant tetravalent dengue envelope domain III vaccine candidate in *insilico* and its properties are studied. To examine the possibility of serotype shift and amplify domain III of all serotype it is for most important to determine the circulating serotype. The primers for this test have been designed to amplify envelope domain III of all serotype and fuse them to construct tetravalent EDIII construct. Only EDIII of DENV1, DENV2 and DENV3 could be amplified as no DENV4 serotype was detected. Fusion of EDIII from DENV1 and DENV2 led to bivalent construct and same approach with other serotype would led to tetravalent construct. Tetravalent envelope domain III construct was constructed using the sequence obtained from sequencing EDIII of DENV1 and DENV2 whereas sequence form NCBI database were utilized for DENV3 and DENV4. *Insilico* analysis of tetravalent construct predicted 11 epitopes that could drive strong immune response with high immunogenicity. Further comprehensive work on cell culture and mice immunization could guide us to exceptional avenue of dengue vaccine development.

Construction of tetravalent EDIII-based vaccine which elicit neutralizing antibodies against the all four serotypes of dengue virus is the broadly accepted approach for development of a desired vaccine. Despite the advent of first licensed dengue vaccine (Dengvaxia®), there are many research which are focused to achieve a safer and effective dengue vaccine. It seems that the current promising researches on dengue vaccines will lead us to achieve a desirable human vaccine in the near future.

LIMITATIONS OF THE STUDY

- Lack of the availability of all four serotype for the construction of recombinant tetravalent construct
- Lack of availability of animal model for the research.
- Lack of research facilities to perform the works of vaccine development.

RECOMMENDATIONS/ FUTURE PERSPECTIVES

- Domain III could be potential vaccine candidate so further elaborative study of Domain III should be done.
- Effective approaches should be taken to develop safe vaccine in time before the severe outbreak happens.
- As covid vaccine was made possible in short period of time, safe dengue vaccine can also be produced if all minds and finance can come together to achieve the goal.

Future Plan:

Fusion of all four serotype in one tetravalent construct and cloning to be used as DNA vaccine.

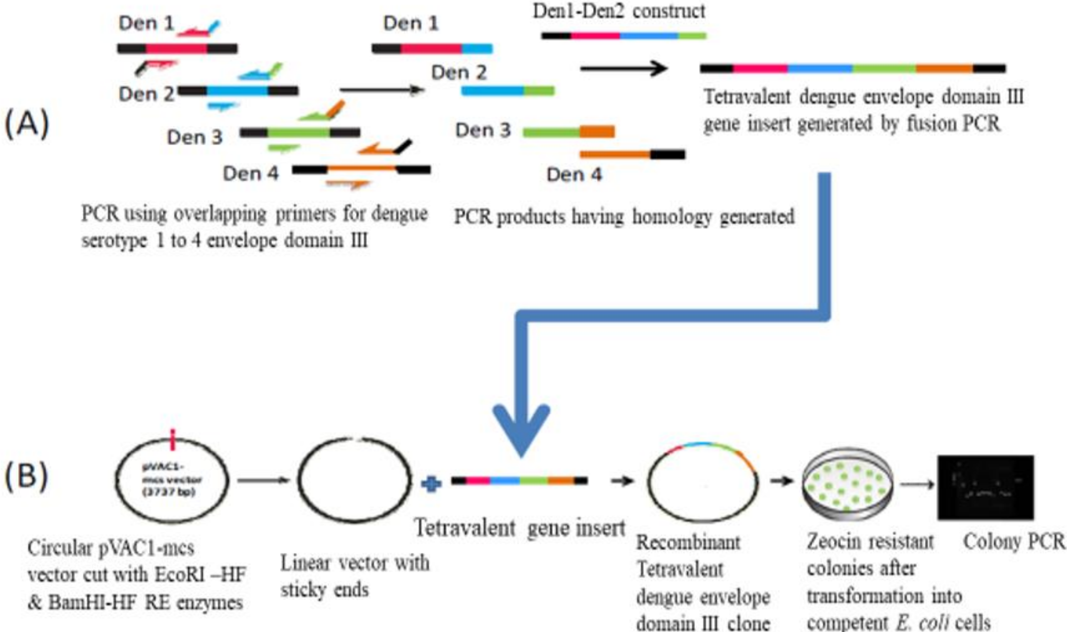


Fig: Schematic diagram of dengue vaccine development

References

AbCAM. (2016). Sandwich ELISA protocol. *Discover*.

Abello, J. E., Gil Cuesta, J., Cerro, B. R., & Guha-Sapir, D. (2016). Factors Associated with the Time of Admission among Notified Dengue Fever Cases in Region VIII Philippines from 2008 to 2014. *PLoS Neglected Tropical Diseases*, *10*(10). https://doi.org/10.1371/JOURNAL.PNTD.0005050

Adhikari, N., & Subedi, D. (2020a). The alarming outbreaks of dengue in Nepal. *Tropical Medicine and Health*, *48*(1), 5. https://doi.org/10.1186/s41182-020-0194-1

Adhikari, N., & Subedi, D. (2020b). The alarming outbreaks of dengue in Nepal. *Tropical Medicine and Health*, *48*(1), 1–3. https://doi.org/10.1186/S41182-020-0194-1/FIGURES/2

Akter, R., Naish, S., Hu, W., & Tong, S. (2017). Socio-demographic, ecological factors and dengue infection trends in Australia. *PLoS ONE*, *12*(10). https://doi.org/10.1371/JOURNAL.PONE.0185551

Anuja, Mathew, Alan, L., & Rothman. (2008). *Understanding the contribution of cellular immunity to dengue disease pathogenesis*.

Arya, S. C., Agarwal, N., Parikh, S. C., & Agarwal, S. (2011). Simultaneous detection of dengue NS1 antigen, IgM plus IgG and platelet enumeration during an outbreak. *Sultan Qaboos University Medical Journal*, *11*(4), 470–476. https://doi.org/10.1016/S1995-7645(11)60171-5

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides*. https://doi.org/10.1016/j.peptides.2015.04.012

Bara, J. J., Clark, T. M., & Remold, S. K. (2013). Susceptibility of Larval Aedes aegypti and Aedes albopictus (Diptera: Culicidae) to Dengue Virus. *Journal of Medical Entomology*, *50*(1), 179–184. https://doi.org/10.1603/ME12140

Bharati, M., & Saha, D. (2018). Multiple insecticide resistance mechanisms in primary dengue vector, Aedes aegypti (Linn.) from dengue endemic districts of sub-Himalayan West Bengal, India. *PLOS ONE*, *13*(9), e0203207. https://doi.org/10.1371/JOURNAL.PONE.0203207

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., William Wint, G. R., Simmons, C. P., Scott, T. W., Farrar, J. J., & Hay, S. I. (2013). The global distribution and burden of dengue. *Nature*. https://doi.org/10.1038/nature12060

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R. W., Simmons, C. P., Scott, T. W., Farrar, J. J., & Hay, S. I. (2013). The global distribution and burden of dengue. *Nature*, *496*(7446), 504–507. https://doi.org/10.1038/nature12060

Bilal Waqar, A. (2016). Dengue Virus: Host-Pathogen Interactions and Emerging Role of DNA Vaccines. *Journal of Human Virology & Retrovirology D*, *3*. https://doi.org/10.15406/jhvrv.2016.03.00091

Blacksell, S. D., Jarman, R. G., Gibbons, R. V., Tanganuchitcharnchai, A., Mammen, M. P., Nisalak, A., Kalayanarooj, S., Bailey, M. S., Premaratna, R., De Silva, H. J., Day, N. P. J., & Lalloog, D. G. (2012). Comparison of seven commercial antigen and antibody enzyme-linked immunosorbent assays for detection of acute dengue infection.

Clinical and Vaccine Immunology. https://doi.org/10.1128/CVI.05717-11

Calvo, E. P., Sánchez-Quete, F., Durán, S., Sandoval, I., & Castellanos, J. E. (2016). Easy and inexpensive molecular detection of dengue, chikungunya and zika viruses in febrile patients. *Acta Tropica*. https://doi.org/10.1016/j.actatropica.2016.07.021

CDC, C. for D. C. and P. (2021). *Clinical Presentation | Dengue | CDC*. Dengue. https://www.cdc.gov/dengue/healthcare-providers/clinical-presentation.html

Chan, M., & Johansson, M. A. (2012). The Incubation Periods of Dengue Viruses. *PLoS ONE*, *7*(11), 50972. https://doi.org/10.1371/journal.pone.0050972

De Paula, S. O., & Fonseca, B. A. L. da. (2004). Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *The Brazilian Journal of Infectious Diseases : An Official Publication of the Brazilian Society of Infectious Diseases*. https://doi.org//S1413-86702004000600002

Dejnirattisai, W., Supasa, P., Wongwiwat, W., Rouvinski, A., Barba-Spaeth, G., Duangchinda, T., Sakuntabhai, A., Cao-Lormeau, V.-M., Malasit, P., Rey, F. A., Mongkolsapaya, J., & Screaton, G. R. (2016). Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nature Immunology*, *17*(9), 1102–1108. https://doi.org/10.1038/ni.3515

Dempsey, L. A., Fehervari, Z., & Visan, I. (2017). Written by CyTOF analysis of anti-tumor responses. *Nature Publishing Group*, *18*. https://doi.org/10.1038/nm.4267

Dhimal, M., Ahrens, B., & Kuch, U. (2015). Climate change and spatiotemporal distributions of vector-borne diseases in Nepal - A systematic synthesis of literature. *PLoS ONE*, *10*(6), 1–31. https://doi.org/10.1371/journal.pone.0129869

Durbin, A. P., Vargas, M. J., Wanionek, K., Hammond, S. N., Gordon, A., Rocha, C., Balmaseda, A., & Harris, E. (2008). Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. *Virology*, *376*(2), 429–435. https://doi.org/10.1016/j.virol.2008.03.028

Dussart, P., Petit, L., Labeau, B., Bremand, L., Leduc, A., Moua, D., Matheus, S., & Baril, L. (2008). Evaluation of two new commercial tests for the diagnosis of acute dengue virus infection using NS1 antigen detection in human serum. *PLoS Neglected Tropical Diseases*. https://doi.org/10.1371/journal.pntd.0000280

Dwivedi, V. D., Tripathi, I. P., Tripathi, R. C., Bharadwaj, S., & Mishra, S. K. (2017). Genomics, proteomics and evolution of dengue virus. *Brifings in Functional Genomics*. https://doi.org/10.1093/bfgp/elw040

EDCD, E. and D. control division. (2019). Prevention, ManageMent and Control of dengue in nePal 2019 government of nepal Ministry of Health and Population department of Health Services. *National Guidlines*.

Endy, T. P., Nisalak, A., Chunsuttiwat, S., Libraty, D. H., Green, S., Rothman, A. L., Vaughn, D. W., & Ennis, F. A. (2002). Spatial and Temporal Circulation of Dengue Virus Serotypes: A Prospective Study of Primary School Children in Kamphaeng Phet, Thailand. *American Journal of Epidemiology*, *156*(1), 52–59. https://doi.org/10.1093/AJE/KWF006

Faheem, M., Raheel, U., Riaz, M. N., Kanwal, N., Javed, F., Zaidi, N. us S. S., & Qadri, I. (2011). A molecular evaluation of dengue virus pathogenesis and its latest vaccine strategies. *Molecular Biology Reports*, *38*(6), 3731–3740. https://doi.org/10.1007/s11033-010-0488-1

Fernandez, S., Shakya, G., Nisalak, A., Upreti, S. R., Dumre, S. P., KC, K., Gibbons, R. V., Na-Bangchang, K., Rudi Grams, H., Ghimire, P., & Eursitthichai, V. (2013). Dengue

Virus and Japanese Encephalitis Virus Epidemiological Shifts in Nepal: A Case of Opposing Trends. *The American Journal of Tropical Medicine and Hygiene*, *88*(4), 677–680. https://doi.org/10.4269/ajtmh.12-0436

Flamand, C., Fritzell, C., Prince, C., Abboud, P., Ardillon, V., Carvalho, L., Demar, M., Boukhari, R., Papaix-Puech, M., Elenga, N., Rousset, D., Matheus, S., Nacher, M., Quenel, P., & Djossou, F. (2017). Epidemiological assessment of the severity of dengue epidemics in French Guiana. *Plus One*. https://doi.org/10.1371/journal.pone.0172267

Gebhard, L. G., Filomatori, C. V., & Gamarnik, A. V. (2011). Functional RNA elements in the dengue virus genome. *Viruses*. https://doi.org/10.3390/v3091739

Gubler, D. J. (1997). Epidemic Dengue and Dengue Hemorrhagic Fever: a Global Public Health Problem in the 21st Century. *Emerging Infections 1*, *21*, 1–14. https://doi.org/10.1128/9781555816940.ch1

Gupta, B. P., Lamsal, M., Chaulagain, S., Rauniyar, R., Malla, R., Shrestha, S., Kurmi, R., & Das Manandhar, K. (2018). Emergence of dengue in Nepal. In *VirusDisease*. https://doi.org/10.1007/s13337-018-0439-3

Gupta, B. P., Singh, S., Kurmi, R., Malla, R., Sreekumar, E., & Manandhar, K. Das. (2015a). Re-emergence of dengue virus serotype 2 strains in the 2013 outbreak in Nepal. *Indian Journal of Medical Research*. https://doi.org/10.4103/0971-5916.176564

Gupta, B. P., Singh, S., Kurmi, R., Malla, R., Sreekumar, E., & Manandhar, K. Das. (2015b). Re-emergence of dengue virus serotype 2 strains in the 2013 outbreak in Nepal. *The Indian Journal of Medical Research*, *142 Suppl*(Suppl 1), 1–6. https://doi.org/10.4103/0971-5916.176564

Gupta, B. P., Tuladhar, R., Kurmi, R., & Manandhar, K. Das. (2018). Dengue periodic outbreaks and epidemiological trends in Nepal. *Annals of Clinical Microbiology and Antimicrobials*, *17*(1). https://doi.org/10.1186/S12941-018-0258-9

Guzman, M. G., Hermida, L., Bernardo, L., Ramirez, R., & Guillén, G. (2010). Domain III of the envelope protein as a dengue vaccine target. *Expert Review of Vaccines*, *9*(2), 137–147. https://doi.org/10.1586/erv.09.139

Guzmán, M. G., & Kourí, G. (2004a). Dengue diagnosis, advances and challenges. *International Journal of Infectious Diseases : IJID : Official Publication of the International Society for Infectious Diseases*, *8*(2), 69–80. https://doi.org/10.1016/J.IJID.2003.03.003

Guzmán, M. G., & Kourí, G. (2004b). Dengue diagnosis, advances and challenges. In *International Journal of Infectious Diseases*. https://doi.org/10.1016/j.ijid.2003.03.003

Hadinegoro, S. R. S. (2012). The revised WHO dengue case classification: does the system need to be modified? *Paediatrics and International Child Health*, *32*(s1), 33. https://doi.org/10.1179/2046904712Z.00000000052

Halstead, S. (2008). *Dengue*.

Hang, V. T., Nguyet, N. M., Trung, D. T., Tricou, V., Yoksan, S., Dung, N. M., Ngoc, T. Van, Hien, T. T., Farrar, J., Wills, B., & Simmons, C. P. (2009). Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses. *PLoS Neglected Tropical Diseases*. https://doi.org/10.1371/journal.pntd.0000360

Henchal, E. A., & Putnak, J. R. (1990). The dengue viruses. *Clinical Microbiology Reviews*.

Hernández-Suárez, C. M., & Mendoza-Cano, O. (2016). Empirical evidence of the effect of school gathering on the dynamics of dengue epidemics. *Global Health Auction*

Vol-9, *9*(1). https://doi.org/10.3402/GHA.V9.28026

Hotta, S. (1952). Experimental Studies on DengueI. Isolation, Identification and Modification of the Virus. *The Journal of Infectious Diseases*, *90*(1), 1–9. https://doi.org/10.1093/INFDIS/90.1.1

Huang, C. H., Tsai, Y. Te, Wang, S. F., Wang, W. H., & Chen, Y. H. (2021). Dengue vaccine: an update. *Expert Review of Anti-Infective Therapy*, *19*(12), 1495–1502. https://doi.org/10.1080/14787210.2021.1949983

Huang, K.-J., Yang, Y.-C., Lin, Y.-S., Liu, H.-S., Yeh, T.-M., Chen, S.-H., Liu, C.-C., & Lei, H.-Y. (2005). Flow Cytometric Determination for Dengue Virus-Infected Cells: Its Application for Antibody-Dependent Enhancement Study. *Dengue Bulletin*, *29*.

Hunsperger, E. A., Yoksan, S., Buchy, P., Nguyen, V. C., Sekaran, S. D., Enria, D. A., Pelegrino, J. L., Vázquez, S., Artsob, H., Drebot, M., Gubler, D. J., Halstead, S. B., Guzmán, M. G., Margolis, H. S., Nathanson, C.-M., Rizzo Lic, N. R., Bessoff, K. E., Kliks, S., & Peeling, R. W. (2009). Evaluation of Commercially Available Anti-Dengue Virus Immunoglobulin M Tests. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid1503.080923

Huntington, M., Allison, J., & Nair, D. (2016). Emerging Vector-Borne Diseases. *American Family Physician*.

Idrees, S., & Ashfaq, U. A. (2012). A brief review on dengue molecular virology, diagnosis, treatment and prevalence in Pakistan. *Genetic Vaccines and Therapy*, *10*(1), 1. https://doi.org/10.1186/1479-0556-10-6

Imad, H. A., Phumratanaprapin, W., Phonrat, B., Chotivanich, K., Charunwatthana, P., Muangnoicharoen, S., Khusmith, S., Tantawichien, T., Phadungsombat, J., Nakayama, E., Konishi, E., & Shioda, T. (2020). Cytokine Expression in Dengue Fever and Dengue Hemorrhagic Fever Patients with Bleeding and Severe Hepatitis. *The American Journal of Tropical Medicine and Hygiene*, *102*(5), 943–950. https://doi.org/10.4269/ajtmh.19-0487

Janeway, C. A. (2000). How the immune system works to protect the host from infection: A personal view. In *National Academy of Sciences*. www.pnas.orgcgidoi10.1073pnas.131202998

Jessie, K., Fong, M. Y., Devi, S., Lam, S. K., & Wong, K. T. (2004). Localization of Dengue Virus in Naturally Infected Human Tissues, by Immunohistochemistry and In Situ Hybridization. *The Journal of Infectious Diseases*, *189*(8), 1411–1418. https://doi.org/10.1086/383043

Katzelnick, L. C., Coloma, J., & Harris, E. (2017). Dengue: Knowledge gaps, unmet needs and research priorities. *The Lancet. Infectious Diseases*, *17*(3), e88. https://doi.org/10.1016/S1473-3099(16)30473-X

King, C. A., Wegman, A. D., & Endy, T. P. (2021). Mobilization and Activation of the Innate Immune Response to Dengue Virus. *Frontiers in Cellular and Infectious Microbiology*. https://doi.org/10.3389/fcimb.2020.574417

Konstantinou, G. N. (2017). Enzyme-linked immunosorbent assay (ELISA). In *Methods in Molecular Biology*. https://doi.org/10.1007/978-1-4939-6925-8_7

Kumar, C Santosh, Sharma, S. (2016). Dengue Virus Infection. *The National Medical Journal of India*. https://nmji.in/view-pdf/?article=e8f0aac1c5161ea53273374e0177a5520msypQ==

Kuno, G., Gómez, I., & Gubler, D. J. (1991). An ELISA procedure for the diagnosis of dengue infections. *Journal of Virological Methods*.

Kuno, G., Gubler, D. J., Valez, M., & Oliver4, & A. (1985). Comparative sensitivity of three

mosquito cell lines for isolation of dengue viruses*. *Bulletin Ofthe World Health Organization*, *63*(2), 279–286.

Kyle, J. L., & Harris, E. (2008). Global Spread and Persistence of Dengue. *Annual Review of Microbiology*, *62*(1), 71–92. https://doi.org/10.1146/annurev.micro.62.081307.163005

Lahariya, C. (2016). Vaccine epidemiology: A review. *Journal of Family Medicine and Primary Care*, *5*(1), 7. https://doi.org/10.4103/2249-4863.184616

Lin, A. V. (2015). Indirect ELISA. *Methods in Molecular Biology*. https://doi.org/10.1007/978-1-4939-2742-5_5

Mackenzie John, S, D. J. G. & L. R. P. (2004). Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nature Medicine*. https://doi.org/10.1038/nm1144

Malla, S., Thakur, G. D., Shrestha, S. K., Banjeree, M. K., Thapa, L. B., Gongal, G., Ghimire, P., Upadhyay, B. P., Gautam, P., Khanal, S., Nisaluk, A., Jarman, R. G., & Gibbons, R. V. (2008a). Identification of all dengue serotypes in Nepal. In *Emerging Infectious Diseases*. https://doi.org/10.3201/eid1410.080432

Malla, S., Thakur, G. D., Shrestha, S. K., Banjeree, M. K., Thapa, L. B., Gongal, G., Ghimire, P., Upadhyay, B. P., Gautam, P., Khanal, S., Nisaluk, A., Jarman, R. G., & Gibbons, R. V. (2008b). Identification of All Dengue Serotypes in Nepal. *Emerging Infectious Diseases*, *14*(10), 1669. https://doi.org/10.3201/EID1410.080432

Marcial-Juárez, E., Yam-Puc, J. C., Cedillo-Barrón, L., García-Cordero, J., Calderón-Amador, J., Maqueda-Alfaro, R. A., Ruiz-Tovar, K., Covarrubias, N. B., Orozco-Uribe, M., Escobar-Gutiérrez, A., & Flores-Romo, L. (2017). Travelling with Dengue: From the Skin to the Nodes. In *Dengue - Immunopathology and Control Strategies*. InTech. https://doi.org/10.5772/intechopen.68338

Modis, Y., Ogata, S., Clements, D., & Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature*. https://doi.org/10.1038/nature02165

Mukhopadhyay, S., Kuhn, R. J., & Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. *Nature Reviews Microbiology 2005 3:1*, *3*(1), 13–22. https://doi.org/10.1038/nrmicro1067

Murugesan, A., & Manoharan, M. (2020). Dengue Virus. *Emerging and Reemerging Viral Pathogens*, 281. https://doi.org/10.1016/B978-0-12-819400-3.00016-8

Mustafa, M. S., Rasotgi, V., Jain, S., & Gupta, V. (2015). Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. *Medical Journal, Armed Forces India*, *71*(1), 67. https://doi.org/10.1016/J.MJAFI.2014.09.011

Ng, W. C., Soto-Acosta, R., Bradrick, S. S., Garcia-Blanco, M. A., & Ooi, E. E. (2017). The 5′ and 3′ Untranslated Regions of the Flaviviral Genome. *Viruses*, *9*(6). https://doi.org/10.3390/V9060137

Organji, S. R., Abulreesh, H. H., & Osman, G. E. H. (2017). Circulation of Dengue Virus Serotypes in the City of Makkah, Saudi Arabia, as Determined by Reverse Transcription Polymerase Chain Reaction. *Canadian Journal of Infectious Diseases and Medical Microbiology*, *2017*. https://doi.org/10.1155/2017/1646701

Pandey, B D, Rai, S. K., Morita, K., & Kurane, I. (2004). First case of Dengue virus infection in Nepal. *Nepal Medical College Journal: NMCJ*, *6*(2), 157–159.

Pandey, Basu D., Nabeshima, T., Pandey, K., Rajendra, S. P., Shah, Y., Adhikari, B. R., Gupta, G., Gautam, I., Tun, M. M. N., Uchida, R., Shrestha, M., Kurane, I., & Morita,

K. (2013). First Isolation of Dengue Virus from the 2010 Epidemic in Nepal. *Tropical Medicine and Health*, *41*(3), 103–111. https://doi.org/10.2149/tmh.2012-17

Pandey, Basu Dev, Morita, K., Khanal, S. R., Takasaki, T., Miyazaki, I., Ogawa, T., Inoue, S., & Kurane, I. (2008). Dengue Virus, Nepal. *Emerging Infectious Diseases*. https://doi.org/10.3201/eid1403.070473

Pandey, Basu Dev, Rai, S. K., Morita, K., & Kurane, I. (2004). First case of Dengue virus infection in Nepal. *Nepal Med Coll J*.

Perera, R., & Kuhn, R. J. (2008). Structural proteomics of dengue virus. In *Current Opinion in Microbiology*. https://doi.org/10.1016/j.mib.2008.06.004

Phommanivong, V., Kanda, S., Shimono, T., Lamaningao, P., Darcy, A. W., Mishima, N., Phaytanavanh, B., & Nishiyama, T. (2016). Co-circulation of the dengue with chikungunya virus during the 2013 outbreak in the southern part of Lao PDR. *Tropical Medicine and Health*, *44*(1), 1–10. https://doi.org/10.1186/S41182-016-0020-Y/FIGURES/4

Piovezan, R., Visockas, A., De Azevedo, T. S., Von Zuben, C. J., & Sallum, M. A. M. (2019). Spatialoral distribution of Aedes (Stegomyia) aegypti and locations of recycling units in southeastern Brazil. *Parasites and Vectors*, *12*(1), 1–13. https://doi.org/10.1186/S13071-019-3794-Z/FIGURES/7

Poudyal, P., Sharma, K., Dumre, S. P., Bastola, A., Chalise, B. S., Shrestha, B., Poudel, A., Giri, A., Bhandari, P., Shah, Y., Poudel, R. C., Khadka, D., Maharjan, J., Ngwe Tun, M. M., Morita, K., Pandey, B. D., & Pandey, K. (2021). Molecular study of 2019 dengue fever outbreaks in Nepal. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *115*(6), 619–626. https://doi.org/10.1093/TRSTMH/TRAA096

Prajapati, S., Napit, R., Bastola, A., Rauniyar, R., Shrestha, S., Lamsal, M., Adhikari, A., Bhandari, P., Ray Yadav, Sanjay, & Manandhar, K. Das. (2020). Molecular phylogeny and distribution of dengue virus serotypes circulating in Nepal in 2017. *PLoS ONE*, *15*(7 July). https://doi.org/10.1371/journal.pone.0234929

Prajapati, S., Napit, R., Bastola, A., Rauniyar, R., Shrestha, S., Lamsal, M., Adhikari, A., Bhandari, P., Ray Yadav, S., & Das ManandharID, K. (2020). Molecular phylogeny and distribution of dengue virus serotypes circulating in Nepal in 2017. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0234929

Pun, S. B. (2011). Dengue: An emerging disease in Nepal. In *Journal of the Nepal Medical Association*.

Rajapakse, S. (2011). Dengue shock. *Journal of Emergencies, Trauma and Shock*, *4*(1), 120. https://doi.org/10.4103/0974-2700.76835

Ramos-Castañeda, J., Barreto dos Santos, F., Martínez-Vega, R., Galvão de Araujo, J. M., Joint, G., & Sarti, E. (2017). Dengue in Latin America: Systematic Review of Molecular Epidemiological Trends. *PLOS Neglected Tropical Diseases*, *11*(1), e0005224. https://doi.org/10.1371/JOURNAL.PNTD.0005224

Rijal, K. R., Adhikari, B., Ghimire, B., Dhungel, B., Pyakurel, U. R., Shah, P., Bastola, A., Lekhak, B., Banjara, M. R., Pandey, B. D., Parker, D. M., & Ghimire, P. (2021). Epidemiology of dengue virus infections in Nepal, 2006–2019. *Infectious Diseases of Poverty*, *10*(1), 1–10. https://doi.org/10.1186/S40249-021-00837-0/TABLES/1

Rodenhuis-Zybert, I. A., Wilschut, J., & Smit, J. M. (2010). Dengue virus life cycle: Viral and host factors modulating infectivity. In *Cellular and Molecular Life Sciences*. https://doi.org/10.1007/s00018-010-0357-z

Rothman, A. L. (2011). Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nature Publishing Group*. https://doi.org/10.1038/nri3014

Saito, M., Tamayose, M., Miyagi, K., Takaragawa, H., Tateyama, M., Tadano, M., & Fujita, J. (2016). Serologic and Virologic Studies of an Imported Dengue Case Occurring in 2014 in Okinawa, Japan. *Japanese Journal of Infectious Diseases*, *69*(1), 60–65. https://doi.org/10.7883/YOKEN.JJID.2015.061

Samsa, M. M., Mondotte, J. A., Iglesias, N. G., Assunção-Miranda, I., Barbosa-Lima, G., Da Poian, A. T., Bozza, P. T., & Gamarnik, A. V. (2009). Dengue virus capsid protein usurps lipid droplets for viral particle formation. *PLoS Pathogens*. https://doi.org/10.1371/journal.ppat.1000632

Sandwich ELISA. (2011). In *Encyclopedia of Cancer*. https://doi.org/10.1007/978-3-642-16483-5_5155

Santos, A. C. M. dos, de Moura, E. L., Ferreira, J. M., de Moura, A. W. A., Ferreira, A. D. S., Bezerra, R. P., de Siqueira Figueiredo, D., de Farias, K. F., de Andrade, T. G., & de Souza Figueiredo, E. V. M. (2017). Association of TNFA (−308G/A), IFNG (+874 A/T) and IL-10 (−819 C/T) polymorphisms with protection and susceptibility to dengue in Brazilian population. *Asian Pacific Journal of Tropical Medicine*, *10*(11), 1065–1071. https://doi.org/10.1016/j.apjtm.2017.10.009

Screaton, G., Mongkolsapaya, J., Yacoub, S., & Roberts, C. (2015). New insights into the immunopathology and control of dengue virus infection. In *Nature Reviews Immunology*. https://doi.org/10.1038/nri3916

Seema, & Jain, S. K. (2005). MOLECULAR MECHANISM OF PATHOGENESIS OF DENGUE VIRUS " ENTRY AND FUSION WITH TARGET CELL. *Indian Journal of Clinical Biochemistry*, *20*(2), 92–103.

Sekhar, A., & Kang, G. (2020). Human challenge trials in vaccine development. *Seminars in Immunology*, *50*, 101429. https://doi.org/10.1016/J.SMIM.2020.101429

Seltenrich, N. (2016). Singapore Success: New Model Helps Forecast Dengue Outbreaks. *Environmental Health Perspectives*, *124*(9), A167. https://doi.org/10.1289/EHP.124-A167

Shim, E. (2016). Dengue Dynamics and Vaccine Cost-Effectiveness Analysis in the Philippines. *Am. J. Trop. Med. Hyg*, *95*(5), 1137–1147. https://doi.org/10.4269/ajtmh.16-0194

Siddiqi, Z., Fatima, J., Karoli, R., Jagirdar, S., Arora, R., & Soni, R. (2019). A STUDY OF TYPICALAND ATYPICAL CLINICAL MANIFESTATIONS IN DENGUE PATIENTS. *Era's Journal of Medical Research*, *6*(2), 57–62. https://doi.org/10.24041/EJMR2019.130

Sim, S., & Hibberd, M. L. (2016). Genomic approaches for understanding dengue: Insights from the virus, vector, and host. In *Genome Biology*. https://doi.org/10.1186/s13059-016-0907-2

Singh, S., Gupta, B. P., Manakkadan, A., Manandhar, K. Das, & Sreekumar, E. (2015). Phylogenetic study reveals co-circulation of Asian II and Cosmopolitan genotypes of Dengue virus serotype 2 in Nepal during 2013. *Infection, Genetics and Evolution*, *34*, 402–409. https://doi.org/10.1016/j.meegid.2015.07.006

St. John, A. L. (2013). Influence of Mast Cells on Dengue Protective Immunity and Immune Pathology. *PLoS Pathogens*, *9*(12), 1–4. https://doi.org/10.1371/JOURNAL.PPAT.1003783

St John, A. L. (2005). Influence of Mast Cells on Dengue Protective Immunity and Immune Pathology. *PLoS Pathogens*. https://doi.org/10.1371/journal.ppat.1003783

Stanaway, J. D., Shepard, D. S., Undurraga, E. A., Halasa, Y. A., Coffeng, L. E., Brady, O. J., Hay, S. I., Bedi, N., Bensenor, I. M., Castañeda-Orjuela, C. A., Chuang, T. W., Gibney, K. B., Memish, Z. A., Rafay, A., Ukwaja, K. N., Yonemoto, N., & Murray, C. J. L.

(2016). The Global Burden of Dengue: an analysis from the Global Burden ofDisease Study 2013. *The Lancet. Infectious Diseases*, *16*(6), 712. https://doi.org/10.1016/S1473-3099(16)00026-8

Stephens, H. A. F. (2009). HLA and other gene associations with dengue disease severity. In *Current Topics in Microbiology and Immunology*. https://doi.org/10.1007/978-3-642-02215-9-8

Suzarte, E., Gil, L., Valdés, I., Marcos, E., Lazo, L., Izquierdo, A., García, A., López, L., Álvarez, M., Pérez, Y., Castro, J., Romero, Y., Guzmán, M. G., Guillén, G., & Hermida, L. (2015). A novel tetravalent formulation combining the four aggregated domain III-capsid proteins from dengue viruses induces a functional immune response in mice and monkeys. *International Immunology*, *27*(8), 367–379. https://doi.org/10.1093/intimm/dxv011

Takasaki, T., Kotaki, A., Nishimura, K., Sato, Y., Tokuda, A., Lim, C. K., Ito, M., Tajima, S., Nerome, R., & Kurane, I. (2008). Dengue virus type 2 isolated from an imported dengue patient in Japan: First isolation of dengue virus from Nepal. *Journal of Travel Medicine*, *15*(1), 46–49. https://doi.org/10.1111/j.1708-8305.2007.00165.x

Tasso De OliveiraMota, M., Fernanda Estofolete, C., Zini, N., Terzian, A. C. B., Gongora, D. V. N., Maia, I. L., & Nogueira, M. L. (2017). Transverse Myelitis as an Unusual Complication of Dengue Fever. *The American Journal of Tropical Medicine and Hygiene*, *96*(2), 380–381. https://doi.org/10.4269/AJTMH.16-0284

Vázquez, S., Lemos, G., Pupo, M., Ganzón, O., Palenzuela, D., Indart, A., & Guzmán, M. G. (2003). Diagnosis of dengue virus infection by the visual and simple AuBioDOT immunoglobulin M capture system. *Clinical and Diagnostic Laboratory Immunology*, *10*(6), 1074–1077. https://doi.org/10.1128/CDLI.10.6.1074-1077.2003

Wang, S. M., & Sekaran, S. D. (2010). Evaluation of a commercial SD dengue virus NS1 antigen capture enzyme-linked immunosorbent assay kit for early diagnosis of dengue virus infection. *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.02142-09

Wasonga, C., Inoue, S., Kimotho, J., Morita, K., Ongus, J., Sang, R., & Musila, L. (2015). Development and evaluation of an in-house IgM-capture ELISA for the detection of Chikungunya and its application to a dengue outbreak situation in Kenya in 2013. *Japanese Journal of Infectious Diseases*. https://doi.org/10.7883/yoken.JJID.2014.482

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. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *9*(4), 523. https://doi.org/10.1016/J.MEEGID.2009.02.003

Wei, K., & Li, Y. (2017). Global evolutionary history and spatio-temporal dynamics of dengue virus type 2 OPEN. *Nature Publishing Group*. https://doi.org/10.1038/srep45505

Whitehead, S. S., Blaney, J. E., Durbin, A. P., & Murphy, B. R. (2007a). Prospects for a dengue virus vaccine. *Nature Reviews Microbiology*, *5*(7), 518–528. https://doi.org/10.1038/NRMICRO1690

Whitehead, S. S., Blaney, J. E., Durbin, A. P., & Murphy, B. R. (2007b). Prospects for a dengue virus vaccine. In *Nature Reviews Microbiology* (Vol. 5, Issue 7, pp. 518–528). https://doi.org/10.1038/nrmicro1690

WHO, W. H. O. (2009). *DENGUE GUIDELINES FOR DIAGNOSIS, TREATMENT, PREVENTION*

AND CONTROL TREATMENT, PREVENTION AND CONTROL TREATMENT, PREVENTION AND CONTROL. www.who.int/tdr

WHO, W. H. O. (2021). *Promoting dengue vector surveillance and control*. Dengue Bulletin, Vol-41. https://www.who.int/activities/promoting-dengue-vector-surveillance-and-control

Wilder-Smith, A. (2019). Dengue vaccine development: status and future. *Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz*, *63*, 40–44. https://doi.org/10.1007/s00103-019-03060-3

Wilder-Smith, A., Ooi, E. E., Vasudevan, S. G., & Gubler, D. J. (2010). Update on dengue: Epidemiology, virus evolution, antiviral drugs, and vaccine development. *Current Infectious Disease Reports*. https://doi.org/10.1007/s11908-010-0102-7

World Health Organisation. (2009). Dengue: guidelines for diagnosis, treatment, prevention, and control. In *Special Programme for Research and Training in Tropical Diseases*. https://doi.org/WHO/HTM/NTD/DEN/2009.1

World Health Organisation. (2020). *Dengue and severe dengue*. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

World Health Organization. Dengue Hemorrhagic Fever: Diagnosis, Treatment, Prevention and Control 2nd edn (WHO Press, 1997). - Google Search. (n.d.).

World Health Organization. (2009). Dengue: guidelines for diagnosis, treatment, prevention, and control. *Special Programme for Research and Training in Tropical Diseases*, x, 147. https://doi.org/WHO/HTM/NTD/DEN/2009.1

World Health Organization (WHO) and the Special Programme for Research and Tropical Diseases (TDR). (2009). Dengue Guidelines for diagnosis, tretment, prevention and control. *Prevention and Control*, 147.

Yung, C.-F., Lee, K.-S., Thein, T.-L., Tan, L.-K., Gan, V. C., Wong, J. G. X., Lye, D. C., Ng, L.-C., & Leo, Y.-S. (2015). Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, singapore. *The American Journal of Tropical Medicine and Hygiene*, *92*(5), 999–1005. https://doi.org/10.4269/ajtmh.14-0628

Yung, C. F., Lee, K. S., Thein, T. L., Tan, L. K., Gan, V. C., Wong, J. G. X., Lye, D. C., Ng, L. C., & Leo, Y. S. (2015). Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, Singapore. *American Journal of Tropical Medicine and Hygiene*. https://doi.org/10.4269/ajtmh.14-0628

Zellweger, R. M., Shresta, S., & DeHaven Dunkle, A. (2014). *Mouse models to study dengue virus immunology and pathogenesis*. https://doi.org/10.3389/fimmu.2014.00151

Zompi, S., & Harris, E. (2012). Animal Models of Dengue Virus Infection. *Viruses*, *4*, 62–82. https://doi.org/10.3390/v4010062

Appendices

Appendix 1.1 Ethical Approval for sample collection.

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 394

8 August 2019

Mr. Ramanuj Rauniyar
Principal Investigator
Central Department of Biotechnology, Tribhuvan University
Kathmandu

Ref: **Approval of thesis proposal** entitled **Role of Selective immunological markers for Dengue Severity in Nepal**

Dear Mr. Rauniyar,

It is my pleasure to inform you that the above-mentioned proposal submitted on **18 February 2019** (**Reg. no. 121/2019**) please use this Reg. No. during further correspondence) has been approved by Nepal Health Research Council (NHRC) Ethical Review Board on **24 July 2019**.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol. Expiration date of this proposal is **January 2021.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their project proposal and **submit progress report in between and full or summary report upon completion.**

As per your thesis proposal, the total research amount is **Rs 8,48,000** and accordingly the processing fee amounts to **Rs 10,000.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

Prof. Dr. Anjani Kumar Jha
Executive Chairperson

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

Appendix 1.2 Consent form for sample collection

अनुसन्धान अध्ययनमा सहभागिता जनाउने मञ्जुरीनामा फाराम

परियोजना शिर्षक : Role of selective immunological markers for dengue severity in Nepal

डेंगु एक लामखुट्टेबाट सर्ने भाइरल रोग हो । डेंगु भाइरस मुख्यत : Aedes aegypti र Aedes albopictus प्रजातिका पोथी लामखुट्टेहरुको टोकाइबाट सर्ने गर्छ । यो रोगको प्रकोप प्राय: गर्मी एवम् उच्च आद्रता र अव्यवस्थित बसोबास भएको क्षेत्रमा बढी देखिन्छ । यस अध्ययनको प्रमुख उद्देश्य नेपालमा फैलिएको डेंगु ज्वरोबारे विस्तृत अध्ययन गर्नु हो । यो अनुसन्धान विभागमा संचालित डेंगु सम्वन्धि राष्ट्रिय तथा अन्तराष्ट्रिय परियोजनाहरु र स्नातकोत्तर तह पूरा गर्नका लागि गर्न लागिएको एक अनुसन्धान हो । तपाईलाई यस अध्ययनमा सहभागी गराउनुको उद्देश्य तपाईबाट संकलित रगतका नमूनामा डेंगु भाइरसका विविध प्रकारहरुको परीक्षण र पुष्टि गर्नुका साथै विविध साइटोकाइन उत्पादनसम्वन्धि अध्ययन गर्नु हो ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाई वा तपाईको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ । यस अध्ययनमा हुने विभिन्न परिक्षणमध्ये डेंगु भाइरसका विविध प्रकारहरुको PCR एवम् ELISA मार्फत वर्गीकरण गरिन्छ जुन नि:शुल्क गरिनेछ र यसले रोगको निदान गर्न मद्दत गर्न सक्छ । यसका अतिरिक्त विविध साइटोकाइन उत्पादन र रोग प्रतिरोधात्मक क्षमता अध्ययनमा पनि यसबाट सहयोग पुग्नेछ जुन भविष्यमा यस रोगसम्वन्धि रोकथामका उपाय खोज्न पनि महत्त्वपूर्ण हुन सक्छ ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको नाम तथा परिचय उल्लेख हुने छैन ।

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैले आफ्नो इच्छाले विना जरिवाना, विना डरत्रास, अनुसन्धानकर्तासमक्ष पूर्व सूचना विनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ । मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु ।

यदि तपाई यस अध्ययनमा सहभागी हुन सहमत हुनुहुन्छ भने तपाईले यस अनुसन्धानको स्वयंसेवकको रुपमा आफ्नो ३-५ मि.लि. रगत दिनुपर्नेछ । तपाइको मेडिकल इतिहास र रगत जांचका प्रयोगशाला परिक्षणका विवरणहरु हामी संकलन र तथ्याङ्क प्रयोग गर्नेछौं । सुरुमा तपाईबाट ३ - ५ मि.लि.रगतको नमुना लिइने छ र दोस्रोपटक रोगको लक्षण देखा परेको ३ - ५ हप्ता भित्र रगतको नमुना लिइने छ । यदि तपाईले यस मञ्जुरीनामामा सही गर्नुभएमा तपाईबाट भविष्यमा एक वा सोभन्दा बढीमा दुई पटक सम्म रगतको नमुना लिन सकिने छ । तपाईबाट लिइएको जैविक पदार्थलाई Central Department of Biotechnology, TU को प्रयोगशालामा PCR, ELISA, Flow Cytometer जस्ता अत्याधुनिक उपकरणहरुको प्रयोग गरि अध्ययन गरिन्छ र प्रश्नपत्र प्रयोग गरेर पनि तथ्याङ्क निकालिन्छ । तपाईबाट लिइएको रगतको नमुना र यसबाट आएको तथ्याङ्क प्रयोग गरिनेछ । तपाईलाई यस अध्ययनमा सहभागी हुन कुनै शुल्क लाग्ने छैन र यसबाट हामीलाई कुनै आर्थिक फाइदा हुने छैन न त व्यापारिकरण गरिनेछ

१. जैविक नमुना र त्यससम्वन्धि तथ्याङ्क डेंगुलगायत डेंगुजस्ता रोगको परीक्षण र त्यस रोगको उपचारका लागि आवश्यक अनुसन्धानकार्यमा प्रयोग गरिने छ ।

२. भविष्यमा यस्तै अन्य अनुसन्धानका लागि मलाई सम्पर्क गर्न सकिने छ ।

हुन्छ   हुँदैन

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा तपाइको सहभागिता स्वेच्छिक हो । तपाईले आफ्नो इच्छाले जरिवाना र डरत्रास विना कुनै पनि समयमा यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नुहुनेछ ।

मैले माथि लेखिएका कुराहरु पढेको छु वा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ। मेरो प्रश्नहरुको जवाफ दिइएको छ र मैले आफ्नो इच्छाले यस फाराममा सही गरेको छु।

यदि तपाई यस अध्ययनमा सहभागी हुन सहमत हुनुहुन्छ भने तल हस्ताक्षर गर्नुहोला।

....	....
मिति	बिरामीको नाम
....	....
ठेगाना :	सम्पर्क नं
....	....
मिति	साक्षीको नाम

(यस अनुसन्धानसम्बन्धि सम्पूर्ण विवरण सहभागिहरुलाई विस्तृत रुपमा बुझाइएको कुरा विश्वस्त गर्न चाहन्छु।)

....	....
मन्जुरी लिनेको नाम	सम्पर्क नं
....	....
मिति	हस्ताक्षर

Appendix 1.3 Case Report Form (CRF) Form

Form No:-......................

Name of Hospital, Place:- .... **Bed no:-** ....

Subject ID:- .... **Site ID:-** .... **Lab ID:-** ....

PATIENT INFORMATION

Home:-... **Current Address:-** ....

Age:- .... **Height:-**..

Weight:-..................................... **Sex:-** Male Female Others

Travel History in 3 weeks:- Yes .... No **Previous history of dengue:-** Yes .... No

Date of onset of fever:- .... **Date of admission:-** ....

Fever:- Intermittent Continuous Remittent ....

PRESENTING SYMPTOMS

Hemorrhagic manifestations:- Yes NO
Severe bleeding:- :- Yes NO
Typeof haemorrhage:- Petechiae Purpura Echymosis Epistaxis Gum bleeding Hematesis Melena
Rash:- :- Yes NO
Itching sensation:- :- Yes NO
Burning/Piercing sensation:- Yes NO
(Mention the whole body/body parts) ....
Shock:- :- Yes NO
Headache:- Yes NO
Nausea:- :- Yes NO
Vomiting:- :- Yes NO
Abdominal pain:- :- Yes NO
Muscles pain:- Yes NO
Lethargy:- Yes NO
Joint pain:- :- Yes NO **Large Small Both** ....
Clinical fluid accumulation:- Yes NO
Respiratory distress:- Yes NO
Impaired consciousness:- :- Yes NO
Organ failure:- Yes NO
Condition of patient:- Stable Critical

CLINICAL COURSE

HE grade at the time of admission:- ....
Condition of patient:- Stable Critical
IV administered:- :- Yes NO
Blood/Platelet/Plasma transfusion given:- Yes NO
Ultrasonography done:- Yes NO Liver/ Spleen/ Gall bladder/ Abdomen/ Kidney

Remarks:- ....

Appendix 2.1 Sample Details regarding site, year of collection and RDT test results

S.N	Sample ID	Sex	Age (yrs)	Address	RDT	Year
1	Nep-1	M	48	Dhading	Positive	2017
2	Nep-2	M	18	Dhading	Positive	2017
3	Nep-3	M	55	Nawalpur	Positive	2017
4	Nep-4	M	38	Rupandehi	Positive	2017
5	Nep-5	M	30	Hetauda	Positive	2017
6	Nep-6	M	47	Jhapa	Positive	2017
7	Nep-7	M	50	Bardiya	Positive	2017
8	Nep-8	F	34	Dhading	Positive	2017
9	Nep-9	M	20	Sarlahi	Positive	2017
10	Nep-10	M	20	Dhading	Positive	2017
11	Nep-11	M	74	Dhading	Positive	2017
12	Nep-12	M	38	Nuwakot	Positive	2017
13	Nep-13	M	14	Sarlahi	Positive	2017
14	Nep-14	M	23	Sarlahi	Positive	2017
15	Nep-15	M	25	Sarlahi	Positive	2017
16	Nep-16	M	20	Sarlahi	Positive	2017
17	Nep-17	M	27	Nuwakot	Positive	2017
18	Nep-18	F	16	Sarlahi	Negative	2017
19	Nep-19	F	60	Kailali	Positive	2017
20	Nep-20	M	50	Rautahat	Positive	2017
21	Nep-21	M	35	Hetauda	Positive	2017
22	Nep-22	M	18	Birgunj	Positive	2017
23	Nep-23	F	27	Makwanpur	Positive	2017
24	Nep-24	F	23	Damauli	Positive	2017
25	Nep-25	M	27	Bharatpur	Positive	2017
26	Nep-26	M	20	Bharatpur	Positive	2017
27	Nep-27	M	13	Khairahani	Negative	2017
28	Nep-28	M	34	Butwal	Positive	2017
29	Nep-29	M	23	Butwal	Positive	2017
30	Nep-30	M	19	Rupandehi	Positive	2017
31	Nep-31	M	44	Kapilvastu	Negative	2017
32	Nep-32	M	35	Butwal	Positive	2017
33	Nep-33	F	20	Bhairahawa	Positive	2017
34	Nep-34	F	40	Butwal	Positive	2017
35	Nep-35	M	28	Bharatpur	Positive	2017
36	Nep-36	M	31	Rupakot	Positive	2017
37	Nep-37	M	38	Bharatpur	Positive	2017
38	Nep-38	F	45	Bharatpur	Positive	2017
39	Nep-39	M	18	Babargunj	Positive	2017

40	Nep-40	M	22	Haripur	Positive	2017
41	Nep-41	F	16	Deurali	Positive	2017
42	Nep-42	F	12	Bharatpur	Positive	2017
43	Nep-43	M	23	Butwal	Positive	2017
44	Nep-44	F	25	Butwal	Positive	2017
45	Nep-45	M	38	Dhading	Positive	2017
46	Nep-46	M	21	Kapilvastu	Positive	2017
47	Nep-47	M	53	Makwanpur	Positive	2017
48	Nep-48	M	42	Jhapa	Positive	2017
49	Nep-49	M	32	Sarlahi	Positive	2017
50	Nep-50	M	30	Sarlahi	Positive	2017
51	KTM/STH_27	M	28	Syangja	Positive	2018
52	KTM/STH_28	M	40	Kathmandu	Positive	2018
53	KTM/STH_29	F	35	Rautahat	Positive	2018
54	KTM/STH_30	M	30	Syangja	Positive	2018
55	KTM/STH_31	M	50	Dhading	Positive	2018
56	KTM/STH_32	F	28	Kathmandu	Negative	2018
57	KTM/STH_33	M	40	Dhading	Negative	2018
58	KTM/STH_34	M	30	Kathmandu	Negative	2018
59	KTM/STH_35	M	42	Chitwan	Negative	2018
60	KTM/STH_36	M	34	Kanchanpur	Negative	2018
61	KTM/STH_37	M	48	Udayapur	Negative	2018
62	KTM/STH_38	M	32	Ramechhap	Positive	2018
63	KTM/STH_39	M	34	Nuwakot	Positive	2018
64	KTM/STH_40	M	32	Kathmandu	Positive	2018
65	KTM/STH_41	F	22	Kathmandu	Negative	2018
66	KTM/STH_42	M	44	Kathmandu	Negative	2018
67	KTM/STH_43	M	26	Kathmandu	Negative	2018
68	KTM/STH_44	M	18	Kathmandu	Negative	2018
69	KTM/SH_1	M	32	Morang	Positive	2018
70	KTM/NOV_1	F	52	Pokhara	Positive	2018
71	KTM/GPC_01	M	54	Kathmandu	Positive	2018
72	KTM/GPC_02	F	35	Kathmandu	Positive	2018
73	KTM/GPC_03	M	32	Kathmandu	Negative	2018
74	KTM/GPC_04	M	52	Kathmandu	Positive	2018
75	KTM/GPC_05	F	23	Kathmandu	Positive	2018
76	STH_19_01	F	34	Kathmandu	Negative	2018
77	STH_19_02	M	34	Kathmandu	Negative	2018
78	STH_19_03	M	25	Kathmandu	Negative	2018
79	STH_19_04	M	48	Kathmandu	Negative	2018
80	STH_19_05	M	19	Kathmandu	Negative	2018
81	DHR/BH_01	F	36	Dharan	Positive	2019
82	DHR/BH_02	F	25	Dharan	Positive	2019

83	DHR/BH_03	M	40	Dharan	Positive	2019
84	DHR/BH_04	M	49	Dharan	Positive	2019
85	DHR/BH_05	F	19	Dharan	Positive	2019
86	DHR/BH_06	M	29	Dharan	Positive	2019
87	DHR/BH_07	F	58	Dharan	Positive	2019
88	DHR/BH_08	M	42	Dharan	Positive	2019
89	DHR/BH_09	F	15	Dharan	Positive	2019
90	DHR/BH_10	F	20	Dharan	Positive	2019
91	DHR/BH_11	M	16	Dharan	Positive	2019
92	DHR/BH_12	M	11	Dharan	Negative	2019
93	DHR/BH_13	F	20	Dharan	Negative	2019
94	DHR/BH_14	M	26	Dharan	Positive	2019
95	DHR/BH_15	F	55	Morang	Negative	2019
96	DHR/BH_16	F	8	Dharan-15	Negative	2019
97	DHR/BH_17	M	77	Dharan-15	Negative	2019
98	DHR/BH_18	F	51	Dharan-7	Positive	2019
99	DHR/BH_19	F	19	Dharan-15	Positive	2019
100	DHR/BH_20	F	28	Udayapur	Negative	2019
101	DHR/BH_21	F	31	Dharan-11	Negative	2019
102	DHR/BH_22	F	19	Dharan-8	Negative	2019
103	DHR/BH_23	M	11	Dharan-10	Positive	2019
104	DHR/BH_24	F	32	Dharan-15	Positive	2019
105	DHR/BH_25	M	18	Dharan-18	Negative	2019
106	DHR/BH_26	F	36	Dharan-18	Negative	2019
107	DHR/BH_27	M	3	Dharan	Negative	2019
108	DHR/BH_28	F	18	Dharan-15	Positive	2019
109	DHR/BH_29	F	65	Dharan-15	Positive	2019
110	DHR/BH_30	F	14	Dharan-7	Negative	2019
111	DHR/BH_31	F	3	Dharan-16	Negative	2019
112	DHR/BH_32	M	40	Dharan-9	Negative	2019
113	DHR/BH_33	M	22	Dharan-10	Negative	2019
114	DHR/BH_34	F	76	Dharan-15	Positive	2019
115	DHR/BH_35	F	18	Dharan	Positive	2019
116	DHR/BH_36	M	31	Dharan-19	Negative	2019
117	DHR/BH_37	M	11	Dharan-16	Negative	2019
118	DHR/BH_38	M	18	Dharan-15	Negative	2019
119	DHR/BH_39	M	28	Dharan- 19	Negative	2019
120	DHR/BH_40	F	26	Dharan-8	Negative	2019
121	DHR/BH_41	M	39	Dharan-16	Negative	2019
122	DHR/BH_42	M	10	Dharan-8	Negative	2019
123	DHR/BH_43	F	37	Dharan-15	Negative	2019
124	DHR/BH_44	F	15	Dharan-15	Positive	2019
125	DHR/BH_45	F	42	Dharan-2	Negative	2019

126	DHR/BH_46	M	9	Dharan-15	Negative	2019
127	DHR/BH_47	M	9	Dharan-8	Negative	2019
128	DHR/BH_48	F	44	Dharan-10	Negative	2019
129	DHR/BH_49	F	37	Dharan-12	Positive	2019
130	DHR/BH_50	M	27	Dharan-12	Negative	2019
131	DHR/BH_51	F	54	Dharan-15	Negative	2019
132	DHR/BH_52	M	17	Dharan-15	Negative	2019
133	DHR/BH_53	M	35	Dharan-15	Negative	2019
134	DHR/BH_54	M	73	Dharan-13	Negative	2019
135	DHR/BH_55	M	26	Morang	Negative	2019
136	DHR/BH_56	F	3	Dharan-15	Negative	2019
137	DHR/BH_57	M	6	Dharan-5	Negative	2019
138	DHR/BH_58	F	3	Dharan-15	Negative	2019
139	DHR/BH_59	M	90	Dharan-11	Negative	2019
140	DHR/BH_60	F	42	Dharan-19	Negative	2019
141	DHR/BH_61	F	70	Dharan-16	Negative	2019
142	DHR/BH_62	M	38	Dharan-3	Negative	2019
143	DHR/BH_63	F	15	Dharan-15	Negative	2019
144	DHR/BH_64	M	17	Dharan	Positive	2019
145	DHR/BH_65	F	25	Dharan	Positive	2019
146	DHR/BH_66	F	25	Dharan-15	Positive	2019
147	DHR/BH_67	F	22	Dharan 15	Positive	2019
148	DHR/BH_68	M	29	Dharan-15	Positive	2019
149	DHR/BH_69	M	32	Dharan-5	Negative	2019
150	DHR/BH_70	F	58	Dharan-15	Positive	2019
151	DHR/BH_71	F	23	Baraha	Positive	2019
152	DHR/BH_72	F	76	Dharan-13	Positive	2019
153	DHR/BH_73	M	33	Dharan-13	Positive	2019
154	DHR/BH_74	M	29	Dharan-9	Positive	2019
155	DHR/BH_75	M	34	Dharan-15	Negative	2019
156	DHR/BH_76	F	28	Dharan-15	Positive	2019
157	DHR/BH_77	M	15	Dharan-12	Positive	2019
158	DHR/BH_78	F	30	Dharan-15	Positive	2019
159	DHR/BH_79	M	19	Dhankuta	Positive	2019
160	DHR/BH_80	F	34	Dharan-15	Negative	2019
161	DHR/BH_81	M	28	Dharan	Negative	2019
162	DHR/BH_82	F	14	Dharan-16	Negative	2019
163	DHR/BH_83	M	65	Dharan	Negative	2019
164	DHR/BH_84	M	67	Dharan-15	Negative	2019
165	DHR/BH_85	M	35	Dharan-1	Negative	2019
166	DHR/BH_86	M	36	Dharan-8	Positive	2019
167	DHR/BH_87	F	28	Dharan-15	Positive	2019
168	DHR/BH_88	M	28	Dharan-8	Positive	2019

169	DHR/BH_89	M	11	Dharan-15	Negative	2019
170	DHR/BH_90	F	17	Dharan-5	Positive	2019
171	DHR/BH_91	F	3	Dharan-15	Negative	2019
172	DHR/BH_92	F	21	Dharan 15	Negative	2019
173	DHR/BH_93	F	23	Dharan-15	Positive	2019
174	DHR/BH_94	M	10	Dharan	Positive	2019
175	DHR/BH_95	M	29	Dharan-15	Negative	2019
176	DHR/BH_96	M	13	Dharan-10	Positive	2019
177	DHR/BH_97	M	12	Dharan	Positive	2019
178	DHR/BH_98	M	37	Dharan	Positive	2019
179	DHR/BH_99	M	16	Dharan	Positive	2019
180	DHR/BH_100	F	8	Dharan	Negative	2019
181	DHR/BH_101	M	38	Jhapa	Positive	2019
182	DHR/BH_102	M	52	Dharan	Positive	2019
183	DHR/BH_103	F	80	Dharan	Positive	2019
184	DHR/BH_104	M	39	Dharan-11	Negative	2019
185	DHR/BH_105	F	32	Dharan-8	Positive	2019
186	DHR/BH_106	F	72	Dharan	Positive	2019
187	DHR/BH_107	F	38	Dharan	Positive	2019
188	DHR/BH_108	M	40	Dharan	Negative	2019
189	DHR/BH_109	M	41	Dharan	Negative	2019
190	DHR/BH_110	M	54	Dharan	Positive	2019
191	DHR/BH_111	M	26	Dharan-9	Positive	2019
192	DHR/BH_112	M	60	Dharan	Positive	2019
193	DHR/BH_113	F	48	Dharan	Positive	2019
194	DHR/BH_114	F	38	Dharan	Positive	2019
195	DHR/BH_115	M	47	Dharan	Positive	2019
196	DHR/BH_116	F	61	Dharan	Positive	2019
197	DHR/BH_117	M	30	Dharan	Positive	2019
198	DHR/BH_118	M	25	Dharan	Positive	2019
199	DHR/BH_119	F	44	Dharan	Positive	2019
200	DHR/BH_120	F	10	Dharan	Positive	2019
201	DHR/BH_121	F	31	Dharan	Positive	2019
202	DHR/BH_122	M	50	Dharan	Positive	2019
203	DHR/BH_123	F	10	Dharan-16	Positive	2019
204	DHR/BH_124	M	43	Dharan	Positive	2019
205	DHR/BH_125	M	28	Dharan	Positive	2019
206	DHR/BH_126	M	35	Dharan-16	Positive	2019
207	DHR/BH_127	M	21	Dharan-13	Positive	2019
208	DHR/BH_128	M	62	Dharan	Positive	2019
209	DHR/BH_129	M	32	Dharan	Positive	2019
210	DHR/BH_130	M	43	Dharan	Positive	2019
211	DHR/BH_131	M	31	Dharan	Positive	2019

212	DHR/BH_132	M	5	Dharan	Positive	2019
213	DHR/BH_133	F	23	Dharan	Positive	2019
214	DHR/BH_134	F	31	Dharan	Positive	2019
215	DHR/BH_135	M	9	Dharan	Positive	2019
216	DHR/BH_136	M	51	Dharan-14	Positive	2019
217	DHR/BH_137	M	66	Dharan-19	Positive	2019
218	DHR/BH_138	M	18	Dharan	Positive	2019
219	DHR/BH_139	M	39	Dharan	Positive	2019
220	DHR/BH_140	F	43	Dharan-17	Positive	2019
221	DHR/BH_141	M	40	Dharan	Positive	2019
222	DHR/BH_142	F	30	Dharan-11	Positive	2019
223	DHR/BH_143	F	15	Dharan-8	Positive	2019
224	DHR/BH_144	F	13	Dharan-9	Positive	2019
225	DHR/BH_145	F	35	Dharan-9	Positive	2019
226	DHR/BH_146	M	17	Pathari	Positive	2019
227	DHR/BH_147	F	14	Dharan-15	Positive	2019
228	DHR/BH_148	F	24	Dharan	Positive	2019
229	DHR/BH_149	F	38	Dharan-16	Positive	2019
230	DHR/BH_150	F	49	Dharan-16	Positive	2019
231	DHR/BH_151	M	43	Dharan	Positive	2019
232	DHR/BH_152	M	10	Dharan	Positive	2019
233	DHR/BH_153	M	35	Dharan	Positive	2019
234	DHR/BH_154	F	43	Dharan	Positive	2019
235	DHR/BH_155	M	3	Dharan	Positive	2019
236	DHR/BH_156	M	19	Dharan-10	Positive	2019
237	DHR/BH_157	M	18	Dharan	Positive	2019
238	DHR/BH_158	M	50	Dharan	Positive	2019
239	DHR/BH_159	F	20	Dharan-12	Positive	2019
240	DHR/BH_160	M	9	Dharan-16	Positive	2019
241	DHR/BH_161	F	20	Kerabari	Positive	2019
242	DHR/BH_162	F	45	Dharan-10	Positive	2019
243	DHR/BH_163	F	34	Dharan	Positive	2019
244	DHR/BH_164	F	2	Dharan-11	Positive	2019
245	DHR/BH_165	F	22	Dharan-15	Positive	2019
246	DHR/BH_166	M	10	Dharan-11	Positive	2019
247	DHR/BH_167	F	27	Dharan-2	Positive	2019
248	DHR/BH_168	M	23	Dharan-9	Positive	2019
249	DHR/BH_169	F	20	Dharan-15	Positive	2019
250	DHR/BH_170	F	35	Dharan-16	Positive	2019
251	DHR/BH_171	F	38	Dharan-19	Positive	2019
252	DHR/BH_172	M	19	Dharan-15	Positive	2019
253	DHR/BH_173	F	21	Dharan-11	Positive	2019
254	DHR/BH_174	M	23	Dharan	Positive	2019

255	DHR/BH_175	F	17	Dharan-11	Positive	2019
256	DHR/BH_176	M	18	Dharan-15	Positive	2019
257	DHR/BH_177	F	40	Dharan-7	Positive	2019
258	DHR/BH_178	F	29	Dharan-4	Positive	2019
259	DHR/BH_179	M	72	Dharan-10	Positive	2019
260	DHR/BH_180	F	66	Dhankuta	Positive	2019
261	DHR/BH_181	F	40	Dharan	Positive	2019
262	DHR/BH_182	M	64	Dharan-8	Positive	2019
263	DHR/BH_183	F	23	Dharan-15	Positive	2019
264	DHR/BH_184	F	36	Dharan-3	Positive	2019
265	DHR/BH_185	F	24	Dharan-3	Positive	2019
266	DHR/BH_186	F	29	Dharan-15	Positive	2019
267	DHR/BH_187	M	32	Dharan-13	Positive	2019
268	DHR/BH_188	F	35	Dharan	Positive	2019
269	DHR/BH_189	M	77	Dharan-10	Positive	2019
270	CMC_19_01	M	35	Bharatpur	Positive	2019
271	CMC_19_02	M	30	Chitwan	Positive	2019
272	CMC_19_03	M	64	Rautahat	Positive	2019
273	CMC_19_04	M	42	Hetauda	Positive	2019
274	CMC_19_05	F	35	Chitwan	Positive	2019
275	CMC_19_06	F	24	Hetauda	Positive	2019
276	CMC_19_07	M	36	Chitwan	Positive	2019
277	CMC_19_08	M	25	Nawalparasi	Positive	2019
278	CMC_19_09	M	60	Hetauda-15	Positive	2019
279	CMC_19_10	M	28	Chitwan	Positive	2019
280	CMC_19_11	M	20	Chitwan	Positive	2019
281	CMC_19_12	F	17	Chitwan	Positive	2019
282	CMC_19_13	M	18	chitwan	Positive	2019
283	CMC_19_14	M	33	Chitwan	Positive	2019
284	CMC_19_15	F	68	Chitwan	Positive	2019
285	CMC_19_16	F	35	Chitwan	Positive	2019
286	CMC_19_17	F	36	Chitwan	Positive	2019
287	CMC_19_18	M	31	Chitwan	Positive	2019
288	CMC_19_19	F	36	Bharatpur 7	Positive	2019
289	CMC_19_20	M	60	Bharatpur 4	Positive	2019
290	CMC_19_21	M	29	chitwan	Positive	2019
291	CMC_19_22	M	39	Hetauda 11	Positive	2019
292	CMC_19_23	F	27	Nawalparasi	Positive	2019
293	CMC_19_24	M	28	Bharatpur 16	Positive	2019
294	CMC_19_25	F	25	Bharatpur	Positive	2019
295	CMC_19_26	M	35	Nawalpur	Positive	2019
296	CMC_19_27	M	69	Palpa	Positive	2019
297	CMC_19_28	M	21	Chitwan	Positive	2019

298	CMC_19_29	F	29	Bharatpur-3	Positive	2019
299	CMC_19_30	M	54	Nawalparasi	Positive	2019
300	CMC_19_31	F	34	chitwan-26	Positive	2019
301	CMC_19_32	M	22	Bharatpur_5	Positive	2019
302	CMC_19_33	M	23	Hetauda 9	Positive	2019
303	CMC_19_34	F	35	Bharatpur	Positive	2019
304	CMC_19_35	F	39	Chitwan	Positive	2019
305	CMC_19_36	F	39	chitwan	Positive	2019
306	CMC_19_37	M	27	Bharatpur 27	Positive	2019
307	CMC_19_38	F	23	Chitwan	Positive	2019
308	CMC_19_39	M	34	Bharatpur	Positive	2019
309	CMC_19_40	F	21	Bharatpur	Positive	2019
310	CMC_19_41	M	48	Bharatpur-10	Positive	2019
311	CMC_19_42	M	20	Chitwan	Positive	2019
312	CMC_19_43	M	45	Hetauda	Positive	2019
313	CMC_19_44	F	29	Chitwan	Positive	2019
314	CMC_19_45	M	17	Hetauda	Positive	2019
315	CMC_19_46	F	55	Chitwan	Positive	2019
316	CMC_19_47	M	20	Chitwan	Positive	2019
317	CMC_19_48	M	20	Rautahat	Positive	2019
318	CMC_19_49	F	48	Bharatpur	Positive	2019
319	CMC_19_50	M	42	Bharatpur 11	Positive	2019
320	CMC_19_51	F	35	Hetauda-2	Positive	2019
321	CMC_19_52	M	39	Bharatpur-10	Positive	2019
322	CMC_19_53	F	29	Bharatpur-7	Positive	2019
323	CMC_19_54	M	29	Nawalparasi	Positive	2019
324	CMC_19_55	F	29	Bharatpur-2	Positive	2019
325	CMC_19_56	M	44	Hetauda-4	Positive	2019
326	CMC_19_57	F	20	Belchowk	Positive	2019
327	CMC_19_58	M	18	Chitwan	Positive	2019
328	CMC_19_59	M	27	Hetauda-7	Positive	2019
329	CMC_19_60	M	55	chitwan	Positive	2019
330	CMC_19_61	F	30	Bharatpur	Positive	2019
331	CMC_19_62	M	26	chitwan	Positive	2019
332	CMC_19_63	M	32	Chitwan	Positive	2019
333	CMC_19_64	M	16	Nawalparasi	Positive	2019
334	CMC_19_65	M	23	Chitwan	Positive	2019
335	CMC_19_66	F	45	Gorkha	Positive	2019
336	CMC_19_67	F	27	Chitwan	Positive	2019
337	CMC_19_68	M	46	Chitwan	Positive	2019
338	CMC_19_69	M	26	Chitwan	Positive	2019
339	CMC_19_70	M	20	Bharatpur 11	Positive	2019
340	CMC_19_71	M	29	Chitwan	Positive	2019

341	CMC_19_72	M	30	Chitwan	Positive	2019
342	CMC_19_73	F	35	Chitwan	Positive	2019
343	CMC_19_74	F	42	Chitwan	Positive	2019
344	CMC_19_75	F	35	Chitwan	Positive	2019
345	CMC_19_76	M	33	Dhading	Positive	2019
346	CMC_19_77	F	20	Bharatpur-17	Positive	2019
347	CMC_19_78	M	33	Chitwan	Positive	2019
348	CMC_19_79	M	32	Chitwan	Positive	2019
349	CMC_19_80	M	20	Chitwan	Positive	2019
350	CMC_19_81	M	31	Chitwan	Positive	2019
351	CMC_19_82	F	22	Chitwan	Positive	2019
352	CMC_19_83	F	30	Chitwan	Positive	2019
353	CMC_19_84	F	72	Hetauda-4	Positive	2019
354	CMC_19_85	M	24	Chitwan	Positive	2019
355	CMC_19_86	M	22	Chitwan	Positive	2019
356	CMC_19_87	F	26	Dang-13	Positive	2019
357	CMC_19_88	M	46	Hetauda-6	Positive	2019
358	CMC_19_89	M	35	Chitwan	Positive	2019
359	CMC_19_90	F	27	Chitwan	Positive	2019
360	CMC_19_91	F	10	chitwan	Positive	2019
361	CMC_19_92	M	33	chitwan	Positive	2019
362	CMC_19_93	M	18	Chitwan	Positive	2019
363	CMC_19_94	M	12	Nawalparasi	Positive	2019
364	CMC_19_95	F	32	chitwan	Positive	2019
365	CMC_19_96	M	38	Chitwan	Positive	2019
366	CMC_19_97	M	20	Chitwan	Positive	2019
367	CMC_19_98	F	9	Chitwan	Positive	2019
368	CMC_19_99	M	31	Nawalparasi	Positive	2019
369	CMC_19_100	M	24	Chitwan	Positive	2019
370	CMC_19_101	F	4	Chitwan	Positive	2019
371	CMC_19_102	F	26	Chitwan	Positive	2019
372	CMC_19_103	M	58	Sunsari	Positive	2019
373	CMC_19_104	F	54	Itahari	Positive	2019
374	CMC_19_105	F	28	Itahari	Positive	2019
375	CMC_19_106	M	14	Sunsari	Positive	2019
376	CMC_19_107	M	5	Terathum	Positive	2019
377	CMC_19_108	M	31	Biratnagar	Positive	2019
378	CMC_19_109	M	36	Biratnagar	Positive	2019
379	CMC_19_110	M	35	Morang	Positive	2019
380	CMC_19_111	F	47	Sunsari	Positive	2019
381	KTM/STH_19_75	F	19	Makwanpur	Positive	2019
382	KTM/STH_19_76	F	24	kapilvastu	Negative	2019
383	KTM/STH_19_77	F	45	Nuwakot	Negative	2019

384	KTM/STH_19_78	F	17	Kathmandu	Negative	2019
385	KTM/STH_19_79	F	43	Dhading	Positive	2019
386	KTM/STH_19_80	F	35	Kathmandu	Positive	2019
387	KTM/STH_19_81	M	30	Kathmandu	Negative	2019
388	KTM/STH_19_82	M	36	Butwal	Positive	2019
389	KTM/STH_19_83	M	15	Kathmandu	Negative	2019
390	KTM/STH_19_84	M	39	Makwanpur	Positive	2019
391	KTM/STH_19_85	F	60	Bhaktapur	Positive	2019
392	KTM/STH_19_86	F	30	Dhading	Positive	2019
393	KTM/STH_19_87	F	30	Kathmandu	Positive	2019
394	KTM/STH_19_88	M	44	Kathmandu	Positive	2019
395	KTM/STH_19_89	M	19	Hetauda	Positive	2019
396	KTM/STH_19_90	M	53	Kathmandu	Negative	2019
397	KTM/STH_19_91	M	26	Illam	Positive	2019
398	KTM/STH_19_92	M	35	Kathmandu	Positive	2019
399	KTM/STH_19_93	M	32	Kathmandu	Positive	2019
400	KTM/STH_19_94	M	19	Kathmandu	Positive	2019

Appendix 2.2. Presentation of Dengue NS1 capture ELISA along with mean OD values at 450nm, ISR and sero-status.

Sample ID	Mean OD	ISR	Status		Sample ID	Mean OD	ISR	Status
Nep-1	1.79	16.42	Positive		KTM/STH_30	3.339	30.63	Positive
Nep-2	2.41	22.11	Positive		KTM/STH_31	3.247	29.79	Positive
Nep-3	3.12	28.62	Positive		KTM/STH_38	3.102	28.46	Positive
Nep-4	0.2	1.83	Positive		KTM/STH_39	0.274	2.51	Positive
Nep-5	0.26	2.39	Positive		KTM/SH_1	3.505	32.16	Positive
Nep-6	3.07	28.17	Positive		KTM/NOV_1	3.477	31.90	Positive
Nep-7	3.07	28.17	Positive		KTM/GPC_01	3.192	29.28	Positive
Nep-8	1.37	12.57	Positive		KTM/GPC_02	3.228	29.61	Positive
Nep-9	3.06	28.07	Positive		KTM/GPC_04	3.199	29.35	Positive
Nep-10	0.78	7.16	Positive		KTM/GPC_05	3.317	30.43	Positive
Nep-11	3.1	28.44	Positive		DHR/BH_01	2.82	25.87	Positive
Nep-12	0.19	1.74	Positive		DHR/BH_02	2.774	25.45	Positive
Nep-13	0.22	2.02	Positive		DHR/BH_03	2.812	25.80	Positive
Nep-14	1.12	10.28	Positive		DHR/BH_04	2.826	25.93	Positive
Nep-15	0.61	5.60	Positive		DHR/BH_05	2.737	25.11	Positive
Nep-16	3.22	29.54	Positive		DHR/BH_06	2.785	25.55	Positive
Nep-17	0.54	4.95	Positive		DHR/BH_07	2.86	26.24	Positive
Nep-19	0.71	6.51	Positive		DHR/BH_08	2.88	26.42	Positive

Nep-20	3.18	29.17	Positive		DHR/BH_09	2.77	25.41	Positive
Nep-21	3.14	28.81	Positive		DHR/BH_10	2.085	19.13	Positive
Nep-22	3.41	31.28	Positive		DHR/BH_11	2.818	25.85	Positive
Nep-23	3.23	29.63	Positive		DHR/BH_14	2.83	25.96	Positive
Nep-24	1.02	9.36	Positive		DHR/BH_18	2.871	26.34	Positive
Nep-25	0.7	6.42	Positive		DHR/BH_19	2.88	26.42	Positive
Nep-26	3.36	30.83	Positive		DHR/BH_23	2.857	26.21	Positive
Nep-28	0.4	3.67	Positive		DHR/BH_24	2.95	27.06	Positive
Nep-29	1.23	11.28	Positive		DHR/BH_28	2.131	19.55	Positive
Nep-30	3.03	27.80	Positive		DHR/BH_29	3.363	30.85	Positive
Nep-32	0.69	6.33	Positive		DHR/BH_34	3.63	33.30	Positive
Nep-33	3.32	30.46	Positive		DHR/BH_35	3.04	27.89	Positive
Nep-34	0.74	6.79	Positive		DHR/BH_44	3.15	28.90	Positive
Nep-35	3.48	31.93	Positive		DHR/BH_49	2.394	21.96	Positive
Nep-36	1.34	12.29	Positive		DHR/BH_64	3.89	35.69	Positive
Nep-37	3.36	30.83	Positive		DHR/BH_65	2.945	27.02	Positive
Nep-38	3.41	31.28	Positive		DHR/BH_66	2.912	26.72	Positive
Nep-39	3.26	29.91	Positive		DHR/BH_67	2.884	26.46	Positive
Nep-40	3.25	29.82	Positive		DHR/BH_68	0.895	8.21	Positive
Nep-41	3.28	30.09	Positive		DHR/BH_70	1.967	18.05	Positive
Nep-42	2.61	23.94	Positive		DHR/BH_71	1.466	13.45	Positive
Nep-43	3.27	30.00	Positive		DHR/BH_90	3.031	27.81	Positive
Nep-44	3.99	36.61	Positive		CMC_19_01	3.05	27.98	Positive
Nep-45	1.22	11.19	Positive		CMC_19_02	2.957	27.13	Positive
Nep-46	0.68	6.24	Positive		CMC_19_03	2.89	26.51	Positive
Nep-47	1.71	15.69	Positive		CMC_19_04	1.466	13.45	Positive
Nep-48	0.84	7.71	Positive		CMC_19_05	3.126	28.68	Positive
Nep-49	2.65	24.31	Positive		CMC_19_06	3.19	29.27	Positive
Nep-50	2.21	20.28	Positive		CMC_19_07	2.763	25.35	Positive
KTM/STH_27	3.193	29.29	Positive		CMC_19_08	3.214	29.49	Positive
KTM/STH_28	2.742	25.16	Positive		CMC_19_09	3.082	28.28	Positive
KTM/STH_29	3.505	32.16	Positive		CMC_19_10	3.101	28.45	Positive

Appendix 2.3. Presentation of Dengue IgM capture ELISA with mean OD for DENRA and NCA, ISR ratio and sero status.

Sample ID	Mean DENRA	Mean NCA	IgM ISR	Status
Nep-1	0.811	0.068	11.93	**Positive**
Nep-2	0.414	0.11	3.76	**Positive**
Nep-3	0.243	0.064	3.80	**Positive**
Nep-4	0.632	0.05	12.64	**Positive**
Nep-5	0.163	0.068	2.40	**Equivocal**
Nep-6	0.712	0.208	3.42	**Positive**
Nep-7	0.155	0.073	2.12	**Equivocal**
Nep-8	1.02	0.121	8.43	**Positive**
Nep-9	0.09	0.06	1.50	**Negative**
Nep-10	0.059	0.055	1.07	**Negative**
Nep-11	0.272	0.127	2.14	**Equivocal**
Nep-12	0.87	0.162	5.37	**Positive**
Nep-13	0.817	0.076	10.75	**Positive**
Nep-14	0.145	0.085	1.71	**Equivocal**
Nep-15	0.201	0.051	3.94	**Positive**
Nep-16	0.229	0.166	1.38	**Negative**
Nep-17	0.065	0.055	1.18	**Negative**
Nep-19	0.057	0.056	1.02	**Negative**
Nep-20	0.363	0.237	1.53	**Negative**
Nep-21	0.625	0.05	12.50	**Positive**
Nep-22	0.366	0.237	1.54	**Negative**
Nep-23	0.223	0.049	4.55	**Positive**
Nep-24	0.72	0.21	3.43	**Positive**
Nep-25	0.07	0.15	0.47	**Negative**
Nep-26	0.05	0.051	0.98	**Negative**
Nep-28	0.413	0.314	1.32	**Negative**
Nep-29	1.123	0.177	6.34	**Positive**
Nep-30	1.532	0.55	2.79	**Equivocal**
Nep-32	0.192	0.052	3.69	**Positive**
Nep-33	0.148	0.072	2.06	**Equivocal**
Nep-34	0.352	0.055	6.40	**Positive**
Nep-35	0.967	0.091	10.63	**Positive**
Nep-36	0.36	0.11	3.27	**Positive**

Nep-37	0.41	0.09	4.56	**Positive**
Nep-38	0.74	0.2	3.70	**Positive**
Nep-39	0.18	0.26	0.69	**Negative**
Nep-40	0.09	0.24	0.38	**Negative**
Nep-41	0.347	0.2	1.74	**Equivocal**
Nep-42	1.07	0.09	11.89	**Positive**
Nep-43	0.13	0.01	13.00	**Positive**
Nep-44	0.51	0.13	3.92	**Positive**
Nep-45	0.25	0.05	5.00	**Positive**
Nep-46	0.32	0.07	4.57	**Positive**
Nep-47	0.78	0.06	13.00	**Positive**
Nep-48	0.15	0.07	2.14	**Equivocal**
Nep-49	0.31	0.15	2.07	**Equivocal**
Nep-50	0.17	0.12	1.42	**Negative**
KTM/STH_27	0.178	0.05	3.56	**Positive**
KTM/STH_28	0.793	0.054	14.69	**Positive**
KTM/STH_29	0.431	0.051	8.45	**Positive**
KTM/STH_30	0.081	0.054	1.50	**Negative**
KTM/STH_31	0.117	0.051	2.29	**Equivocal**
KTM/STH_38	0.072	0.049	1.47	**Negative**
KTM/STH_39	0.079	0.049	1.61	**Negative**
KTM/SH_1	0.095	0.072	1.32	**Negative**
KTM/NOV_1	0.173	0.05	3.46	**Positive**
KTM/GPC_01 Acute	0.74	0.049	15.10	**Positive**
KTM/GPC_02 Acute	0.083	0.057	1.46	**Negative**
KTM/GPC_04 Acute	0.069	0.058	1.19	**Negative**
KTM/GPC_05 Acute	0.481	0.053	9.08	**Positive**
DHR/BH_01	0.459	0.057	8.05	**Positive**
DHR/BH_02	0.062	0.05	1.24	**Negative**
DHR/BH_03	0.917	0.047	19.51	**Positive**
DHR/BH_04	0.92	0.049	18.78	**Positive**
DHR/BH_05	0.076	0.057	1.33	**Negative**
DHR/BH_06	0.104	0.05	2.08	**Equivocal**
DHR/BH_07	0.459	0.047	9.77	**Positive**
DHR/BH_08	0.063	0.05	1.26	**Negative**
DHR/BH_09	0.096	0.055	1.75	**Equivocal**
DHR/BH_10	0.273	0.069	3.96	**Positive**

DHR/BH_11	0.08	0.05	1.60	**Negative**
DHR/BH_14	0.83	0.071	11.69	**Positive**
DHR/BH_18	0.147	0.052	2.83	**Equivocal**
DHR/BH_19	0.205	0.068	3.01	**Positive**
DHR/BH_23	0.414	0.054	7.67	**Positive**
DHR/BH_24	0.086	0.055	1.56	**Negative**
DHR/BH_28	0.189	0.081	2.33	**Equivocal**
DHR/BH_29	0.073	0.056	1.30	**Negative**
DHR/BH_34	0.142	0.059	2.41	**Equivocal**
DHR/BH_35	0.662	0.059	11.22	**Positive**
DHR/BH_44	0.668	0.053	12.60	**Positive**
DHR/BH_49	0.068	0.053	1.28	**Negative**
DHR/BH_64	0.293	0.063	4.65	**Positive**
DHR/BH_65	0.369	0.061	6.05	**Positive**
DHR/BH_66	0.345	0.056	6.16	**Positive**
DHR/BH_67	0.25	0.052	4.81	**Positive**
DHR/BH_68	0.789	0.068	11.60	**Positive**
DHR/BH_70	0.165	0.056	2.95	**Positive**
DHR/BH_71	0.17	0.052	3.27	**Positive**
DHR/BH_90	0.364	0.056	6.50	**Positive**
CMC_19_01	0.253	0.052	4.87	**Positive**
CMC_19_02	0.306	0.137	2.23	**Equivocal**
CMC_19_03	1.707	0.12	14.23	**Positive**
CMC_19_04	0.484	0.05	9.68	**Positive**
CMC_19_05	0.078	0.054	1.44	**Negative**
CMC_19_06	0.165	0.055	3.00	**Positive**
CMC_19_07	0.158	0.064	2.47	**Equivocal**
CMC_19_08	0.375	0.055	6.82	**Positive**
CMC_19_09	0.311	0.059	5.27	**Positive**
CMC_19_10	0.187	0.053	3.53	**Positive**

Appendix 2.4.Presentation of IgG ELISA the mean OD for DENRA and NCA, ISR and serostatus.

Sample ID	Mean DENRA	Mean NCA	ISR	Status
Nep-1	0.16	0.05	3.20	**Positive**
Nep-2	0.12	0.19	0.63	**Negative**
Nep-3	0.163	0.061	2.67	**Equivocal**
Nep-4	0.184	0.064	2.88	**Positive**
Nep-5	0.214	0.059	3.63	**Positive**
Nep-6	0.09	0.05	1.80	**Equivocal**
Nep-7	0.173	0.09	1.92	**Equivocal**
Nep-8	0.11	0.05	2.20	**Equivocal**
Nep-9	0.07	0.05	1.40	**Negative**
Nep-10	0.06	0.05	1.20	**Negative**
Nep-11	0.18	0.06	3.00	**Positive**
Nep-12	0.05	0.07	0.71	**Negative**
Nep-13	0.09	0.07	1.29	**Negative**
Nep-14	0.22	0.05	4.40	**Positive**
Nep-15	0.21	0.05	4.20	**Positive**
Nep-16	0.177	0.05	3.54	**Positive**
Nep-17	0.168	0.05	3.36	**Positive**
Nep-19	0.06	0.06	1.00	**Negative**
Nep-20	0.199	0.05	3.98	**Positive**
Nep-21	0.075	0.075	1.00	**Negative**
Nep-22	0.32	0.05	6.40	**Positive**
Nep-23	0.322	0.05	6.44	**Positive**
Nep-24	0.08	0.05	1.60	**Negative**
Nep-25	0.183	0.123	1.49	**Negative**
Nep-26	0.066	0.05	1.32	**Negative**
Nep-28	0.107	0.06	1.78	**Equivocal**
Nep-29	0.062	0.05	1.24	**Negative**
Nep-30	0.066	0.05	1.32	**Negative**
Nep-32	0.23	0.06	3.83	**Positive**
Nep-33	0.06	0.05	1.20	**Negative**
Nep-34	0.071	0.054	1.31	**Negative**
Nep-35	0.245	0.057	4.30	**Positive**
Nep-36	0.247	0.057	4.33	**Positive**

Nep-37	0.172	0.055	3.13	**Positive**
Nep-38	0.05	0.05	1.00	**Negative**
Nep-39	0.168	0.05	3.36	**Positive**
Nep-40	0.156	0.05	3.12	**Positive**
Nep-41	0.075	0.062	1.21	**Negative**
Nep-42	0.106	0.07	1.51	**Negative**
Nep-43	0.054	0.05	1.08	**Negative**
Nep-44	0.079	0.061	1.30	**Negative**
Nep-45	0.187	0.045	4.16	**Positive**
Nep-46	0.186	0.05	3.72	**Positive**
Nep-47	0.173	0.05	3.46	**Positive**
Nep-48	0.08	0.04	2.00	Equivocal
Nep-49	0.14	0.05	2.80	Equivocal
Nep-50	0.275	0.056	4.91	**Positive**
KTM/STH_27	2.428	0.094	25.83	**Positive**
KTM/STH_28	0.637	0.117	5.44	**Positive**
KTM/STH_29	0.148	0.108	1.37	**Negative**
KTM/STH_30	0.091	0.095	0.96	**Negative**
KTM/STH_31	0.124	0.112	1.11	**Negative**
KTM/STH_38	0.106	0.123	0.86	**Negative**
KTM/STH_39	0.093	0.085	1.09	**Negative**
KTM/SH_1	0.086	0.097	0.89	**Negative**
KTM/NOV_1	1.855	0.095	19.53	**Positive**
KTM/GPC_01	1.203	0.098	12.28	**Positive**
KTM/GPC_02	0.103	0.081	1.27	**Negative**
KTM/GPC_04	0.085	0.09	0.94	**Negative**
KTM/GPC_05	0.707	0.083	8.52	**Positive**
DHR/BH_01	0.178	0.05	3.56	**Positive**
DHR/BH_02	0.793	0.054	14.69	**Positive**
DHR/BH_03	0.431	0.051	8.45	**Positive**
DHR/BH_04	0.081	0.054	1.50	**Negative**
DHR/BH_05	0.117	0.051	2.29	Equivocal
DHR/BH_06	0.084	0.047	1.79	Equivocal
DHR/BH_07	0.076	0.055	1.38	**Negative**
DHR/BH_08	0.081	0.053	1.53	**Negative**
DHR/BH_09	0.081	0.047	1.72	Equivocal
DHR/BH_10	0.062	0.053	1.17	**Negative**

DHR/BH_11	0.067	0.048	1.40	**Negative**
DHR/BH_14	0.072	0.049	1.47	**Negative**
DHR/BH_18	0.079	0.049	1.61	**Negative**
DHR/BH_19	0.091	0.052	1.75	**Equivocal**
DHR/BH_23	0.093	0.048	1.94	**Equivocal**
DHR/BH_24	0.072	0.063	1.14	**Negative**
DHR/BH_28	0.078	0.048	1.63	**Negative**
DHR/BH_29	0.102	0.056	1.82	**Equivocal**
DHR/BH_34	0.095	0.072	1.32	**Negative**
DHR/BH_35	0.173	0.05	3.46	**Positive**
DHR/BH_44	0.74	0.049	15.10	**Positive**
DHR/BH_49	0.083	0.057	1.46	**Negative**
DHR/BH_64	0.091	0.051	1.78	**Equivocal**
DHR/BH_65	0.069	0.058	1.19	**Negative**
DHR/BH_66	0.481	0.053	9.08	**Positive**
DHR/BH_67	0.427	0.048	8.90	**Positive**
DHR/BH_68	0.501	0.057	8.79	**Positive**
DHR/BH_70	0.075	0.057	1.32	**Negative**
DHR/BH_71	0.495	0.061	8.11	**Positive**
DHR/BH_90	0.108	0.057	1.89	**Equivocal**
CMC_19_01	0.072	0.094	0.77	**Negative**
CMC_19_02	0.668	0.053	12.60	**Positive**
CMC_19_03	0.068	0.063	1.08	**Negative**
CMC_19_04	0.293	0.061	4.80	**Positive**
CMC_19_05	0.369	0.056	6.59	**Positive**
CMC_19_06	0.345	0.052	6.63	**Positive**
CMC_19_07	0.25	0.068	3.68	**Positive**
CMC_19_08	0.789	0.056	14.09	**Positive**
CMC_19_09	0.165	0.052	3.17	**Positive**
CMC_19_10	0.08	0.056	1.43	**Negative**

Appendix 2.5. Cycle threshold (CT) values for Real time PCR positive samples

Sample ID	Flurochrome	Serotype	Ct value
Nep-7	Texas Red	DENV3	23.33
Nep-9	VIC	DENV2	22.37
Nep-26	VIC	DENV2	21.06
Nep-33	FAM	DENV1	26.23
Nep-39	VIC	DENV2	28.62
Nep-40	VIC	DENV2	31.21
Nep-41	VIC	DENV2	33.68
Nep-43	FAM	DENV1	20
Nep-45	VIC	DENV2	29.73
Nep-46	VIC	DENV2	30.72
Nep-47	VIC	DENV2	30.5
Nep-48	VIC	DENV2	22.2
Nep-49	VIC	DENV2	29.41
Nep-50	VIC	DENV2	28.43
KTM/STH_27	VIC	DENV2	25.23
KTM/STH_28	VIC	DENV2	24.34
KTM/GPC_01	VIC	DENV2	22.17
KTM/GPC_02	VIC	DENV2	28.73
KTM/GPC_04	VIC	DENV2	22.18
KTM/GPC_05	VIC	DENV2	23.51
DHR/BH_02	VIC	DENV2	22.71
DHR/BH_07	VIC	DENV2	21.71
DHR/BH_14	VIC	DENV2	20.32
DHR/BH_18	VIC	DENV2	21.39
DHR/BH_19	VIC	DENV2	21.65
DHR/BH_23	VIC	DENV2	28.63
DHR/BH_24	VIC	DENV2	22.07
DHR/BH_28	VIC	DENV2	26.01
DHR/BH_29	VIC	DENV2	22.06
DHR/BH_34	VIC	DENV2	21.04

Appendix 3.2 Sequence used in Phylogeny of D1DIII

Accession no.	Country	Year
AB111071	Combodia	2001
AB597966	Indonesia	2010
AM746212	Saudi Arabia	2006
AY732390	Thailand	1983
EU069598	Singapore	2003
FJ882559	Vietnam	2007
JF297571	India	1962
JF297583	India	2005
JF967939	India	2010
JN029811	China	2010
AB111454	Japan	2001
MK209645	Nepal	2017

Appendix: 3.3 Sequence used for Phylogeny of D2DIII

Accession no.	Country	Year
MH594926	INDIA	2017
MG840600	CHINA	2019
KT781518	INDIA	2009
MW290491	CHINA	2020
MK858112	INDIA	2021
KJ806779	SINGAPORE	2015
MW512370	SINGAPORE	2021
MH594906	INDIA	2018
MZ520906	SPAIN	2021

Appendix 4.1.

4.1.1 Mean of Non endemic control (NEC), endemic control (EC) and cut off values for DENV-1 and DENV-2.

DENV1			**DENV2**		
	Mean	SD		Mean	SD
Blank	0.028	0	Blank	0.026	0.001414214
1 Ab neg	0.0835	0.019091883	1 Ab neg	0.1035	0.000707107
2 Ab neg	0.0505	0.000707107	2 Ab neg	0.052	0.001414214
Pos control	1.365	0.14283557	Pos control	2.543	0.169705627
Neg control	1.033	0.04384062	Neg control	1.716	0.070710678
Non Endemic control	Mean	SD	**Non Endemic control**	Mean	SD
NEC1	1.2335	0.054447222	NEC1	2.594	0.073539105
NEC2	1.0025	0.006363961	NEC2	1.022	0.011313708
NEC3	1.1235	0.126572114	NEC3	1.3215	0.123743687
NEC4	1.411	0.002828427	NEC4	1.367	0.067882251
NEC5	1.1465	0.027577164	NEC5	2.249	0.038183766
Average	1.1834	0.043557778	Average	1.7107	0.062932504
Endemic Control	Mean	SD	**Endemic Control**	Mean	SD
EC1	1.501	0.004242641	EC1	2.38	0.025455844
EC2	0.837	0.016970563	EC2	0.412	0.049497475
EC3	1.3215	0.075660426	EC3	1.334	0.096166522
EC4	1.2085	0.139300036	EC4	1.188	0.029698485
EC5	1.327	0.207889394	EC5	1.348	0.052325902
EC6	1.139	0.134350288	EC6	2.3885	0.013435029
EC7	1.6905	0.08131728	EC7	1.657	0.141421356
EC8	0.613	0.079195959	EC8	0.682	0.021213203
EC9	1.018	0.060811183	EC9	0.4225	0.000707107
EC10	0.3265	0.012020815	EC10	1.004	0.014142136
Average	1.0982	0.081175858	Average	1.2816	0.044406306
Cut off NEC	1.314073333		Cut off NEC	1.899497511	
Cut off EC	1.341727575		Cut off EC	1.414818918	
Cut off Control	**1.32790045**		**Cut off Control**	**1.657158214**	

4.1.2 Mean of Non endemic control (NEC), endemic control (EC) and cut off values for DENV-3 and DENV-4.

	DENV3					**DENV 4**
	Mean	SD			Mean	SD
Blank	0.0285	0.002121		Blank	0.025	0.001414
1 Ab neg	0.096	0.059397		1 Ab neg	0.0945	0.019092
2 Ab neg	0.0485	0.002121		2 Ab neg	0.054	0.002828
Pos control	1.9315	0.061518		Pos control	1.5795	0.1492
Neg control	1.699	0.114551		Neg control	0.7945	0.116673
Non Endemic control	Mean	SD		**Non Endemic control**	Mean	SD
NEC1	1.645	0.048083		NEC1	0.907	0.19799
NEC2	2.075	0.101823		NEC2	1.6885	0.089803
NEC3	1.3045	0.045962		NEC3	1.0505	0.072832
NEC4	1.8925	0.120915		NEC4	1.4265	0.135057
NEC5	1.9345	0.082731		NEC5	1.452	0.031113
Average	1.7703	0.079903		Average	1.3049	0.105359
Endemic Control	Mean	SD		**Endemic Control**	Mean	SD
EC1	2.05	0.083439		EC1	1.546	0.073539
EC2	1.3225	0.016263		EC2	0.793	0.08061
EC3	2.516	0		EC3	2.072	0.031113
EC4	1.8265	0.02192		EC4	1.466	0.018385
EC5	1.901	0.055154		EC5	1.465	0.035355
EC6	1.7345	0.000707		EC6	1.3125	0.003536
EC7	1.4775	0.051619		EC7	1.0935	0.120915
EC8	0.9365	0.003536		EC8	0.711	0.039598
EC9	2.209	0.039598		EC9	1.845	0.056569
EC10	0.8505	0.019092		EC10	0.4155	0.101116
Average	1.6824	0.027632		Average	1.27195	0.056074
Cut off NEC	**2.010009**			Cut off NEC	**1.620977**	
Cut off EC	**1.765296**			Cut off EC	**1.440171**	
Cut off Control	**1.8876 528**			**Cut off Control**	**1.530574**	

4.1.3 Mean of Samples and status of presence of different antibody for dengue in samples

	DENV1		DENV2		DENV 3		DENV 4	
Sample ID	**Mean**	**status**	**Mean**	**status**	**Mean**	**status**	**Mean**	**Status**
1	1.001	Negative	1.5885	Negative	1.5115	Negative	1.185	Negative
2	0.886	Negative	1.507	Negative	1.5815	Negative	1.2475	Negative
3	0.6755	Negative	0.831	Negative	1.0565	Negative	0.716	Negative
4	1.783	**Positive**	2.717	**Positive**	2.2385	**Positive**	1.0975	Negative
5	2.2345	**Positive**	2.494	**Positive**	2.397	**Positive**	1.852	**Positive**
6	0.7805	Negative	2.3285	**Positive**	1.617	Negative	1.257	Negative

7	0.4855	Negative	2.04	Positive	1.3555	Negative	1.0945	Negative
8	0.933	Negative	2.0755	Positive	1.1385	Negative	0.755	Negative
9	0.43	Negative	0.6245	Negative	1.158	Negative	1.008	Negative
10	1.255	Negative	1.21	Negative	1.44	Negative	1.1875	Negative
11	1.4025	Positive	2.4005	Positive	1.487	Negative	1.0575	Negative
12	1.509	Positive	1.814	Positive	1.7375	Negative	1.0915	Negative
13	0.6225	Negative	1.5325	Negative	0.95	Negative	0.682	Negative
14	1.6485	Positive	2.8165	Positive	2.1315	Positive	1.365	Negative
15	1.486	Positive	2.5945	Positive	1.833	Negative	1.1035	Negative
16	0.972	Negative	2.5395	Positive	1.81	Negative	1.3565	Negative
17	1.4	Positive	2.001	Positive	1.657	Negative	1.2115	Negative
18	1.6045	Positive	1.569	Negative	1.9775	Positive	1.6235	Positive
19	1.443	Positive	1.2315	Positive	1.7065	Negative	1.468	Negative
20	0.823	Negative	2.8565	Positive	1.5365	Negative	0.844	Negative
21	1.114	Negative	1.011	Negative	0.986	Negative	0.785	Negative
22	0.7385	Negative	2.4595	Positive	1.5445	Negative	1.1245	Negative
23	0.5835	Negative	2.3965	Positive	1.5475	Negative	1.0115	Negative
24	1.342	Positive	1.7735	Positive	1.68	Negative	1.4755	Negative
25	0.814	Negative	1.956	Positive	1.3955	Negative	0.783	Negative
26	1.016	Negative	1.019	Negative	1.5735	Negative	1.39	Negative
27	2.184	Positive	2.0475	Positive	1.96	Positive	1.5265	Negative
28	1.2355	Negative	1.999	Positive	1.4825	Negative	1.048	Negative
29	1.5915	Positive	1.92	Positive	1.4625	Negative	1.3725	Negative
30	0.9735	Negative	1.345	Negative	0.7965	Negative	0.62	Negative
31	1.2765	Negative	2.4485	Positive	1.2795	Negative	0.726	Negative
32	2.129	Positive	2.6165	Positive	2.0265	Positive	1.2955	Negative
33	0.3395	Negative	0.53	Negative	0.8375	Negative	0.7625	Negative
34	2.163	Positive	2.5765	Positive	2.108	Positive	1.641	Positive
35	0.947	Negative	1.577	Negative	0.87	Negative	0.8755	Negative
36	1.0525	Negative	2.255	Positive	1.52	Negative	1.339	Negative
37	1.8205	Positive	2.3745	Positive	1.6355	Negative	1.5395	Positive
38	0.9705	Negative	1.6385	Negative	1.572	Negative	1.5685	Positive
39	0.4015	Negative	2.144	Positive	0.4905	Negative	0.261	Negative
40	0.435	Negative	2.147	Positive	1.0745	Negative	0.9445	Negative
41	0.84	Negative	1.729	Positive	0.06	Negative	0.0545	Negative
42	1.1285	Negative	1.905	Positive	0.9595	Negative	0.9085	Negative
43	1.795	Positive	1.607	Negative	0.935	Negative	1.0205	Negative
44	2.377	Positive	2.376	Positive	1.3855	Negative	1.3025	Negative
45	0.7795	Negative	2.279	Positive	1.339	Negative	1.3275	Negative
46	2.04	Positive	2.457	Positive	1.783	Negative	1.3395	Negative
47	1.2695	Negative	2.318	Positive	1.4965	Negative	1.352	Negative
48	0.786	Negative	2.007	Positive	0.973	Negative	0.9095	Negative
49	0.874	Negative	2.2495	Positive	0.9385	Negative	0.859	Negative
50	0.989	Negative	1.94	Positive	1.3425	Negative	1.337	Negative

4.1.4. Comparison of In-House ELISA results with In-Bios kit ELISA.

	DENV-1	DENV-2	DENV-3	DENV-4	In-Bios kit
Sample ID					
1	Negative	Negative	Negative	Negative	**Positive**
2	Negative	Negative	Negative	Negative	Negative
3	Negative	Negative	Negative	Negative	Equivocal
4	**Positive**	**Positive**	**Positive**	Negative	**Positive**
5	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
6	Negative	**Positive**	Negative	Negative	Equivocal
7	Negative	**Positive**	Negative	Negative	Equivocal
8	Negative	**Positive**	Negative	Negative	Equivocal
9	Negative	Negative	Negative	Negative	Negative
10	Negative	Negative	Negative	Negative	Negative
11	**Positive**	**Positive**	Negative	Negative	**Positive**
12	**Positive**	**Positive**	Negative	Negative	Negative
13	Negative	Negative	Negative	Negative	Negative
14	**Positive**	**Positive**	**Positive**	Negative	**Positive**
15	**Positive**	**Positive**	Negative	Negative	**Positive**
16	Negative	**Positive**	Negative	Negative	**Positive**
17	**Positive**	**Positive**	Negative	Negative	**Positive**
18	**Positive**	Negative	**Positive**	**Positive**	Negative
19	**Positive**	**Positive**	Negative	Negative	Negative
20	Negative	**Positive**	Negative	Negative	**Positive**
21	Negative	Negative	Negative	Negative	Negative
22	Negative	**Positive**	Negative	Negative	**Positive**
23	Negative	**Positive**	Negative	Negative	**Positive**
24	**Positive**	**Positive**	Negative	Negative	Negative
25	Negative	**Positive**	Negative	Negative	Negative
26	Negative	Negative	Negative	Negative	Negative
27	**Positive**	**Positive**	**Positive**	Negative	Equivocal
28	Negative	**Positive**	Negative	Negative	Negative
29	**Positive**	**Positive**	Negative	Negative	Negative
30	Negative	Negative	Negative	**Positive**	Negative
31	Negative	**Positive**	Negative	Negative	**Positive**
32	**Positive**	**Positive**	**Positive**	Negative	**Positive**
33	Negative	Negative	Negative	Negative	Negative
34	**Positive**	**Positive**	**Positive**	Negative	**Positive**
35	Negative	Negative	Negative	Negative	Negative
36	Negative	**Positive**	Negative	Negative	**Positive**
37	**Positive**	**Positive**	Negative	**Positive**	**Positive**
38	Negative	Negative	Negative	**Positive**	Negative

39	Negative	**Positive**	Negative	Negative	**Positive**
40	Negative	**Positive**	Negative	Negative	**Positive**
41	Negative	**Positive**	Negative	Negative	Negative
42	Negative	**Positive**	Negative	Negative	Negative
43	**Positive**	Negative	Negative	Negative	Negative
44	**Positive**	**Positive**	Negative	Negative	Negative
45	Negative	**Positive**	Negative	Negative	**Positive**
46	**Positive**	**Positive**	Negative	Negative	**Positive**
47	Negative	**Positive**	Negative	Negative	**Positive**
48	Negative	**Positive**	Negative	Negative	Equivocal
49	Negative	**Positive**	Negative	Negative	Equivocal
50	Negative	**Positive**	Negative	Negative	**Positive**

COMPOSITION OF REAGENTS

1. Coating Buffer:
i. Sodium Carbonate (Na_2CO_3) = **5.3 g**
ii. Sodium bicarbonate ($NaHCO_3$) = **4.2 g**
iii. Total volume = 1000 ml (**1 litre**) in **Distilled water**
iv. PH adjustment = **9.6**
v. Sterilization by autoclaving

2. Wash Buffer:
1 X PBS (Phosphate Buffered Saline) + **0.05 % Tween 20**

3. 1 X PBS (Phosphate Buffered Saline):
i. Sodium Chloride (NaCl) = **8 g**
ii. Potassium chloride (KCl) **= 0.2 g**
iii. Disodium phosphate (Na_2HPO4) = **1.44 g**
iv. Potassium dihydrogen Phosphate (KH_2PO_4) = **0.24**
v. Total volume = 1000 ml (**1 litre**) in **Distilled water**.
vi. PH Adjustment **= 7.4** (with HCl.)
Sterilization by autoclaving.

4. Blocking Buffer: 1 % Casein in 1 X PBS, Sterilization by filtration