

**MIMICKING NATURAL ANTI-DENV ANTIBODY
RESPONSE IN MICE MODEL USING DENGUE
ENVELOPE PROTEIN DOMAIN 3**

Thesis Submitted for the Award of the Degree of

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In

Biochemistry

Submitted By

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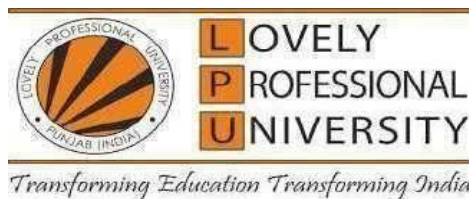
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August, 2023

CANDIDATE DECLARATION

I hereby declared that the presented work in the thesis entitled **“Mimicking natural anti-DENV antibody response in mice model using dengue envelope protein domain 3”** in fulfillment of degree of Doctor of Philosophy (Ph. D) is outcome of research work carried out by me under the supervision Dr. Anjuvan Singh, working as Professor , in the Department of Biotechnology at Lovely Professional University, Punjab, India. And Co-supervisor Dr. M. Monirul Islam working as Professor ,in the Department of Biochemistry and Molecular Biology at University of Chittagong, Chittagong, Bangladesh in the In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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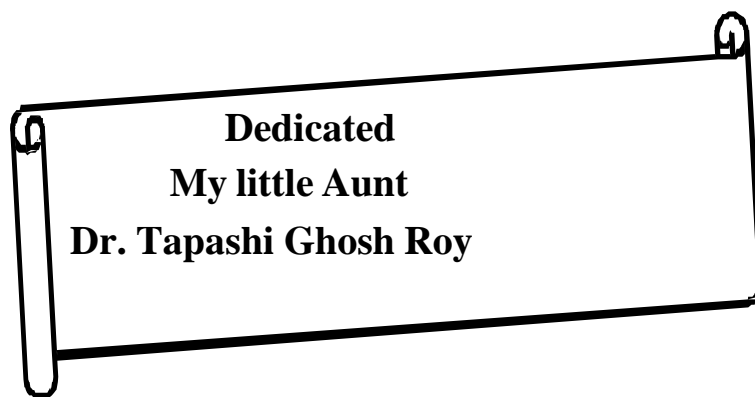
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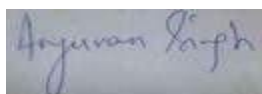
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**Dedicated
My little Aunt
Dr. Tapashi Ghosh Roy**

CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **“Mimicking natural anti-DENV antibody response in mice model using dengue envelope protein domain 3”** submitted in fulfillment of the requirement for the reward of degree of Doctor of Philosophy (Ph.D.) in the Department of Biochemistry, is a research work carried out by Moushumi Ghosh Roy (Registration No.11816342), is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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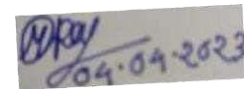
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A small rectangular stamp containing a handwritten signature and the date '04.04.2023'.

The Author

Abstract

Four dengue virus serotypes, DENV1-4, cause dengue disease in almost all over the tropical and sub-tropical countries worldwide and till to date, almost over 40% of population of the globe are at risk of having DENV infections in each year. Moreover, in recent years, both the incidence and severity of dengue infections have been increasing significantly affecting millions of lives each year. In addition, there is no dengue-specific treatment and/or effective dengue vaccine available. This is perhaps due to inadequate understanding of DENVs and dengue disease etiology in details. In this study, we first looked into the prevalence of the dengue virus serotypes (DENV1-4), and then we investigated the infecting DENV serotype-specific dengue disease manifestations (clinical signs and symptoms along with clinical/blood parameters) and anti-DENV IgG and IgM antibodies in dengue patients in Chittagong, Bangladesh. Finally, we investigated the DENV serotype-specific antibodies following DENV Envelop Protein Domain 3 (DENV ED3) immunization in BALB/c and Swiss albino mice models.

First, the DENV serotyping based on RT-PCR revealed that 42 out of 112 samples tested were positive for DENV where 76% samples had single DENV serotype infections and remaining 24% had been concurrently infected with multiple DENV serotypes. Among the single DENV serotypes infections, DENV4 was the most prevailed serotype, followed by DENV2, DENV1 and DENV3, what was clearly in contrast with previous observation that DENV3 was the most prevailed DENV serotype in Bangladesh and India. Interestingly, although the DENV1 was not prevalent in single DENV serotype infection, DENV1 was found common in 90% of samples concurrently infected with multiple DENVs. A detail investigation of the clinical signs and symptoms recorded from patients infected and hospitalized following DENV infections suggested that DENV1 and DENV2 serotype infections resulted in very similar signs and symptoms, however, signs and symptoms of DENV3 and DENV4 serotypes were slightly different. More specifically Fever, Vomiting, M-Pain, Ache & Pain, Myalgia, Re-o-pain, Rash, Diarrhea, Nausea and Headache observed following DENV1 and DENV2 infections, but Ache & pain and

Diarrhea were absent in DENV3 and DENV4 infected patients. However, postulation of a rationale for infecting DENV serotype with serospecific clinical dengue manifestation is difficult, but inclusion of more samples may help developing the rationale in future. Similarly, investigation of dengue infection associated blood parameters that are routine use, namely, platelet, RBC and WBC counts in blood following DENV infections indicated that, first, regardless of the infecting DENV serotypes, very rapid drops in platelet counts observed soon after DENV infection and dropping of platelet counts continued for 3-4 days of infection. However, following DENV3 infection decreasing trends of platelet counts continued further. Similar rapid drops in platelet counts were also observed in patients infected with concurrent multiple DENV serotypes. There was no DENV-serotype specific changes and/or discrepancies observed for hematocrit values and WBC counts. These observations together might suggest that the DENV3, both as a single serotype and co-infecting DENV in concurrent multiple DENV infections may results severe dengue disease compared to other DENV serotypes.

Detection of anti-DENV ED3 IgG and IgM antibodies in DENV infected sera indicated that natural DENV infections generated mostly DENV sero-cross-reactive anti-DENV antibodies and only in some cases DENV-sero-specific antibody responses were observed. Therefore, interpreting any rationale for infecting DENV serotypes and DENV-specific antibody responses seemed quite difficult. These observations were in contrast to what we anticipated and reported in literature that following natural DENV infections might generate infecting DENV-serotype-specific long-lasting anti-DENV antibodies. Furthermore, detection of anti- DENV IgM and IgG antibodies in natural DENV infections suggested for confirmation and differentiation of primary and secondary DENV infections, respectively. However, differentiation of DENV infection into primary and secondary infections based on the detection of anti-DENV IgM and IgG ratio though supported the RT-PCR-based DENV serotyping to some extent, but deviated a lot as well. For examples, RT- PCR serotyping showed single DENV serotype infections, but IgM/IgG-based serotyping indicated multiple DENV serotype infection, in addition to being RT-PCR negative but IgG/IgM positive.

To this end, we investigated anti-DENV ED3 specific IgG antibodies following artificial DENV ED3 immunization in Swiss albino and BALB/c mice models and compared the anti-DENV ED3 responses in natural DENV infections and artificial immunization in mice. First, the similarities, both the natural DENV1 infection and DENV1 ED1 (1ED3) immunization in mice generated DENV1-2-3 ED3 cross-reactive anti-ED3 IgG responses. The large discrepancies were the immune responses against remaining three DENVs; DENV2 ED3 (2ED3), DENV3 ED3 (3ED3) and DENV4 ED3 (4ED3) generated mostly DENV-ED3 specific (DENV sero-specific) IgG responses in mice model, which were in contrast to what have been observed in natural DENV infections. Secondly, the levels of sero-cross-reactive anti-DENV ED3 antibodies in natural DENV infections might suggestive for large number of secondary DENV infections, fully corroborated with the high frequencies of secondary DENV infections observed following IgG/IgM testing compared to DENV serotyping by RT-PCR of the same samples.

Later on we tried to mimic secondary DENV infection in mice model through re-injecting a secondary DENV ED3 to the mice primarily injected with a different DENV ED3s. For example, a primarily 1ED3 injected mice was secondarily injected with 2ED3, 3ED3 and 4ED3 in separate mice groups. Interestingly, such secondary immunization studies clearly indicated that level of sero-cross-reacting anti-DENV IgG increased with heterogeneous secondary ED3 immunization, except the 4ED3 as secondary ED3s that did not cross-stimulated primary anti-1ED3 response. Similarly while a primarily 2ED3 injected mice were secondarily injected with 1ED3, 3ED3 and 4ED3, the secondary 1ED3 and 4ED3 injections did not have much effect on primary anti-2ED3 response, while secondary 3ED3 immunization resulted 2ED3-3ED3 cross-reacting anti-ED3 IgG response. Very similar observations made for the secondary immunization using 1ED3, 2ED3 and 4ED3 in mice primarily injected with 3ED3. Interestingly, when mice primarily injected with 4ED3 (that generated almost absolute DENV4-serospecific IgG antibodies) were secondary injected with remaining DENV1 ED3s no boosting of anti-4ED3 IgG response was observed, rather secondary 1ED3 immunizations generated primary-like anti-ED3 IgG responses as observed following primary DENV1-3 ED3 immunizations.

These observations clearly suggested that the primary DENV1-3 ED3 immunization generated mostly sero-cross-reactive IgG responses while 4ED3 generated serospecific IgG response. The level of sero-cross-reactive IgG antibodies increased following secondary DENV ED3 immunization.

Then the mice immunization studies using multivalent ED3s in the same dose formulation to mimic concurrent multiple DENV infections showed that DENV1 ED3, DENV2 ED3 and DENV3 ED3 were co-dominantly immunogenic though the magnitudes of antibody titers were different for different ED3s. Interestingly, the poorly immunogenic DENV4 ED3 became almost non-immunogenic when injected with 2ED3 and/or 3ED3. This is perhaps for the first time observed that the immunogenicity of 4ED3 could be regained only when the multivalent ED3 formulation 1ED3 and 4ED3 together. Such adjuvanting effects of 1ED3 on 4ED3 is a new observation. Therefore, we suggest that a future DENV ED3-based vaccine formulation should consider the differential immunogenicity of four ED3s and the adjuvanting effects of 1ED3 on 4ED3 to have, at least, similar neutralizing antibodies.

To this end, to assess the impact of ED3 immunization on the expression of CD markers, namely CD44, CD62L) and cytokines, namely, IL-4 and IFN on CD4⁺ Th cells and CD8⁺ Tc cells in circulation were analyzed following DENV ED3 immunization at weekly intervals by flowcytometry. The DENV1 ED3 immunization generated the highest level of CD44, CD62L, IL-4 and IFN- γ on both Th and Tc cells, followed by 2ED3, and 3ED3 while the least immunogenic 4ED3 immunization resulted the lowest expression. The differential cytokine expression followed by immunization fully corroborated with their anti-ED3 IgG responses.

Altogether, these observations clearly suggested that despite of having very similar sequences and structures, four DENVs are different from their immunogenicity viewpoint. The discrepancies observed among four DENV ED3s in mice studies and discrepancies observed between the natural DENV infection and mice immunization studies could be originated from unexplored intrinsic properties of four DENVs. To this

end, we therefore, suggest that further studies towards the understanding of dengue etiology and future dengue vaccine design must consider the differential immunogenicity of four DENVs in a single tetravalent vaccine formulation.

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List of Abbreviations

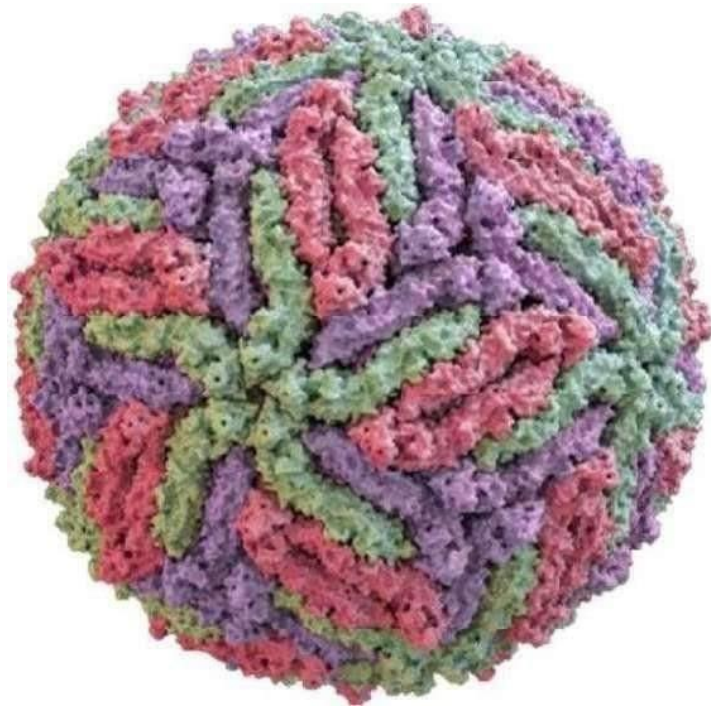
Ab	Antibody
ADE	Antibody-dependent enhancement
Amp	Ampicillin
APC	Antigen-presenting cell
ARB	Animal research branch
BSA	Bovine Serum Albumin
C	Capsid
C protein	Core/Capsid protein
Cp	Chloramphenicol
CRL	Carbohydrate recognizing lectin
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin
DENV	Dengue virus
DENV-1	Dengue serotype 1
DENV-1ED3	Dengue serotype 1 envelope protein domain 3
DENV-2	Dengue serotype 2
DENV-2ED3	Dengue serotype 2 envelope protein domain 3
DENV-3	Dengue serotype 3
DENV-3ED3	Dengue serotype 3 envelope protein domain 3
DENV-4	Dengue serotype 4
DENV-4ED3	Dengue serotype 4 envelope protein domain 3
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
E	Envelope
E protein	Envelope protein

ED1	Envelope protein domain 1
ED2	Envelope protein domain 2
ED3	Envelope protein domain 3
EDTA	Ethylene di-amine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fc-γR	Fc-gamma receptor
GAGS	Sulfated glycosaminoglycans
Gdn-HCl	Guanidine hydrochloride
GSL	Glycosphingolipid
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
ICDDR, B	International Centre for Diarrhoeal Disease Research, Bangladesh
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Ig-like domain	Immunoglobulin-like domain
IPTG	Isopropyl β -D-1-thiogalactopyranoside
M	Membrane
M protein	Membrane protein
mAb	Monoclonal antibody
MW	Molecular weight
Ni-NTA	Nickel-Nitro acetic acid column
LBP	Laminin binding proteins
NS	Non-structural protein

OD	Optical Density
OPD	O-phenyl Di-amine
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	PBS-Tween
PCR	Polymerase chain reaction
(pr)M	(pre-) Membrane
RdRps	RNA-dependent RNA polymerases
RNA	Ribonucleic acid
SC	Subcutaneous
SI	Secondary infection
TB	Transformation Buffer
TFA	Trifluoro acetic acid
TGN	Trans-Golgi network
WHO	World Health Organization
mg	Milligram
mL	Milliliter
µg	Microgram
µL	Microliter
°C	Degree Celsius

Chapter 01

Introduction



1.1 Introduction

Mosquitoes in hot and nearly tropical areas of the universe spread the dengue virus. Four dengue virus serotypes (DENV1-4) are responsible for causing the disease, which are structurally similar but antigenically different [1–5]. For DENVs, only humans are the known hosts. DENV infections have been reported to result a wide disease spectrum, from a little nonspecific fever to a serious dengue hemorrhagic fever. A unique high temperature known as "dengue fever (DF)" is the first sign of dengue, and in extreme cases, severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are symptoms of DENV infections [6]. The typical dengue fever includes a high temperature, myalgia, headache, rash, and arthralgia. Furthermore, hemorrhagic manifestation, vascular fluid leakage, shock, thrombocytopenia, and severe complement activation are signs of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), respectively [7]. The DHF and DSS are severe dengue manifestation, which usually require immediate hospitalization. Despite the fact that the DHF was first noticed more than 50 years ago in Southeast Asia, it is now only sometimes detected during current dengue outbreaks in other countries [8, 9]. Over 40% of the global population is thought to be susceptible to dengue due to the DENV infection, which accounts for around 390 million cases of illness annually. As a result, a notable example of the severe viral infections around the globe today is dengue, particularly in hot and humid summers' regions [1].

DENV is a positive-sense RNA virus with a single strand and an envelope. Only 10 gene products are encoded by the DENV RNA genome, including the capsid protein (C-protein), premembrane protein (prM-protein), envelope protein (E-protein), and seven nonstructural proteins (NS-proteins) [10]. The outermost surface structure of mature DENVs is composed of the three ED1, ED2, and ED3 domains [11]. Again, the ED3 is the most immunogenic part of the E-protein and antibodies against ED3 alone could prevent dengue infection in in vitro cell culture studies [12- 14]. The ED3 alone might determine DENV sero-specificity, despite the four serotypes sharing a great deal of

similarities [15, 16]. In order to fully comprehend DENV and dengue disease etiology, the ED3 may serve as an alternate simplified system and therefore, it may pave a way for future dengue disease management.

The finding of the unique residue determinants for each DENV serotype still eludes researchers despite several attempts to examine the atomic-level elements that determine the DENV-specific residues. This is due to the vast differences in sequencing, dengue pathophysiology, production of anti-DENV antibodies that are both neutralizing and sub-neutralizing during a real DENV infection, and immunogenicity of various DENV serotypes in various geographical areas. However, model systems are still lacking available for the dengue study community to use in order to thoroughly examine DENV infection. Consequently, creating a model to investigate DENV-sero-specificity would be worthwhile in order to learn more about DENV infection and aid in the management of the disease in the future.

12 Epidemiology

Dengue fever was first documented in 1779 in Cairo, Egypt, and Jakarta, Indonesia [17]. A year later, dengue fever cases in Philadelphia, United States, were also reported. Between 1823 and 1905, outbreaks were reported in Hong Kong, the Caribbean, and India. In the Philippines, the first significant DHF outbreak occurred in 1953–1954 [18]. All epidemics have been reported to be originated from four serologically distinct dengue serotypes [19]. Interestingly, the dengue epidemics showed a very large degree of serotype distribution and disease etiology from geolocation and outbreak time viewpoints [20]. For example, DENV-2 was the most common serotype between 1980 and early 1990 outbreaks, later DENV-3 serotype outbreak was reported [21, 22]. The Indian subcontinent is where the DENV-3 serotype first emerged, and it eventually spread to other continents [23]. Although there were a few minor DHF outbreaks in

South Asia between 1964 and 1966 [24], the first significant DHF pandemic took place in Sri Lanka in 1989. The DENV-3 and DENV-2 serotypes were responsible for the initial and Virus outbreaks in Sri Lanka later on [25]. The first significant DHF outbreak in India was caused by DENV-2 in 1996, and nearly ten years later, in 2003, DENV-3 caused a second DF pandemic in the nation [26]. However, a DHF outbreak has not yet been observed in Bangladesh, where DF was documented from the middle of the 1960s to the middle of the 1990s [27, 28]. South Asia's epidemiology trend for DHF is now comparable to Southeast Asia's. The causes of the universal revival of pandemic of DF and DHF are not entirely evident, despite the fact that sporadic DF has been known for more than 200 years [8]. The spread of more severe forms of the dengue virus, however, might have been aided by the virus's microevolution. Actually, There is evidence that the virus is displacing older genotypes with more advanced ones, which could illustrate the rise in dengue infections worldwide (Figure 1.1) [29,30]. Over 50% of people on earth reside in at-risk locations, and over 50% do so in nations where dengue is an endemic disease [31-32]. The likelihood of the dengue virus and its vectors spreading will likely increase due to changes in temperature, precipitation, and the density of natural catastrophes, in addition to non-climatic events connected to population expansion and migration, urbanization, and international trade and travel [33].

13 Dengue Infection in Human

Dengue virus is maintained in two transmission cycles: one involving monkeys and rainforest mosquitoes are non-human primates (enzootic cycle), and the cycle of epidemic transmission from one person to another that takes place in cities is the other (urban cycle) (Figure 1.2). Human hosts are affected by female mosquitoes, primarily *Aedes aegypti*, but also *Aedes albopictus* and some endemic vectors like *Aedes polynesiensis* in French Polynesia [34-36]. As a result of being transferred from humans when the two species interact, the virus is controlled in ongoing human-mosquito cycles in or close to human population centers. The daytime biting mosquito *Aedes aegypti*

loves to breed in home and peridomestic water sources. It has been able to thrive in urban areas and thanks to its ability to adapt to human surroundings and its desiccation-resistant eggs. Again, the Asian tiger mosquito, commonly known as *Aedes albopictus*, is a secondary dengue vector. Its role as a semi-tropical dengue vector was first noted in Taiwan in 1917 [37], and it is the primary dengue vector in countries when *Aedes aegypti* is absent. When both species cohabit in rural areas, the *Aedes albopictus* also serves as a maintenance vector [38,39]. Needlestick wounds, bone marrow transplants, and mucocutaneous contact with infected blood from aviremic patient are all examples of ways that the dengue virus can spread without using a mosquito as a vector [40].

14 Dengue Virus

Antigenically distinct but genetically closely related four serotypes (DENV1-4) [Figure 1.1] between 100 and 1,500 years ago, the dengue virus separately developed from ancestor sylvatic viruses [41]. Small errors in RNA transcription during the period of isolation and accumulation and differential genetic recombination might have contributed to the evolution of the four distinct serotypes [42,43]. This classification was later supported by molecular data, which also helped to clarify the phylogeny of the four serotypes. First DENV-4, then DENV-2, then DENV-1, and finally DENV-3 separated from the common progenitor [44] (Figure 1.3) (Table 1.1).

1.4.1 Dengue Virus Diversity

As for all RNA viruses, during replication, the DENV RNA-dependent RNA polymerase generates mistakes that lead to variability in the virus genome. Hence, dengue viruses live as a community of genetic variants, known as "quasispecies" [45]. Additional variability in DENV genomes is derived from recombination [46]. Dengue virus

serotypes (DENV1-4) from a phylogenetic group that, compared to other flaviviruses, are more intimately connected to one another [47]. All sero-groups share a high degree of genetic similarity and little compositional variance. Studies comparing sequences revealed a 30–40% change in amino acids among four DENV serotypes [48]. Although smaller, the changes in amino acids between each serotype are significant enough to be referred to as clusters of DENV variants [49]. But such minute genetic variation results a small but significant antigenic differentiation and variation in their serum reactivity. Moreover, the overall structures and individual protein structures are also very similar. Despite the above mentioned similarities, all serotypes could generate both serotype-specific as well as sero-cross reactive immune responses in the hosts.

1.4.2 Serotype Association in Disease Severity

There are controversies over the association of dengue diseases severity and infecting DENV serotypes [50,51]. It has been reported that DENV2 could produce more severe diseases than DENV1, for example, DENV1 infection was linked with signs of heightened vascular permeability and the DENV2 infection resulted shock to a greater extent in previous dengue outbreak [52]. Therefore, fluid replacement was less effective in treating DENV2 infections, and DENV2-induced pathophysiological responses were more aggressive [52]. Furthermore, a 25 years long dengue research in Thailand revealed that the most common serotype detected from DHF/DSS cases (35%), was DENV2, followed by DENV3 (31%), DENV1 (24%), and DENV4 (10%), suggesting DENV2 might cause more severe diseases than DENV1 or DENV4 [50]. However, DENV serotype-dengue severity association in Indonesia and Brazil showed that DENV3 resulted more severe dengue than the severity resulted from DENV2 infections [51, 53-54]. Moreover, only patient being infected with DENV1 and DENV3 had primary immune responses [51], but patients infected with DENV2 and DENV4 displayed a secondary antibody response [51]. Therefore, at present it is very difficult to assign dengue disease severity to the infecting DENV serotypes.

1.4.3 Virion Morphology

The dengue virus, either as a mature or immature particle, is roughly spherical and is comprised of a nucleocapsid core (containing viral genome), C proteins surrounded by a lipid bilayer, and an envelope protein (mediating virus attachment, fusion, and penetration to the host T-cells). The viral envelope spans through the lipid bilayer with the membrane (M) and envelope (E) proteins added. In the majority of flaviviruses, the E-protein is exposed on the outside of the virion and is glycosylated. Mature dengue virions exhibit a reasonably smooth surface with the icosahedral scaffold is made up of 180 copies of the envelope protein [55](Figure 1.4).

1.4.4 Dengue Virus Life Cycle

The pathogenic progression of dengue fever and the transportation of the virus depend on direct connection between host receptor molecules and the virus transmitted by a mosquito bite. As DENV receptors on host cells in mammalian cells, sulfated glycosaminoglycans (GAGs), lectins that identify carbohydrates, glycosphingolipids (GSL), laminin-binding proteins, and proteins with chaperone function have all been identified [56]. Via receptor-mediated endocytosis, the virus first binds to a specific receptor, after which it is internalized into an endosomal vesicle. Viral glycoproteins facilitate the union of the viral and cellular membranes in the endosome's acidic pH, enabling virion deconstruction and viral RNA release into the cytoplasm, where it controls the creation of viral proteins. Once all the essential proteins are synthesized, the non-structural proteins mediate viral RNA replication and subsequent production of new virions. On the surface of the endoplasmic reticulum (ER), structural proteins and newly produced RNA are assembled into viruses. Immature, infectious viral and subviral

particles are created as a result, and these particles are carried via the trans-Golgi network (TGN) (Figure 1.5) and broken by the host protease furin. Exocytosis is then used to release mature virions and subviral particles [57]. A single viral molecule can produce tens of thousands of DENV copies within just a few hours of infection, leading to cell damage and, in the worst cases, death. The cycle of dengue virus infection is catalyzed by RNA-dependent RNA polymerases (RdRps), which are expressed by the virus [58].

1.4.5 Genomic Organization

The 11 kb single-stranded genomic RNA of the dengue virus possesses an open reading frame (ORF) that contains over 10,000 nucleotides and is catalytically translated into ten viral proteins (Table 1.2). The genomic RNA has a type I cap (m7GpppAmp) at its 5' end but no 3' terminal polyadenyl tract. The DENV genome encodes ten viral proteins, three of which are structural proteins and are situated at the 5' end of the ORF (C: Capsid; prM/M: Precursor of Membrane protein; E: Envelope) (Figure 1.6) and at the 3' end of the DENV genome, there are seven non-structural (NS) multifunctional proteins in the following order: 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' [59,60].

1.4.6 Structural Organization

C, M, and E proteins are the three structural proteins that are encoded on the 5' quarter of the genome, while the other genomic sequences encode nonstructural (NS) proteins. [59, 61-63] (Table 1.3).

1.4.6.1 Core/Capsid Protein (C-Protein)

The capsid protein, which makes up the majority of the nucleocapsid's protein structure, is a brief protein with a length of 120 amino acids. The first protein to be created was protein C, which has a molecular weight of 13,500 Daltons [64]. In order to neutralize the negatively charged viral RNA in such a compact structure and facilitate the binding of the RNA genome for virus assembly, the C protein's greater Arginine and Lysine contents impart a net positive charge [61]. Each monomer of the tight dimer formed by the folding of the C-protein has four alpha helices [65]. The mature C protein (virion C), which serves as a signal peptide for prM ER translocation, NS3/NS2B, a viral serine protease, cleaves from the mature C protein (intracellular C) [66].

1.4.6.2 Membrane Associated Protein (M-Protein)

The prM protein is a precursor glycoprotein to the structural M-protein, which functions as a component of the nucleocapsid and aids the envelop protein in the formation of mature virions. It appears to be important for virus morphogenesis, which increases the infectivity of the virus [67]. When a virus is maturing in golgi vesicles, the prM protein functions as a chaperon protein to stop the irreversible inactivation of the E protein at low pH [68]. It also aids appropriate folding, membrane attachment, and assembly of the E protein [69]. The prM protein undergoes a delayed cleavage to create the M protein, a process that is associated to viral budding/maturation to prevent immature virions from merging with host T-cell membranes [70].

1.4.6.3 Envelope Protein (E-Protein)

The "class II" viral fusion protein known as envelope protein [71] is found on the exterior of the virus and is only involved in receptor-mediated virus attachment with host T-cell, low pH triggers the virus's fusion with the endosomal membranes of the host cell [72], and hemagglutination of erythrocytes [73]. It is also the main target of antibodies that neutralize viruses [74-79]. It is the protein that is most important for viral entry into cells [74,75]. The main protein component on the outside of the virion is the primary envelope glycoprotein (E), whose molecular weight varies between serotypes and ranges from 51,000 to 59,000 Dalton (494 to 501 amino acids) [80]. Moreover, it has been claimed that DENVs' E-protein is what makes them immunogenic [81,82]. Thus, a thorough knowledge of the dengue disease etiology depends greatly on the structural and functional structure of the E-protein [81]. The 400 amino acids at the E protein's N-terminal region, which has a length of about 500 amino acids, make up the ectodomain. The three structural domains that make up the E-protein ectodomain are Domain 1, Domain 2, and Domain 3 (ED1, ED2, and ED3, respectively [85, 86]. (Figure 1.7).

Domain 1: With 120 residues (residues 1-51, 132-191, 278-293) serving as a flexible hinge region that is crucial in fusing, domain 1 is the core eight-stranded beta-barrel domain [85,86]. The conformational modifications required for viral entrance and nucleocapsid escape from the endosomal compartment are mediated by domain 1 (ED1) [89–92].

Domain 2: The highly conserved fusion peptide is located at the distal end of domain 2, which is extended long and finger-like protrusion from ED1 and a second N-linked glycosylation that targets DC-SIGN [75,91-93]. The dimerization domain, ED2, is made up of two components (residues 52-131 and residues 192-277). In order for viral RNA to be released into the cell cytoplasm and fusion with the endosomal membrane to be induced by low pH, the ED2 fusion peptide is required [94].

Domain 3: Domain 3 is made up of the E protein's C terminal portion and has an immunoglobulin (Ig)-like structure (residues 294-395). A 15 residue linker connects ED3 to ED1 and one intra-monomer disulfide bridge holds them together. The ED3 lies perpendicular to the virion surface and is projected to the furthest [90] and reported to mediate host-virus interactions as well as virus internalization [70, 84, 95-97]. These interpretations were further corroborated with the observations that murine-derived monoclonal antibodies (MAb) targeting ED3 could prevent virus attachment [98] and recombinant ED3 could efficiently bind vertebrate target T-cells through interactions with heparan sulfates, inhibiting viral infection [99,100]. The ED3-induced antibodies were also capable of totally neutralizing homologous dengue serotypes, though not as effectively against heterologous serotypes. These results together open an era of dengue research to be focused on to explore the residue determinants of dengue sero- specificity and dengue eradication strategy.

1.4.6.4 Non-Structural Protein (NS-Protein)

The virus life cycle depends heavily on the seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Cell-associated, cell-surface, and extracellular non-virion variants of the NS1 protein can be detected [57] that gives rise to antibodies with complement-fixing activity to the host upon infection with DENV [101]. Protease activity and triphosphate/helicase activity are the two main functions of NS3 [59, 102-103] while viral RNA-dependent RNA polymerase is represented by the NS5 protein [61][59] and also has methyltransferase activity [104]. Other remaining NS-protein (NS2a, NS2b, NS4a, and NS4b) may be involved in internal localization and processing of other protein [105-107].

1.4.6.5 Receptor Recognition and Attachment

The E glycoprotein's interactions with cellular surface receptor molecules mediate virus entry into the target host cells. Multiple cellular receptor molecules are likely to be used in conjunction or sequentially during the process of receptor recognition and attachment.

15 Dengue Pathogenesis

According to reports, the natural long-term protective immunity is largely restricted to the DENV serotype that is causing infection, with little to no cross-protection to other DENV serotypes that are still present [108]. Cross-reactive antibodies with one serotype typically do not result in protection against other serotypes, but rather increase the severity of the disease after a secondary dengue infection with a different serotype [109]. Although the causes of severe dengue sickness are not fully known, they are probably multifactorial. Following secondary dengue infection with a DENV serotype other than the primary serotype, a number of theories, including the antibody-dependent enhancement theory (ADE), virulent virus theory, T-lymphocyte activation theory, and molecular mimicry theory (Figure 1.8; Table 1.4), have been put forth.

1.5.1 Antibody-Dependent Enhancement Theory

Neutralizing homotypic immunoglobulin G (IgG) antibodies that are produced after a primary DENV infection often offer long-lasting protection against the infecting serotype [110], but they can also produce a limited amount of cross-protective immunity against the other three serotypes [111]. To create antigen-antibody complexes, the patient's already present antibodies attach to the secondary dengue virus, but they may not be avid enough to neutralize a second serotype during secondary dengue infections caused by a serotype other than the primary infecting serotype. Hence, a greater number of cells could become infected with the dengue

virus as a result of such heterotypic secondary dengue infection [112], leading to more severe and aggressive dengue [113].

1.5.2 Virulence Virus Theory

Dengue pathogenicity (virulence) can also be associated with specific dengue strains [114-117]. For example, during the outbreak, a transition in hosts may have allowed increasing virulence of the prevalent DENV-2 [115] and according to reports; primary DENV-1 infection followed by DENV-2 or DENV-3 infection is associated with increased occurrences of DHF [116].

1.5.3 T-Lymphocyte Activation Theory

Memory DENV-specific T cells are faster than naive cells at recognizing DENV-infected cells upon secondary infection, activating quickly and secreting large amounts of cytokines that promote vascular permeability and plasma leakage [118]. According to a theory put out [119], Reactivation of cross-memory T cells that are focused on the initial DENV infection rather than the secondary virus infection results from a delayed immune response to the secondary virus infection. TLR-expressing monocytes grew in moderate dengue, but not in severe cases, and plasmacytoid dendritic cells declined more quickly in DHF patients than in DF patients. Depending on the combination of boosting and neutralizing antibodies present in the host, the overall impact on IFN response may differ from patient to patient [120].

1.5.4 Molecular Mimicry Theory

The alternative way to describe the etiology of DHF and DSS is autoimmune reactions resulting from molecular mimicry. A sequence similarity has been found between 20-amino acid sequences of E-protein and a group of clotting agents that includes plasminogen [118]. During DENV infections, cross-reactive antibodies to plasminogen emerged [121]. These results suggest a link between the emergence of hemorrhagic symptoms and these cross-reactive antibodies. Additionally, it has been discovered that antibodies to the NS1 protein attach to endothelial cells and human blood coagulation factors and integrin epitopes cross-react [122] contributing to dengue disease severity.

1.6 Secondary Dengue Infection

The severe infection brought on by various dengue viruses in a person who has already had dengue fever is known as secondary dengue. The body's stronger immunological reactions are what cause this. After recovering from the first infection, immunity to a certain serotype is believed to last for the rest of one's life. Only temporary and limited cross-immunity to the other serotypes exists after recovery [123,124]. After recurrent infections (secondary infection, SI) by different serotypes, the risk of getting severe dengue increases [109]. During an initial infection, dengue cross-reactive antibodies are generated, and these two components combine to form infectious immune complexes that infiltrate cells with Fc receptors. As a result, there are more infected cells overall, and each infected cell produces more viral material. In the late stages of the sickness, elevated cytokine concentrations, likely as an outcome of T cell clearance of diseased cells, create blood vessel permeability, which results in shock and death [125,126]. A second case of dengue infection of a different serotype and contributions from the circulating virus are risk factors for DHF/DSS [127]. Although DHF/DSS can result from SI brought on by any of the four DENV serotypes, not all infection sequences have the same likelihood of doing so. Early studies carried out in Thailand found that secondary DENV-2 infection had a 5-7 times greater likelihood of being connected to DHF as opposed to subsequent DENV-

1 or DENV-3 infection, with the largest risk occurring when DENV-1 is followed by DENV-2 [7]. In the Cuban experience, the DENV-1/DENV-2 and DENV-1/DENV-3 viruses have been linked to the highest severity. The DENV-2/DENV-3 sequence demonstrated low pathogenicity [128,129,130]. Secondary infections can cause DHF even 20 years after a first infection [128].

17 Clinical Manifestation of Natural Dengue Infection

An incubation period of 4 to 7/14 days follows before symptoms appear after the DENV transmission. The dengue symptoms are categorized into three forms, namely: Dengue Fever, Dengue Haemorrhagic Fever, and Dengue Shock Syndrome (Figure 1.9).

1.7.1 Dengue Fever (DF)

Most cases of dengue fever are self-limiting and not fatal. Infections that are either primary or secondary might result in dengue fever. The symptoms appear suddenly and include a high fever, severe headache (particularly behind the eyes), arthralgia, myalgia, anorexia, abdominal discomfort, and occasionally a maculopapular rash. The duration of the fever, which might be biphasic, ranges from 2 to 7 days [131]. On the face, neck, and chest, the characteristic feature of flushing is frequently seen [132]. Elevated levels of liver enzymes, thrombocytopenia, and absolute granulocytopenia are all linked to DF. Even though haemorrhagic signs of dengue fever are rare, few people have experienced petechiae/purpura, gastrointestinal bleeding, epistaxis, and gingival bleeding [133]. Dengue fever normally passes without a trace, though it can take longer in adulthood [134].

1.7.2 Dengue Hemorrhagic Fever (DHF)

DHF typically come after primary dengue infections, especially in young children, however, it generally follows secondary infection. It is believed that maternally acquired dengue antibodies encourage initial infections in such infants [135,136]. High fever, hemorrhagic events, and circulatory failure symptoms are the characteristics of DHF. Once the fever has subsided, hemorrhagic symptoms typically take place [137]. Gum bleeding and petechiae are less severe bleeding symptoms. Hematemesis or melaena, followed by epistaxis, are frequent symptoms of hemorrhage in the gastrointestinal system [138,139]

1.7.3 Dengue Shock Syndrome (DSS)

A very high death rate (about 9.3%, and up to 47% in cases of deep shock) is linked to dengue shock syndrome [133]. Circulatory abnormalities, circumoral cyanosis, and chilly blotchy skin are symptoms of severe plasma leakage that results in dengue shock syndrome. Early warning indications of impending shock include persistent vomiting and acute abdominal discomfort [135]. The start of severe shock may be indicated by sudden hypotension [138]. Due to metabolic or electrolyte abnormalities, encephalopathy may occur along with dengue shock syndrome [135].

18 Serotype Specificity, Sero-Cross-Reactivity and the ED3

Virus T-cell interaction involves ED3 [79]. Monoclonal antibodies that are specific to ED3 have been reported to be more effective at neutralizing DENVs in mice over antibodies that are specific to ED1 or ED2 [140,141]. Twenty-two hydrophobic and aromatic residues, of which ten are not totally conserved among the four serotypes, make up the core of ED3, a structurally independent domain. This implies that the Interdigitation of the side-chains in the core of ED3 can accommodate some sequence

changes [142]. Additionally, ED3 can even trigger sero-specific immunological reactions [15] and can be separated from the remainder of the envelope protein without changing the native structure identified by monoclonal antibodies [143], making it a useful model for research in immunology and biophysics. Moreover, for new subunit vaccines, the ED3 has long been thought of as a promising immunogen [144,145]. The four dengue serotypes, the specificity of all four ED3s (representing all four DENV serotypes) must be integrated into a single diagnostic test to give appropriate sensitivity for global use (Table 1.5). The ED3 has been successfully used to increase the specificity of DENV IgG antibodies since it has the least resemblance with any other proteins of other flaviviruses [108,146,147]. Additionally, interacting with all four DENV serotypes is the mouse monoclonal antibody 2H12 and neutralizes DENV-1, 3, and 4 encounters a much conserved epitope in the AB loop (residues 314–317) of ED3 and has a conserved manner of binding [148]. The fact that, this binding varied by serotype and was temperature-dependent which suggests that the virus's stability is an important consideration in virus neutralization. Similar to this, another monoclonal antibody (mAb), 4E11 produced against DENV1 [149], detects only viruses of the dengue group and no other flaviviruses [150] and has different degrees of efficacy in neutralizing the four serotypes of DENV [81]. Importantly, the mAb 4E11's epitope residues are found in the E-ED3 protein's domain [150].

19 Rationale for selecting DENV ED3s

Despite being fragmented domain of the whole DENV envelop protein, the ED3s of DENV1-4 could retained DENV1-4 virus serospecificity. Therefore, DENV1-4 ED3s offer a promising and very simplified tool, both for studying dengue disease etiology and intervening anti-dengue strategy [148]. Interestingly, all four DENV ED3s have been reported to be immunogenic in mice model without having any noticeable toxicity. In addition, the ED3s mediate DENV-host interactions and reported to contain all the putative epitope residues conferring DENV serospecificity, and sero-cross-specificity and importantly, DENV neutralizing monoclonal antibodies had also been reported to be on ED3s [15,71,140,151-155]. Altogether, these observations suggested that DENV1-4 ED3s would be model systems representing the whole live DENV1-4, therefore, use of DENV specific ED3s might overcome the complexity associated with working using live viruses. Accordingly, in this study we selected DENV1-4 ED3s as alternative to the live

DENV1-4 for both studying natural anti-dengue antibody responses following dengue infection and for studying anti-dengue antibody responses against single and multiple ED3 immunization in mice model.

1.9.1 Importance of ED3 in Dengue Diagnosis

The E-protein, which is also regarded to be the primary antigenic site of the DENV, induces neutralizing antibodies and cell-mediated immune responses in hosts infected with DENV [156-160]. The most important E-Protein characteristics from the perspectives of vaccination and sero-diagnosis are connected to ED3 [161,162]. The crucial and predominate virus-specific neutralizing sites are present in the well-characterized ED3 [163]. Most DENV serotype-specific antibodies bind to ED3, according to studies using mice mAbs [78,98,145,153,163,164] Additionally, because ED3 exhibits reduced sequence closeness with other Flaviviruses co-circulating in the similar native locations, it has been suggested as an emerging tool for the reliable diagnosis of dengue-infected patients [165-167]. It is discovered that the mAbs against ED1/ED2 or prM neutralize ineffectively and exhibit significant cross-reactivity with the four DENV serotypes [157]. The ED3s of 4 serotypes could potentially be employed for serological typing of the 4 dengue strains despite having 70–89% sequence identity [168], according to several studies [169,170]. In the acute and convalescent phases of dengue disease, a combination of the four distinct dengue serotype ED3s or a chimeric ED3 giving sero-specificity of all dengue ED3s has already been demonstrated to be a reliable recombinant protein [147,165,171,172]. Despite this, the existence of the four dengue serotypes suggests that a single diagnostic test must combine the specificity of all four ED3s (representing all four DENV serotypes) in order to provide sufficient sensitivity for application worldwide [167].

1.9.2 Diagnosis of Dengue Infection

Since dengue virus infection produces several nonspecific syndromes, a solely clinical symptom-based diagnosis is unreliable. Therefore the preferred methods for diagnosing DENV infections include the disclosure of dengue-specific IgM antibodies, DENV NS1 antigen assays, and viral RNA amplification in patient serum samples and virus neutralization assays [173]. Presently, the three primary methods used by most laboratories are viral isolation and characterization, genetic sequence discovery utilizing a nucleic acid amplification technology assay, and the detection of dengue virus-specific antibodies to determine the presence of dengue virus infection [174].

1.9.3 Virus Isolation and Characterization

In order to diagnose DENV infection, the conventional diagnostic technique has been virus isolation. [175]. It is still the "gold standard" when using cell culture and mosquitoes, despite the RT-PCR method for quick diagnosis rapidly displacing it. According to reports, the cultivated virus can be detected at day 1 with improved sensitivity using RT-PCR and cell culture [176]. With cultivated mosquito cells like the AP-61, Tra-284, C6/36, AP-64, and CLA-1 cell lines or mammalian cells like the LLCMK2, Vero, and BHK-21 cell lines, it is simple to isolate viruses from clinical samples [177]. Early in the acute period of the illness, the rapid anamnestic production of cross-reactive antibodies that create immunological complexes with the circulating virus, however, makes viral extraction from secondary infected patients more challenging [178]. Although DENV can only be definitively detected by virus isolation, the process is not very practical because isolation might last for days or weeks [179].

1.9.4. Serological Diagnosis

The five serological techniques that have been used to determine dengue infection are complement fixation (CF), hemagglutination inhibition (HI), neutralization test (NT), indirect immunoglobulin G (IgG) ELISA and immunoglobulin M (IgM) capture enzyme linked immunosorbent assay (MAC-ELISA). Because to its high level of

sensitivity and simplicity, the HI has become recognized as the gold standard technique [180], although it lacks specificity, requires paired samples, and cannot identify the viral serotype that is causing the infection. The NT has a relatively low specificity and can only be used to determine the serotype that is infected in situations of early dengue infections [180]. However, because the four separate DENV strains must be propagated in a BSL2 laboratory [181], in addition to the challenges associated with the standardization of the methodologies [182,183] it is both time-consuming and challenging to manage. Additionally, the IgM capture ELISA (MAC-ELISA) for identifying acute phase antibodies is sensitive enough to detect anti-dengue IgM antibodies which is the most popular technique for diagnosing dengue. Convalescent samples are not required [184-186] due to the fact that anti-dengue IgM antibodies appear five days after the onset of the initial clinical symptoms. MAC- ELISA offers the benefit of quickly detecting the spread of transmission during epidemics. This IgM antibody, however, strongly reacts with other flaviviruses, leading to large levels of "false positive" predictions and extremely low detection levels, particularly in secondary infections [187]. It has also been reported to use IgG- ELISA to find anti-dengue IgG antibodies. It can be utilized to distinguish between primary and secondary dengue illnesses [188]. The test is straightforward and simple to perform, and it may be used to analyze a large number of samples [189-192]. However, it lacks specificity for the different flavivirus sero complex groups.

1.9.5. NS1-based Diagnosis:

The dengue NS1 antigen test is an easy, rapid diagnostic procedure for dengue virus infection [193]. This antigen has been found in DENV-infected individuals' serum as early as 1 day after the onset of symptoms (DPO) and as late as 18 DPO, prior to the formation of antibodies. [194-197]. The diagnostic effectiveness for the early diagnosis of dengue infection may be improved by combining the NS1 antigen and antibody tests [193]. Additionally, sensitive, simple, and specific anti-NS1 serotype IgG ELISA is available [174] and anti-dengue virus IgM antibodies with specificity for both E/M and NS1 show no cross-reactivity. These assays, which are not type-specific at the moment, are being tested in various settings for their efficacy and affordability as diagnostic tools. Additionally, this approach will no longer produce a positive result a few weeks after the onset of the first manifests [198].

1.9.6. PCR-based Diagnosis:

Dengue virus detection by molecular techniques, such as RT-PCR, is strongly suggestive of acute dengue virus infection in human serum or plasma [199-201]. The single step multiplex RT-PCR [202] and two-step nested RT-PCR [201] are two RT-PCR techniques that are well known for the detection and typing of dengue virus. By analyzing the various amplicon sizes in the Agarose gel, they have the benefit of being able to identify and distinguish the four dengue virus serotypes. For the purpose of detecting the dengue virus in acute-phase serum samples, numerous researchers have recently reported on real-time RT-PCR methods that are entirely automatic [203-206]. In comparison to traditional PCR or RT-PCR procedures, the real-time PCR or qRT-PCR assay has numerous benefits, including rapidity, the capacity to produce quantitative values, a reduced rate of contamination, higher sensitivity, higher specificity, and ease of standardization. As a result, viral RNA can be found even before symptoms appear. Thus, PCR-based diagnosis is more accurate, swift, simple, and affordable than virus isolation approaches [207], but it stops working a few weeks after an infection starts [208].

1.10 Immuno-Pathogenesis of Dengue

When the innate and adaptive immune response swiftly clears the virus from host tissues—rather than when the viral burden is highest in vivo then dengue characteristics are discovered. As a result, it has been suggested that the etiology of clinically significant problems is intimately tied to the immune response of the host [209].

1.11 The Humoral Immune Response and Antibody-Dependent Enhancement

In the majority of acute viral infection scenarios, antibodies—neutralizing and non-neutralizing—are linked to control, elimination, and eventually protection. Homotypic immunity, which lasts for a lifetime against that serotype, can be acquired through infection, whereas heterotypic immunity, which lasts only temporarily against other serotypes, cannot [210]. On virus-neutralizing antibodies, homotypic immunity is thought to be based. Very likely as a result of E-protein-specific antibodies that are

cross-reactive, which provide protection when concentrations rise over a certain threshold, heterotypic immunity is transitory. The person becomes more vulnerable to infection by different DEN virus serotypes as time goes on because these antibody levels decline [211-213]. Several prospective cohort studies conducted in Asia and Latin America have shown that secondary infection is an epidemiological risk factor for severe dengue [209-213]. The most typical explanation for the increased risk of illness in subsequent infections is that the virus is bound by non-neutralizing, cross-reactive antibodies created by an initial infection, and allows it to enter Fc-receptor containing cells with greater potential. This process, known as antibody-dependent enhancement (ADE), increases the number of virus-infected cells, may increase the likelihood of developing a serious illness [214-223]. According to concerns about ADE, a vaccination could worsen a disease by causing re-infection if one or more serotypes are only partially protected.

1.11.1 The Cellular Immune Response:

It is conceivable that cellular immune responses have a part in both the resolution of viral infection and the beginning of severe disease. Numerous DENV proteins have been found to include CD4+ and CD8+ T cell epitopes that are unique to DENV [215-217]. Although T cell epitopes on structural proteins have been described, non-structural proteins have also been found to contain the great majority of them (Table-1.6). However, it is believed that the development of plasma leakage is influenced by memory-activated T lymphocytes that may detect both conserved and changed peptide ligand epitopes [218]. After a subsequent heterologous infection, cross-reactive T cells that are mostly specific for a major DENV serotype are said to arise, according to T cell original antigenic sin [219]. The increase in preexisting cross-reactive and low-affinity memory T cells results in ineffective viral control. These cells also promote immunopathology and severe dengue sickness by overproducing inflammatory cytokines [219]. Some hypotheses propose a correlation between disease severity and the magnitude of the T cell response [220]. Contrary to the predictions of primordial antigenic sin, a growing body of evidence suggests that T cells may play a role in the regulation of DENV infection [221]. Numerous studies in mice and human samples demonstrate that dengue-specific CD4+ and CD8+ T lymphocytes offer protection from serious sickness [222].

1.12 Current Dengue Research Status

The success of dengue study, *in spite of* countless efforts throughout the last 30 years, is still at a bottleneck. This is due to four distinct DENVs' enigmatic intrinsic immunogenicity, Anti-DENV antibody sero-cross-talks that are complex, different seroprevalence, and serotype-dependent dengue severity [223]. Vaccines are the most effective method for preventing this crippling disease because previous attempts to reduce the mosquito vector have little success [224]. Despite the fact that there is currently no dengue vaccine, numerous effective options are in the middle to late phases of development [225]. The biggest problem is that there isn't a dengue vaccine formulation that can make DENV-specific antibodies that can neutralize all four DENV serotypes [226]. The two vaccines with the highest potential are Sanofi Pasteur's Dengvaxia [227], demonstrated disputed efficacy in several geographic locations and dengue-endemic areas [227]. For instance, it was unable to generate comparable levels of type-specific antibodies against each of the four DENV serotypes, instead inducing a skew anti-DENV antibody response against DENV1 serotype [227,228]. Otherwise, the National Institute of Allergy and Infectious Diseases' TV003/005 [229] reported that all four DENV serotypes have been found to induce substantial levels of anti-DENV antibodies [230], although there are still few details on its effectiveness in a variety of ethnic groups in various areas where dengue is common [231]. ED3 has been implicated in mediating host-virus interactions, and monoclonal antibodies raised against ED3s have been shown to virtually fully neutralize DENVs in both in vitro and in vivo settings [232]. Consequently, a good candidate for a dengue vaccine would be the fragmented DENV-specific ED3s [233]. This is because DENV ED3s have immunogenic properties and could keep their serospecificity [234,235] in spite of being a portion of envelop protein in its entirety. The recombinant ED3s may also fold naturally into an enduring native-style building. The ED3s collectively could therefore act as a straightforward good dengue research model [235] and, in turn, could be used as a prototype for a potential dengue vaccine as opposed to live, attenuated DENVs [236].

1.13 Aims and Objectives:

Though ED3 is a fragmented domain of the whole dengue envelope protein, it can maintain DENV sero-specificity. Therefore, it is likely that it can be used to mimic the natural anti DENV response in mice model. Therefore, in this research project we aimed at

1. Investigation of natural dengue infection

- a) DENV Serotyping and DENV seroprevalence in natural DENV infection in Chittagong, Bangladesh;
- b) Investigation of anti-DENV IgM and anti-DENV IgG antibody responses in natural DENV infections;
- c) Exploring the correlation of infecting DENV serotype and dengue disease severity.

2. Mimicking natural anti-DENV antibody responses in micemodel

- a) Anti-DENV-serotype-specific antibody responses in primary DENV-ED3 immunization (primary responses) in mice model following artificial immunization using DENV-serotype-specific ED3s:
 - i) Evaluation of DENV-ED3 serospecificity;
 - ii) Evaluation of DENV-ED3 sero-cross-reactivity.
- b) Investigation of serospecific and sero-cross-reactive secondary anti-ED3 antibody responses in mice following secondary heterotypic DENV ED3 immunization;
- c) Anti-ED3 antibody responses in mice following concurrently immunized with different combinations of ED3s mimicking anti-DENV responses following natural concurrent multiple DENV infections.

3. Investigation of the immune statuses (T cell memory status) of mice injected with DENV-serotype-specific ED3s by flow-cytometer.

4. Establishing the correlation (if there is any) between anti-DENV responses in natural DENV infections and anti-ED3 responses in mice model and proposing a model system for future dengue research.

Table 1.1: Estimated sequence homology among dengue viruses*.

DENVs	Sequence homology (%)			
	DENV1	DENV2	DENV3	DENV4
DENV1		42	39	73
DENV2	36		24	23
DENV3	30	34		52
DENV4	73	34	55	

*The sequence homology table was generated from Chang AY et al., [33]

Table 1.2: Typical lengths of the ten DENV proteins. Data were reproduced from multiple sequence alignments of amino acid sequences derived from complete genome sequences in GenBank.*

Proteins	DENV-1	DENV-2	DENV-3	DENV-4
C	114	114	113	113
prM/M	166	166	166	166
E	495	495	495	495
NS1	352	352	352	352
NS2A	218	218	218	218
NS2B	130	130	130	130
NS3	619	618	619	618
NS4A	150	150	150	150
NS4B	249	248	248	245
NS5	899	900	900	900
Length of CDS	3392	3391	3390	3387

* The table of dengue genome sequences was reproduced from Osatomi K et al., [238]

Table 1.3: Structural and functional properties of DENV proteins.

Protein	M.W (KDa.)	Localization	Known Modifications	Known Function	Protein Interactions
Capsid	13	Lipid droplets, Cytoplasm, Nucleus	None	RNA binding, nucleo-capsid Precursor	Daxx, Sec3
Membrane	8	ER membrane	Glycosylation	Virion morphogenesis, transport	Envelope, v-ATPase, Claudin-1
Envelope	54	ER membrane	Glycosylation	Virion assembly, receptor binding, membrane –fusion	prM, Nkp44, Bip, Calnexin, Caltri-Culin
NS1	46	ER lumen, Plasma membrane	Glycosylation, GPI anchor	Replication, virus maturation	NS4A, hnRNP C1/C2, Clusterin, STAT3
NS2A	22	ER membrane	None	Replication	NS3
NS2B	14	ER membrane	None	Replication, co-factor of NS3 protease	NS3
NS3	69	Cytoplasm	None	Protease, helicase, NTPase, RTPase	NS2B, NS5, NS4B, NRBP, La
NS4A	16	ER membrane	None	Replication, anti-STAT1	NS3
NS4B	27				
NS5	104	Cytoplasm, Nucleus	Phosphorylation	Methyltransferase, guanyltransferase, RdRp, anti-STAT2	NS3, STAT2, Importin, ZO-1

*The table was reproduced from Rey FA et al., [90]

Table 1.4: Existing hypothesis to explain DENV pathogenesis*

Hypothesis	Mechanism	Results
ADE	Immune-complex	Enhanced infection of target T-cell
T-cell mediated	Cytokine production	Increased vascular permeability
Virus virulence	Highly virulent strains	Increased infection of target T-cells
Molecular mimicry	Autoimmune reactions	Hemorrhagic manifestations

*This table was reproduced from White horn J et al [238].

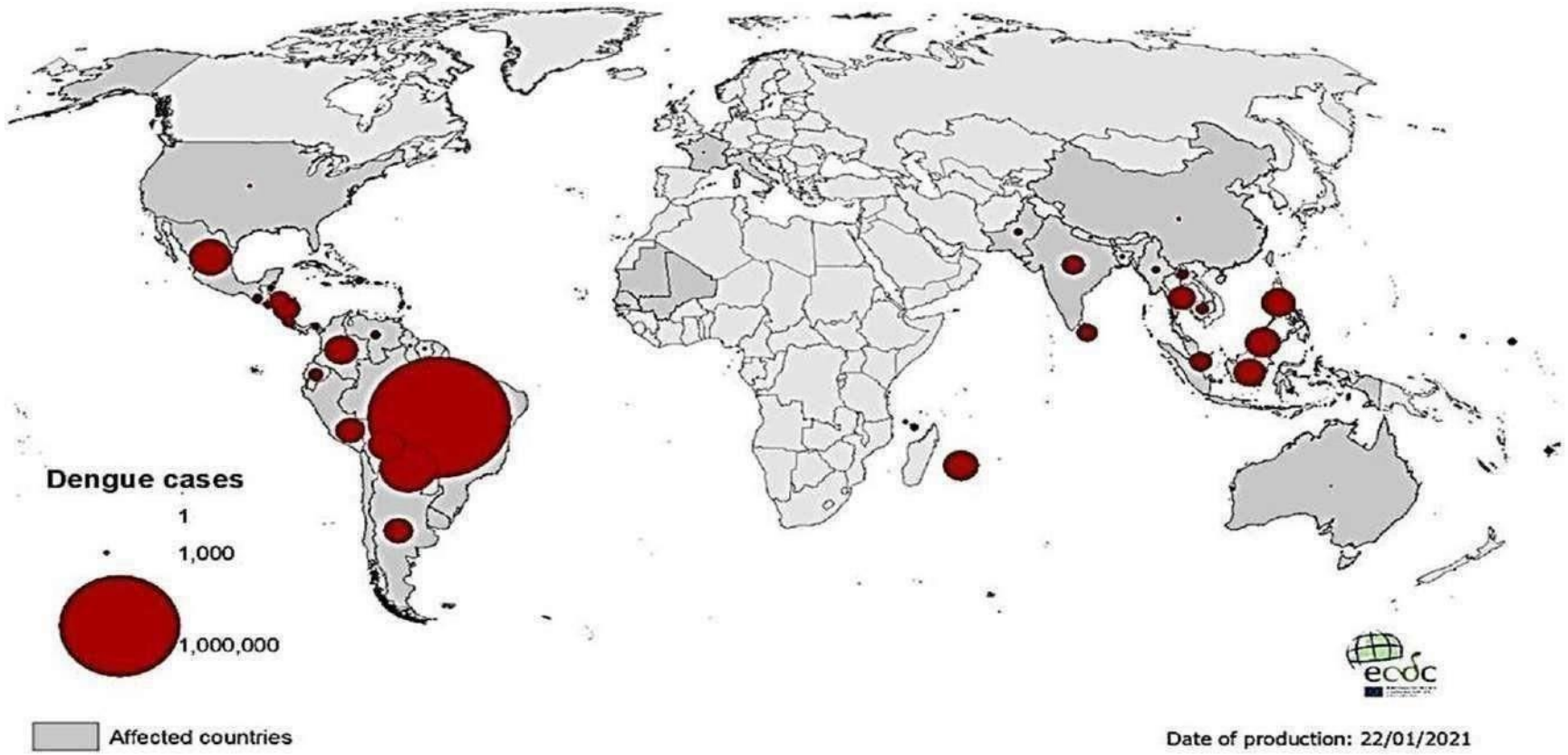


Figure 1.1: Geographical distribution of dengue cases reported worldwide (from 01 Jan 2020 - 31 Dec 2020) [239]

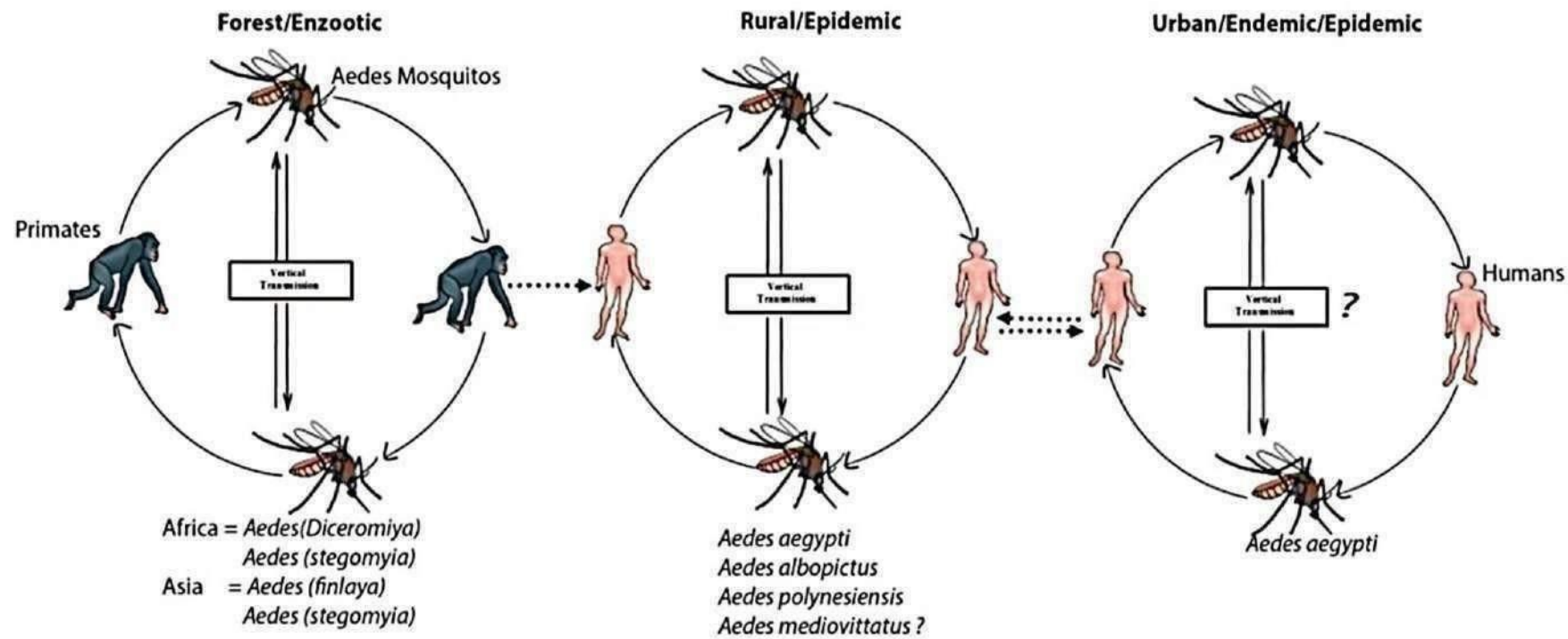


Figure 1.2: Transmission of dengue viruses' [240]

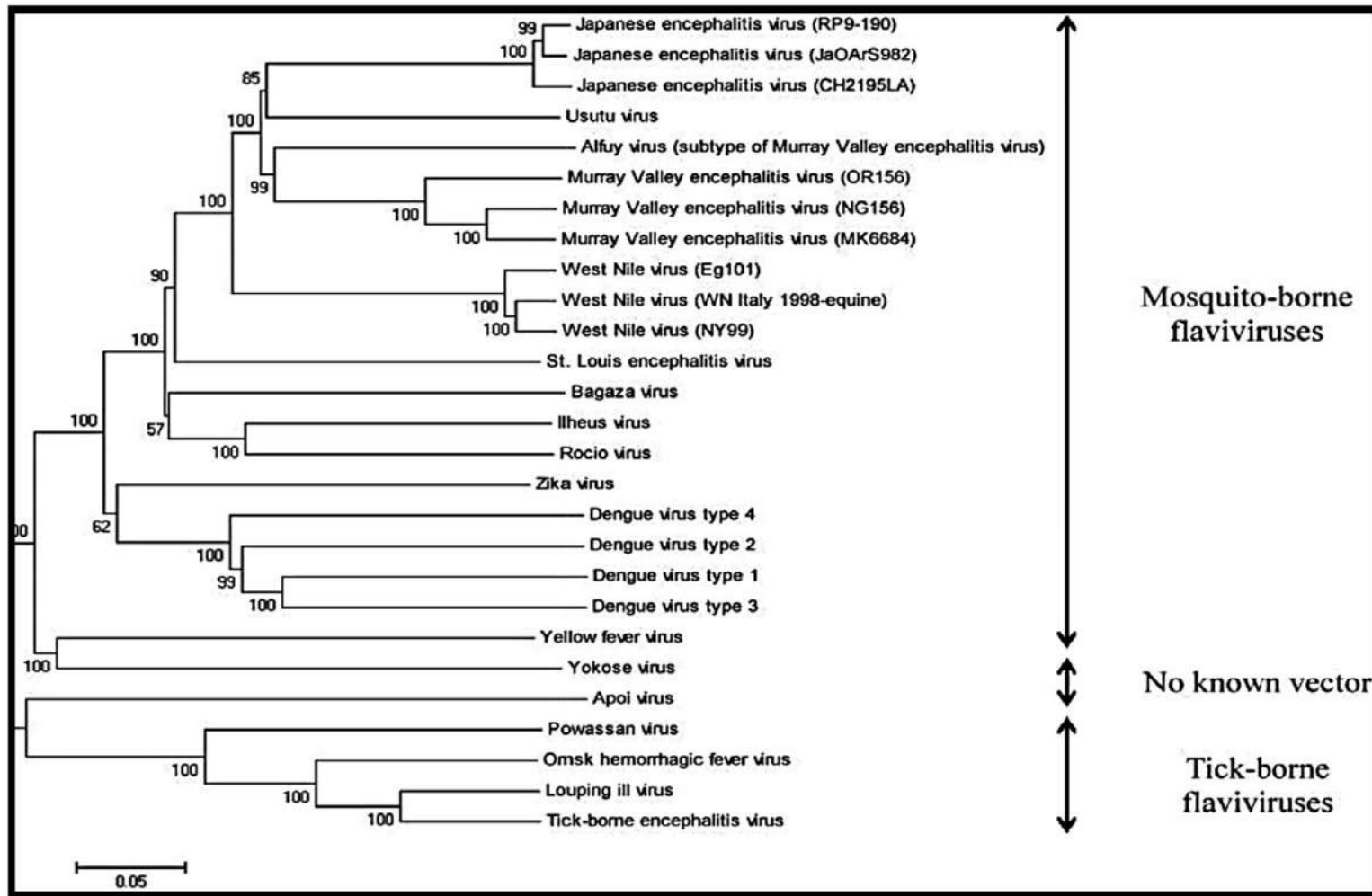


Figure 1.3: Maximum likelihood tree of dengue virus with other flaviviruses [241]

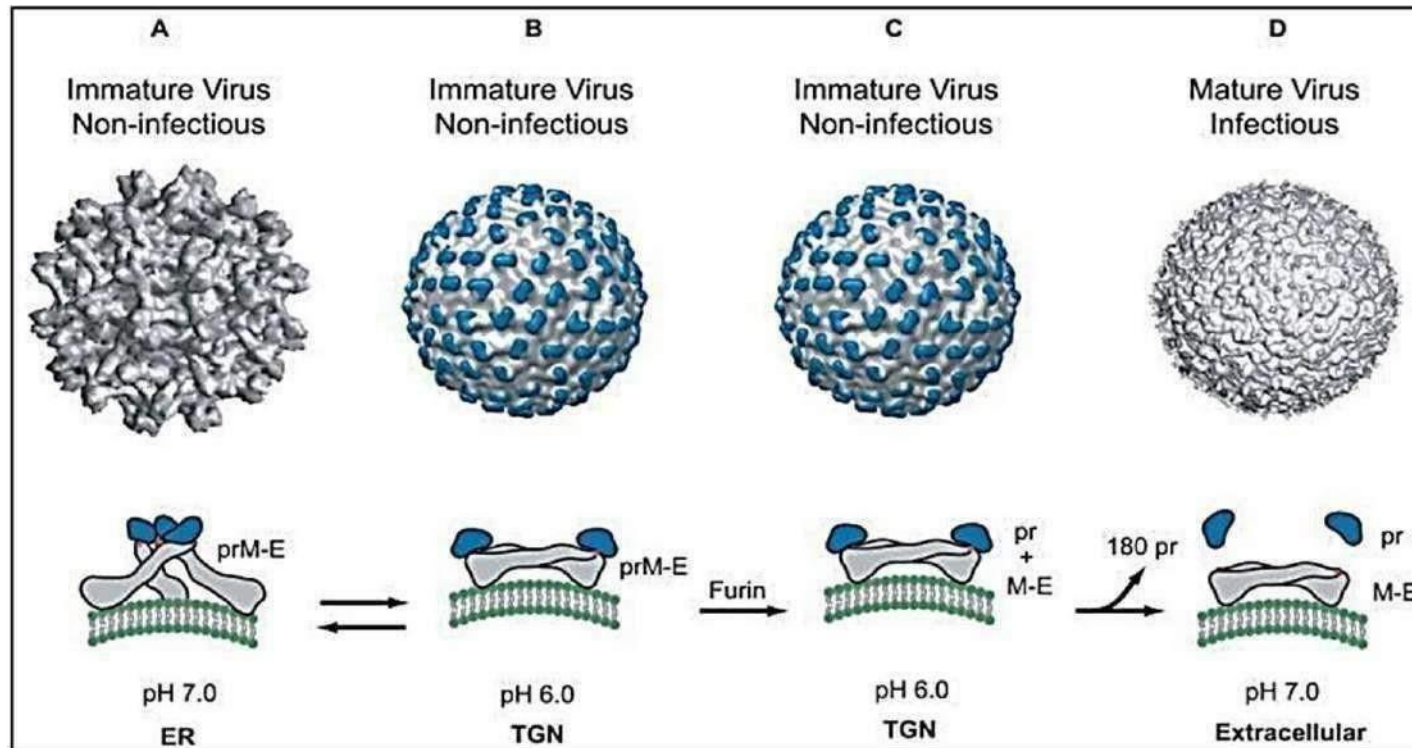


Figure 1.4: Structure of the dengue virion and conformations of the E protein. ER, TGN and PrM stand for endoplasmic reticulum, trans-golgi network and precursor of the membrane protein respectively [242]

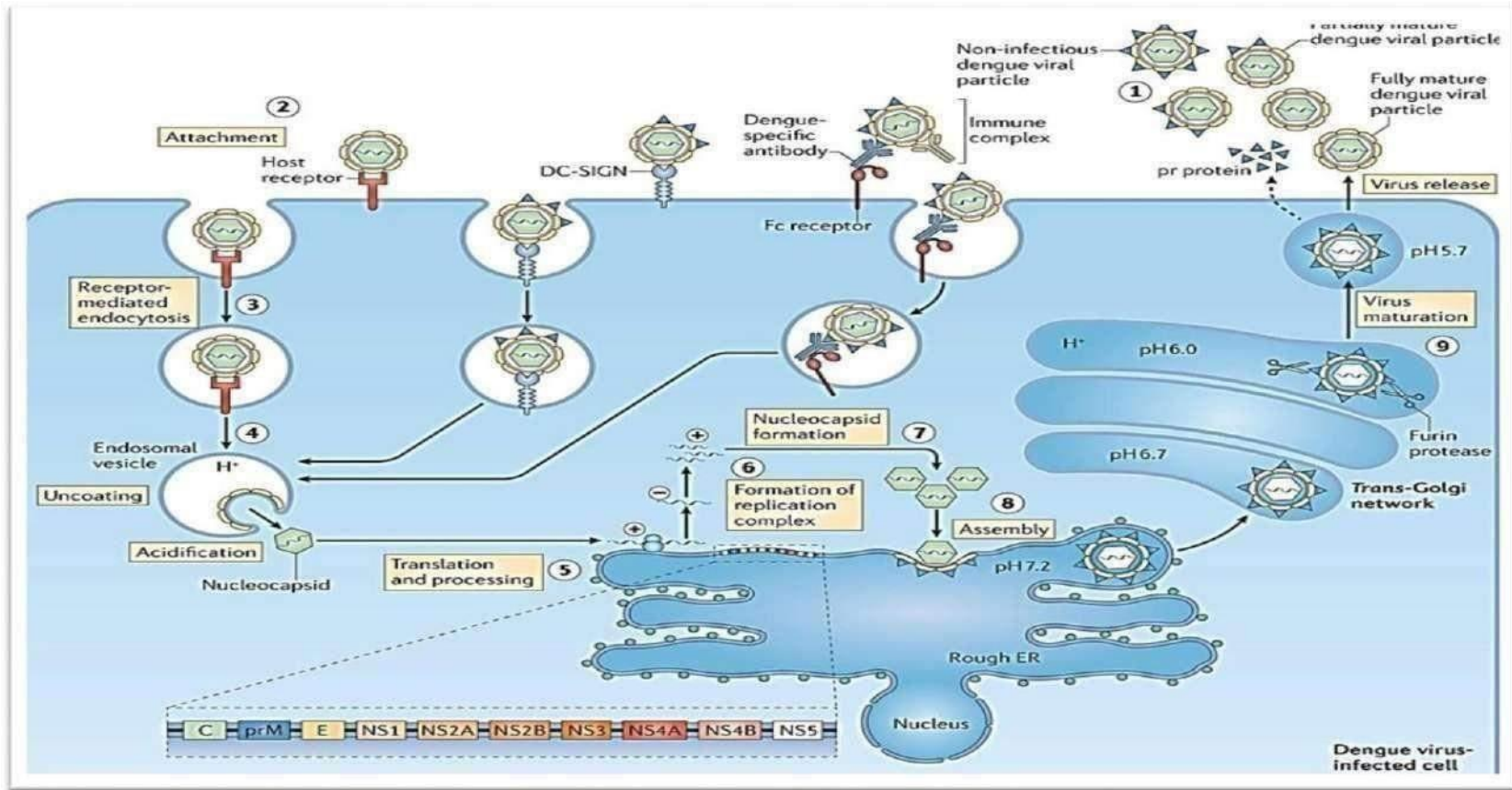


Figure 1.5: The life cycle of DENV [243]

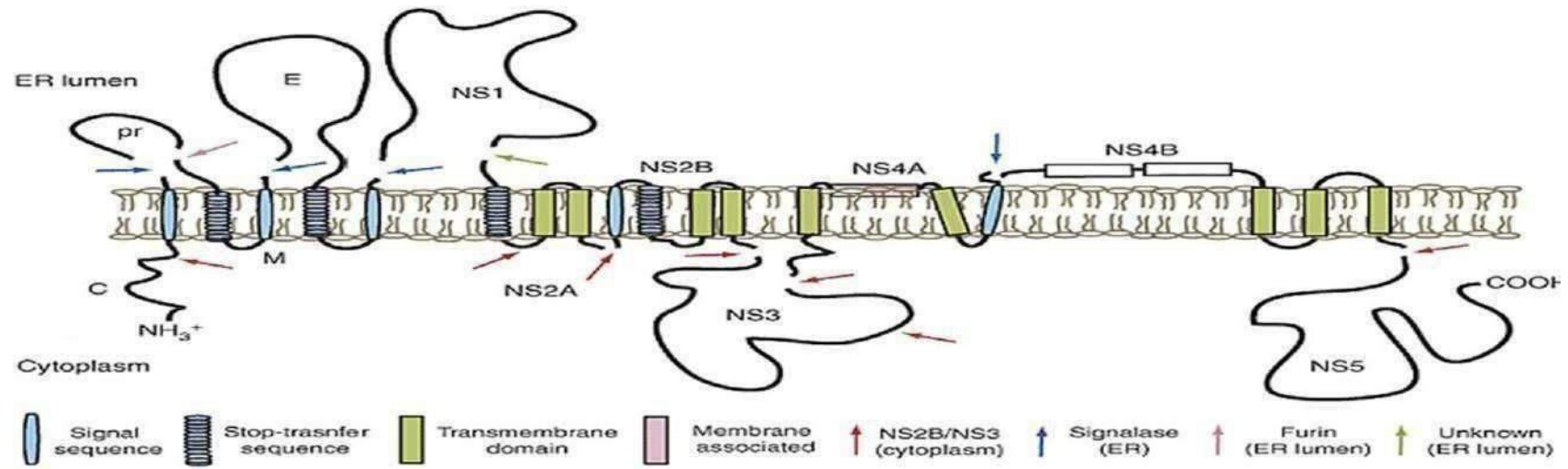
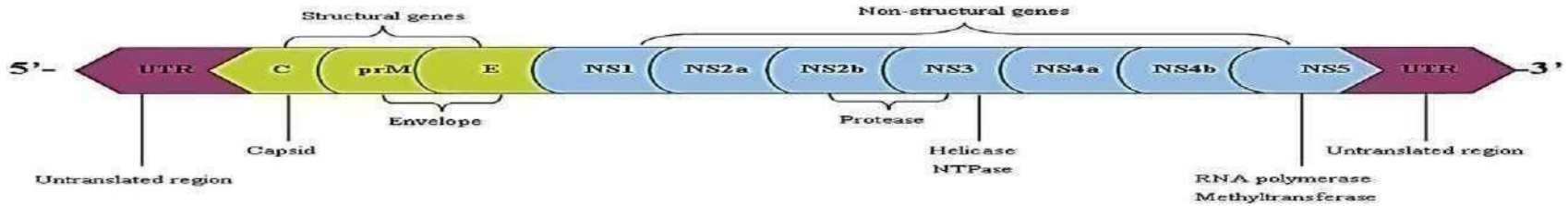


Figure 1.6: Genomic organization of dengue RNA genome. Schematic diagram showing: (top) Schematic representation of the DENV genome [244], (bottom) the membrane topology and proteolytic cleavage sites of the transcribed polyprotein. Cellular and viral proteases (denoted by arrows) process the immature polyprotein into ten separate proteins [242].

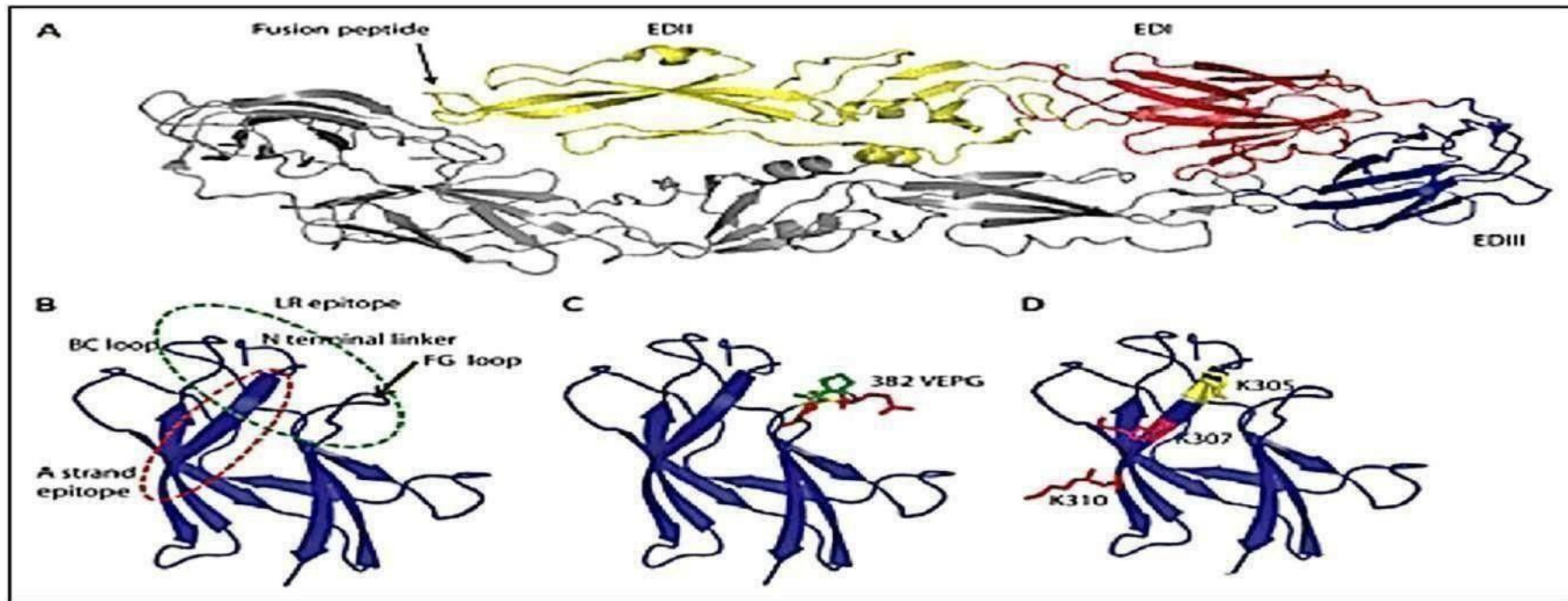


Figure 1.7: The E-protein structure of DENV-2. (A) The natural protein forms a head-to-tail homo-dimer that lays flat on the surface of the virus, and it is made up of three beta-barrel domains, denoted domains I (ED1; red), II (ED2; yellow), and III (ED3; blue). ED3 view in greater detail (B). The BC loop, the N linker region, and the FG loop of the ED3 are all included in the lateral ridge (LR) epitope. On the A strand, the epitope is located. (C) To create the viruses DV2IC20 and DV2IC21, the lateral ridge epitope was disrupted by removing four amino acids from the FG loop. (D) The A strand epitope was broken by substituting glutamic acid for lysine at positions 305, 307, and 310 [245].

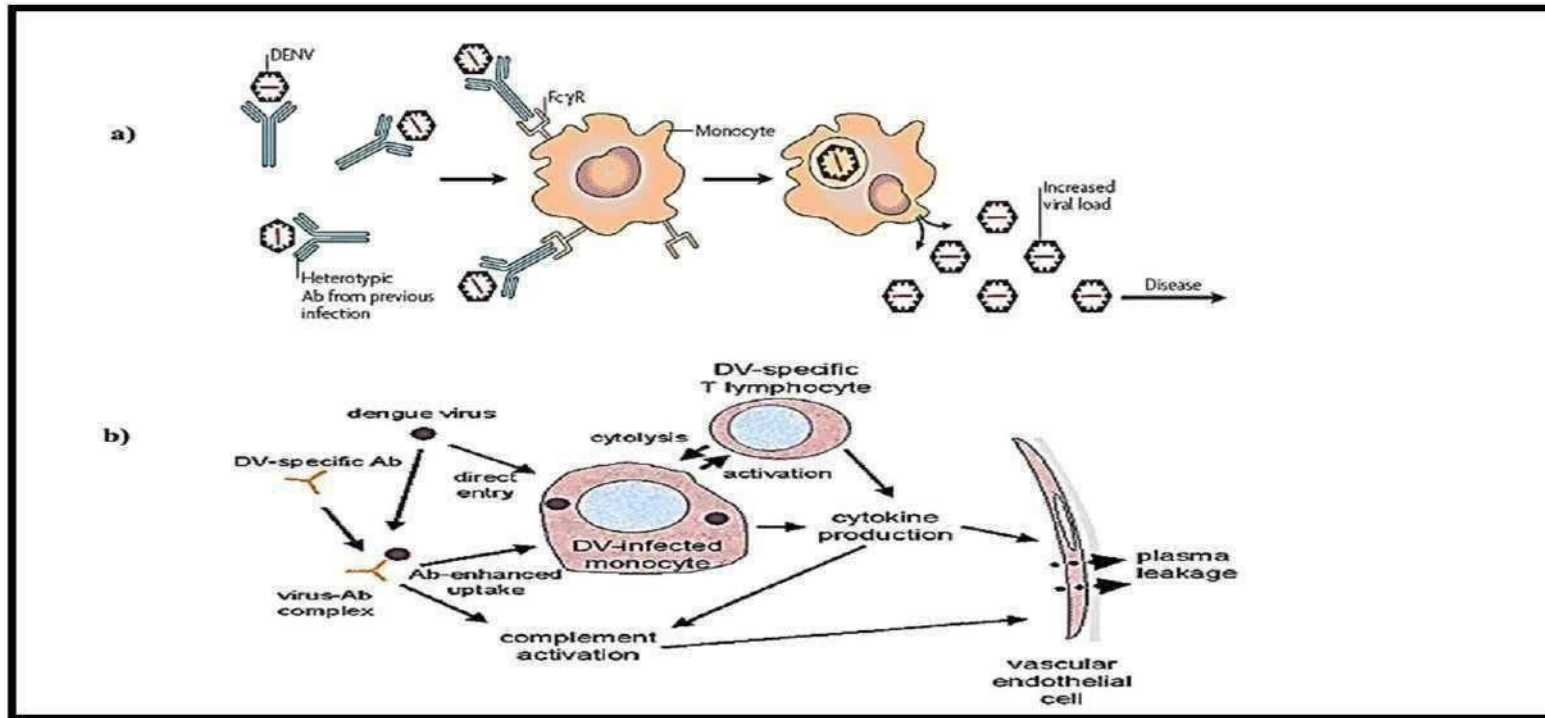


Figure 1.8: Dengue pathogenesis. (a) The antibody-dependent enhancement (ADE) of dengue virus replication [246] and (b) The T-cell-mediated immunopathogenesis in DHF leading to plasma leakage.

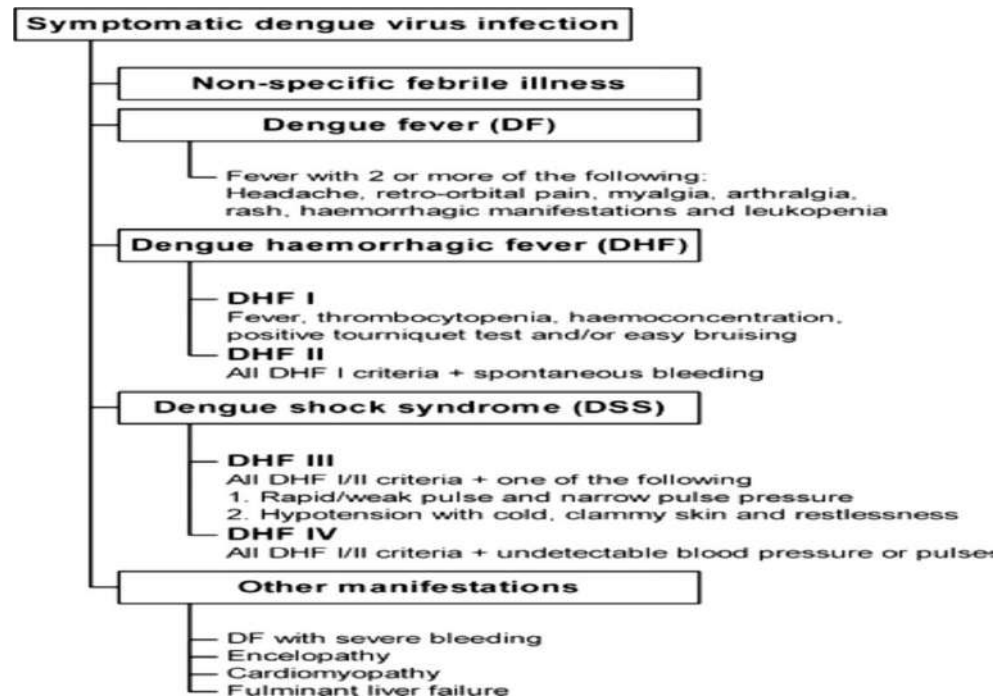


Figure 1.9: Clinical aspects of DENV infections [247]

Table 1.5 Sequence and structural similarities among DENV ED3s.

	DENV1	DENV2	DENV3	DENV4
DENV1		0.87 Å^a	0.57Å	1.01 Å
DENV2	79.0%		0.74 Å	0.76 Å
DENV3	88.6%	78.0%		1.07 Å
DENV4	71.4%	71.1%	69.5	

The values in the right top half and bottom left half represent, respectively, the C α -RMSD and percentage identity between two serotypes ED3s [142].

Table 1.6: Antigenic peptides recognized by T cells from the patients with secondary DENV infection [248]

Sequence	Location	Subset
TAGILKRWGTIKKSKAINVL	Capsid ₆₂₋₈₁	CD4
IKKSKAINVLRGFRKEIGRM	Capsid ₇₂₋₉₁	CD4
RGFRKEIGRMLNILNRRRRS	Capsid ₈₂₋₁₀₁	CD4
LGELCEDTITYKCPLLRQNE	preM ₄₁₋₆₀	CD4
RMAILGDTAWDFGSL	Env ₄₁₁₋₄₂₅	CD8
TFHTMWHVTRGAVLM	NS3 ₄₅₋₅₉	CD4
IEPSWADVKKDLISY	NS3 ₆₅₋₇₉	CD8
FSPGTSGSPIIDKKG	NS3 ₁₃₀₋₁₄₄	CD8
KVVGLYGNGVVTRSG	NS3 ₁₄₅₋₁₅₉	CD4
TKRYLPAIVREAIKR	NS3 ₂₀₀₋₂₁₄	CD4
ILAPTRVVAEMEEA	NS3 ₂₂₀₋₂₃₄	CD8
EMEEALRGLPIRYQT	NS3 ₂₃₀₋₂₄₄	CD8
EHTGREIVDLMCHAT	NS3 ₂₅₀₋₂₆₄	CD4
EIVDLMCHATFTMRL	NS3 ₂₅₅₋₂₆₉	CD4
EAHFTDPASIAARGY	NS3 ₂₈₅₋₂₉₉	CD8
LDNINTPEGIIPSMF	NS3 ₄₉₅₋₅₀₉	CD8
TPEGIIPSMFEPERE	NS3 ₅₀₀₋₅₁₄	CD8
VAAEGINYADRRWCF	NS3 ₅₅₀₋₅₆₄	CD8

Chapter 02

Materials and Methods



2. Materials and Methods:

2.1 Investigation on natural DENV infections:

2.1.1 Study setting and design:

In total, 112 dengue serum samples were used in this retrospective observer-based research, which was performed at the Chittagong Medical College Hospital in Chittagong, Bangladesh. The serum samples were taken from individuals who were mostly hospitalized for dengue treatment during July and August 2019 and identified as such (NS1-based DENV diagnosis) [249].

2.1.2 Ethics statement:

This study has been conducted under ethical approval from the Ethical Review Board of Chittagong Medical College Hospital (CMC/PG/2018/580), and ethical approval from Chittagong University (AERB-FBSCU-20221031-(1)) with the participants' signed consent. All patients' data were totally anonymous and kept confidential.

2.1.3 Collection of DENV infected human sera:

As part of this investigation, 112 serum samples infected with DENV were used. These were the source from the Chittagong Medical College Hospital (receiving signed consent from the subjects and ethical approval from the Chittagong University's Ethical Review Committee). There were 78.57% male and 21.42% female participants in the age group of 19 to 50, and 40.47% and 59.52% of them lived in urban and rural regions, respectively. The serum samples were provided by the patients with the dengue virus (DENV NS1 positive; July–August 2019) who were admitted to the Chittagong Medical Hospital for medical care. A 100- μ L aliquot of each serum sample was heat inactivated and stored at -80°C until it was required.

2.1.4 DENV serotyping

Using an RNA extraction kit, 100 mL of serum samples were first used to separate and purify total RNAs (Promega Corporation). The cDNAs were created by the use of primers specific to DENV [254]. In more detail, the reaction mixture (which included RNA extract (7.8 μ L), Oligo dT (1 μ L), random primer (1 μ L), and conserved primer for DENV (0.8 μ L) was incubated at 70°C for 5 minutes, then rapid freezing was performed on ice. After adding the PCR master mix (9.4 L), a thermal cycler (Thermo Fisher Scientific, USA; 25°C for 5min, 42°C for 60min, and 70°C for 15min) was used to conduct the PCR. At last, infection-causing DENV serotypes were determined using DENV serotype-specific primers (DENV1= 5'-CCC-CGT-AACACT-TTG-ATC-GCT-CCA-TT-3'; DENV2= 5'-CGC-CACAAG-GGC-CAT-GAA-CAG-3', DENV3=5'-GCA-CAT-GTTGAT-TCC-AGA-GGC-TGT-C-3'; DENV4=5'-GTT-TCCAAT-CCC-ATT-CCT-GAA-TGT-GGT-GT-3') utilizing the standard cyber-green PCR procedure. [cDNA (4 μ L); DENV-specific and DENV conserved primers 0.5 μ L+0.5 μ L); and master mix (10 μ L); H₂O (5 μ L). The PCR methodology utilized the following procedure: initial denaturation at 96°C for 2 minutes, followed by denaturation at 96°C for 15 seconds, annealing at 55°C for 33 seconds, and extension at 72°C for 1 minute. Steps 2 through 4 were iterated 45 times with fluorescence data being recorded at the extension step.] The DENV serotypes present in the infected samples were confirmed utilizing a one-step qRT-PCR serotyping kit (Fast Track Dengue Diagnostics), and the qPCR data was collected using the qTower instrument (Analytik Jena) (Figure 2.2).

2.1.5 Clinical Manifestation of Dengue Infection

According to professional clinical doctors at Chittagong Medical College Hospital in Chittagong, Bangladesh, the clinical signs and symptoms of dengue infections may include vomiting, fever, myalgia, musculoskeletal pain, rash, retro-orbital discomfort, headache, nausea, diarrhea, ache-pain, , jaundice, and photophobia. After diagnosing dengue infections, further clinical tests such as platelet count, hematocrit

measurement, and white blood cell count are typically performed. These tests are often performed using a haematology cell counter, such as the Mindray BC-5150 [251].

2.1.6 The ED3 in dengue research: DENV ED3 design, expression and purification

The envelop protein domain 3 (ED3) according to reports containing all the putative epitope residues conferring DENV-sero-specificity [252]. and has long been considered as a streamlined model for DENV sero-specificity [253]. The ED3 sequences of DENV1 (1ED3), DENV2 (2ED3), DENV3 (3ED3), and DENV4(4ED3) were retrieved from Uni-Prot IDs P17763, P14340, P27915.1 and P09866, respectively. The nucleotide sequences encoding ED3s along with a thrombin cleavage site and a His6-tag were cloned into pET15b expression vector using NdeI and BamHI restriction enzymes as reported previously [168,253,254] All four DENVED3s were overexpressed through IPTG induction in JM109(DE3) pLysS cell line as inclusionary bodies [243]. Cells were collected through centrifugation, lysis buffer, then lysed (150 mM NaCl, 0.5% sodium deoxycholate, and 1% SDS in 50 mM Tris- HCl pH 8.5) and lysis wash buffer (lysis buffer plus 1% v/v NP-40) using sonication keeping on ice. The Cys residues oxidized in the air in 6M guanidine hydrochloride long-term dialysis was performed in 50 mM Tris-HCl, pH 8.7 at 4°C and purification through Ni-NTA (Wako, Japan) chromatography. Finally, His6-tag N-terminus was cut using thrombin proteolysis [168] and then separated by a second round of Ni-NTA chromatography and ED3s were purified by reversed-phase HPLC. Protein identities were verified by MALD-TOP mass and preserved as lyophilized powder at -30°C until used.

2.1.7 Detection of anti-DENV ED3 antibodies in human by Immunological assay

According to earlier reports [168,253,254] anti-DENV ED3 IgM and IgG antibodies were examined by ELISA on 96-well microtiter plates (Nunc) in naturally infected human samples. Overnight at room temperature, the plates were coated with 2.5 g/mL of pure ED3s in 100 μ L/well of phosphate buffered saline (PBS, pH 7.4). PBS was used to remove unbound ED3s, and then 1% BSA in PBS was used to block the plates for 45 minutes at 37°C. Anti-dengue sera were applied after washing with PBS at dilution of 1:50 in 0.1% BSA in PBS initially, then by three times dilution, and then incubated for two hours at 37°C. The samples were washed with PBS-0.05% Tween-20 three times. followed by a final wash with PBS, unbound antibodies were thoroughly eliminated. Anti-human IgG-HRP conjugate at a 1:4000 dilution and anti-human IgM-HRP conjugate at a 1:10000 dilution were added to microtiter plates, which were then blot dried before 90 minutes of incubation at 37°C. Through three washings with PBS-0.05% Tween-20 and one with PBS, the unbound conjugates were eliminated. Color intensity was evaluated using a microplate reader after 20 minutes of incubation at 450 nm (OD450nm) by introducing 0.4 mg/ml of the substrate OPD (o-phenylenediamine), in addition to (100 μ L/well) of 4 mM H₂O₂ (Dynatech Microplate Reader). To determine the antibody titers, a power fitting was applied to the OD450nm values plotted against the antisera dilution's reciprocal. The cutoff values for IgM and IgG were set at OD450nm = 0.1 and 0.4, respectively, above the background values. (Figure 2.3) [254].

2.1.8 Differentiation of DENV infections

Categorization of DENV infections into initial infections and secondary infections was done according to the anti-ED3 IgM and IgG ratio, as previously reported [156,188,255,256] To be more precise, anti-ED3 IgG and IgM antibody titers of anti-dengue sera were determined against ED3s of four DENVs. Then anti-ED3 IgM titers

were divided by anti-ED3 IgG titers and if the anti-ED3 IgM antibody titers were ≥ 1.5 fold over anti-ED3 IgG titers the DENV infections were categorized as primary DENV infection and otherwise as secondary DENV infections. Similarly, ≥ 1.5 fold higher anti-ED3 IgM titers over anti-ED3 IgG titers against a single DENV ED3 and against multiple DENV ED3s were considered as one DENV serotype and simultaneous infections with numerous DENV serotypes, respectively. Such DENV serotyping based on anti-ED3 IgG and IgM antibodies was furthermore validated with RT-PCR based DENV serotyping data [257].

2.1.9 Methodology for achievement of the objectives

Investigation on natural DENV infections

Blood samples collected from symptomatic dengue patients confirmed by NS1-based dengue diagnosis. Presence of infecting DENV serotype and anti-dengue ED3 antibody were confirmed by RT-PCR and ELISA (IgM, IgG) respectively (Figure 2.1)

Natural anti-DENV antibody response in DENV-infected sera will be monitored using ELISA and DENV serotyping will be done using RT-PCR.

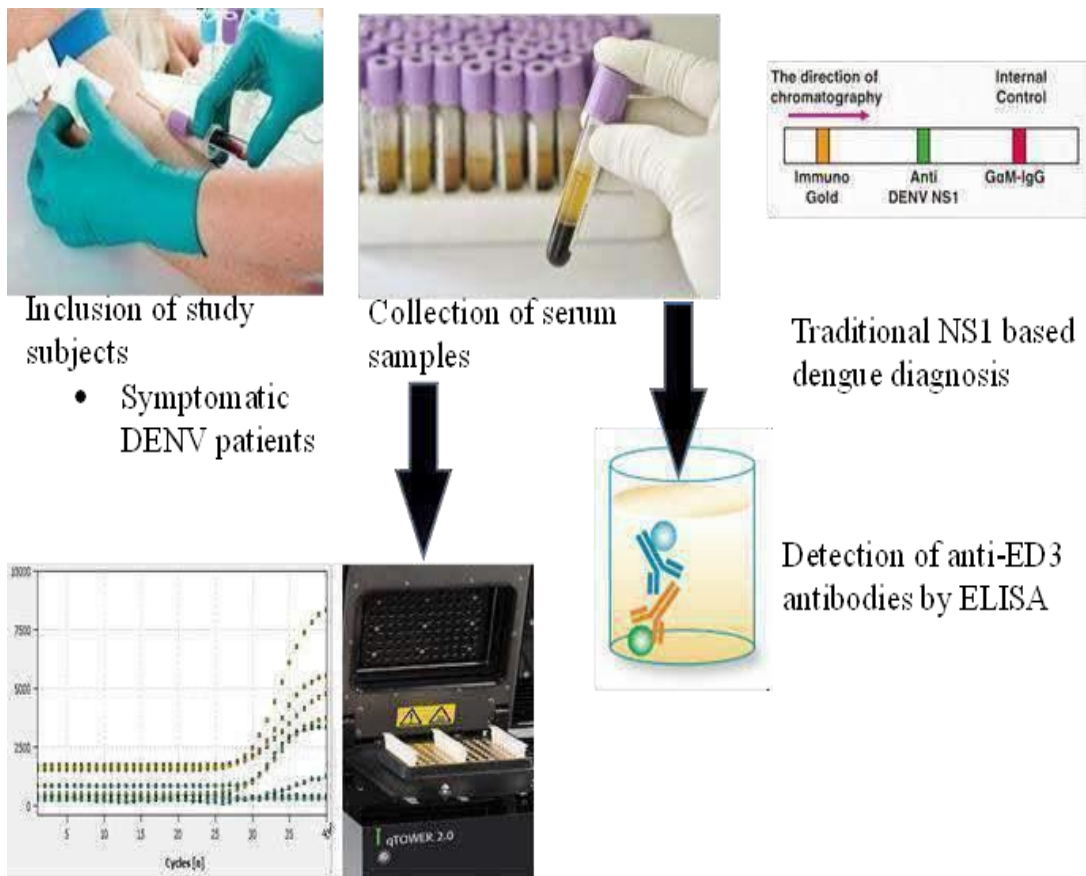


Figure 2.1: Flowchart for investigating natural DENV infections

Flowchart for the Dengue specific RT-PCR to confirm Dengue Serotyping

DENV Serotyping:

(a) Blood collection from dengue patient



b) Collection of DENV infected human sera



d) DENV serotyping by RT-PCR



c) Preparation of CDNA



e) RT-PCR Result

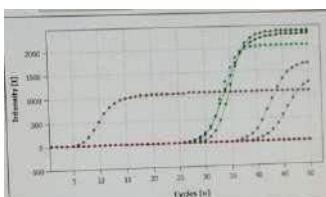


Figure 2.2: **Sample collection from natural infection and RT-PCR.** a) Blood collection from dengue patient b) Collection of DENV infected human sera c) Preparation of CDNA d) DENV serotyping by RT-PCR e) RT-PCR Result.

Flowchart for the Detection anti-DENV ED3 antibodies

a)



b)



c)



d)



Figure 2.3: Sample collection from natural infection and ELISA. (a): Blood collection from dengue patient; (b): ELISA plate; (c): Performing ELISA; and (d): ELISA plate in ELISA reader.

2.2 Immunological Studies

2.2.1 Immune Response Studies: Artificial Immunization Study

2.2.1.1 Animal Model

As previously reported [258], the Swiss Albino mice of 3-4 weeks age were used in all experiments which were purchased from the Animal Resource Branch, ARB of International Centre for Diarrhoeal Research, Bangladesh, ICDDR, B (Figure 2.5). All mice were accommodated and treated with an animal care facility in compliance with the regulations of the Department of Biochemistry and Molecular Biology in the University of Chittagong with a pathogen-free hygienic area. Free access to sterilized food and water was provided to all mice.

2.2.1.2 Antigen Dose Formulation and Immunization

First all four DENV1-4 ED3s (1ED3, 2ED3, 3ED3, and 4ED3 where 1-4 stand for DENV1, DENV2, DENV3, and DENV4, respectively; ED3 stands for Envelope protein domain 3), the immunogens were individually dissolved in de-ionized water at 1 mg/ml concentration (protein stock). Doses were formulated at 30µg protein/dose per mice in PBS (Phosphate Buffered Saline, pH7.4) just prior to injection (Figure 2.6). Then the protein solutions in PBS were mixed with the same amount of Freund's incomplete adjuvants and injected subcutaneously (SC) (1st dose) (Figure 2.7a). The repeated doses were also formulated in the same manner (1:1 ration in Freund's adjuvants) but with complete Freund's Adjuvants and injected interperitonally at monthly intervals (Figure 2.7b). We maintained the same living standard for both the experimental and control mice. It is worth mentioning that mice were immunized with 200µL of diluted protein antigen (30µg/mice). Swiss-Albino mice of 3-4 weeks were divided into four groups at random, each group containing five experimental mice and two control mice. The experimental mice were repeatedly given all four DENV in a heterogeneous combination:

Group 1: The first dose was 1ED3, followed by 2ED3 (second dose), 3ED3 (third dose) and 4ED3 (fourth dose); Group 2: The first dose was 2ED3, followed by 1ED3 (second dose), 3ED3 (third dose) and 4ED3 (fourth dose); Group 3: The first dose was 3ED3, followed by 1ED3 (second dose), 2ED3 (third dose) and 4ED3 (fourth dose); Group 4: The first dose was 4ED3, followed by 1ED3 (second dose), 2ED3 (third dose) and 3ED3 (fourth dose)(Table 2.2). In the control mice, single immunogen was injected in every dose (Group 1: 1ED3; Group 2: 2ED3; Group 3: 3ED3; Group 4: 4ED3). Total four doses were given. The initial dose delivered subcutaneously using incomplete Freund's adjuvant (IFA), while doses 2 to 4 were given intraperitoneally with complete Freund's adjuvant (CFA) (Table 2.3).

2.2.2 Serum Sample Collection

2.2.2.1 Tail Bleeding

For the collection of serum samples, live mice were restrained mechanically, to allow blood collection by puncturing the tail smoothly so that bleeding occurs very slowly. From zero to before the next immunization, blood was collected through tail bleeding at weekly intervals and tested for anti-ED3 IgGs using ELISA, both for sero-specific and sero-cross-reacting antibodies.

Blood sample was taken from the tail vein (and artery) by cutting a small (3 mm) slit in the tail's terminal and gently massaging the tail with the thumb and finger of one hand to enhance blood flow into the collecting vial (Figure 2.8). On average 20 µl of blood were collected in PBS (pH 7.4) and diluted 50 times with further addition of PBS. Centrifugation (at 3000 x g for 5 minutes) was used to separate the serum. Before usage, serum samples were stored at -40°C as a 50% glycerol stock.

2.2.2.2 Whole Blood Collection

All mice were sacrificed after the fourth/ final dose, and direct cardiac blood samples were obtained. The mice have been sacrificed by spinal dislocation; direct access to the heart was used to collect blood samples, using a 23G needle and a 5 mL syringe (Figure 2.9). After mixing the blood samples with an equal volume of PBS, the mixture was left to stand 20 minutes at room temperature before being 10 minutes of centrifuging at 3000 rpm. Until usage, supernatants (serum samples) were collected and stored at -40°C.

2.2.2.3 Analysis of Anti-ED3 Antibody Responses

The sero-specificity and anti-ED3 IgG sero-cross-reactivity generated following immunization were monitored at weekly intervals using ELISA. In brief, all anti-ED3 sera raised were tested against all four DENV ED3s (DENV1-4 ED3s) and antibody titers were calculated and compared.

2.3 Serum Antibody Response Study by ELISA

2.3.1 Enzyme-Linked Immunosorbent Assay (ELISA) in mice.

The dengue-specific (both DENV cross-reactive and sero-type-specific) anti-ED3 IgG reaction of antibodies were studied using ELISA. First, 100 µL/well of the 96-well microtiter plates were coated with dengue antigens at 1.5 µg/mL concentration and incubated overnight at temperature of the living space. After PBS washing three times to remove unbound proteins, unfilled areas were filled with 1% BSA in PBS (200 µL/well; for 45 minutes at 37 °C). Following a 1X PBS wash, 150µL of serum sample (at different dilutions; 1:100 to 1:200) was applied in the first well, followed by three-fold dilutions incubated with 0.1% BSA-PBS for two hours at 37°C. After two hours of incubation at 37°C, three times in 1X PBS-Tween 20 (PBST) were used to wash the plates and once in PBS. After washing, a 100 µL/well of α -mice IgG-HRP

conjugate was used as the secondary antibody, at 1:4000 dilution in 0.1% BSA in PBS-Tween and incubated for 90 minutes at 37 °C. Following the removal of the unbound conjugates using three washes in 1X PBS Tween-20 and one in 1X PBS, coloring was carried out using the substrate OPD (O-phenyl Di-amine; 0.4 mg/ml supplemented with 4 mM H₂O₂; 100ul/well) (Figure 2.10). Following a 20-min incubation time at room temperature, (Thermo Scientific Multiscan®EX Primary EIA V 2.3) microplate reader was used to measure color intensity at 450 nm.

2.3.2 Data Analysis

The ELISA data were calculated, compared, and analyzed through the spreadsheet application Microsoft Excel. Using a cutoff of 0.1 above the background reading, the power of the antibodies was calculated from the power relationship (fittings) of OD450nm vs the serum dilution factors' reciprocal (OD450). Pymol graphics was used to generate the structure models (www.pymol.org).

2.4 Immune Response Studies: Concurrent Artificial Immunization Study

2.4.1 Animal Model

Balb/c mice aged 3-4 weeks were used in all tests and were obtained from the Animal Resource Branch (ARB) of the International Centre for Diarrheal Research, Bangladesh, ICDDR. All mice were housed and maintained in a pathogen-free hygienic space in accordance with the rules of the Department of Biochemistry and Molecular Biology at the University of Chittagong. All mice were given unrestricted access to sterilized food and water.

2.4.2 Antigen Dose Formulation and concurrent heterotypic multiple ED3 Immunization

In this study, 11 experimental groups with total 33 mice (Group A, B, C, D, E, F, G, H, I, J, K) were taken and 3 experimental mice were placed in each Group. After immunization small amount of blood samples were collected through tail-bleeding at day 7, 14, 21, 28 and 35 and tested for ED3-specific IgG antibodies using ELISA. However, for 28th day, antibody level was high in this study. So I am trying to discuss only ELISA result of 1 dose of 28th days, 2nd dose of 21 days and 3rd dose of 7 days. On day 28, mostly non-specific responses i.e mostly heterogeneous antibodies were observed

This Artificial immunization studies conducted in the absence of Freund's adjuvants in Balb/c mice model to mimic anti-DENV antibody responses against concurrent heterotypic dengue infections in mice model. Here we used three combinations comprising six bivalent ED3s (1ED3-2ED3, 1ED3-3ED3, 1ED3-4ED3, 2ED3-3ED3, 2ED3-4ED3, 3ED3-4ED3), four trivalent ED3s (1ED3-2ED3-3ED3, 1ED3-2ED3-4ED3, 1ED3-3ED3-4ED3, and 2ED3-3ED3-4ED3) and one tetravalent combination (1ED3-2ED3-3ED3-4ED3).

2.4.3 Data Analysis

The ELISA results were generated, compared, and analyzed using Microsoft Excel, a spreadsheet tool [254]. We determined the antibody titers by fitting the power relationship between OD₄₅₀ and the reciprocal of the serum dilution factors (with a 0.1 threshold above the background reading) (OD₄₅₀).

2.5 Cell surface CD Marker Analysis by Flowcytometry.

2.5.1 Investigation circulating T-cell for CD markers

2.5.1.1 Flow Cytometry:

2.5.1.2 Cell surface CD marker analysis: Whole blood was drawn from the tail and gently incorporated into FACS buffer (PBS supplemented with 2% FBS, 1 mM EDTA, and 0.1% sodium azide). Red blood cells (RBCs) were dissolved in RBC lysis solution (contains 0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA) for 5 minutes at room temperature. After two rounds of FACS buffer washing (1600 rpm, 4 °C, 5 min), the pellets (cells) were resuspended in 100 µL of FACS buffer that had already been chilled. Anti-CD3-Pcy5, anti-CD4-Pcy7, anti-CD44-FITC, and anti-CD62L-PE conjugated antibodies (0.5µg/100µL cell suspension) were used to stain the Th cell lineage in the dark for 30 minutes, while anti-CD3-Pcy5, anti-CD8-Pcy7, anti-CD44-FITC, and anti-CD62L-PE conjugated antibodies were used to stain the Tc cell lineage for the same amount of time. To remove excess conjugated antibodies that were not attached to the cells, FACS buffer was applied to the cells. After that, cells were resuspended in a 300 L FACS buffer, and data were collected using CytoFlex (Beckman Coulter) (Fig. 2.11).

2.5.1.3 Internal marker analysis:

We used the same method used for surface CD markers, with the addition of 0.05% Tween 20 during the labeling of the markers with fluorescence-conjugated antibodies, to examine the levels of IL-4 and IFN- in circulating T-cells (collected through tail-bleeding) at weekly intervals after immunization. In particular, CD3-Pcy5, CD4-Pcy7, CD8-Pcy7, and IL4-PE were used in one tube, and CD3-Pcy5, CD4-Pcy7, CD8-Pcy7, and INF-PE were utilized in another tube. At least three mice's worth of flow cytometry data were gathered, averaged, and displayed with standard deviations.

2.5.4 Data Analysis

The CytoFLEX data were analyzed using CytExpert software as (figure 2.12). In brief, firstly the CD3⁺ cell cluster was selected from cell scattering graph followed by selecting CD4⁺/CD8⁺ cells (for Th or Tc cell respectively in different experiment) from CD3⁺ cell cluster. Finally, CD3⁺ CD44⁺CD4⁺ cells or CD3⁺CD44⁺CD8⁺ cells were sort out followed by sorting out CD3⁺CD4⁺CD44⁺CD62L⁺ cells or CD3⁺CD8⁺CD44⁺CD62L⁺cells and CD3⁺CD4⁺IL4⁺INF- γ or CD3⁺CD8⁺IL4⁺INF- γ (Figure 2.12). The data were compared with the help of spreadsheet application Microsoft Excel as well.

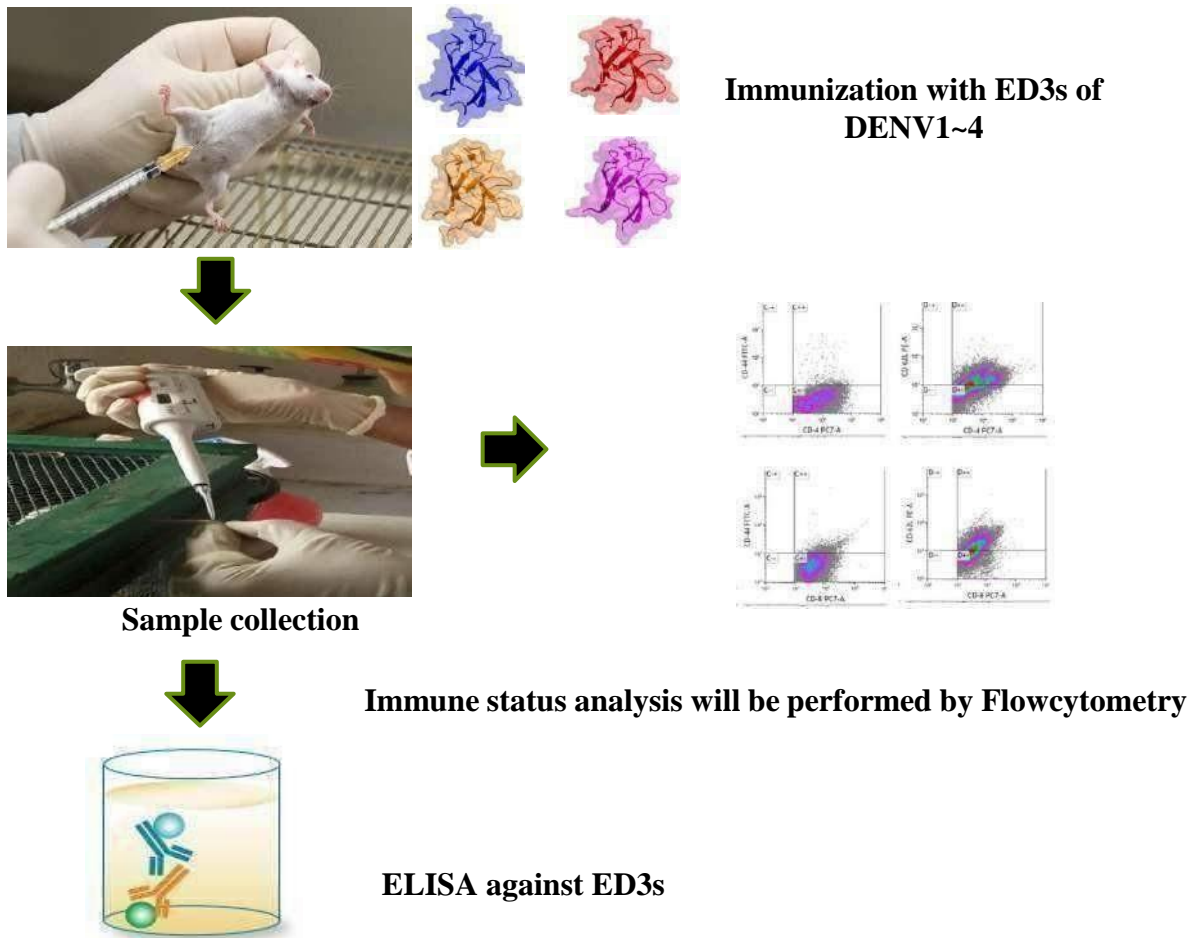


Figure 2.4: Flowchart of Immunization studies will be conducted in Swiss albino mice model.

Table 2.1: The sequence details of E-protein domain 3 (ED3) of DENV1-4.

Mutant IDs	Uniprot ID	Sequence details
DENV-1ED3	P17763	HMGMSYVMCTGSFKLEKEVAETQHGTVLVQVKYEGT DAPCKIPFSTQDEKGVTONRLITANPIVTDKEKPVNIET EPPFGESYIVVGAGEKALKQCWFKKGSSIGK
DENV-2ED3	P14340	HMGMSYSMCTGKFKIVKEIAETQHGTIVIRVQYEGDG SPCKIPFEIMDLEKRHVLGRLITVNPIVTEKDSPVNIEAE PPFGDSYIIIGVEPGQLKLNWFKKGSSIGQ
DENV-3ED3	P27915.1	GSGMSYAMCLNTFVLKKEVSETQHGTILIKVEYKGED APCKIPFSTEDGQGAHNGRLITANPVVTKKEEPVNIE AEPPF GESNIVIGIGDKALKINWYRKGSSIGK
DENV-4ED3	P09866	GSGMSYTMCSGKFSIDKEMAETQHGTTVVKVKYEGA GAPCKVPIEIRDVNKEKVVGRISSTPLAENTNSVTNIEL EPPFGDSYIVIGVGNLALTLHWFRKGSSIGK

Table 2.2: Designing of experiment.

Mice Group	Immunogen		Route of administration
	Experimental	Control	
Group 1	1ED3→2ED3→3ED3→4E D3	1ED3	Subcutaneous
Group 2	2ED3→1ED3→3ED3→4E D3	2ED3	Intraperitoneal
Group 3	3ED3→1ED3→2ED3→4E D3	3ED3	Intraperitoneal
Group 4	4ED3→1ED3→2ED3→3E D3	4ED3	Intraperitoneal

Table 2.3: Immunization scheme.

Day/Dose	Immunization			Sample Collection
	Immunogen (µg)	Administration Route	Fruend's Adjuvant	
7/1 st	30	Subcutaneous	Incomplete	Tail Bleed
14				Tail Bleed
21				Tail Bleed
28				Tail Bleed
35/2 nd	30	Interperitoneal	Complete	Tail Bleed
42				Tail Bleed
49				Tail Bleed
56				Tail Bleed
63/3 rd	30	Interperitoneal	Complete	Tail Bleed
70				Tail Bleed
77				Tail Bleed
86				Tail Bleed
93				Tail Bleed
100/4 ^t h	30	Interperitoneal	Complete	Tail Bleed
107				Tail Bleed
114				Tail Bleed
121				Tail Bleed
128				Heart Blood



Figure 2.5: Swiss Albino mice model which was used in this research work.



Figure 2.6: Handling and restraining the mouse before immunization.



(a)



(b)

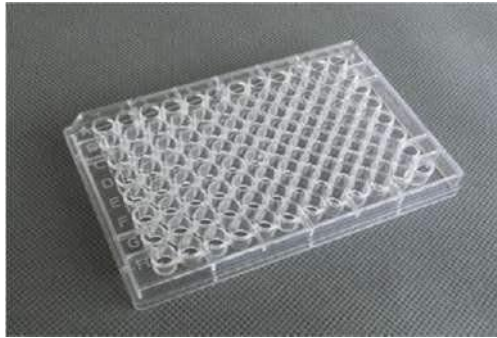
Figure 2.7: Immunization (a) Subcutaneous immunization, (b) Interperitoneal immunization.



Figure 2.8: Performing tail-bleed to collect blood sample from live immunized mice.



Figure 2.9: Collection of heart blood after sacrificing the immunized mice.



(a)



(b)



(c)



(d)

Figure 2.10: Detection of anti-dengue antibodies in serum samples. a) ELISA was performed using 96-well microtiter plates; (b) Washing ELISA plates; (c) Antibody titer calculated through 3-fold serial dilution procedure and (d) ELISA plates read using a microtiter plate reader.

Table 2.4: Concurrent heterotypic multiple ED3 immunization Experiment design

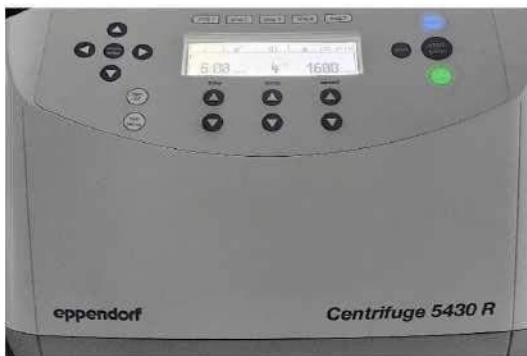
Mice groups	Immunogen	Amount in μg	Route of administration
Group 1	1ED3-2ED3	15+15	Subcutaneous
Group 2	1ED3-3ED3	15+15	
Group 3	1ED3-4ED3	15+15	
Group 4	2ED3-3ED3	15+15	
Group 5	2ED3-4ED3	15+15	
Group 6	3ED3-4ED3	15+15	
Group 7	1ED3-2ED3-3ED3	10+10+10	
Group 8	1ED3-2ED3-4ED3	10+10+10	
Group 9	1ED3-3ED3-4ED3	10+10+10	
Group 10	2ED3-3ED3-4ED3	10+10+10	
Group 11	1ED3-2ED3-3ED3-4ED3	7.5+7.5+7.5+7.5	



(a)



(b)



(c)



(d)

Figure 2.11: Detection of CD44+, CD44+CD62L+ and IL-4 , IFN γ Tc and Th cells from blood sample. (a) CD marker (b) Sample processing. (c) Centrifuge machine and (d) CytoFLEX Flow Cytometer used in this research work.

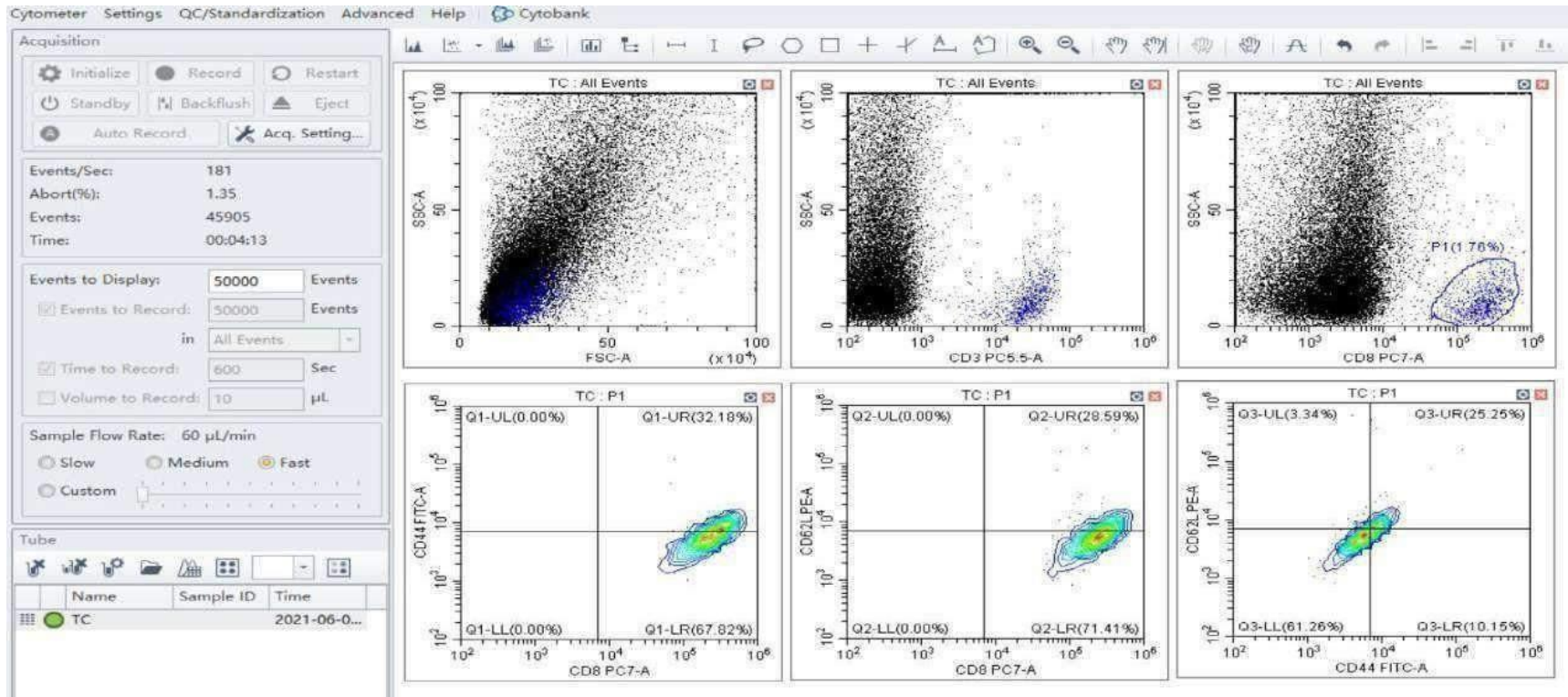


Figure 2.12: Analyzing flow cytometry data using CytExpert software.

Chapter 03

Results and Discussions



3. Results and Discussions

Objective 1 : Investigation of natural dengue infection and DENV Serotyping and DENV seroprevalence in Chittagong, Bangladesh.

3.1 DENV Seroprevalence in Chittagong

112 samples were analyzed, and 42 of them tested positive for DENV by RT-PCR, with 10 (24%) having concurrent infection with two or more DENV serotypes and 32 (76%) having single DENV serotype (Figure 3.1) [257]. Among the single DENV serotype infections, DENV4 was the most prevailed serotype, followed by DENV2, DENV1, and DENV3 serotypes which was in contrast to what had been reported earlier in Bangladesh [259]. Infection rates for DENV4, DENV2, DENV1, and DENV3 were, correspondingly, 41, 25, 22, and 12, to be more specific (Figure 3.1). Nonetheless, several earlier studies showed that Bangladesh [259] and India [260] had a high prevalence of DENV3 serotypes. Demonstrating periodical and/or geographical fluctuations in the DENV serotypes that transmit infection from year to year, may have contributed to the present increase in both the prevalence and severity of dengue.

Interestingly, among concurrent multiple DENV infections in Chittagong, DENV1 was the common prevalent infectious DENV serotype, then by DENV3, DENV4, and DENV2 (Figure 3.1), which is remarkably comparable to what has been observed globally regarding current dengue outbreaks [261-264]. Although we currently lack a conclusive explanation for these observations, we predicted that a normal immune response to DENV would be cross-reactive; in both mouse models and actual DENV infections, immunological reactions to DENV1 produced DENV1-DENV2-DENV3 cross-reactive IgG.

Such sero-cross-reacting anti-DENV1 IgG might facilitate additional DENV serotype infections, either as concurrent multiple DENV infections or heterotypic secondary infections [265-266]. Further research utilizing artificial immunization and

The identification of naturally occurring antibodies against dengue might investigate the reasons for the increased incidence of DENV1 in many concurrent DENV infections.

I also carried out same investigated on the samples collected from Dhaka Medical College Hospital and got the same results. Such as all 95 subjects were screened by NS1-based dengue diagnosis and among them 35 samples were NS1 positive (DENV infected) and 60 were NS1 negative (dengue suspects). However Dengue serotyping study was carried out on 35 NS1 positive samples and 17 DENV positive samples (tested by RT-PCR). All of them were single DENV serotype where DENV4 constituted the highest prevalent (35%) followed by DENV2 (29%), DENV1 (24%) and DENV3 (12%).

3.2 DENV Infections: Serotype-Specific Clinical Manifestation

Nearly all DENV infections were accompanied by symptoms like fever, musculoskeletal pain, vomiting, re-o-pain, myalgia, rash, headache and nausea (Table 3.1) [257]. An inclusive observation showed that DENV1 and DENV2 infections produced clinical signs and symptoms that were remarkably similar to each other but slightly distinct from those caused by DENV3 and DENV4 infections. For instance, ache-pain and diarrhea were not observed with DENV3 and DENV4 infections, but observed in DENV1 and DENV2 infections [257]. However, the number of samples tested in each categories were relatively small and further investigations including large number of samples may help identifying the DENV serotypes that are causing an infection based on the clinical symptoms. However, it is clear that treatment of dengue infected patients can be started after appearance of common dengue sign and symptoms rather than waiting for time-consuming and expensive diagnosis process for dengue infections.

3.3 Clinical Results on DENV Infection

Signs and symptoms of dengue start when a virus replicates in the afflicted persons, proceeded by cytokine release that boosts RBC levels in the bloodstream, increases vascular permeability, and decreases platelet and leucocyte counts while raising plasma leakage [267]. As a result, platelet counts in all 42 RT-PCR positive samples dropped immediately after diagnosis and continued to fall until day 4 after DENV infections (Figure 3.2a). However, trend of decreasing in platelet count were much slower following DENV1, DENV2, and DENV4 infections and quickly began to rise (after day 3). On the other hand, platelet counts continued decreasing, even after day 0 of infection in patients infected with DENV3 (Figure 3.2a). Furthermore, decreased in platelet counts was also observed in concurrent DENV infections where DENV3 was present along with other DENVs (Figure 3.2a). These findings made it abundantly evident that DENV3 perhaps the fetal-specific most DENV serotype, causing plasma leakage, thrombocytopenia, dengue shock syndrome and dengue hemorrhagic fever both when it is present alone and when other DENV infections are present simultaneously. Additionally, overt plasma leakage has been linked to severe thrombocytopenia ($<100,000/\text{mm}^3$) as a factor in the severity of dengue [268].

However, the leucocyte counts (WBC) (Figure 3.2c), and hematocrit value (RBC) (Figure 3.2b), were nearly same regardless of the DENV serotypes that were infected except a little increase in WBC counts on day 4 following DENV3 infections, which may led to leukocytosis (Figure 3.2c). In addition, Days 0–3 after DENV2 infections as well as days 0–2 after concurrent DENV1, 3, 4 infections that resulted in leucopenia had extremely low leucocyte counts. When leukopenia <5000 cells/mL, an infection has advanced to the critical stage (Figure 3.2c), and it has been hypothesized that patients who have DENV2 infection may also experience greater severity [269]. However, no clear trend was found, hence additional research with a large number of samples is important to take into account in future studies.

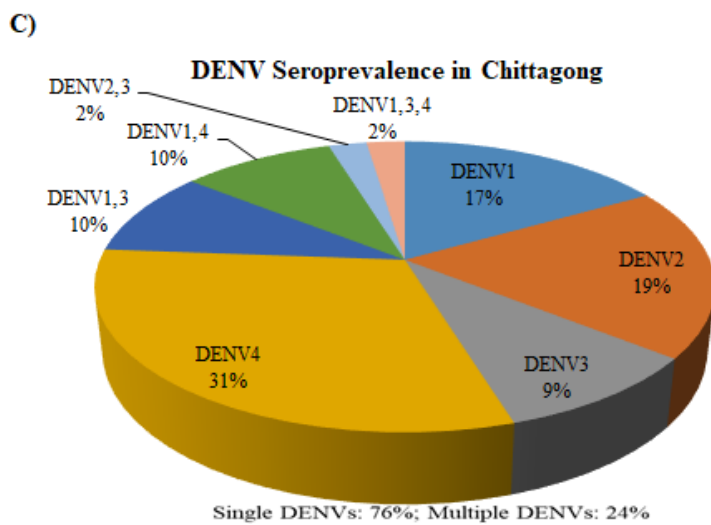
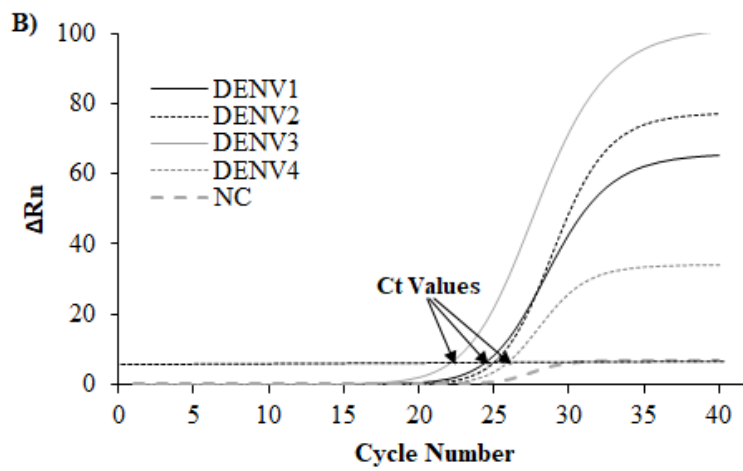
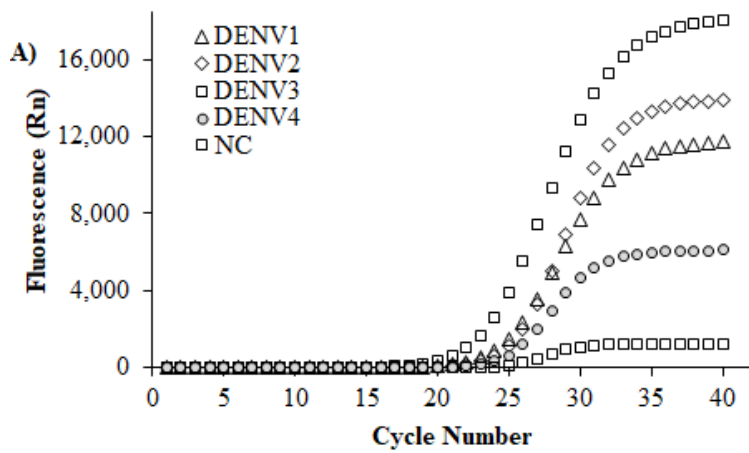


Figure 3.1: DENV Seroprevalence in Chittagong, Bangladesh. RT-PCR was used for serotyping. In panels A and B, RT-PCR normalized and fitted data are presented. Panel C displays the seroprevalence of DENV in Chittagong, Bangladesh. 42 out of the 112 samples that were analyzed were DENV serotype positive. The figure shows the relative percentage of DENV serotypes. While only 24% of samples were simultaneously infected with multiple DENV serotypes, the remaining 76% of samples were infected with a single DENV serotype [257].

Table 3.1: Clinical outcome of Dengue infection [257].

Serotype (Number, n)	Fever %	Vomiting %	M-pain %	Ache & Pain %	Myalgia %	Re-o- pain %	Rash %	Diarrhea %	Nausea %	Headac he%
DENV1 (n=7)	100	42	42	42	42	28	14	14	28	28
DENV2 (n=8)	100	25	25	12	50	37	25	12	25	62
DENV3 (n=4)	100	25	50	--	100	50	50	25	25	100
DENV4 (n=13)	100	30	23	38	84	30	15	--	53	100
DENV1,4 (n=4)	100	66	33	100	66.66	66	-	-	100	100
DENV1,3 (n=4)	100	66		16	66	33	16	16	83	100
DENV1,3,4 (n=1)	100	100			100				100	100

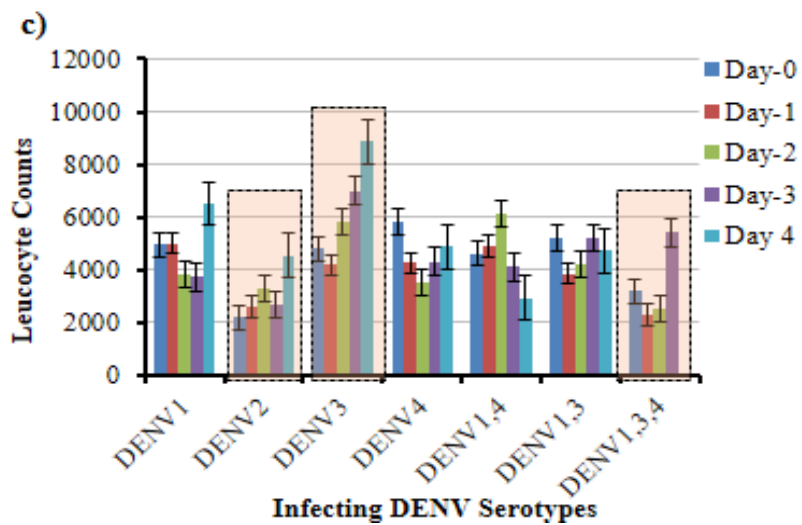
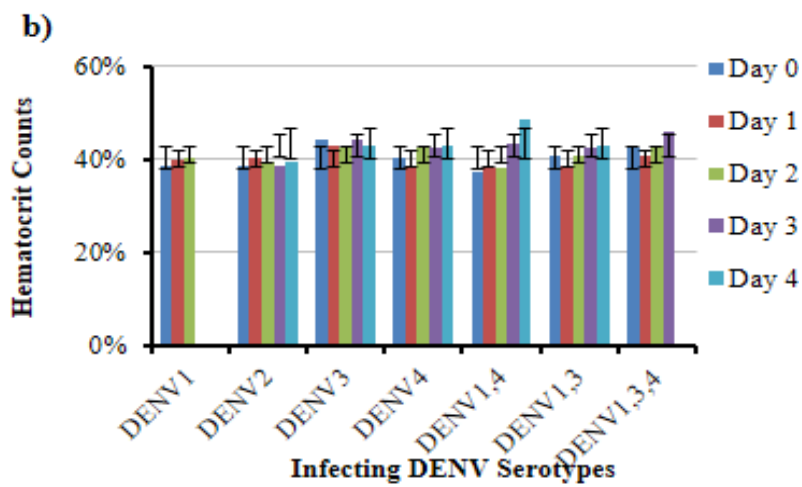
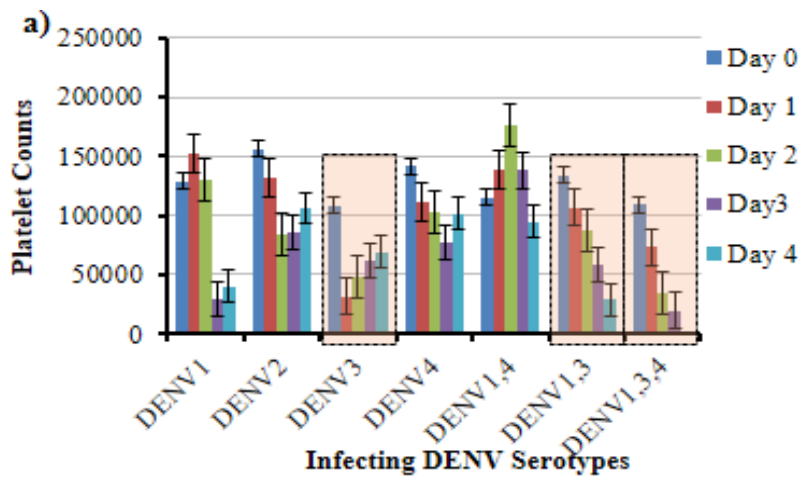


Figure 3.2: Clinical manifestation of dengue versus infecting DENV serotypes: The effects of DENV infection on platelet counts (per microliter of blood), hematocrit counts (%), and leucocyte counts (per microliter of blood) are illustrated in (a), (b), and (c), respectively. The legends are displayed on the right side of each panel and are the same for all of them. When patients were simultaneously infected with numerous DENV serotypes, including DENV3 and any additional DENV serotypes, platelet counts continued to decline shortly after DENV infections. The values of the hematocrit remained practically unchanged in each case. Leukocyte count, however, remained extremely low after DENV2 and DENV1, 3, 4 infections [257].

3.4 Anti-DENV IgG and IgM Antibodies in Natural DENV infections:

Only 39 of the 112 samples tested exhibited anti-ED3 IgM titers >1.5 times higher over anti-ED3 IgG titers, indicating that only 35% of the samples might have experienced primary DENV infections, and the remaining 73 samples (65%) had been secondary infected with DENVs (Figure: 3.3a) [270]. Of the primary DENV-infected samples, it is interesting to note that 20 (51%) of the samples tested positive for a single DENV serotype infection, whereas the remaining 19 (49%) tested positive for multiple DENV serotype infections (Figure: 3.3b) [270]. Additionally, among the primary DENV-infected samples, DENV4 (26%), became the most prevalent serotype, the next DENVs are DENV1 (13%), DENV2 (8%), and DENV3 (5%). Such high prevalence of DENV4 serotype was in contrast to the DENV seroprevalence recorded in Dhaka, Bangladesh in 2019 [264,271-272] but almost fully corroborated with our previous study based RT-PCR of the same samples (Figure 3.1C) [257].

A detailed comparison both RT-PCR and anti-DENV IgM/IgG-based and DENV serotyping of the same DENV-infected samples showed that 10 and 19 out of 112 samples (24% and 49%), respectively, were concurrently infected with multiple DENV serotypes (Figure.3.1c,3.3b). In addition, nine of the ten samples with concurrent infections have DENV1 infections (Figure.3.1c). Even though the precise cause of the high prevalence of DENV1 serotype in concurrent DENV infections is still unknown,

The high sequence similarity among DENV1, DENV2, and DENV3 [142, 273]. and anti- DENV1 ED3 IgG has DENV1-DENV2-DENV3 sero-cross-reactivity in both natural DENV infections and mice models [274]. may together contribute to the observed concurrent multiple DENV infections.

3.5 DENV serotyping: RT-PCR versus anti-DENV IgM/IgG

In DENV serotyping, RT-PCR is the gold standard for detecting viral RNA in infected serum during the acute phase of infection [275]. Nevertheless, viral RNA, in most cases, can only be detected at the very early stages of infection [276,277]. and therefore, RT-PCR-based DENV serotyping may experience very high false negative predictions [278]. As a result, dengue diagnosis cannot be made with RT-PCR alone. Here, we report data on anti-DENV ED3 IgG and IgM antibodies in DENV-infected sera and contrast RT-PCR results for DENV serotyping [257]. The DENV4 serotype predominated in the most recent dengue outbreaks in Chittagong, according to both anti-DENV IgM/ IgG antibody-based and RT-PCR serotyping (26% versus 31%). However anti-DENV IgM/IgG antibody and RT-PCR, the percentages of single and multiple DENV serotypes were, (51% and 49%) and (76% and 24%) respectively. (Figure 3.3b; 3.1c). Such significant discrepancies were most likely caused by the fact that RT-PCR relies simply on the presence of damaged-prone viral RNA, but anti-DENV antibody testing depends on the presence of stable antibodies produced in response to both current and earlier DENV infections. In the following section we discuss the discrepancies observed between DENV IgG/IgM antibody and RT-PCR based DENV serotyping.

First, 9 samples tested positive for DENV by IgM/IgG antibodies and RT-PCR(Figure 3.4a), although anti-DENV IgM antibodies typically revealed the presence of multiple DENV serotype infections rather than the primarily single DENV serotype infection shown by RT-PCR (Figure 3.1c). Similarly, anti-DENV antibodies revealed that 27 samples shown positive DENV infections, however, among them 20 and

7 samples positive for single DENV serotype and multiple serotypes, respective infection by RT-PCR (Figure 3.4b). This suggests that all 27 samples may have experienced a heterotypic secondary DENV infection and may have been previously infected with DENVs in addition to being re-infected in the recent outbreaks.

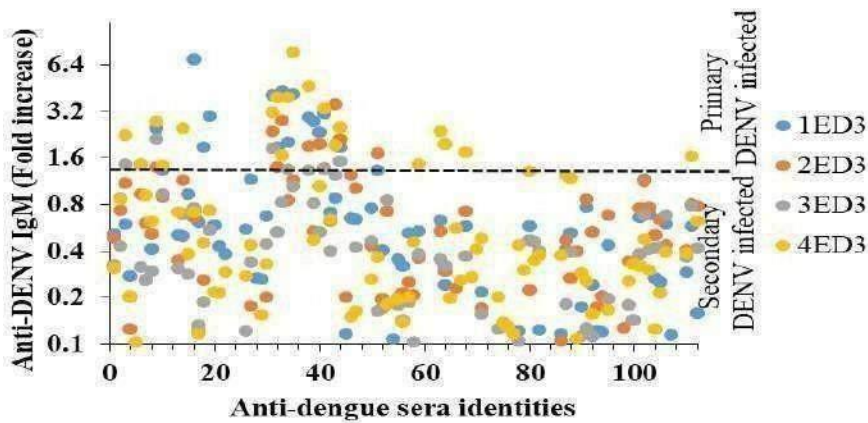
The samples used in this investigation were confirmed dengue cases, yet 70 out of 112 were found to have positive anti-DENV IgM/IgG antibodies while being negative by RT-PCR (Figure 3.4c). These findings made it abundantly evident that RT-PCR alone cannot be used for effective dengue surveillance, and they also raised the possibility that secondary and multiple concurrent DENV infections are far more common in Bangladesh than previously believed and reported [264,271]. In order to accurately assess DENV surveillance, seroprevalence, and DENV infection status in dengue endemic regions, we advise taking into consideration RT-PCR in addition to NS1-based diagnosis and anti-DENV antibody testing.

Table 3.2: Comparative view of DENV serotyping by RT-PCR and anti-DENV ED3 IgM/IgG.

Samples	RT-PCR serotyping*		Anti-DENV ED3 IgM/IgG serotyping		
112	DENV Positive	Single DENV Infection	32	Single DENV Infection	5
				Multiple DENV Infection	6
				Secondary DENV Infection	21
	DENV Negative	DENV Negative	70	Single DENV Infection	2
				Multiple DENV Infection	1
				Secondary DENV Infection	7
	DENV Negative	DENV Negative	70	Single DENV Infection	11
				Multiple DENV Infection	12
				Secondary DENV Infection	47

*RT-PCR based DENV serotyping data were reproduced from our previous report^[26]. In anti-DENV ED3 IgM/IgG-based DENV serotyping was done based on

3.3 a)



3.3 b)

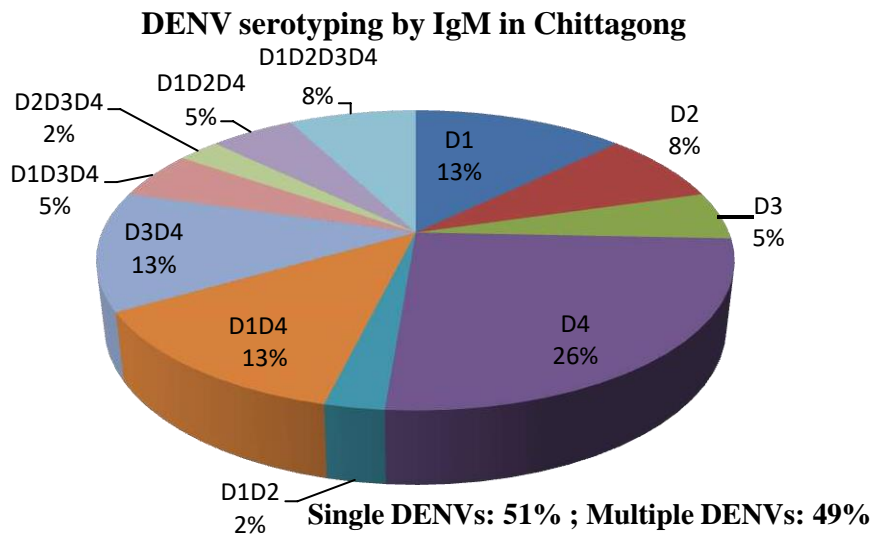


Figure: 3.3 DENV infection status in Chittagong, Bangladesh. (a) Anti-ED3 IgM and anti-ED3 IgG were found to have an IgM/IgG ratio greater than 1.5 against a primary DENV serotype and less than 1.5 against a secondary DENV serotype, respectively (b) The results of serotyping using anti-ED3 IgM and anti-ED3 IgG revealed that 39 out of 112 samples had anti-ED3 IgM/IgG titers that were 1.5 times higher, indicating that only 39 samples are DENV positive. And only 20 of the 39 samples, or 51%, showed an IgM/IgG ratio over 1.5 against a single DENV serotype, indicating that they were serospecific, or primary infections. The remaining 19 samples, or 49%, each showed an IgM/IgG ratio over 1.5 against multiple DENV serotypes, indicating that they were multiple DENV infections.

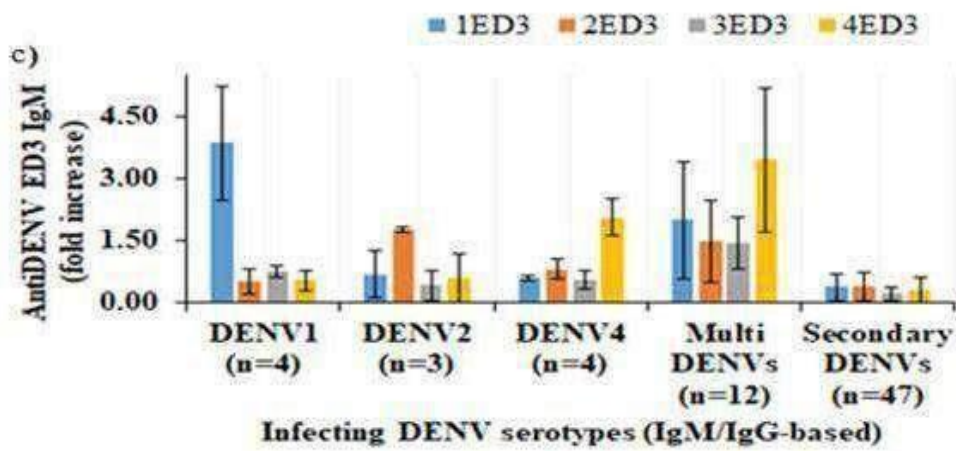
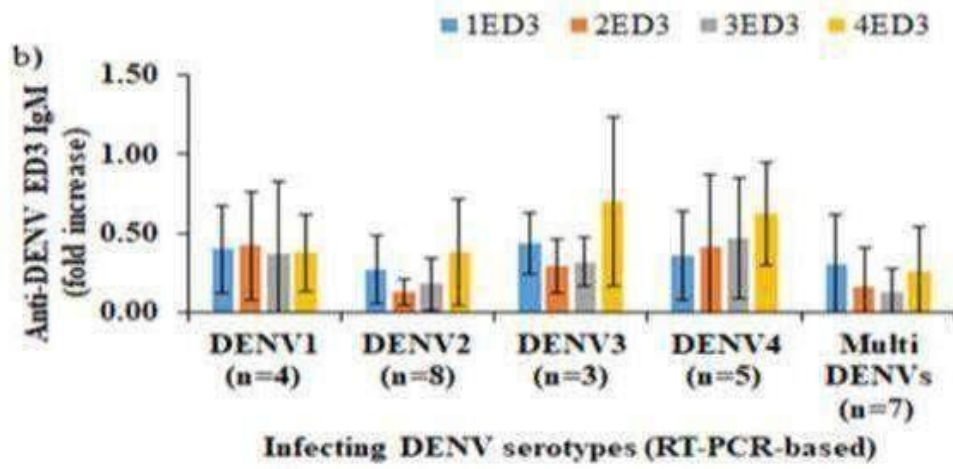
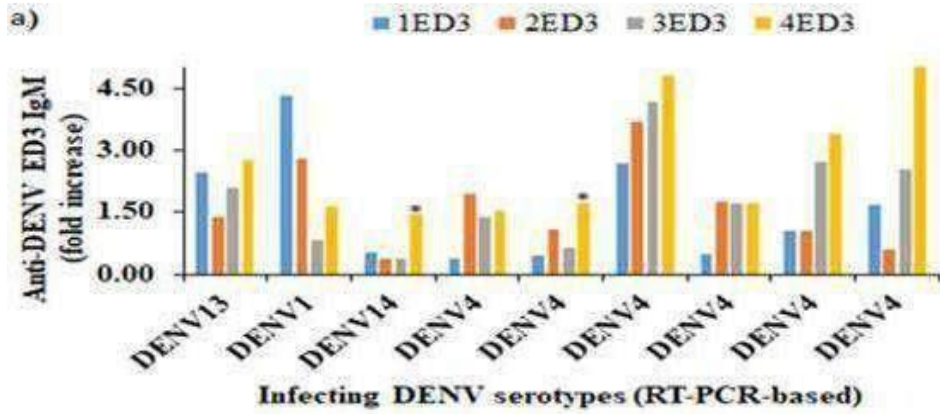


Figure 3.4: Comparison DENV serotyping by RT-PCR and anti-DENV IgM/IgG antibodies. (a) Anti-DENV IgM/IgG responses (by ELISA) versus infecting DENV serotypes (by RT-PCR) are shown. Anti-DENV ED3 IgM/IgG ratio results for 9 samples that were positive for DENVs by RT-PCR were also positive for DENVs. However, 7 samples positive for single DENV serotypes by RT-PCR but were positive for multiple DENV serotypes by anti-DENV ED3 IgM/IgG. These findings revealed that multiple DENVs were quite common in a single dengue session. (b) 27 samples, which were DENV positive by RT-PCR, were found positive for secondary DENV infection by anti-ED3 IgM/IgG ratio. These suggested that these 27 samples had been previously infected with DENVs and recently they might experience secondary DENV infections. (c) Among the 70 samples tested DENV negative by RT-PCR, 11, 12 and 47 were single DENV, multiple DENVs and secondary DENV positive, respectively by anti-DENV ED3 IgM/IgG antibodies.

Objective 2: Mimicking natural anti-DENV antibody responses in mice model

3.6 Artificial Immunization

Here we investigated the effects of primary, heterotypic secondary, heterotypic tertiary, and heterotypic quaternary ED3 immunization on the generation of responses from anti-ED3 IgG antibodies that are (ED3-specific) and sero-cross-reactive in the Swiss Albino mice model. Anti-ED3 IgG antibodies' sero-specificity and sero cross recognition were investigated by raising serum antibodies against injected ED3s and analyzed by ELISA against all four wild-type ED3s. The antibody titers were calculated from the power relationship (fittings) of OD450nm versus the reciprocal of the serum dilution.

3.6.1 Primary anti-DENV ED3 IgG responses against DENV1-4 ED3s in mice

For artificial immunization 4 groups of mice were taken, each group containing 7 mice. 1st group was immunized with 1ED3, 2nd group with 2ED3, 3rd group with 3ED3, 4th group with 4ED3 respectively. Immunization using DENV1 ED3 (1ED3) generated 1ED3-2ED3-3ED3 cross-reacting anti-1ED3 IgG (Figure 3.5). However, anti-3ED3 and anti-4ED3 sera were mainly 3ED3 and/or 4ED3-specific, respectively, with little to no cross-recognition of other ED3s (Figure 3.5). Anti-2ED3 sera were primarily serotype-specific, with some cross-recognition of 3ED3 but no cross-recognition of 1ED3 (Figure 3.5). However, anti-1ED3 antibodies identify 2ED3 and 3ED3 is still a mystery. One possible explanation could be the epitopes on 1ED3, 2ED3, and 3ED3 may be of similar configuration, however, relative accessibility to the antibody might be different, for example, the epitopes on 1ED3 might be partially buried compared to those on 2ED3 and 3ED3 (Figure 3.5). On the other hand, strict serospecificity of anti-4ED3 indicated that epitope structure on 4ED3 is completely different from those on other ED3s [279]. Moreover, these similarities in sequence and structure were fully supported by the cross-recognition of 1ED3, 2ED3, and 3ED3 by anti-1ED3 IgG antibodies, but not by the serospecific recognition of 4ED3 or by anti-4ED3 sera (Figure 3.5).

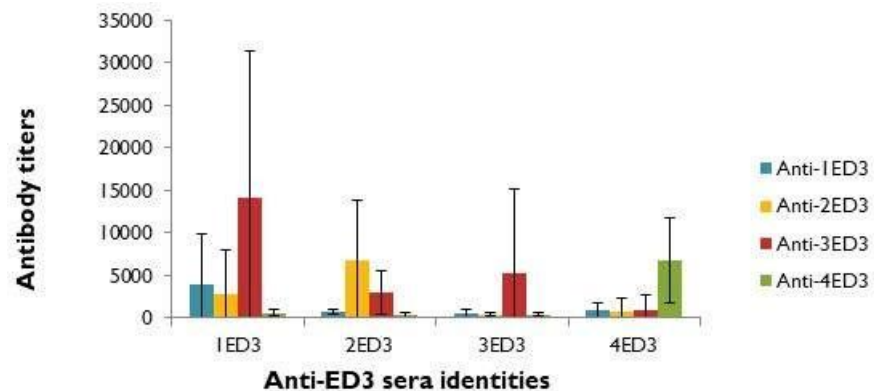


Figure 3.5: The anti-ED3 IgG antibody responses in mice models. Four different mice groups were injected with four different ED3s (individual ED3s at 30µg/mice in the presence of incomplete adjuvants (Swiss albino mice). Generation of anti-ED3 IgG antibodies was monitored at weekly intervals for 4 weeks through ELISA against all four ED3s and anti-ED3 IgG titers on day 21 are shown. First, all four ED3s were immunogenic and generated serotype-specific anti-ED3 IgG responses. Second, anti-1ED3 IgG was 1ED3-2ED3-3ED3 cross-reactive, and anti-2ED3 were 2ED3-3ED3 cross-reacting. On other hand, anti-3ED3 and anti-4ED3 was mostly serotype-specific.

3.7 Primary anti-ED3 IgG effects on the results of secondary heterotypic ED3immunization

A primary 1ED3 generated 1ED3-2ED3-3ED3 cross-reacting anti-ED3 IgG (Figure 3.5). Interestingly, a secondary immunization using 2ED3 further boosted up anti-2ED3 and anti-3ED3 IgG responses, indicating that anti-2ED3 and anti-3ED3 memory generated following primary 1ED3 injection recognized 2ED3 (Figure 3.6). Similarly, when a primarily 2ED3 and 3ED3 injected mice groups were secondarily injected with 1ED3 respectively, did not have any noticeable effect on anti-1ED3 IgG response. Generated almost equivalent 2ED3- 3ED3 cross-reacting IgG responses in 2ED3 and 3ED3 mice group. The second dose with 1ED3 in primarily 3ED3 injected mice group did not much affect the anti-3ED3 antibody status (Figure 3.6). Primary immunization With 4ED3 response generated 4ED3 specific without any cross recognition of other remaining ED3s 2nd dose with 1ED3 as a secondary infection 1ED3 remained 1ED3-2ED3-3ED3 cross-reactive so 4ED3-1ED3 infection shows all antibody (1ED3-2ED3-3ED3-4ED3) (Figure 3.6). It only happens when mice are immunized first with anti 4ED3.

On the other hand, as expected from primary anti-ED3 IgG responses, a primary anti-4ED3 IgG affected the outcome of any secondary anti-ED3 responses (Figure 3.5). Quite unexpectedly, the poorly immunogenic 4ED3 became almost completely non-immunogenic when it is used as a secondary immunization. The most peculiar observations made were that anti-2ED3 and anti-3ED3 sera did not exhibit any cross-recognition of 1ED3, despite the fact that anti-1ED3 serum was 1ED3-2ED3-3ED3 cross-recognizing (Figure 3.6).

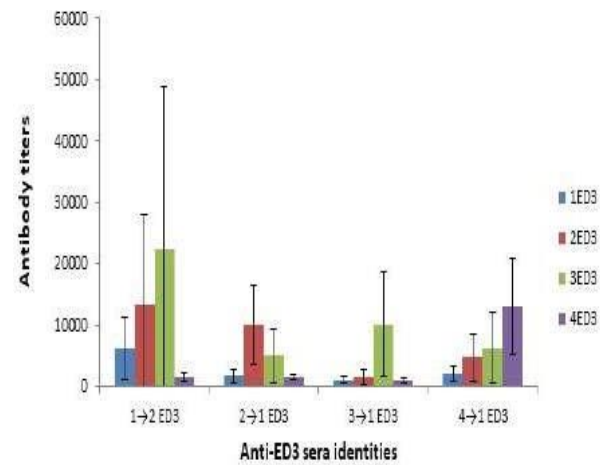


Figure 3.6: Secondary heterotypic infections like 1ED3-2ED3, 3ED3-1ED3 are sero cross reactive where anti 3ED3 antibodies were the highest. This observation may be due to presence of anti 1ED3 in these cases. Where anti-1ED3 is highly cross reactive with anti-3ED3. 4ED3-1ED3 infection remained shows all antibody.

3.8 Tertiary heterotypic DENV infection

In this Tertiary heterotypic DENV infection, ED3s were injected in secondary immunized mice. For the purpose mice group 1st (primarily with 1ED3, secondarily with 2ED3) & 2nd (primarily 2ED3 secondarily with 1ED3) were immunized with 3ED3, and group 3rd (primarily with 3ED3, secondarily with 1ED3) & 4th (primarily with 4ED3, secondarily with 1ED3) were immunized with 2ED3 (Figure 3.7). Further, 1st group generated the third dose with 3ED3 increased anti-2ED3-3ED3 IgG response with slight recognition of 1ED3, fully corroborated with the cross- recognition of 2ED3-3ED3 observed with 2ED3 and 3ED3 immunization. 2nd group third dose with 3ED3 further boosted up anti-2ED3-3ED3-cross-reactive IgG response. 3rd group while the third dose with 2ED3 further increased the 3ED3-specific immune response as we expected. 4th group third dose with 2ED3 further boosted up 2ED3 response (Figure 3.7).

Tertiary heterotypic infections like 1ED3-2ED3-3ED3, 2ED3-1ED3-3ED3, 3ED3-1ED3-2ED3 are in fact having the same immunization but different combinations, such as in 1ED3-2ED3-ED3 all antibodies were the highest compared to other combinations (Figure 3.7). Similarly 2ED3-1ED3-3ED3 immunization shows high anti- 2ED3 and 3ED3 antibodies but anti-4ED3 antibody was minimal. In 3ED3-1ED3-2ED3 immunization, only anti-3ED3 antibody was observed as the highest one but anti-1ED3 antibody was not observed in all cases. 4ED3-1ED3-2ED3 group remained mostly cross-reactive with 2ED3-4ED3. This group shows low antibody compare to others.

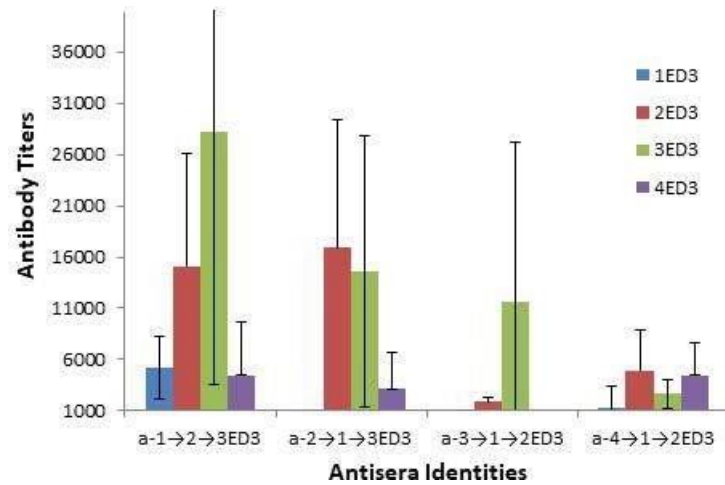


Figure 3.7: Tertiary heterotypic infections like 1ED3-2ED3-3ED3, 2ED3-1ED3-3ED3, 3ED3-1ED3-2ED3 are in fact having the same immunization but different combinations, such as in 1ED3-2ED3-ED3 all antibodies were the highest compared to other combinations.

3.9 Quaternary heterotypic DENV infection

1st (1ED3- 2ED3-3ED3), 2nd (2ED3-1ED3-3ED3) and 3rd (3ED3-1ED3-2ED3) groups were immunized with 4ED3. 4ED3 did not improve the IgG level for either of the four ED3s. These observations clearly indicated that 4ED3 sequence is at the distal from other ED3s, fully in agreement with their sequence and structural similarities [279]. Moreover, 4ED3 could be very poorly immunogenic [280]. 4th (4ED3- 1ED3 - 2ED3) group immunized with 3ED3 did not affect the 4ED3-specific response but elicited the 3ED3-specific response. This might be originated from the high immunogenicity of 3ED3 over other ED3s.

Regarding Quaternary heterotypic DENV infections, 3ED3 antibodies were observed as the highest antibody in all groups because DENV3 is the more immunogenic. We also observed that though group 1-3 are immunized with 4ED3 even then 4ED3 antibody is very low in all cases but 4ED3 antibody is only observed when mice are immunized first with anti 4ED3. In 4th group anti 3ED3 antibodies were the highest where mice of this group were lastly immunized with 3ED3. It is noted that anti-3ED3 antibody is always observed if mice are immunized with anti-3ED3 in any time. This is in contrast with 4ED3 where anti-4ED3 antibody response is observed only when mice are immunized first with anti-4ED3.

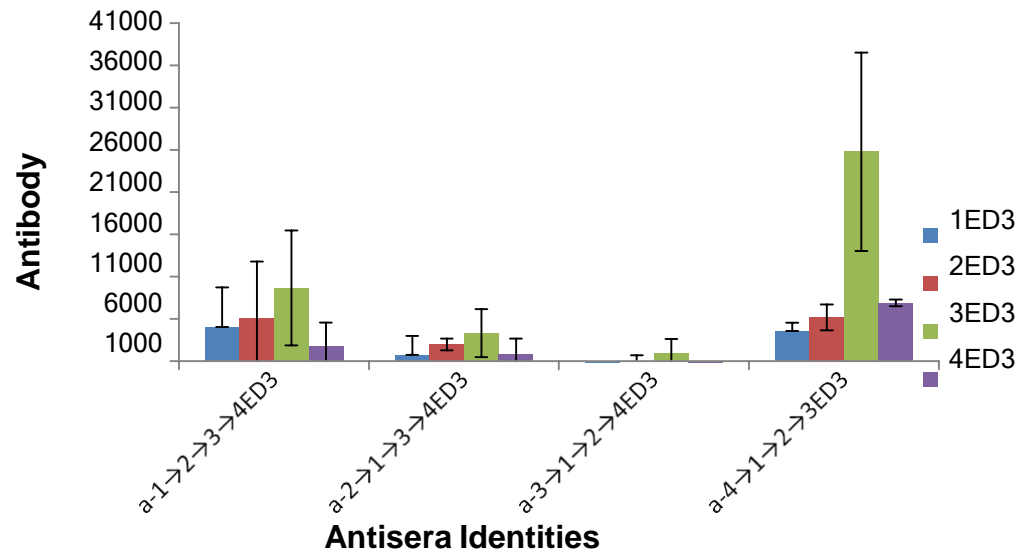


Figure 3.8: Regarding Quaternary heterotypic DENV infections: 3ED3 antibodies were observed as the highest antibody in all groups because DENV3 is the more immunogenic

3.10 : Immunogenicity, Sero-specificity, and Sero-Cross-Specificity of DENV1-4 ED3s

Although the DENV1-4 ED3s share a great deal of sequence and structural similarities, they are very much different from each other from sero-specificity, sero-cross-specificity and immunogenicity viewpoints. For instance, "classical immune response patterns" were almost exactly followed by the immunization with 2ED3 and 3ED3, and immunological responses (anti-ED3 antibody levels) increased the amount of dosages. Otherwise, anti-1ED3 and anti-4ED3 antibody responses increased only following 1st and 2nd doses for 1ED3 and 4ED3 but rather decreased following dose 4 and dose 3 (Figure 3.5 and 3.6). These observations clearly indicated that the immunogenicity of four ED3s was different, i.e. levels of IgG antibody and immunological memory status developed against different ED3s were different. To be more precise, all four ED3s could evoke anti-ED3 antibody responses along with different levels of immunological memory. The immunological memory being generated following the very first dose could be sustained a couple of months (during the study period) for 2ED3 and 3ED3. On the other hand, the anti-ED3 memory generated following 1ED3 and 4ED3 immunization perhaps sustained only for a couple of weeks. Furthermore, 2ED3 and 3ED3 had similar immunogenicity (in terms of sero-specific antibody titers) and the immunogenicity of 4ED3 was moderate and 1ED3 was the lowest.

From sero-specificity viewpoint, 1ED3 (anti-1ED3 IgG) was the most sero-cross-reactive, 2ED3 (anti-2ED3) was 2ED3- 3ED3 cross-reactive. The 3ED3 (anti-3ED3) and 4ED3 (anti-4ED3) was solely sero-specific. These interpretations were furthermore in line with observations with heterotypic secondary ED3 immunization. More specifically, 2ED3 and 3ED3-specific cross memory generated following 1ED3 challenges were boosted up further following secondary immunization with 2ED3 and 3ED3.

On the other hand, 4ED3 immunization did not generate any sero- cross-responses and did not have any effect on sero-cross-responses following heterotypic secondary ED3 immunization (Figure 3.5, 3.6, 3.7, 3.8). These results altogether indicated that a similar phenomenon may appear in natural DENV infections as well and may open a door for formulating four ED3-based vaccine candidates in the future. Therefore DENV serotype-specific ED3s could be worth considering models for investigating DENV infections in details and could open a new era for future dengue research and dengue disease management.

Objective 2: Anti-ED3 antibody responses against concurrent multiple DENV ED3s in mice.

3.11 Anti-ED3 IgG Responses against Concurrently Injected Heterotypic ED3s

3.11.1 Introduction

In our study it has been noted that natural DENV infection shows 24% concurrent heterotypic DENV infection, so we attempt to mimic different combinations artificially in Mice model, so that we can compare the natural and artificial infection and find any clue to make four ED3s based dengue vaccine candidate in future. In this research, the Artificial immunization study has been conducted in the absence of Freund's adjuvants in Balb/c mice model to mimic anti-DENV antibody responses against concurrent heterotypic dengue infections in mice model. Here we used three combinations comprising six bivalent ED3s (1ED3-2ED3, 1ED3-3ED3, 1ED3-4ED3, 2ED3-3ED3, 2ED3-4ED3, 3ED3-4ED3), four trivalent ED3s (1ED3-2ED3-3ED3, 1ED3-2ED3-4ED3, 1ED3-3ED3-4ED3, 2ED3-3ED3-4ED3) and one tetravalent combination (1ED3-2ED3-3ED3-4ED3).

3.11.2 Anti-ED3 IgG Responses against the Bivalent ED3 Combinations

The anti-ED3 IgG responses against bivalent, trivalent, tetravalent, combinations of ED3s were investigated using all possible bivalent, trivalent and tetravalent combinations of ED3s as immunogens in Swiss albino mice. Now let us first consider anti-ED3 IgG responses against bivalent of combinations ED3s. The very first dose (dose 1) with 1ED3--2ED3 bivalent combination generated 1ED3-2ED3-3ED3 cross-reacting anti-ED3 IgGs (Figure 3.9a).

However, following dose 2 and dose 3 the immune responses shifted to 2ED3-specificity (Figure 3.9a), indicating that, first, the immunogenicity of 1ED3 might not so prolonged as observed with 2ED3 and second, the immunogenicity of 2ED3 prevailed over 1ED3; 2ED3 is more immunogenic over 1ED3 (Figure 3.9a).

Very similar, if not identical, the anti-ED3 IgG titers against bivalent 1ED3-3ED3 immunization also generated 1ED3-2ED3-3ED3 cross-reacting anti-ED3 IgG responses(Figure 3.9b) as expected from their individual immunization study (Figure 3.5), where antibody titer against 3ED3 was the highest, indicating that immunogenicity of 3ED3 was higher over 1ED3. Recognition of 2ED3 could be originated from cross- recognition of 2ED3 by anti-1ED3 and anti-3ED3 IgG. Interestingly, sero-specific recognition of 3ED3 increased following successive doses (Figure 3.9b). Mice concurrently injected with bivalent 1ED3-4ED3 generated 1ED3-2ED3-3ED3-4ED3 cross-reacting anti-ED3 IgG response (Figure 3.9c) as expected from their individual immunization studies; 1ED3 and 4ED3 are co-immunogenic (Figure 3.6). However, the anti-4ED3 IgG titer was the least, suggesting that the immunogenicity of 4ED3 was lower than that of 1ED3.

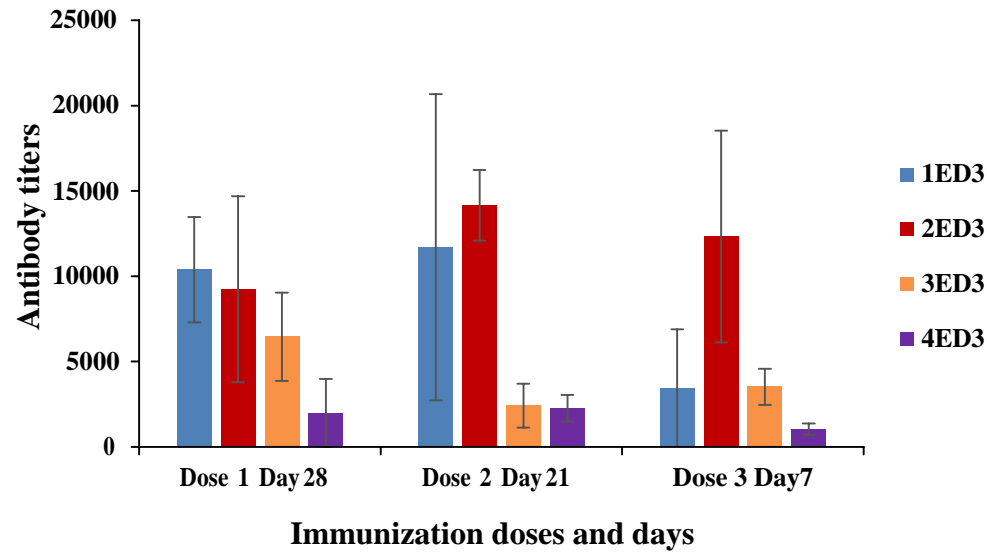


Figure-3.9a: Immune responses against the bivalent 1ED3-2ED3 combination. The first dose generated anti-1ED3-2ED3-3ED3 cross-reacting anti-ED3 IgG. Cross-recognition of 3ED3 decreased significantly following dose 2 and responses become almost 2ED3-specific following dose 3. These observations suggested that 1ED3 induced unstable cross-reacting responses and over the time 2ED3-specific responses prevailed and suggested that 2ED3 is more immunogenic and sero-specific than 1ED3.

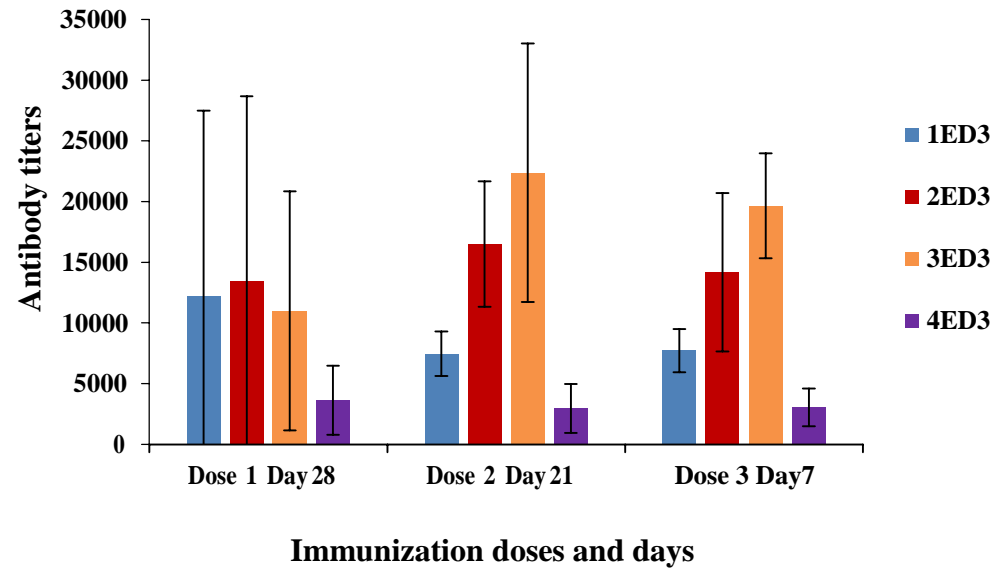


Figure 3.9b: Immune responses against the bivalent 1ED3-3ED3 combination. Dose 1 induced unstable 1ED3-2ED3-3ED3 cross-reacting anti-ED3 IgGs. However, anti-3ED3 IgG responses increased with successive doses, indicating that the immunogenicity of 3ED3 was higher over 1ED3.

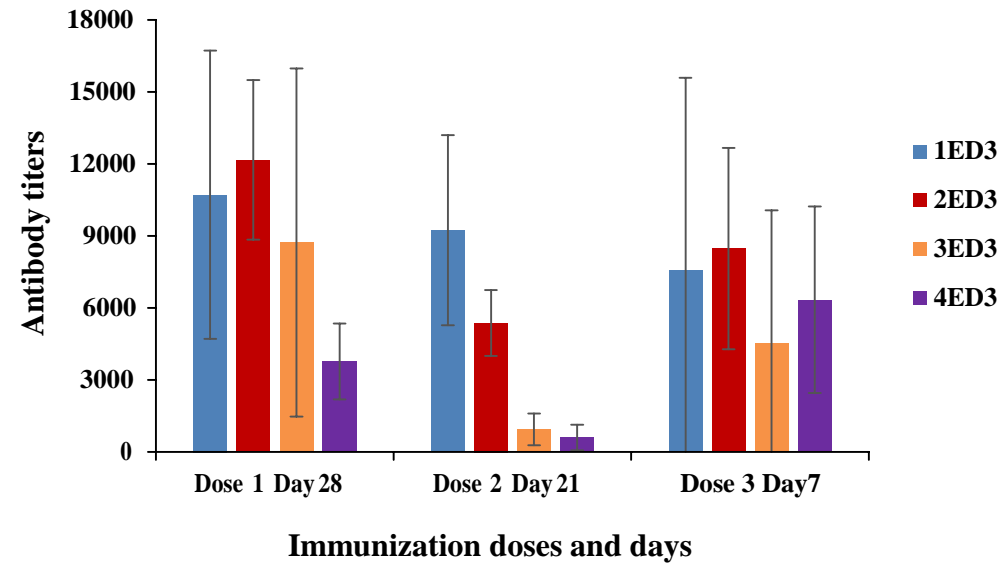


Figure 3.9c: Immune responses against the bivalent 1ED3-4ED3 combination. Both 1ED3 and 4ED3 were co-dominantly immunogenic. Better recognition of 1ED3 and 2ED3 originated from anti-1ED3 and reduced recognition of 4ED3 by anti-4ED3. Minimal cross-recognition of 3ED3 might be come from 1ED3 which corroborated the data found in the single ED3 study.

Similarly, mice injected with bivalent 2ED3-3ED3 had 2ED3-3ED3 sero-cross-reactive anti-ED3 IgG (Figure 3.9d). The very similar recognition of 2ED3 and 3ED3 (similar antibody titers) suggested that both 2ED3 and 3ED3 are co-dominantly immunogenic and are of similar immunogenicity (Figure 3.9d). Moreover, 2ED3-3ED3 cross-reacting anti-ED3 IgG responses sustained and/or remained very similar over doses.

However, following immunization using bivalent 2ED3-4ED3 and 3ED3-4ED3 generated mostly 2ED3-specific and 3ED3-specific IgG responses, respectively with no cross-recognition of 4ED3 (Figure 3.9e; 3.9f). These findings indicated that both 2ED3 and 3ED3 were not only more immunogenic over 4ED3, but suppressed the immunogenicity of 4ED3 by both bivalent ED3 combinations.

Furthermore, the immunogenicity of 4ED3 was further suppressed compared to that observed with individual 4ED3 immunization (Figure 3.5) and with 1ED3-4ED3 immunization (Figure 3.9c). Altogether, these observations clearly indicated that 2ED3 and 3ED3 are of similar immunogenicity, co-dominantly immunogenic and their immunogenicity is noticeably higher than those of 1ED3 and 4ED3. Furthermore, the immunogenicity of 4ED3 was suppressed in the presence of 2ED3 and 3ED3 and interestingly 4ED3 showed immunogenicity only in the presence of 1ED3 in antigen preparation (Figure 3.12d).

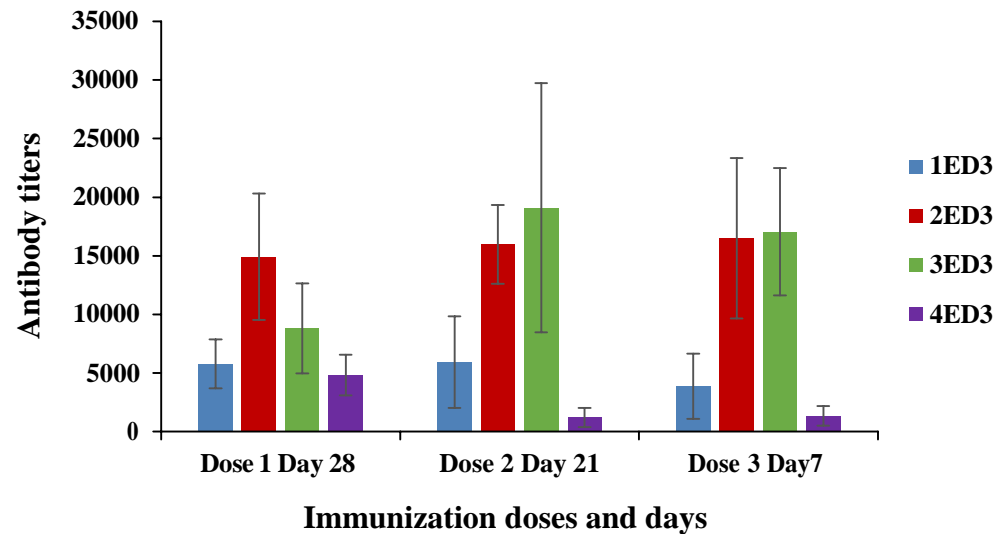


Figure 3.9d: Immune responses against the bivalent 2ED3-3ED3 combination. Almost similar recognition of 2ED3 and 3ED3, and minimal cross-recognition of 4ED3, corroborating with the previous observations that anti-2ED3 and anti-3ED3 cross-recognize 2ED3 and 3ED3, respectively. In addition, anti-3ED3 could cross-recognize neither 1ED3 nor 4ED3. Interestingly, throughout the immunization protocols, both 2ED3 and 3ED3 remained co-dominantly immunogenic.

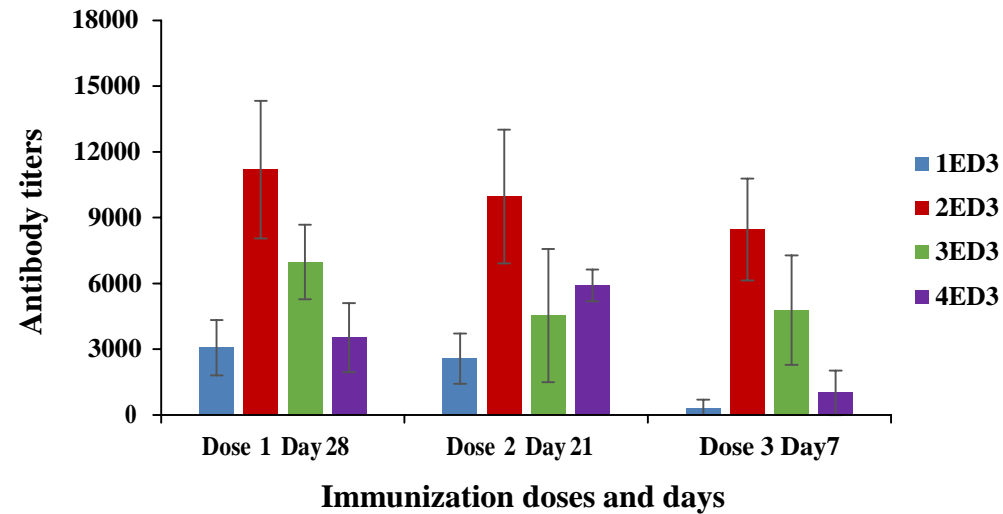


Figure 3.9e: Immune responses against the bivalent 2ED3-4ED3 combination. Immunization with bivalent 2ED4-4ED3 generated mostly 2ED3-specific anti-ED3 IgG with cross-recognition of 3ED3 and minimal responses against 4ED3. Anti-ED3 IgG titers suggested that 2ED3 was not only highly immunogenic over 4ED3, but also suppressed the immunogenicity of 4ED3.

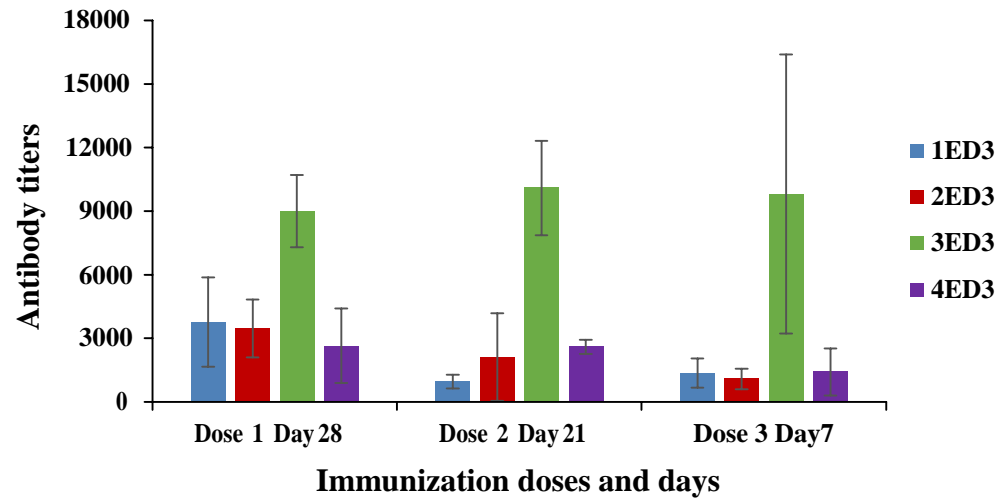


Figure 3.9f: Immune responses against the bivalent 3ED3-4ED3 combination. Immune response against concurrently injected bivalent 3ED3-4ED3 was mostly 3ED3-specific with a minimal 4ED3-specific response. High anti-3ED3 IgG titers suggested that 3ED3 is more immunogenic than 4ED3 and immunogenicity of 4ED3 was furthermore suppressed in the presence of 3ED3.

3.11.3 Anti-ED3 IgG Responses against the Trivalent ED3 Combinations

Now let us consider anti-ED3 IgG responses against the trivalent combination of ED3s. As expected from monovalent (Figure 3.5) and bivalent (Figure 3.9) combination studies, immunization using trivalent 1ED3-2ED3-3ED3 generated 1ED3-2ED3-3ED3 cross-recognizing IgG responses (Figure 3.10a) where anti-1ED3 IgG titer was the lowest with no cross-recognition of 4ED3. These results indicated that the immunogenicity of 1ED3 was lower compared to that of 2ED3 and 3ED3, corroborating with bivalent combinations (Figure 3.9).

Injection of bivalent 1ED3-2ED3 and 2ED3-4ED3 generated mostly anti-2ED3 IgG (Figure 3.9a; 3.9e) and 1ED3-4ED3 injection generated anti-1ED3-2ED3-3ED3-4ED3 cross-reacting IgG responses (Figure 3.9c). As expected from cumulative effects of 1ED3, 2ED3 and 4ED3, the trivalent 1ED3-2ED3-4ED3 formulation generated 1ED3-2ED3-4ED3 cross-reacting IgG with a minimal cross-recognition of 3ED3 (Figure 3.10b). These results clearly indicated that immunogenicity of 4ED3 suppressed in the bivalent combination of 2ED3-4ED3 could be at least partially regained when injected with 1ED3. Such observations were furthermore corroborated with anti-ED3 IgG responses observed against the trivalent combination of 1ED3-3ED3-4ED3 (Figure 3.10c). To be more precise, anti-ED3 IgG against 3ED3-4ED3 was mostly 3ED3-specific (Figure 3.9f), however, the responses were shifted to 1ED3-3ED3-4ED3 cross-recognizing with lower recognition of 2ED3 when the 1ED3 was added in antigen formulation (Figure 3.10c).

Our last trivalent combination was 2ED3-3ED3-4ED3 where only 1ED3 was missing. We previously discussed that 2ED3 and 3ED3 were of similar immunogenicity (Figure 3.9d) and suppressed the immunogenicity of 4ED3 in bivalent combinations (Figure 3.9e; 3.9f). Injection of trivalent 2ED3--3ED3-4ED3 combination also generated almost equivalent anti-2ED3-3ED3 cross-recognizing IgG responses with minimal and/or no recognition of 4ED3; 4ED3 remained very poorly immunogenic (Figure 3.10d), fully corroborating previous observations (Figure 3.9).

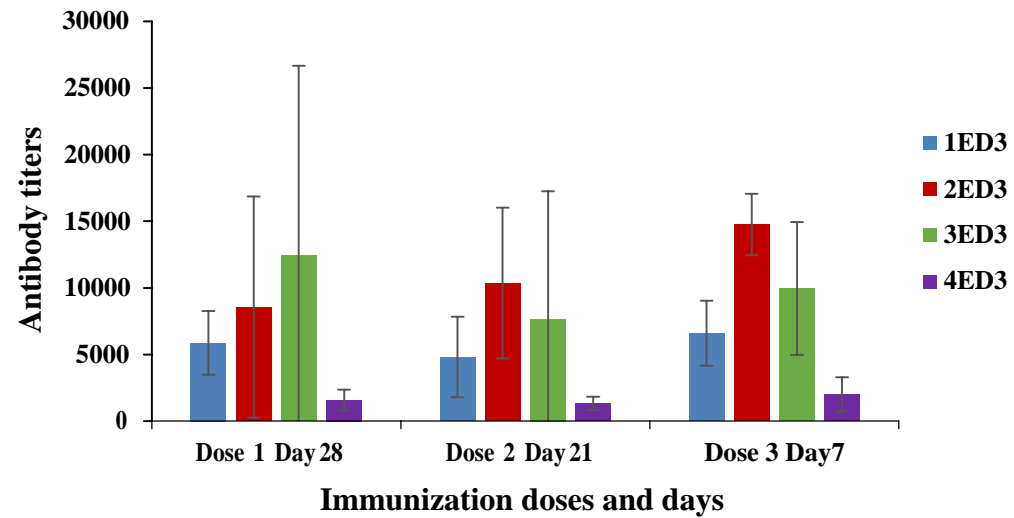


Figure 3.10a: Immune responses against the trivalent 1ED3-2ED3-3ED3 combination. The dose 1 generated 1ED3-2ED3-3ED3-cross-recognizing anti-ED3 IgG, however, anti-2ED3-3ED3 cross-recognizing IgG responses prevailed.

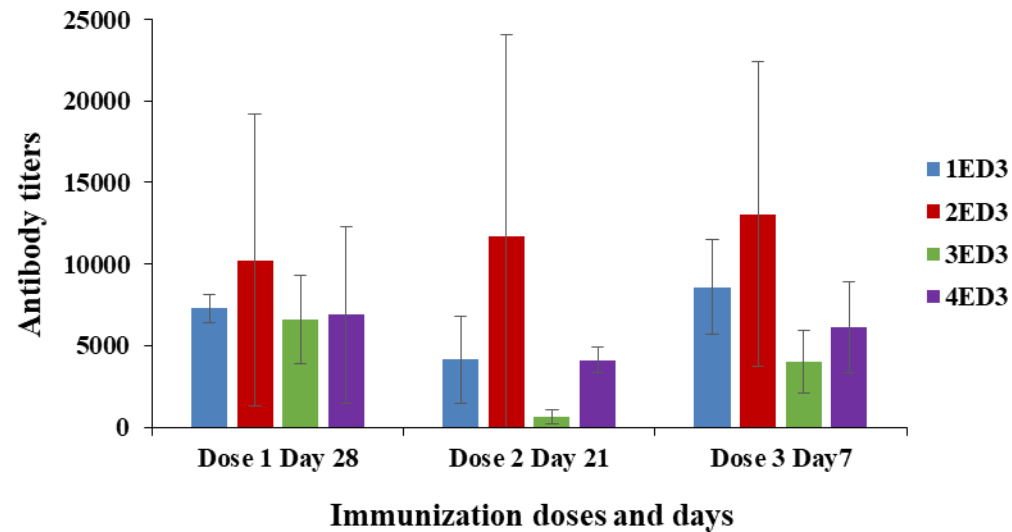


Figure 3.10b: Anti-ED3 IgG responses against the trivalent 1ED3-2ED3-4ED3 combination. Injection of 1ED3-2ED3-4ED3 initially (dose 1) generated 1ED3-2ED3-3ED3-4ED3 cross-recognizing IgG responses. However, following successive doses recognition of 3ED3 reduced, as expected. The cross-recognition of 1ED3 and 2ED3 originated from anti-1ED3 and anti-2ED3 while the sero-specific recognition of 4ED3 was solely by anti-4ED3 IgG.

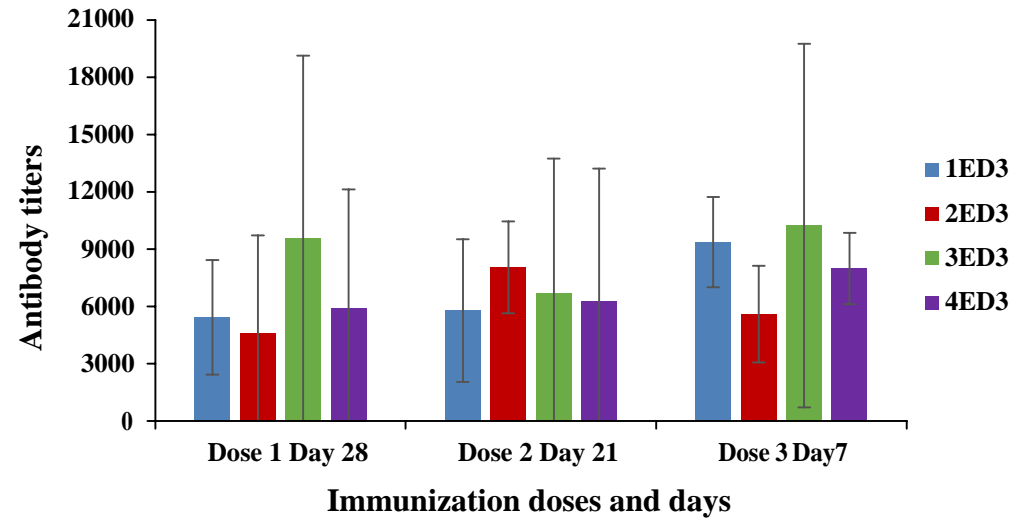


Figure 3.10c: Anti-ED3 IgG responses against the trivalent 1ED3-3ED3-4ED3 combination. From the very first dose to the last dose of immunization, the anti-ED3 IgG responses remained 1ED3-2ED3-3ED3-4ED3 Sero-cross-reactive. However, recognition of 3ED3 (antibody titers against 3ED3) was the highest, suggesting that antigenicity of 3ED3 was clearly higher over 1ED3 and 4ED3.

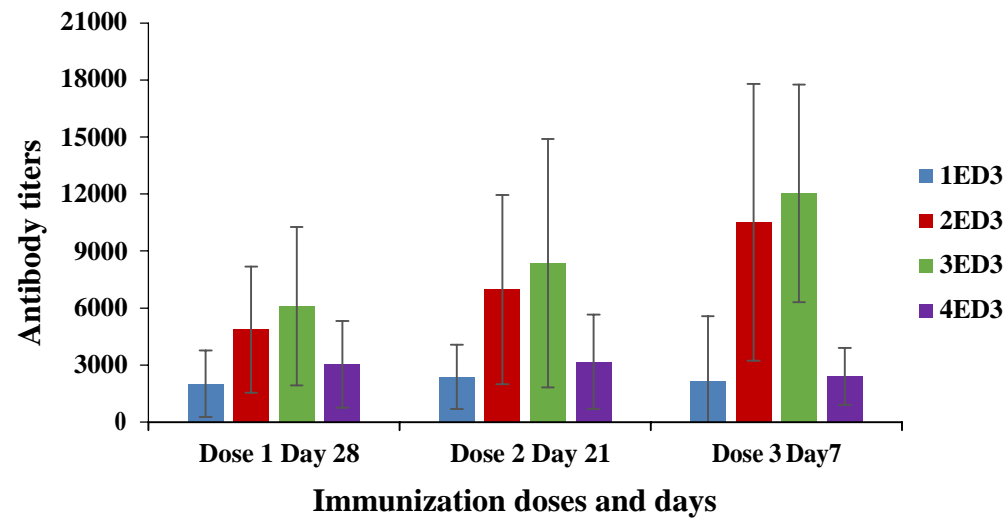


Figure 3.10d: Anti-ED3 IgG responses against the trivalent 2ED3-3ED3-4ED3 combination. Anti-ED3 IgG showed almost similar recognition of 2ED3 and 3ED3 and reduced recognition of 4ED3, indicating that 2ED3 and 3ED3 are of similar immunogenicity, co-dominantly immunogenic and the immunogenicity of 4ED3 was furthermore suppressed in the presence of 2ED3 and 4ED3 as observed in bivalent combinations as well (Figure 3.9e).

3.11.4 Anti-ED3 IgG Responses against the Tetravalent ED3 Combinations

Immunization against tetravalent composition of all four ED3s at equimolar concentration generated 1ED3-2ED3-3ED3-4ED3 cross-reacting anti-ED3 IgG responses (Figure 3.11). However, the antibody titer against 4ED3 was the lowest indicating that the immunogenicity of 4ED3 is very poor. The order of immunogenicity was 2ED3 > 3ED3 > 1ED3 > 4ED3 (Figure 3.5).

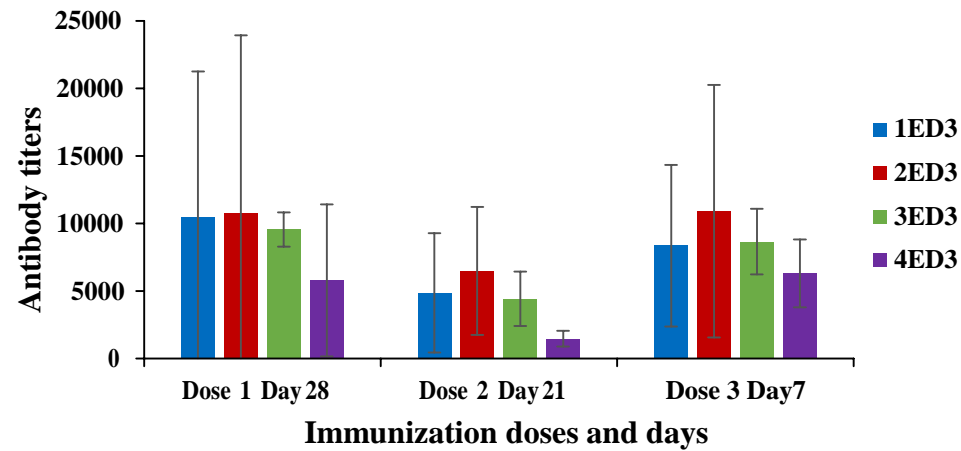


Figure 3.11: Anti-ED3 IgG responses against the tetraivalent 1ED3-2ED3-3ED3-4ED3 combination. Injection of tetraivalent 1ED3-2ED3-3ED3-4ED3 generated 1ED3-2ED3-3ED3-4ED3 cross-reacting anti-ED3 IgG responses. Cross-recognition 1ED3-2ED3-3ED3 was quite similar (similar antibody titers against 1ED3, 2ED3, and 3ED3), but recognition of 4ED3 was relatively low.

3.12 Co-dominant Immunogenicity of ED3s

Immunization against 1ED3 generated 1ED3-2ED3-3ED3 cross-reacting IgG response (Figure 3.12a). However, in bivalent combinations with 2ED3 (1ED3-2ED3) mostly 2ED3-specific and 3ED3 (1ED3-3ED3) was mostly 1ED3-2ED3-3ED3 cross-reacting. However, anti-ED3 IgG responses shifted mostly to 2ED3 and 3ED3 specificity, respectively with repeated doses (Figure 3.9; 3.10). This indicated that both 2ED3 and 3ED3 were more immunogenic than 1ED3. Similarly, 1ED3-2ED3-3ED3 remained co-dominantly immunogenic with 1ED3-2ED3-3ED3 cross-reacting anti-ED3 IgG. However, in 1ED3-4ED3 combination both 1ED3 and 4ED3 remained co-dominantly immunogenic and generated 1ED3-2ED3-3ED3-4ED3 cross-recognizing anti-ED3 IgG. Interestingly, anti-4ED3 IgG appeared only when 4ED3 was present in antigen preparation along with 1ED3 (Figure 3.12a).

In the mice group where 2ED3 was in common in heterogeneous combinations of ED3s, 2ED3 remained the most immunogenic and remained co-dominantly immunogenic with 3ED3 in any combinations (Figure 3.12b). To be more precise, the immune responses remained 2ED3 and/or 3ED3 directional if 2ED3 and/or 3ED3 were present in any combinations of ED3s, from bivalent to tetravalent formulation (Figure 3.12b and 3.12c). Similarly, 3ED3 also remained the most immunogenic one in all combinations of 1ED3, 3ED3 and 4ED3 and become co-dominantly immunogenic when the antigen formulation had 2ED3 (Figure 3.12c). Interestingly, all mice anti-4ED3 IgG antibodies were observed only when they were immunized using 1ED3-4ED3 together in bivalent (Figure 3.9), trivalent (Figure 3.10) and tetravalent (Figure 3.11) composition of ED3s.

3.13 Adjuvanting Effects of 1ED3 on 4ED3

The DENV4 ED3 (4ED3) showed poor immunogenicity in both primary and concurrent heterotypic immunization. The immunogenicity of 4ED3 was further reduced after successive doses when 2ED3/3ED3 was present in the combinations (Figure 3.12d).

However, when the ED3 combination formulation had 1ED3 and 4ED3 together in any combination of ED3s 4ED3 became immunogenic (Figure 3.12d). Though we do not have a good explanation for reduced immunogenicity of 4ED3 in the presence of 2ED3 and 3ED3 and increased immunogenicity of 4ED3 in the presence of 1ED3 (Figure 3.12d), such adjuvanting effects of 1ED3 on 4ED3's immunogenicity is a new observation. Altogether, we suggest that considerable differences in immunogenicity and adjuvanting effects of 1ED3 boosting the immunogenicity of poorly immunogenic 4ED3 may pave a way for developing effective subunit-based dengue vaccine design in the future.

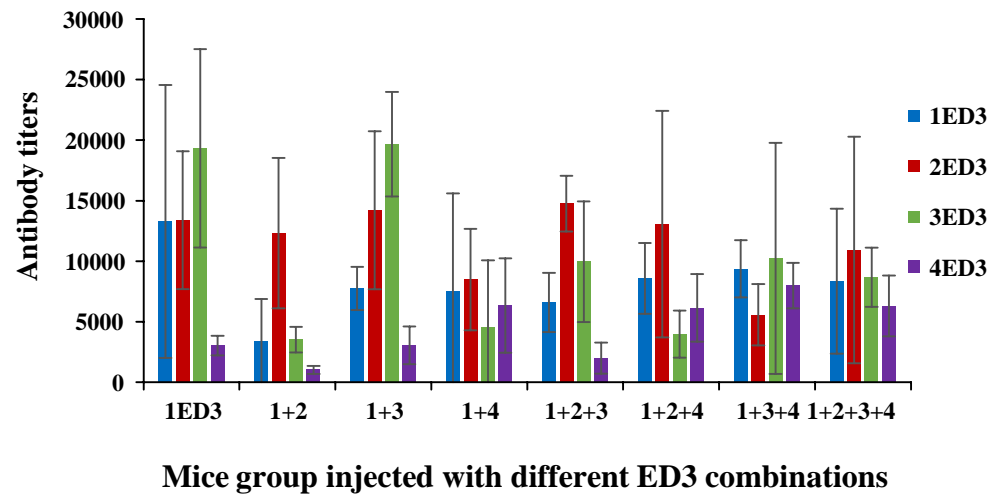


Figure 3.12a: Anti-ED3 IgG responses against when 1ED3 was present in different ED3 combinations. First, 1ED3 remained immunogenic with any combinations of ED3s. However, the immunogenicity of 1ED3 (anti-1ED3 IgG response) was lower than that of 2ED3 and 3ED3. Interestingly, 4ED3 remained very poorly immunogenic with any combinations of 2ED3 and 3ED3 and become immunogenic only when 4ED3 is present with 1ED3 in any combinations of all four ED3s. Antibody titers after dose 3 are shown.

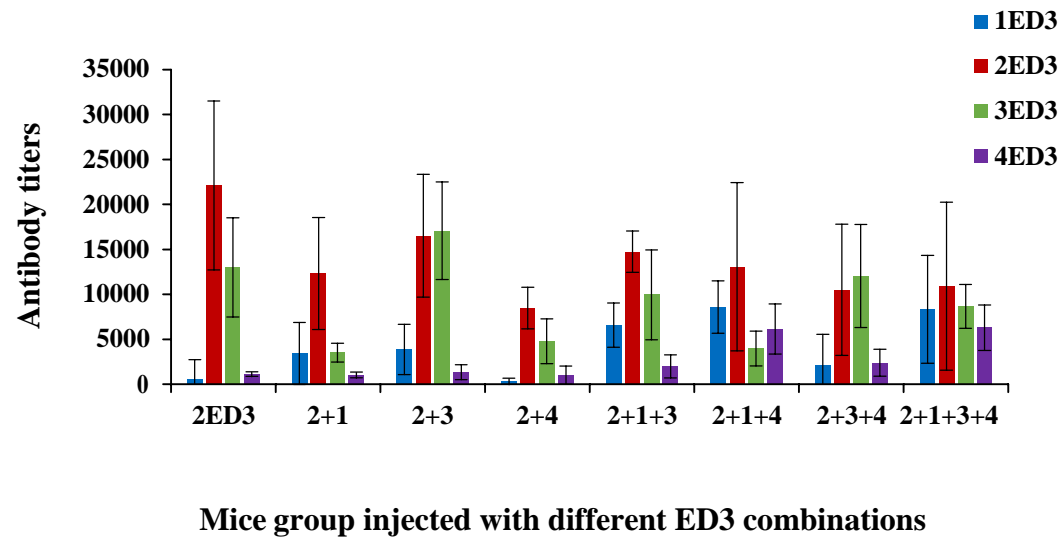


Figure 3.12b: Immune responses against different combinations of ED3s where 2ED3 was common. In any combinations 2ED3 remained the most immunogenic (over 1ED3 and 4ED3) and co-dominantly immunogenic when presented with 3ED3, suggesting that 2ED3 and 3ED3 are of similar immunogenicity and are co-dominantly immunogenic in any combinations of ED3s. Antibody titers after dose3 are shown.

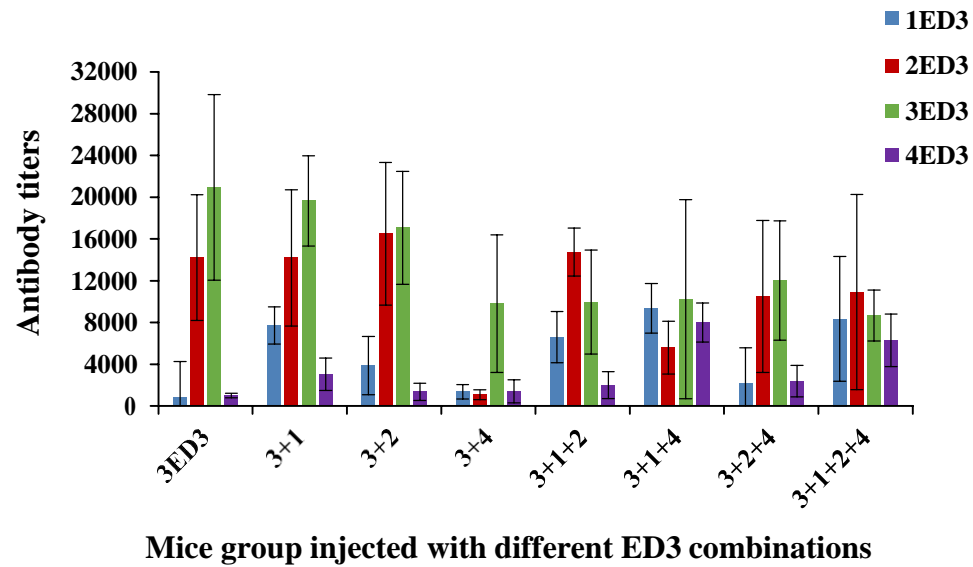


Figure 3.12c: Anti-ED3 IgG responses against different combinations of ED3s where 3ED3 was common. In any combinations, 3ED3 remained the most immunogenic (over 1ED3 and 4ED3) and co-dominantly immunogenic when 3ED3 was present with 2ED3. Antibody titers after dose 3 are shown.

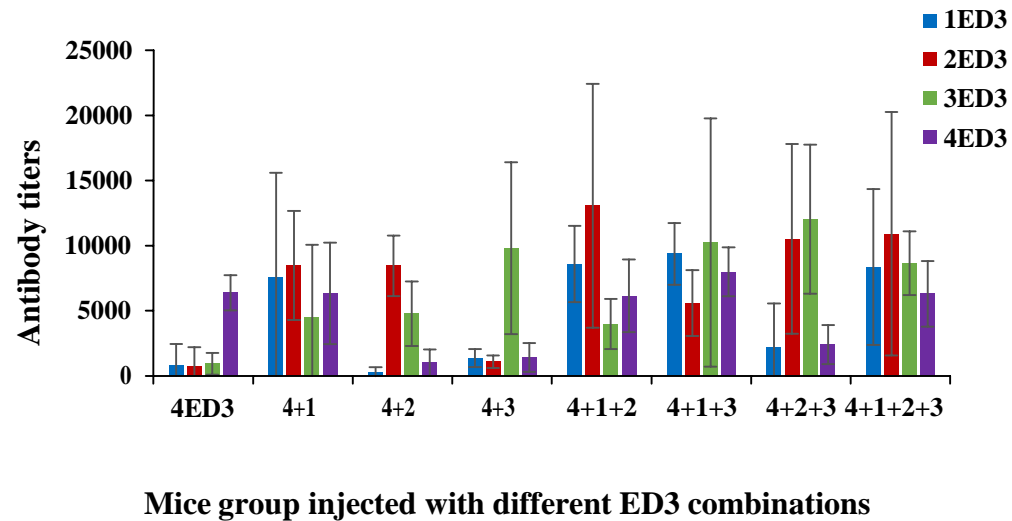


Figure 3.12d: Anti-ED3 IgG responses against different combinations of ED3s where 4ED3 was in common. In any combinations, 4ED3 remained very poorly immunogenic (compared to other ED3s) and the immunogenicity of 4ED3 was further reduced when injected with 2ED3 and 3ED3. Interestingly, 4ED3 became immunogenic only when 1ED3 was present in antigen preparation.

3.14 Heterogeneous Immunogenicity of Four DENV ED3s

Here we also report two mysterious observations with IgG antibody reactions against ED3. First, the anti-1ED3 IgG response was quite unstable cross-recognizing 2ED3 and 3ED3 in addition to its sero-specific recognition. However, over the course of immunization and successive doses, the responses became 1ED3-2ED3 cross-reacting. Furthermore, anti-2ED3 and anti-3ED3 IgG did not exhibit any cross-recognition of 1ED3, anti-1ED3 sera exhibited cross-recognition of 2ED3 and 3ED3. According to these findings, though 1ED3, 2ED3, and 3ED3 shared similar epitope conformation, their relative accessibility to the anti-DENV antibodies was different, fully corroborating with their modeled structure analysis that epitopes of 2ED3 and 3ED3 might readily be accessible while those on 1ED3 was partially buried.

Finally, the 4ED3 was poorly immunogenic as reported previously, however, its immunogenicity reduced further when injected with 2ED3 and/or 3ED3. Interestingly, the anti-4ED3 IgG antibody responses appeared only when 4ED3 was injected along with 1ED3; 1ED3 could boost up the immunogenicity of 4ED3. Though we do not have any good explanation for the adjuvanting effects of 1ED3 on the immunogenicity of 4ED3, 1ED3 could be a good choice for making poorly immunogenic 4ED3 into an immunogenic one. Such diverse heterogeneous immunogenicity of four ED3s might be restricting the success of available dengue vaccine candidates. To this end, we, therefore, suggest a differential and careful designing of a tetravalent ED3 formulation based on four ED3s have different immunogenic properties and adjuvanting effects of 1ED3 on 4ED3 may pave the way to develop a potent ED3-based vaccine against dengue virus in a single formulation in the future.

Objectives 3: Investigation of the immune statuses (T cell memory status) of mice injected with DENV-serotype-specific ED3s by flow-cytometer.

3.15 Investigated Immune status by Flow-Cytometer

To assess the potentiality of anti-dengue ED3 for confirming a long-term immune response, here we investigated the generation of central T cells memory and effector cells response by injecting all four wild-type dengue ED3s in the BALB/c mice model (Figure 3.13, Figure 3.14, Figure 3.15, and Figure 3.16). We have taken three mice in each group injecting with same DENV ED3 along with two mice as control injecting with PBS only. We performed a CD marker analysis in mice blood cell by using CytoFLEX flow cytometer and analyzed all data in CytExpert software without any compensation. Finally, we compared the T cell memory response data with antibody response evaluated by ELISA for confirming an effective immunity.

3.16 Generation of T cell Memory Response against Individual ED3s

To investigate anti-ED3 memory responses against injected DENV-specific ED3s, we have monitored the level of CD markers on T-lymphocytes on 14th and 21st day after immunization. First, immunization using DENV1-4 ED3s produced both Tc cell and Th cell memory responses confirming both cellular and humoral immunity for long-term. However, the memory T cell response against DENV1 ED3 (1ED3) was the highest, followed by DENV2 ED3 (2ED3), DENV3 ED3 (3ED3) and the minimal response against DENV4 ED3 (4ED3) [274]. (Figure 3.13,

Figure 3.14, Figure 3.15, and Figure 3.16). Interestingly, all DENV memory responses on day 21 were almost nearly to day 14 responses confirming an elevated response for a long period of time (Figure 3.17a and Figure 3.17b).

3.17 T cell Memory against 1ED3

The majority of CD44⁺ and CD44⁺CD62L⁺ cells were seen in mice treated with 1ED3 (for both Tc and Th) cell indicating effector memory and central memory T-cell responses, respectively.

3.17.1 Responses on Day 14

On day 14 of immunization 34.57% Tc cells were CD44⁺ compared to 16.35% positive in control mice injected with only PBS. This observation suggested the generation of effector memory Tc cell memory against 1ED3 (Figure 3.13a). Similar arising in the mutual-expression of CD44 and CD62L (CD44⁺CD62L⁺) was also observed; percent CD44⁺CD62L⁺ Tc cell on day 14 was 33.33 (Figure 3.13b). These results clearly indicated the generation of a central memory Tc cell response. Similarly, CD44 was expressed on 59.49% of Th cells (CD4⁺ T cells) on day 14 after immunization (Figure 3.15a). Furthermore, alike Tc cells responses, CD44 and CD62L (CD44⁺CD62L⁺ cells) mutual expression was found on 47.18% of total Th cells on day 14.

3.17.2 Responses on Day 21

The percent of CD44⁺ Tc cells were almost unchanged on day 21, to be more precise, 35.45% (Figure 3.14a). In addition, the co-expression of CD44 and CD62L (CD44⁺CD62L⁺) was also observed; percent CD44⁺CD62L⁺ Tc cell on day 21 was 28.62, altogether indicating that, the generated memory response is long lasting (Figure 3.14b). Similarly, third week later after immunization, CD44 was expressed on 48.32% of Th cells remaining nearly the expression on day 14 (Figure 3.16a). At the same time, alike Tc cells responses, CD44 and CD62L (CD44⁺CD62L⁺ cells) mutual expression was found on 41.59% of total Th cells day 21, suggesting that, the Th cell mediated immune response is also long lasting (Figure 3.16b).

3.18 T cell Memory against 2ED3

Injection of DENV2 ED3 (2ED3) into BALB/c mice also produced both effector and humoral T cell memory as observed with 1ED3 immunization. However, the T cell memory response was slightly lower than that observed against 1ED3.

3.18.1 Responses on Day 14

Following 2ED3 injection, 34.62% of Tc cells expressed CD44 on day 14, while number of Th cells bearing CD44 was 46.18% (Figure 3.13a and Figure 3.15a). On the other hand, the numbers of CD44⁺CD62⁺ Tc cells and Th cells were 29.49% and 39.12%, respectively on the very same day (Figure 3.13b and Figure 3.15b).

3.18.2 Responses on Day 21

The responses (both Tc and Th memory cells) on day 21 were very close to the responses on day 14. To be more precise, following 2ED3 injection, 33.09% of Tc cells expressed CD44 on day 14 while number of Th cells bearing CD44 was 43.36% (Figure 3.14a and Figure 3.16a). On the other hand, the numbers of CD44+CD62+ cells Tc cells and Th cells were 26.05% and 33.78%, respectively on that day (Figure 3.14b and Figure 3.16b).

3.19 T cell Memory against 3ED3

Immunization using DENV3 ED3 (3ED3) also generated reasonably T cell memory response in mice model.

3.19.1 Responses on Day 14

On day 14 of immunization, 25.52% of Tc cells and 30.17% of Th cells had CD44+ on their surface (Figure 3.13a and Figure 3.15a). Among CD44+ T cells, 21.78% of Tc cells and 28.21% of Th cells had co-expression of CD44 and CD62L(CD44+CD62L+) on their surfaces (Figure 3.13b and Figure 3.15b).

3.19.2 Responses on Day 21

As observed with 3ED3 immunization, expression of CD44 and CD62L, both as single markers and co-expressed, remain nearly on day 21. More precisely, on day 21, the number of CD44+ T cells, CD44+ Th cells, CD44+CD62L+Tc cells and CD44+CD62L+Th cells were 22.48%, 33.93%, 16.41%, 25.85%, respectively.

Altogether, these observations suggested a long-term T cell memory response against 3ED3. (Figure 3.14 and Figure 3.16).

3.20 T cell Memory against 4ED3

Several previous studies suggested that DENV4 ED3 (4ED3) had the poorest immunogenicity when compared with other remaining three ED3s [280-282]. from the development of the anti-ED3 IgG antibody responses in mice model. Here, we also observed the lowest T-cell memory responses against 4ED3 when compared with the T cell memory responses against 1ED3, 2ED3 and 3ED3 (Figure 3.17a and Figure 3.17b).

3.20.1 Responses on Day 14

In 4ED3 injected mice, 17.19% of Tc cells and 23.48% of Th cells had CD44 on their surface on day 14 (Figure 3.13a and Figure 3.15a). Similarly, only 15.11% of Tc cells and 19.68% of Th cells had co-expression of CD44 and CD62L (CD44+CD62L+ cells) on that day (Figure 3.13b and Figure 3.15b).

3.20. 2 Responses on Day 21

On day 21 after 4ED3 injection, only 16.26% of Tc cells and 18.09% of Th cells had CD44 on their surface (Figure 3.14a and Figure 3.16a). Moreover, the numbers of CD44+CD62+ Tc cells and Th cells were 12.37% and 13.60%, respectively on that day, which were much lower than those observed with 1ED3, 2ED3 and 3ED3 (Figure 3.14b and Figure 3.16b). These observations suggested that through 4ED3 generated T cell memory 4ED3 is the least immunogenic, fully corroborating with previous studies [280-282].

3.21 Overall Tc cell memory status against individual ED3s

Tc cell response is inescapable to combat any viral disease including dengue disease through developing cellular and humoral immunity against the invading viruses. Interestingly, four different DENV ED3s (DENV1-4 ED3s) have very similar sequences and structures, however, immune responses that they generated in mice were different; the magnitudes of both effector and central T cell memory were different for different ED3s. To be more precise, 1ED3 generated that highest of effector memory Tc cell (CD44+CD8+ cells) and central memory Tc cell (CD44+CD62+CD8+ cells), followed by 2ED3, and 3ED3 (Figure 3.17a). On the other hand, 4ED3 generated the lowest number of effector memory Tc cell (CD44+CD8+ cells) and central memory Tc cell (CD44+CD62+CD8+ cells) (Figure 3.17a). These findings indicated that 1ED3, 2ED3 and 3ED3 were of similar immunogenicity while the 4ED3 was the least immunogenic.

3.22 Overall Th cell memory status against individual ED3s

While injecting with individual ED3s, here again 1ED3 produced highest number of CD44+ and CD44+CD62L+ Th cell followed by 2ED3, 3ED3 and 4ED3 (Figure 3.17b). But the Th memory response for 1ED3, 2ED3 and 3ED3 was very similar and high especially even on day 21 confirming a strong long-term immunity against all these three ED3s. As observed with Tc cell memory formation, the 4ED3 produced a poor response when compared to other ED3s (Figure 3.17b).

3.23 Comparing T cell memory response data with antibody response

We have compared T cell memory responses data with antibody responses measured by ELISA after injecting the mice with individual DENV ED3s on day 28 after immunization (personal communication). The ELISA data suggest that

1ED3 is highly sero-cross-reactive recognizing both 2ED3 and 3ED3, in addition to its sero-specific recognition of 1ED3. Thus, it produced the highest antibody responses (highest antibody titers) in total (Figure 3.18). Therefore, the highest memory T cell responses data found during CD marker analysis fully corroborated with the anti-1ED3 IgG responses in mice model. Anti-2ED3 sera mostly recognized 2ED3 with slight cross-recognition of 3ED3 and anti-3ED3 sera solely recognized 3ED3 (Figure 3.18). However, Anti-2ED3 sera had somewhat lower titers than anti-1ED3 sera, but very slightly higher titers than anti-3ED3 sera (Figure 3.18). Otherwise, anti-4ED3 sera was mostly 4ED3-specific without cross-recognizing other ED3s and anti-4ED3 antibody titers were the lowest (Figure 3.18). Accordingly, the 4ED3 also generated the lowest number of T cell memory in mice model (Figure 3.17a and Figure 3.17b). Interestingly, the vigor of T cell memory responses (number of CD44+CD62L+ T cells) also followed the same order, 1ED3>2ED3>3ED3>4ED3. Furthermore, a very high correlation of antibody responses i.e. anti-DENV ED3 IgG titers versus the numbers of CD44+ Th cells (R^2 value 0.87 and 0.95 on 14 and 21 days respectively) and CD44+CD62L+ Th cells (R^2 value 0.93 and 0.98 on 14 and 21 days respectively) (Figure 3.20a and Figure 3.20b), clearly suggested that all four ED3s may produce DENV-specific ED3s long-lasting antibody responses with reasonable immunological memory. The antibody responses along with T cell memory functions generated following ED3 immunization in mice model clearly claimed that 1ED3 has the highest immunogenicity, both from the production of antibody responses and memory functions of effector T cells. Therefore, both the cellular and humoral immunity against DENV1 might be long-lasting. The DENV2 ED3 and DENV3 ED3 were of very similar immunogenicity, both from generation of ED3-specific IgG and memory T-cell viewpoints. Otherwise, the immunogenicity of 4ED3 seemed strange, both from cellular and humoral responses perspectives. Such heterogeneous immunogenicity of DENV1-4 ED3s might be very similar to what have been reported with natural DENV infections as well [283-284] and may

be contributing to the very limited success for most of the dengue vaccines [285,286]. At this end we believe that this study will help understanding immune responses against individual DENVs and may open the door for formulating a four ED3-based vaccine candidate with equal/similar immunogenicity of all four DENVs in future.

Table 3.3: Percentage of CD44+ and CD44+CD62L+ Tc and Th cells generated against all DENV wild-type ED3s and control mice (injecting with PBS only) on day 14 and day 21 of immunization.

Cells	CD44+ T cells				CD44+CD62L+ T cells			
T cells	Tc		Th		Tc		Th	
Day after immunization	Day 14	Day 21	Day 14	Day 21	Day 14	Day 21	Day14	Day 21
1ED3	34.57	35.45	59.49	48.32	33.33	28.62	47.18	41.59
2ED3	34.62	33.09	46.18	43.36	29.49	26.05	39.12	33.78
3ED3	25.52	22.48	30.17	33.93	21.78	16.41	28.21	25.85
4ED3	17.19	16.26	23.48	18.09	15.11	13.37	19.68	13.6
Control	16.35	6.7	15.73	9.04	11.25	6.45	12.31	7.29

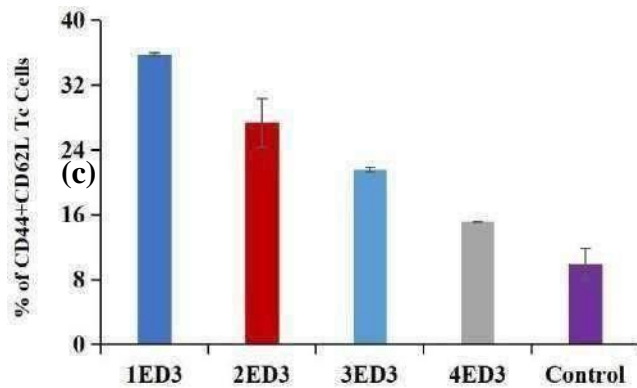
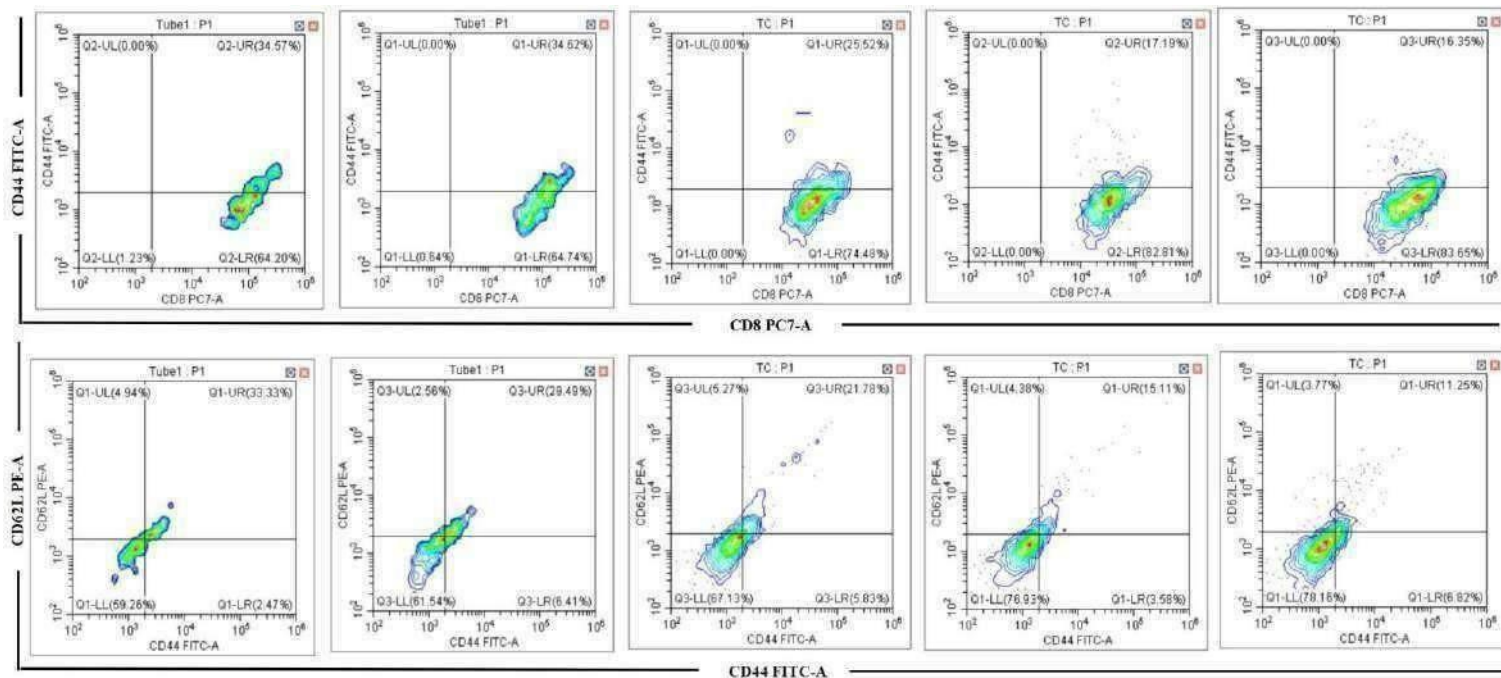


Figure 3.13: Generation of memory Tc cell ((a) CD44+, (b) & (c) CD44+CD62L+) in DENV1-4 ED3 injected mice on day 14 after immunization. (a) 1ED3 and 2ED3 produced highest number of CD44+ Tc cell followed by 3ED3 and 4ED3 compared to control mice (injected with PBS only). (b) & (c) 1ED3 produced highest number of CD44+CD62L+ Tc cell followed by 2ED3, 3ED3 and minimal response by 4ED3.

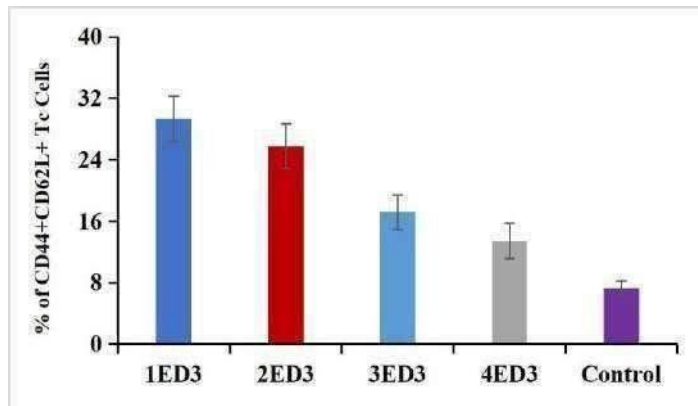
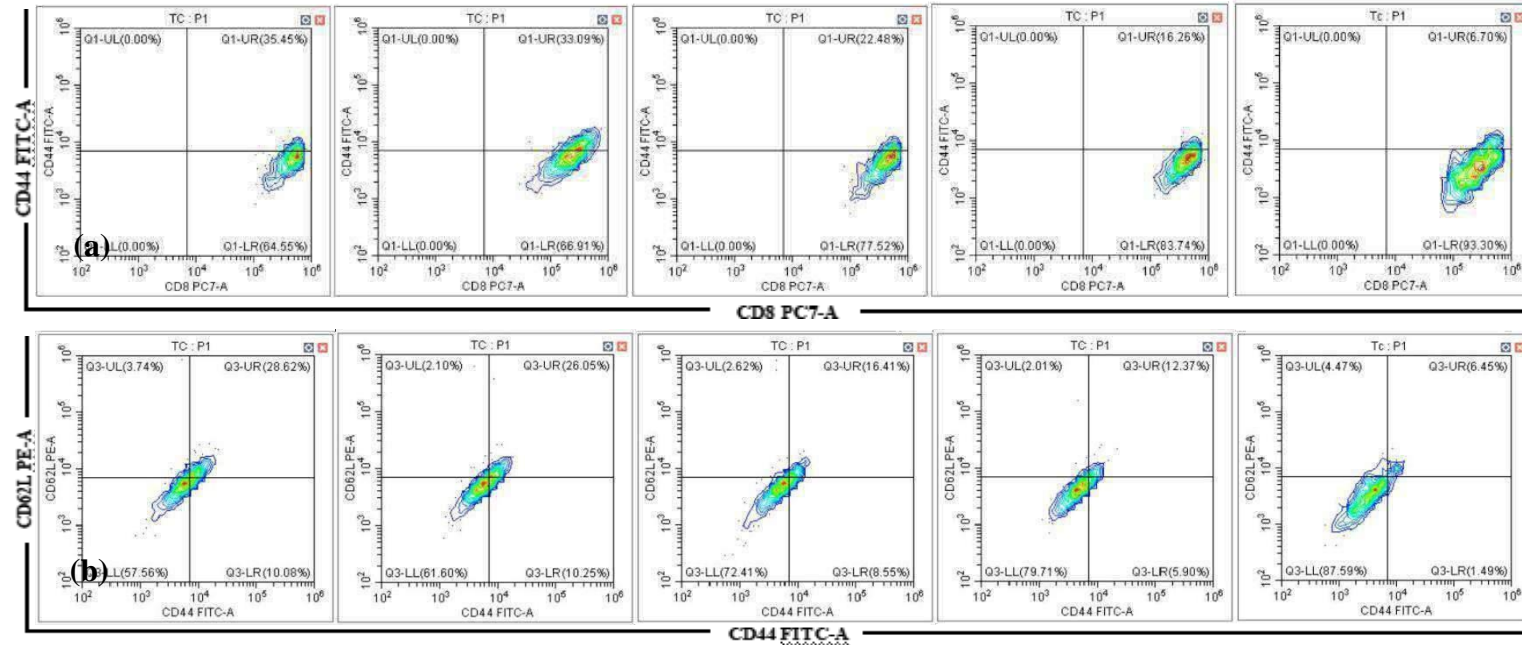


Figure 3.14: Generation of memory Tc cell ((a) CD44+, (b) & (c) CD44+CD62L+) in DENV1-4 ED3 injected mice on day 21 after immunization. (a) 1ED3 produced highest number of CD44+ Tc cell followed by 2ED3, 3ED3 and 4ED3 compared to control mice. (b) & (c) 1ED3 produced highest number of CD44+CD62L+ Tc cell as well followed by 2ED3, 3ED3 and 4ED3.

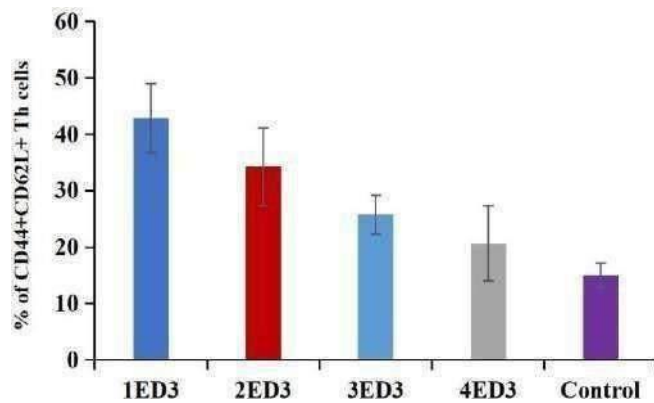
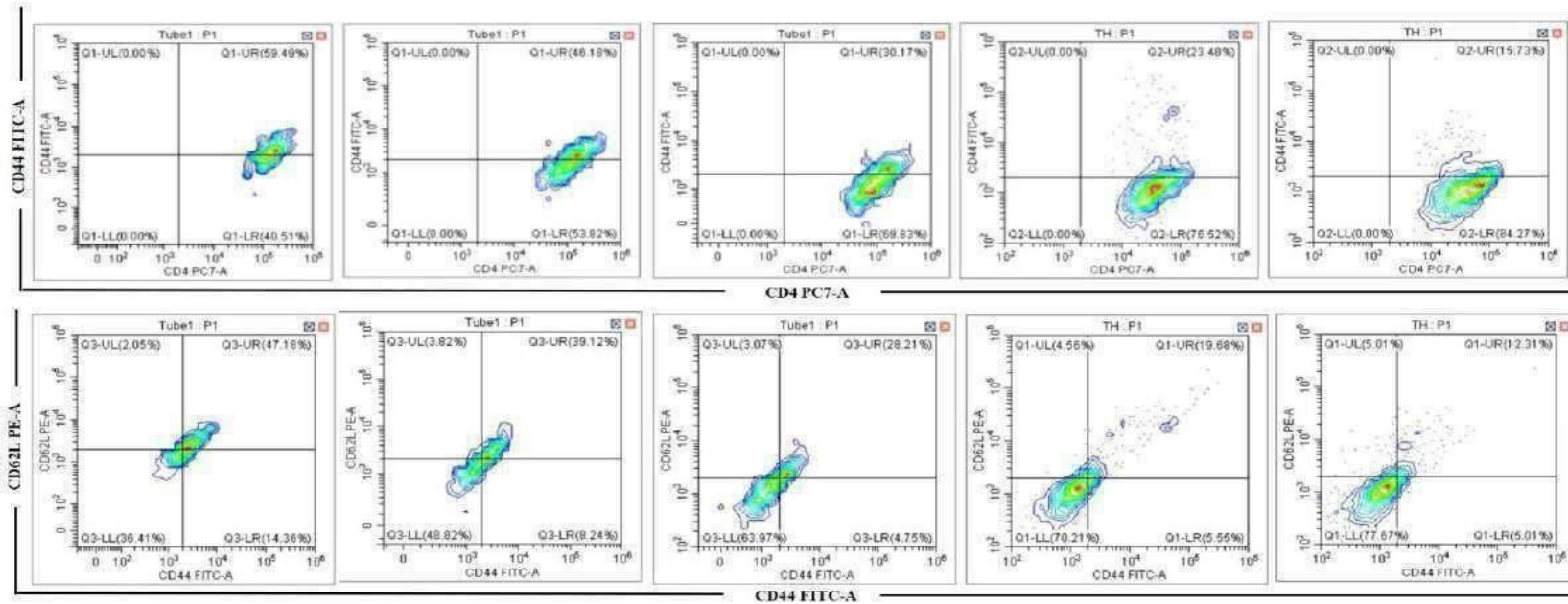


Figure 3.15: Generation of memory Th cell ((a) CD44+, (b) & (c) CD44+CD62L+) in DENV1-4 ED3 injected mice on day 14 after immunization. (a) 1ED3 produced highest number of CD44+ Th cell followed by 2ED3, 3ED3 and 4ED3 compared to control mice. (b) & (c) 1ED3 produced highest number of CD44+CD62L+ Th cell as well followed by 2ED3, 3ED3 and 4ED3.

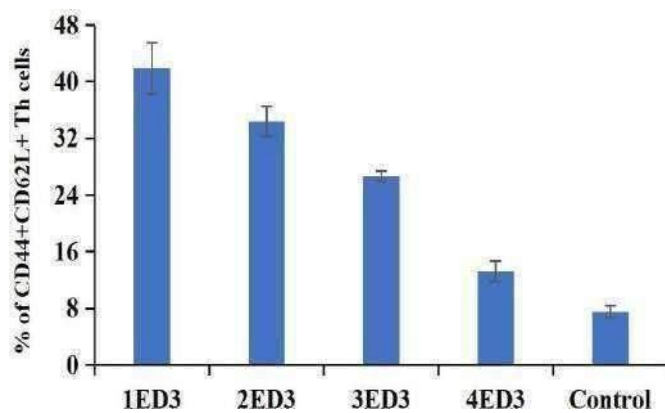
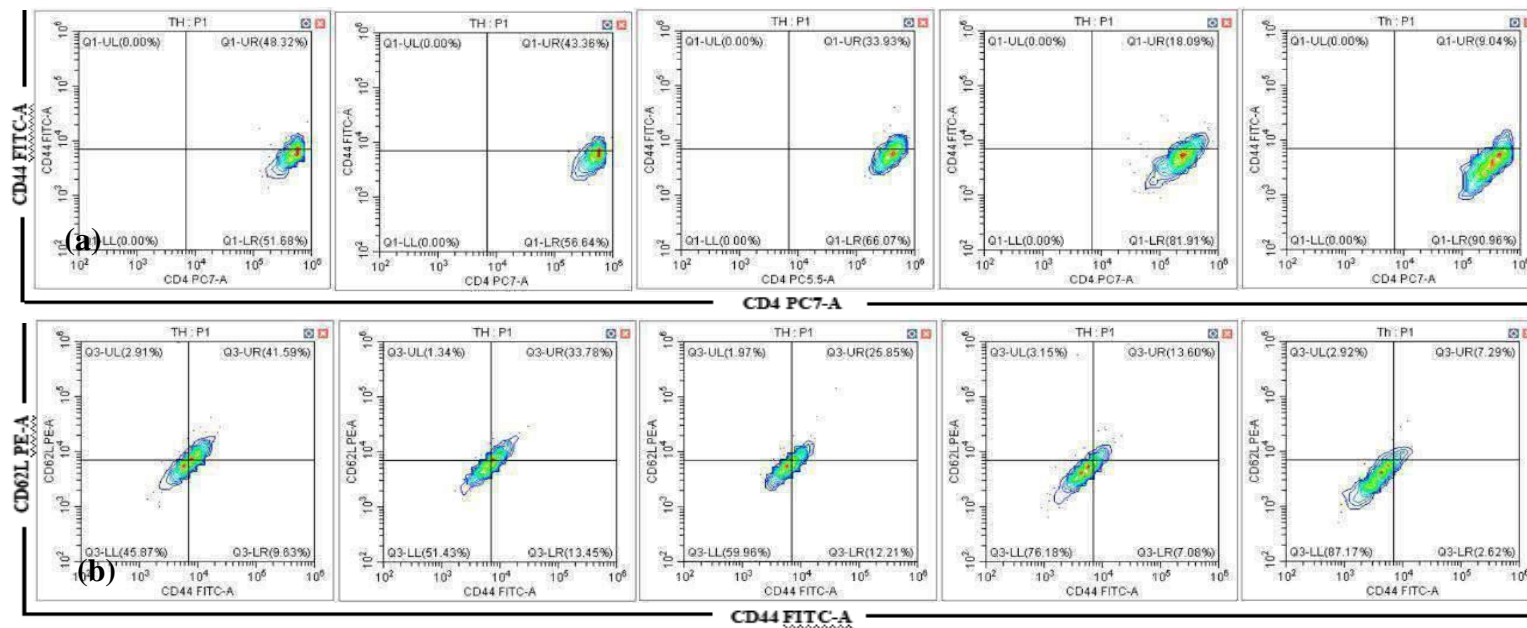


Figure 3.16: Generation of memory Th cell ((a) CD44+, (b) & (c) CD44+CD62L+) in DENV1-4 ED3 injected mice on day 21 after immunization. (a) 1ED3 produced highest number of CD44+ Th cell followed by 2ED3, 3ED3 and 4ED3 compared to control mice. (b) & (c) 1ED3 produced highest number of CD44+CD62L+ Th cell as well followed by 2ED3, 3ED3 and 4ED3.

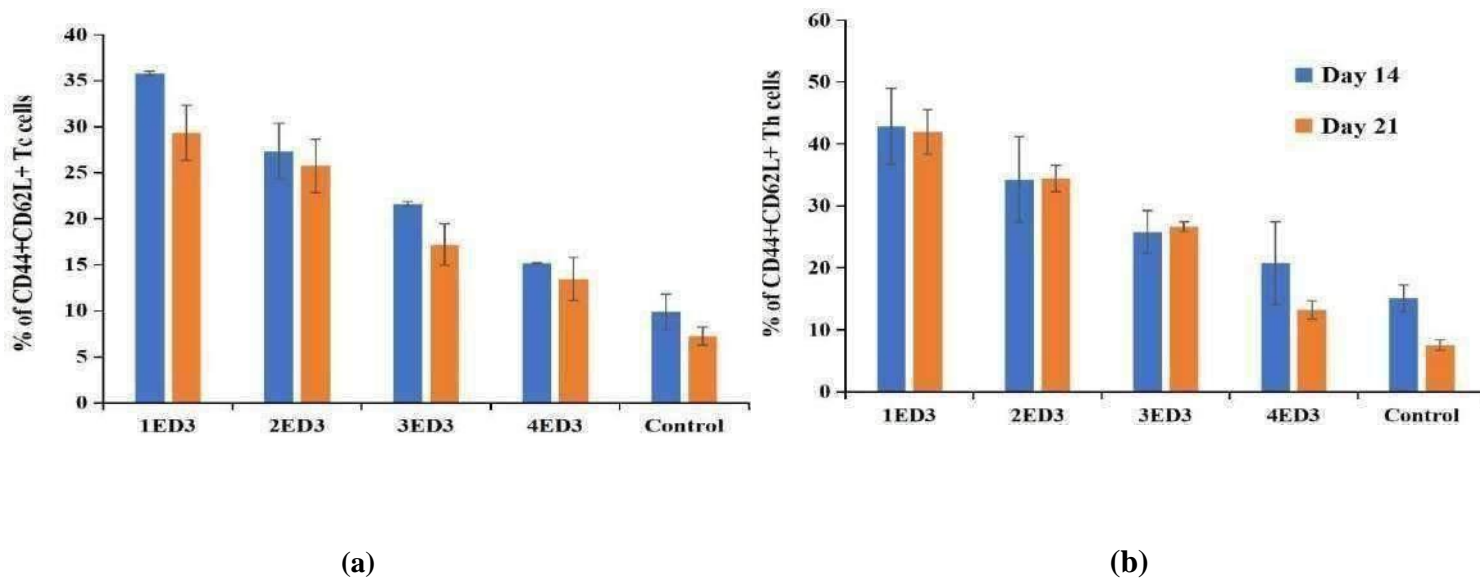


Figure 3.17: Generation of CD44+CD62L+ (a) Tc cells, and (b) Th cells against DENV1-4 ED3s on day 14 and day 21 of immunization. (a) The 1ED3 highest number of memory Tc cells (CD44+CD62L+ Tc cells) followed by 2ED3, 3ED3, and 4ED3 on both day 14 and 21. (b) The 1ED3 also generated highest number of CD44+CD62L+ Th cells and a similar response were observed from 2ED3 and 3ED3, although slightly low. Again, the 4ED3 produced minimal CD44+CD62L+ Th cells on both day 14 and day 21

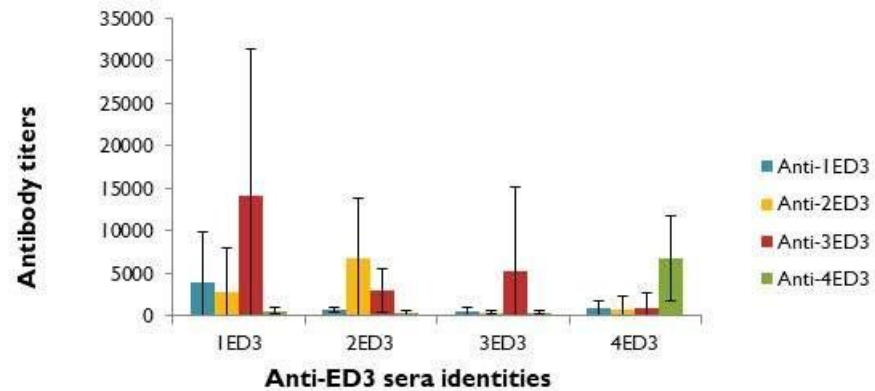


Figure 3.18: The anti-dengue antibody response in DENV1-4 ED3 injected mice. The 1ED3 had highest sero-cross-reactivity generating antibody against 2ED3 and 3ED3 along with 1ED3. Thus, 1ED3 produced highest antibody response in total. 2ED3 showed sero-cross-reactivity and 3ED3 producing antibody more specific. But still 2ED3 produced a little bit more antibody than 3ED3 in total number. The 4ED3 showed no cross-reactivity generating antibody only for itself and thereby minimal in total number. The antibody response fully matches with the memory T cell number.

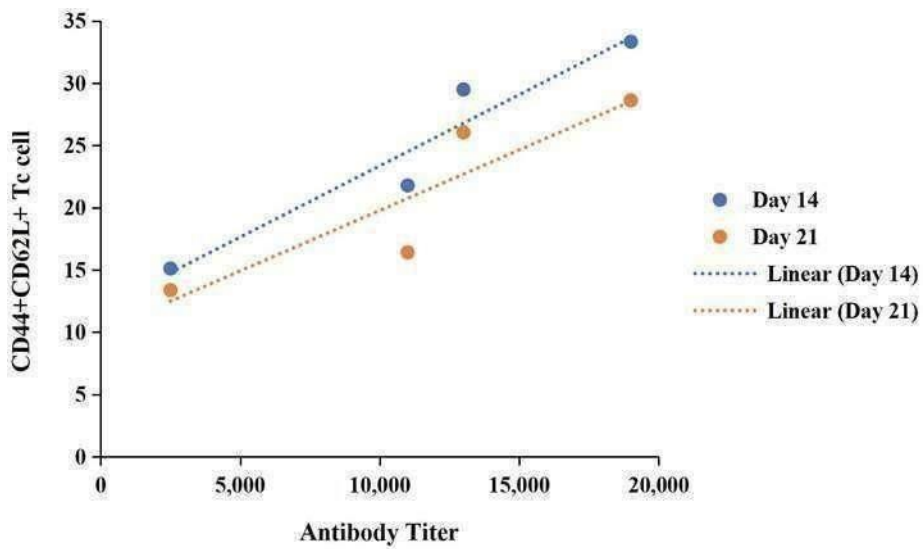
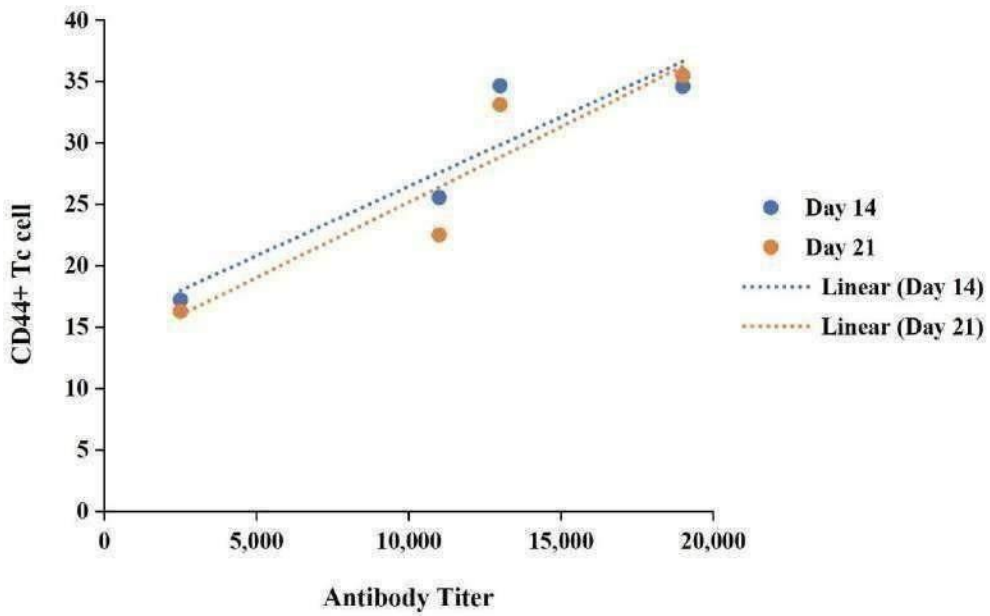


Figure 3.19: Correlation of antibody responses (anti-DENV ED3 IgG titers) versus the numbers of (a) CD44+ Tc cells and (b) CD44+CD62L+ Tc cells on day 14 and day 21.

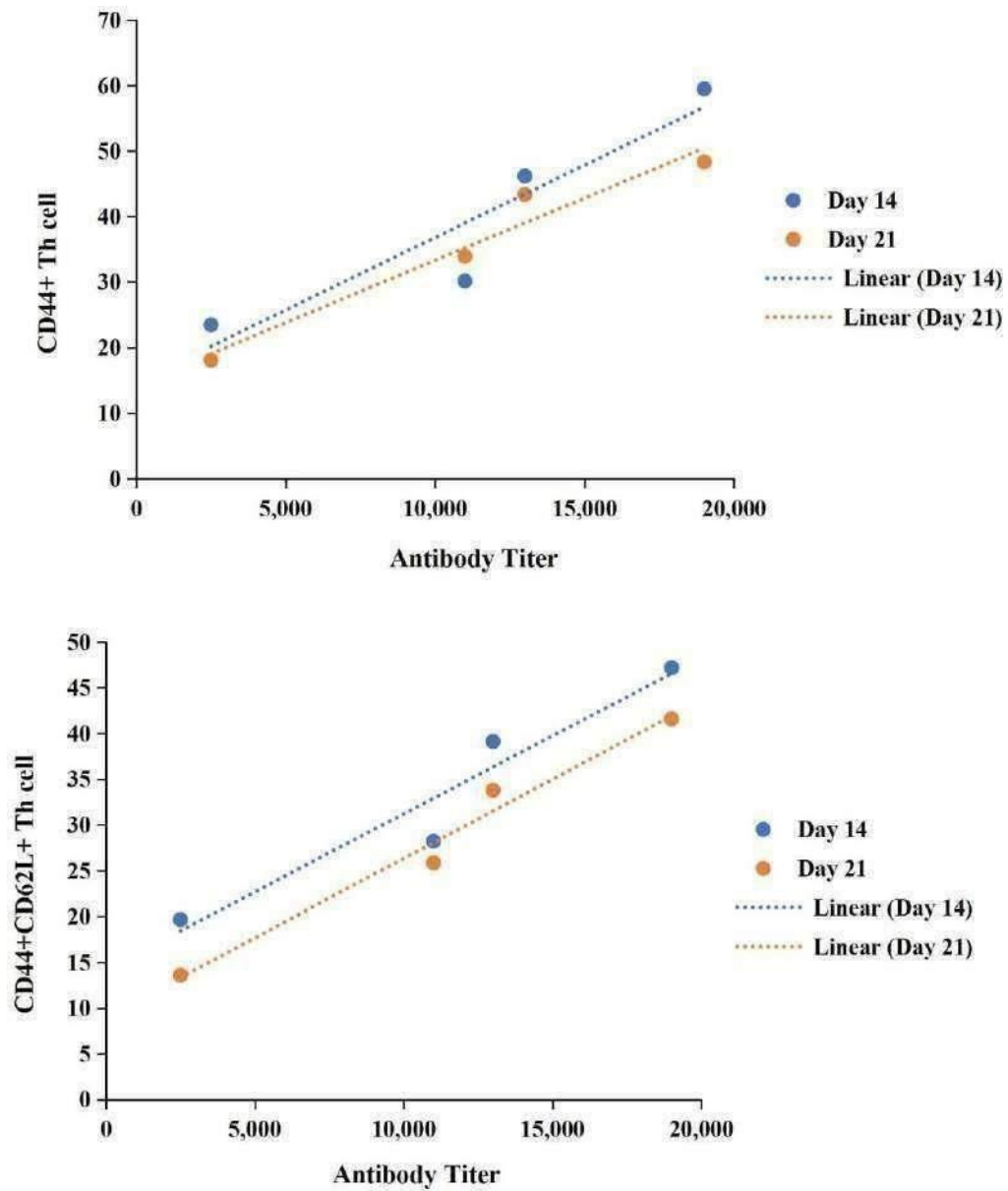
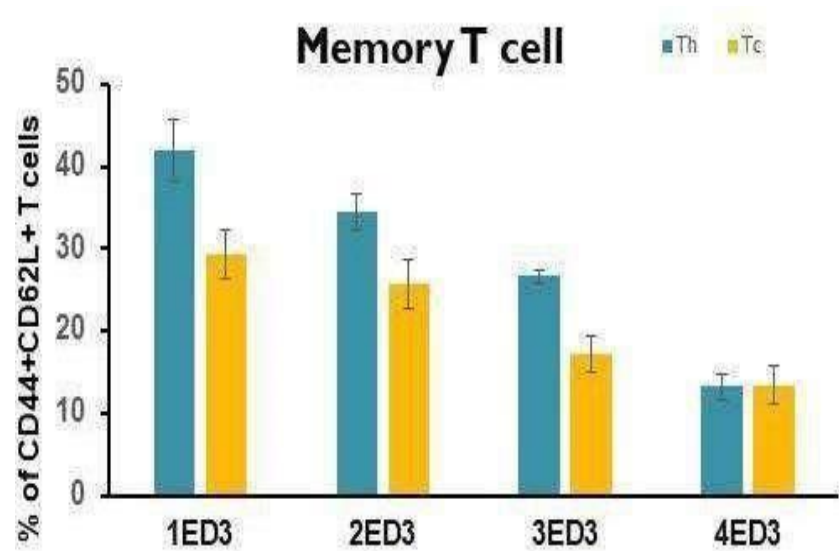
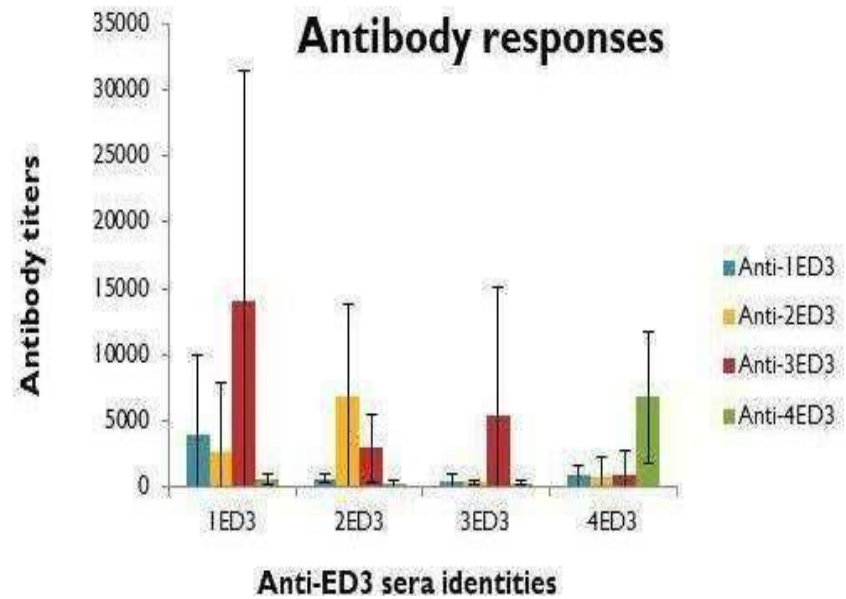


Figure 3.20: Correlation of antibody responses (anti-DENV ED3 IgG titers) versus the numbers of (a) CD44+ Th cells and (b) CD44+CD62L+ Th cells on day 14 and day 21.



(a)



(b)

Figure 3.21: Correlation between memory cell and antibody responses in mice model a) Memory T cell b) Antibody responses.

3.24 Correlation between memory cell and antibody responses

DENV1 ED3 (1ED3) was the most immunogenic[3.21a]; Anti-1ED3 sera was sero-cross-reactive; as observed in natural dengue infections; , 1ED3 produced highest antibody response in total [Figure 3.21b] 2ED3 showed sero-cross-reactivity to each other producing antibody against both 2ED3 and 3ED3. 3ED3 and 4ED3 showed more sero-specific. But still 2ED3 produced a little bit more antibody than 3ED3 in total number which are similar immunogenicity. 4ED3 was the least immunogenic; the 4ED3 showed no cross-reactivity generating antibody only for itself and thereby minimal in total number [Figure 3.21a]. Heterogeneous immunogenicity of four different DENV ED3s must be in consideration in future DENV vaccine design.

3.25 Different Memory responses

These observations unfolded that regardless of being fragmented into a single domain and synthesized artificially as a recombinant protein in *E. coli*, the ED3 domain could generate ED3-specific memory Tc and Th cells (Table 3.3). Very recent studies showed that four different DENV ED3s are different from the vigor of anti-DENV ED3 IgG responses they generated in mice model (personal communication), i.e. 1ED3 the most immunogenic, then comes 2ED3, 3ED3 and 4ED3 (Figure 3.18). In this study, we showed that 1ED3 generated the highest memory T cells (both Tc cells and Th cell) with highest numbers of CD44+ T cells and CD44+CD62L+ T cells, the next are 2ED3, 3ED3 and 4ED3. Furthermore, a very high correlation of antibody responses (anti-DENV ED3 IgG titers) versus the numbers of CD44+ Th cells and CD44+CD62L+ Th cells (Figure 3.20a and Figure 3.20b), both on day 14 and day 21 clearly suggested that all four ED3s could generate DENV ED3-specific long-lasting antibody responses with reasonable immunological memory. However, the vigor and quality of humoral (IgG antibodies; CD44+/CD44+CD62L+ Th cell) and cellular immunity

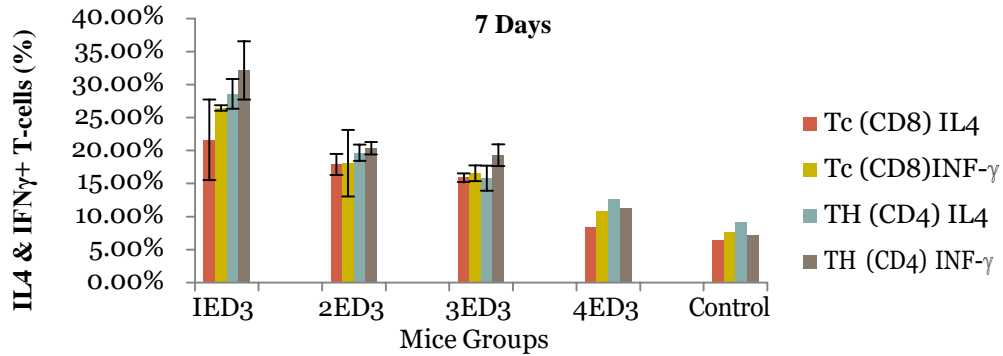
(CD44+/CD44+CD62L+ Tc cell) were different for different DENV ED3s (Figure 3.19a, Figure 3.19b, Figure 3.20a, and Figure 3.20b).

However, it is evident that all four ED3s could generate both effector and central memory following immunization in mice and suggested that a ED3-based tetravalent vaccine formulation with equal/similar anti-ED3 IgG titers and similar T cell memory for all four ED3s may pave a way combating recurrent dengue infections in near future.

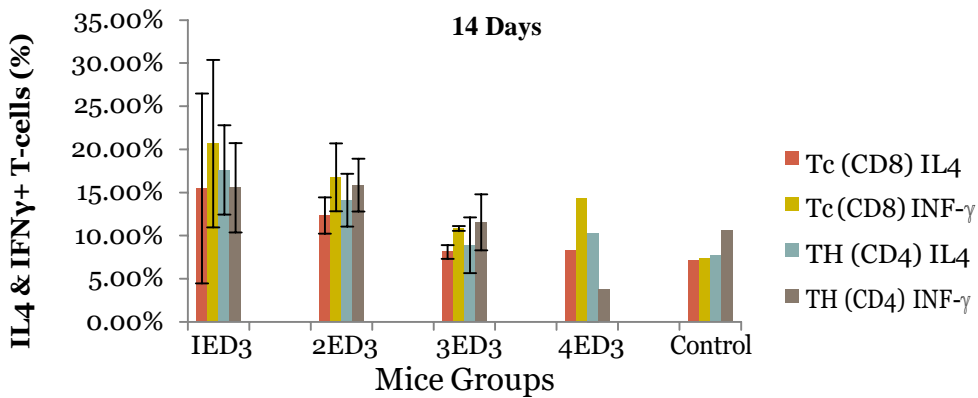
3.26 Anti-dengue cytokine responses against four different DENVs in mice model

We investigated the generation IL-4 & INF- γ response by injecting all four wild-type dengue ED3s in the **Swiss Albino mice** model. For artificial immunization 3 groups of mice were taken. Each group contained 5 mice. 1st mouse was immunized with 1ED3, 2nd mouse with 2ED3, 3rd mouse with 3ED3, 4th mouse with 4ED3 respectively, 1 mouse was taken also which are not immunized. This experiment monitoring IL-4 & INF- γ responses at weekly intervals like 7th, 14th 21st days. We performed IL-4 & INF- γ marker analysis in mice blood cell by using CytoFLEX flow cytometer and analyzed all data in CytExpert software without any compensation.

a)



b)



c)

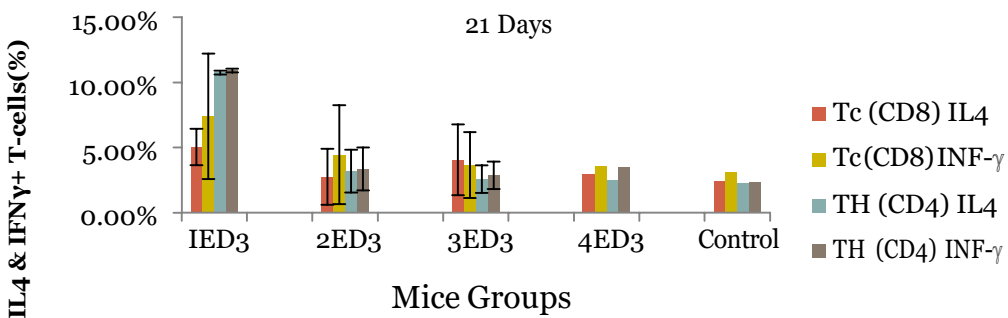


Figure 3.22 : The order of IL4 & INF- γ expression a) It is observed that 1ED3 has IL4 & INF- γ expression 38% in day 7, b) 30% in day 14 C) 11% in day 21. So It is clear that day by day this expression is decreasing.

3.27 Generation of IL-4 & INF γ responses against individual ED3s

To investigate anti-ED3 memory responses against injected DENV-specific ED3s, we have monitored the level of IL-4 & INF- γ markers on T-lymphocytes on 14th and 21st day after immunization. First, immunization using DENV1-4 ED3s produced both Tc cell and Th cell memory response. However, the memory T-cell response against DENV1 ED3 (1ED3) was the highest, followed by DENV2 ED3 (2ED3), DENV3 ED3 (3ED3) and the minimal response against DENV4 ED3 (4ED3) [278]. Interestingly, all DENV IL-4 & INF- γ responses on day 7 interleukin secretion is very high 38%, follow by 14 days 30%, 21 days 11% in 1ED3 responses gradually decreases Interleukin responses day by day (Figure:3.22).

Differential expression CD markers and cytokines (CD44, CD62L, IFN- γ , and IL-4) following ED3 immunization indicated that 1ED3 resulted the highest percentage of effective and central T-cell memory followed by 2ED3, 3ED3 and 4ED3, fully in line with their vigor of antibody responses. These observations clearly suggested that while sharing a great deal of the same sequences and structures, the immunogenicity and epitope configuration of all four ED3s are different. Therefore, a rational designing including differential immunogenicity of individual ED3s in tetravalent dengue vaccine formulation and epitope configuration of all ED3s conferring sero-specificity and/or sero-cross-recognition of all for ED3s is worth considering for developing potent ED3-based dengue vaccine.

3.28 Effects of individual ED3s on differential expression of CD marker and cytokines

In order to assess the outcomes of ED3 immunization, we examined the intracellular

expression of IL4 and IFN- γ by circulating T cells at days 7, 14, and 21 after the initial dose of immunization. We also looked at the surface CD markers CD3, CD4, CD8, CD44, and CD62L. Following 1ED3, which also produced the highest numbers of CD44+ and CD44+CD62L+ T-cells (both Tc and Th-cells), 2ED3, 3ED3, and 4ED3 immunization produced the highest levels of IL-4 and IFN- γ (Figure 3.23), which is fully supported by the corresponding anti-ED3 IgG titers. The number of memory T-cells and the level of cytokines steadily decreased over the course of many weeks. The formation of both efficient humoral and cellular immunity was clearly indicated by the high levels of CD44+CD62L+ T-cells, IL-4, and IFN- γ , which were highest against 1ED3, followed by 2ED3, 3ED3, and 4ED3 [274]. (Figure 3.23). Similar to what had been shown in the control mice, the 4ED3 had the fewest or smallest number of CD44+ and CD62L+ Tc and Th cells, as well as the least quantity of IL-4 and INF- (Figure 3.23).

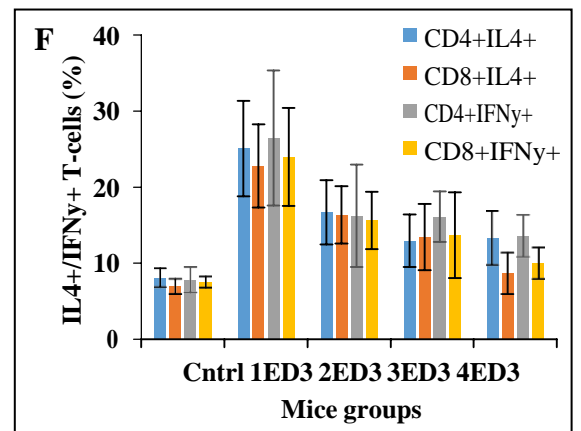
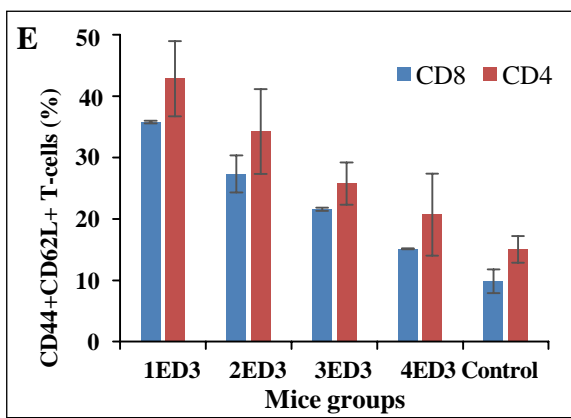
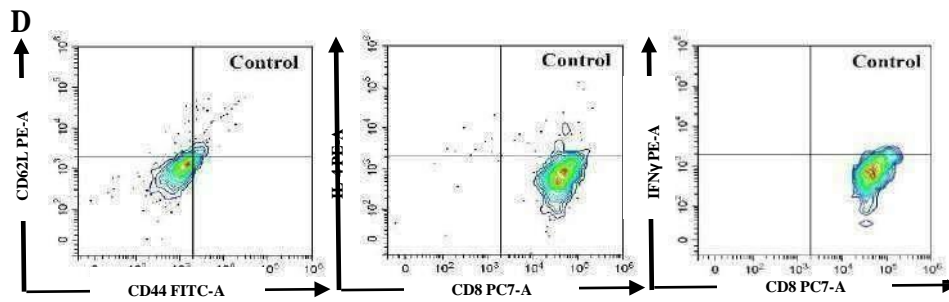
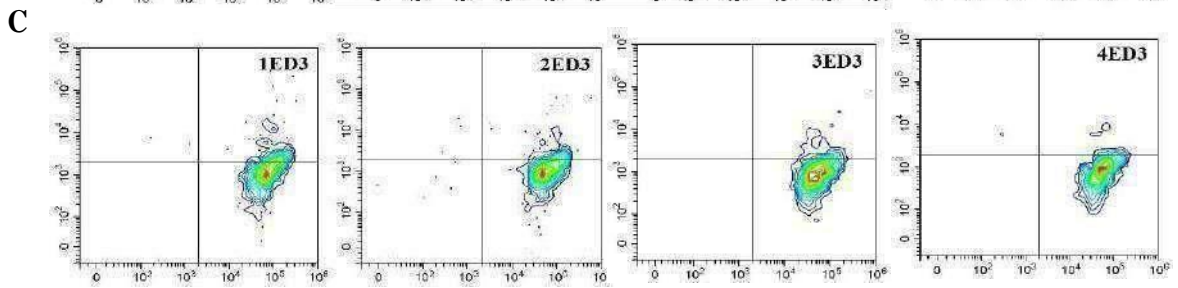
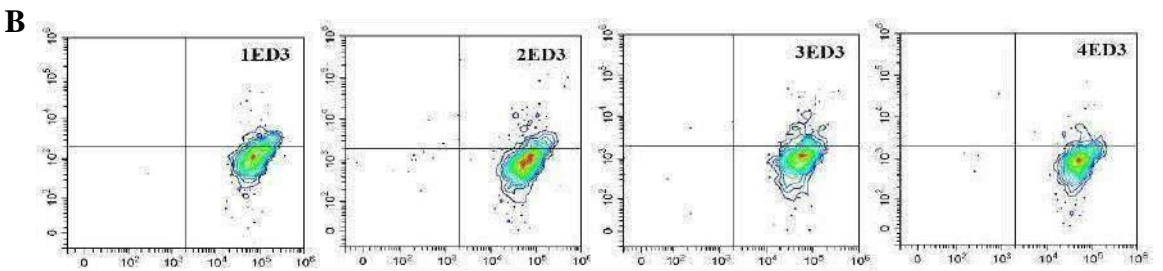
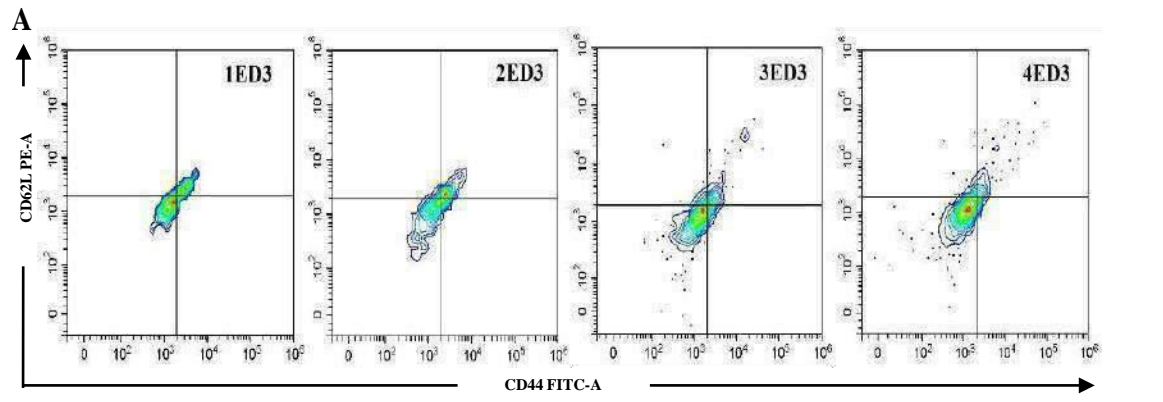


Figure 3.23: Effects of ED3 immunization on T-cell memory status in mice.

The co-expression of CD44 and CD62L (A), IL-4 (B), and IFN- γ (C) by Tc-cells are displayed. (D) Expression of CD44/CD62L, IL-4, and IFN in control mice that have not been inoculated. Panels E and F, respectively, depict the relative proportions of CD44+CD62L+ T-cells and T-cells producing IL-4 and IFN- γ in ED3-immunized mice. Following 1ED3 immunization, 2ED3, 3ED3, and 4ED3 immunization showed the highest co-expression of CD44+CD62L+ on T-cells and the highest intracellular expression of IL-4 and IFN- γ by T-cells. Results indicated that DENV4 ED3 was the least immunogenic and 1ED3 the most immunogenic.

3.29 Differential immunogenicity

All DENV1-4 ED3s may maintain the distinct DENV serospecificity as seen in the production of ED3-specific IgG antibodies in mice models, despite having a great deal of sequence and structural similarity and being produced as a single, broken-down domain of the whole E-protein. According to both the levels of T-cell memory produced after ED3 immunization and the ED3-specific IgG titers, 1ED3 was highly immunogenic, 2ED3 and 3ED3 were similar and moderately immunogenic, and 4ED3 was the least immunogenic. Understanding the sero-cross-talk of anti-DENV antibodies in research using artificial immunization and actual dengue infections is further complicated by the puzzling sero-cross-recognition of 1ED3-2ED3-3ED3 by anti-1ED3 sera but not by anti-2ED3 and anti-3ED3 sera. Due to varying immunogenicity and mysterious sero-cross-talks of anti-DENV antibodies, tetravalent dengue vaccinations may not be as successful as they may be. For the first time, DENV1 is described as the most mysterious DENV serotype, both from producing serospecific and sero-cross-reactive anti-DENV antibody responses. While DENV4 has long been regarded as a "black ship" in the DENV family. Therefore, we suggest that future tetravalent dengue vaccine formulation must consider the sero-cross-talks anti-dengue antibody in tetravalent composition as well as the antigenicity of various DENVs.

Objectives 4 : Establishing the correlation (if there is any) between anti-DENV responses in natural DENV infection and anti-ED3 responses in mice model and proposing a model system for future research.

3.30 Correlation between outcome of natural and artificial infections

Among 112 human samples, 42 DENV positive samples were investigated for antibody by ELISA IgG and IgM. These types of antibodies were compared with mice concurrent artificial infection by ELISA IgG. From the observations, it is noted that if a patient is infected with DENV1, highly 1ED3-2ED3-3ED3 cross-reacting anti-ED3 IgM & IgG antibodies are generated; which is very similar in mice study [Figure 3.24]. Anti-2ED3 sera was mostly serotype-specific with slight cross-recognition of 3ED3 in mice and DENV2 in natural infection mostly sero-cross reactive. Anti 3ED3 sera mostly serotype-specific but DENV3 in natural infection mostly sero-cross reactive. Anti-4ED sera was mostly serotype specific in artificial infection which is in contrast in natural dengue infection [Figure 3.24]. Patients infected with DENV1,4 and DENV1,3 generated antibodies against all DENV serotypes, which is very similar in mice study. [Figure 3.25]. Such as mice concurrently bivalent immunized with 1ED3-4ED3 results in cross reactive with 1ED3-2ED3-3ED3-4ED3, so it is observed that in both cases, results of DENV1, 4 and 1ED3-4ED3 infections are similar. 4ED3 remained very poorly immunogenic with any combinations of 2ED3 and 3ED3 in mice study. 4ED3 with 1ED3 caused increase of immunogenicity, such adjuvanting effects of 1ED3 and 4ED3 immunogenicity is a new observation in this study. Applying this concept, future Dengue model system can be developed. However only 3 patients were available with DENV1,4 infection and 5 patients with DENV1,3 infection. If more human samples would have been available, the concept for development of Dengue model system would be stronger [Figure 3.25]. Mice trivalent infection was mostly serotype-specific but natural study was mostly sero cross-reactive [Figure 3.26]. This observation may be due to the fact that the study on mice was in fixed/controlled environment. We have no idea whether humans have been affected by any other dengue virus before. Only one patient was available who was affected with trivalent infection DENV1, 3, 4 in 112 dengue infected patients during the dengue outbreak in Chittagong Bangladesh 2019 [Figure 3.26].

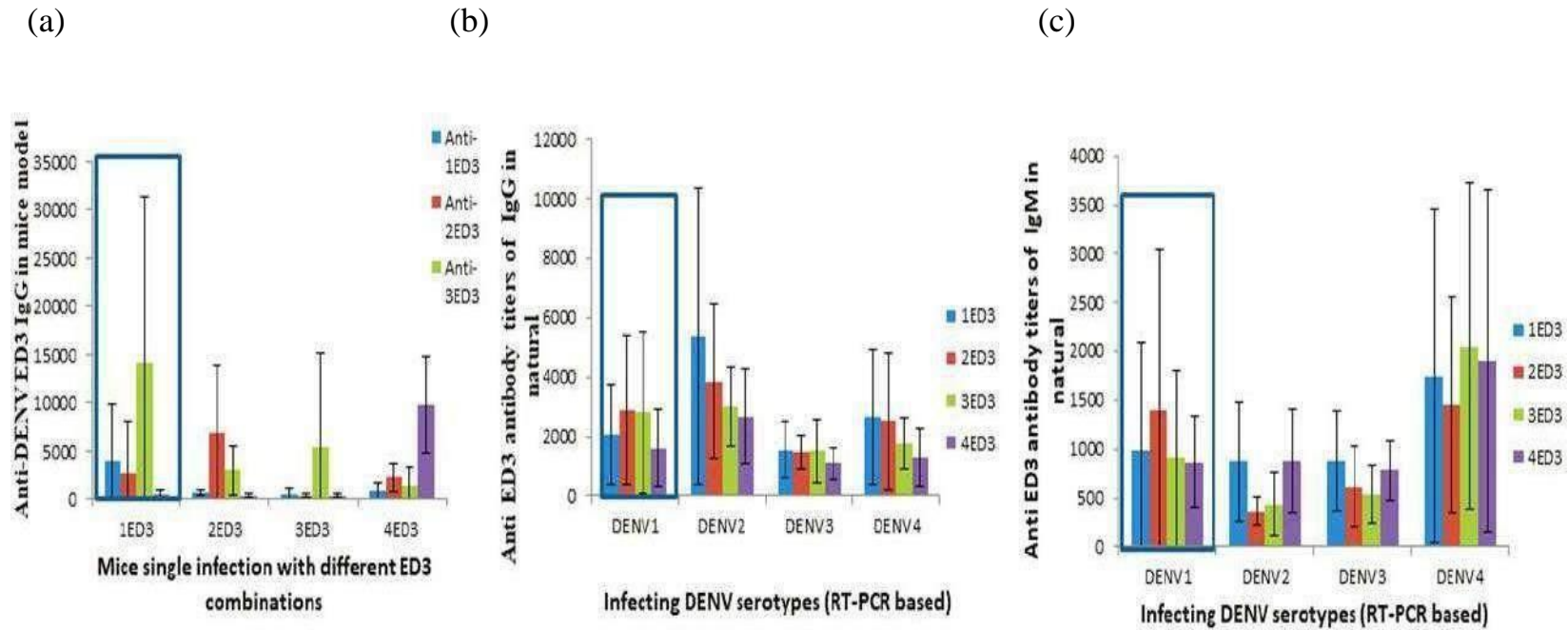
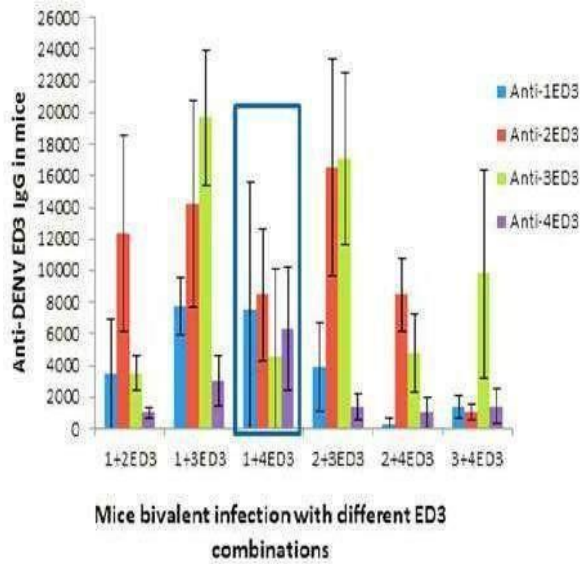
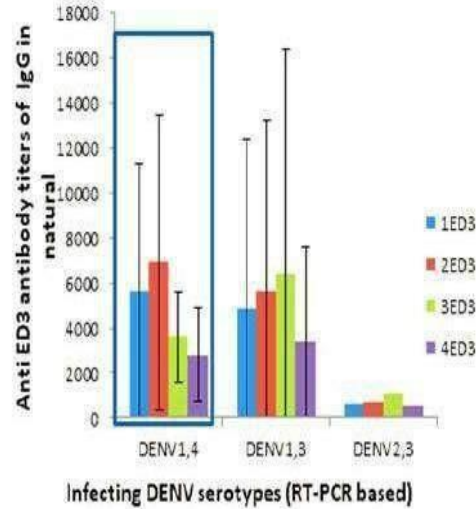


Figure 3.24: Correlation between artificial and natural DENV infections with single combination (a). Mice single infection with different ED3 combinations (b) Anti ED3 antibody titers of IgG in single natural infection (c) Anti ED3 antibody titers of IgM in single natural infection.

(a)



(b)



(c)

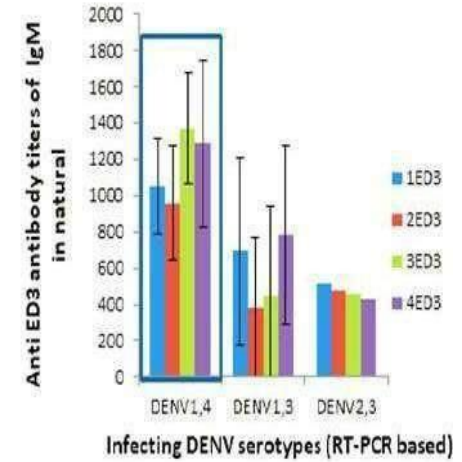
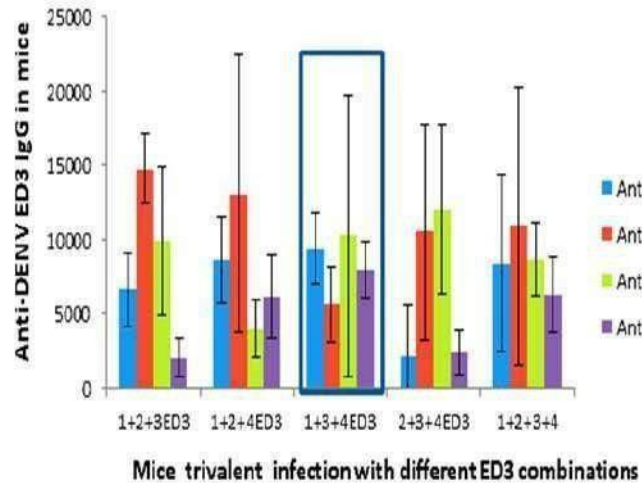
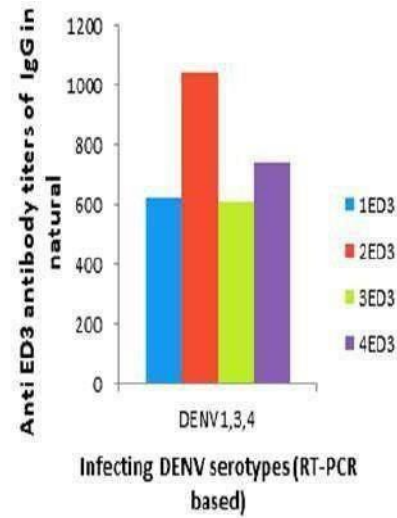


Figure 3.25: Correlation between artificial and natural DENV infections with bivalent combination: (a) Mice bivalent infection with different ED3 combinations. (b) Anti ED3 antibody titers of IgG in bivalent natural infection (c) Anti ED3 antibody titers of IgM in bivalent natural infection.

(a)



(b)



(c)

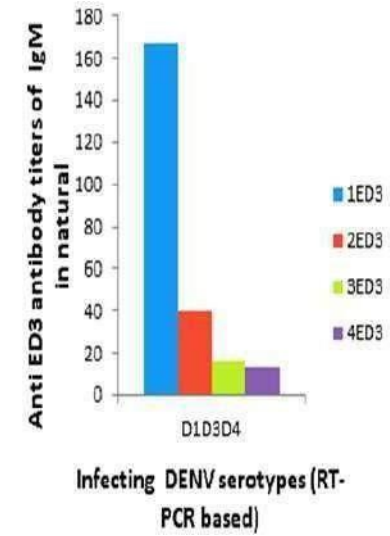


Figure 3.26: Correlation between artificial and natural DENV infections with trivalent combination : (a) Mice trivalent infection with different ED3 combinations (b) Anti ED3 antibody titers of IgG in trivalent natural infection (c) Anti ED3 antibody titers of IgM in trivalent natural infection.

Chapter 04

Conclusions



04: Conclusion

During the 2019 dengue outbreak, all four DENV serotypes circulated simultaneously, according to DENV serotyping results in Chittagong, Bangladesh. Moreover, multiple DENV infections occurred concurrently in 24% of the afflicted populations. Contrary to what has been reported, this may be the first instance in Bangladesh where we identify most prevalent serotype DENV4. In several concurrent DENV infections in the past, DENV1 was the serotype that was most common (present in 90% of instances of multiple DENV infections). Our findings strongly suggested that DENV seroprevalence can vary yearly based on geolocations. The recent outbreak of severe dengue in Bangladesh may have been brought on by the high frequency of several concurrent DENV infections. It became abundantly clear from the clinical signs, symptoms, and blood counts on 112 samples that the most fetal DENV serotype may be DENV3, when acts as a single serotype and present in the multiple infection at the same time and cause plasma leakage thrombocytopenia, dengue shock syndrome and dengue hemorrhagic fever. Future studies should consider including additional samples in order to better understand the causes of the dengue illness's intensity and clinical appearance.

The artificial immunization study clearly indicated that 1ED3 (ED3 of DENV1) generated 1ED3-2ED3- 3ED3 sero-cross- reactive antibody responses, while the 4ED3 generated only 4ED3-specific anti-4ED3 IgG response. 4ED3 is solely sero-specific when mice are first immunized with 4ED3 in following heterotypic secondary ED3 immunization. Moreover, Sero-cross-reactive 1ED3, 2ED3, and 3ED3 are with low cross-recognition of 4ED3. It is clear that 4ED3 sequence is at the distal from other ED3s. A similar phenomenon also appeared in natural DENV infections. In concurrent study, when the ED3 combination formulation had 1ED3 and 4ED3 together, then 4ED3 became immunogenic.

In this study, we also attempted to make correlation of memory cell (CD44 +Tc cell, CD62L+Tc cell and CD44 +Th cell, CD62L+Th cell) with cytokine responses (IL4 + Tc cell, INF- γ Tc cell and IL4+ Th cell, IFN- γ Th cell) by Flow Cytometry against antibody responses in mice. This study concluded that, DENV1 ED3 (1ED3) was the most immunogenic; Anti-1ED3 sera was sero-cross-reactive; as observed in natural dengue infections; 1ED3 produced highest antibody response in total. 2ED3 revealed sero-cross-reactivity with 2ED3 and 3ED3. Still 2ED3 produced a little bit more antibody than 3ED3 in total number which are of similar immunogenicity. 4ED3 was the least immunogenic. The 4ED3 showed no cross-reactivity generating antibody only for itself and thereby minimal in total number. Heterogeneous immunogenicity of four different DENV ED3s must be in consideration in future DENV vaccine design.

Correlation between outcome of natural and artificial infection have also been evaluated. From the observation, it can be concluded that in natural and artificial infection, DENV1,4 infection showed 1ED3-2ED3-3ED3-4ED3 sero-cross reactivity. Thus based on this concept, we can formulate a four ED3s-based vaccine candidate in future by using only 1ED3 and 4ED3. This concept helps to produce polyclonal antibodies in future.

Chapter 05

List of Publications

Publication based on thesis:

1. Moushumi Ghosh Roy, Kutub Uddin, Din Islam, Mohammad Monirul Islam, Anjuvan Singh -Secondary DENV infections along with concurrent multiple DENV serotypes in a single dengue session contributing to dengue severity in Chittagong Bangladesh, Journal of Survey in Fisheries Sciences, Page 01-13, 2023.
2. Moushumi Ghosh Roy, Anjuvan Singh and Mohammad Monirul Islam, Prevalence of dengue virus, their infection, diagnosis, and challenges, Journal of Applied Biology & Biotechnology, page 1-8, 2023.
3. Moushumi Ghosh Roy, Anjuvan Singh and Mohammad Monirul Islam, Prevalence of dengue virus, their infection, diagnosis, and challenges, Journal of Applied Biology & Biotechnology, page 1-8, 2023.
4. Md. Din Islam, Tahmina Sharmin, Imrul Hassan Tipo, Antara Saha, Sanjida Yesmin, Moushumi Ghosh Roy, Subbiam Brindha, Yutaka Kuroda, M. Monirul Islam, -The immunogenicity of DENV1-4 ED3s strongly Differ despite their almost identical three dimensional structures and high sequence similarities, International Journal of Molecular Sciences, page 08-11, 2023.

Paper presented at International/National Symposia/Conferences based on thesis:

I attended six conferences and presented my research works in different Conferences as mentioned below

- 1) Oral presentation on the topic -Secondary DENV infections along with concurrent multiple DENV serotypes in a single dengue session contributing to dengue severity in Chittagong, Bangladesh at the BSBMB-SABC-OMC International conference held on 03-05 February 2023 at Nabab Nawab Ali Chowdhury Senate Bhaban. University of Dhaka.
- 1) Oral presentation on the topic, -Serotype-specific clinical manifestations of dengue in Chittagong, Bangladesh at the International Conference on Biomacromolecules and Cellular Interface (ICBCI-2021) held virtually on 9-10 January 2021 at Dr. B.R Ambedkar National Institute of Technology, Jalandar-144011, Punjab, India.
- 3) Oral presentation on the topic, -2019 Dengue Outbreak in Chittagong: Seroprevalence and natural anti-dengue antibody responses at the Sixth Conference (Virtual) of Bangladesh Crystallographic Association held on 15-16 January 2021.
- 4) Poster presentation on the topic, -Prevalence of Dengue Virus, Their Infection, Diagnosis and Challenges at International Conference on Sustainability: Life on Earth 2021 (ICSLOE 2021) held on 17-18 December 2021 organized by Department of Botany and Zoology, School of Bio-engineering and Biosciences, and Institute of Forest Productivity, Ranchi, Jharkhand at Lovely Professional University, Punjab.
- 5) Poster presentation on the topic, -Anti-dengue antibody responses in concurrent dengue infections at the 1st research symposium organized by the Institutional Quality Assurance Cell (IQAC) of the University of Chittagong Bangladesh held on 24 March 2022.

- Ø Poster presentation on the topic, -Four Different DENV ED3s Generated Different T- cell Memory in Artificial Immunization in Mice Modell at the 1st Jamal Nazrul Islam National Conference for young researchers organized by University of Chittagong Bangladesh held on 21 May 2022.

Chapter 06

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