MOLECULAR UNDERSTANDING OF DENGUE VIRUS PATHOGENESIS AMONG EASTERN INDIAN PATIENTS AND DETERMINATION OF EFFECT OF QUERCETIN AGAINST DENGUE

Thesis submitted for the Degree of

Doctor of Philosophy (Ph.D.) (Science)

By

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"MOLECULAR UNDERSTANDING OF DENGUE VIRUS PATHOGENESIS AMONG EASTERN PATIENTS AND DETERMINATION OF EFFECT OF QUERCETIN AGAINST DENGUE"** submitted by **Sri Saikat Mukherjee** who got his name registered on **03.05.2016** for the award of Ph. D. (Science) Degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Anusri Tripathi** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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DECLARATION

I declare that the work in this thesis entitled **"MOLECULAR UNDERSTANDING OF DENGUE VIRUS PATHOGENESIS AMONG EASTERN PATIENTS AND DETERMINATION OF EFFECT OF QUERCETIN AGAINST DENGUE"** has been carried out by me in the Department of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine, under the supervision of **Dr. Anusri Tripathi.**

.....

Saikat Mukherjee

DEDICATION

I dedicate this thesis

To

My Beloved Parents

ACKNOWLEDGEMENTS

My work, the entirety of this thesis would not have been possible without the guidance and support of my guide and supervisor, **Dr. Anusri Tripathi**, Assistant Professor (Molecular Biology), Dept. of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine. I am and will be forever grateful and indebted to her for providing me the opportunity, and in turn guiding, motivating and mentoring me through all possible nooks and crannies of research work and manuscript or thesis writing. I offer my deepest gratitude to her and wish this guidance continues.

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Saikat Mukherjee

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LIST OF ABBREVIATIONS

µg	Micro gram
µl	Micro liter
Alb	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
Ang2	Angiopoietin2
ANOVA	Analysis of variance
ApoA1	Apolipoprotein A1;
APP	Acute phase proteins
APR	Acute phase response
AST	Aspartate aminotransferase
B	Basophils
BEB	Bengali Bangladeshis
BR	Bilirubin
C	Caspsid
CRP	C-reactive protein
C_T	Cycle threshold
DENV	Dengue virus
DHF	Dengue Hemorrhagic Fever
DNA	Deoxy ribonucleic acid
dNTP	Deoxynucleoside triphosphates
DSS	Dengue shock syndrome
DwoWS	dengue patients without warning signs;
DwWS	dengue patients with warning signs
E	Envelope
E	Eosinophils
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

FB	Fibrinogen
FDA	U.S. Food and Drug Administration
HCV	Hepatitis C virus
HD	High dose
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HPI	Hours Post Infection
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
HVL	High viral load
HWE	Hardy-Weinberg equilibrium
I.P.	Intraperitoneal
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-6	Interleukin-6
Kg	Kilo gram
L	Leucine
L	Lymphocytes
LD	Linkage disequilibrium
LD	Low dose
LD_{50}	Lethan Dose 50%
LDL	Low density lipoprotein
LVL	Low viral load
M	Methionine
M	Monocytes
MAC	Monoclonal antibody coated
MAF	Minor allele frequency
MD	Mid dose
MEGA	Molecular Evolutionary Genetics Analysis
mg	Mili gram

MTase	Methyl transferase
N	Neutrophils
NCBI	National Center for Biotechnology Information
NCDCP	National Center for Vector Borne Diseases Control
ng	Nano µgram
NIH	National Institute of Health
NIV	National Institute of Virology
NS1	Non structural protein 1
NS2A	Non structural protein 2A
NS2b	Non structural protein 2B
NS3	Non structural protein
NS4A	Non structural protein
NS4B	Non structural protein
NS5	Non structural protein
OECD	Organisation for Economic Cooperation and Development
PAGE	Polyacrylamide gel electrophoresis
PCA	Principle component analysis
PCR	Polymerase chain reaction
pg	Pico gram
prM	Pre membrane
PRR	Pattern Recognition Receptors
Pt	Platelets
PVDF	polyvinylidene fluoride
Q	Glycine
qRT-PCR	Quantitative real time PCR
RBC	Red blood cell
RDRP	RNA dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid

ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
SAA2	Serum amyloid A2
SAP	Serum amyloid P
SAS	South Asian population
SD	Severe dengue
SDS	Sodium dodecyl-sulfate
SNP	Single-nucleotide polymorphism
TC	Total cholesterol
TG	Triglyceride
TLR	Toll like receptor
TMB	3,3,5,5′-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor Alpha
UTR	Un-translated region
V	Valine
VEGF	Vascular endothelial growth factor
VL	Viral load
WB	Western blot
WBC	White blood cell
WHO	World Health Organization

ABSTRACT

Currently Dengue virus (DENV) infection has been one of the major public health problems, ranging from self limiting mild febrile illness to severe and fatal disease outcome (DHF/DSS). Pathophysiology of severe dengue is still unclear, viral factors and host immune response might play crucial role in determining disease severity. In India, scenario has been found to be very much complex and also frequently changed in each outbreak in terms of severity of the disease. Currently, there are no prognostic markers which could predict disease severity of dengue infected patients and no anti-viral therapy is currently available that could inhibit DENV replication at early stage of infection. Hence, this study aimed to evaluate importance of viral factors viz. circulation serotypes, viral load; and host factors: immunogenetic make up of host innate immune genes viz. polymorphic variants of toll like receptors (TLR3, 7, and 8), C-reactive protein (CRP) and serum protein levels in determining disease susceptibility and severity. Identification of reliable dengue severity predictive markers in patient serum and evaluation of antiviral effect of Quercetin, a plant derived bioactive compound, against DENV replication will be helpful in dengue disease management.

In this study, total 51.5% (309/600) patients were found to be dengue infected. Among the circulating serotypes, D2 and D2-D4 were most predominant mono and co-infected serotypes of which D2 serotype was also found to be more prevalent among severe dengue patients. Non-synonymous polymorphisms of TLR7 [rs179008 (gly11leu)] and TL8 [rs3764880 (met1val)] genes were found to be associated with disease susceptibility and severity. The TT genotype of CRP polymorphism rs3091244 was found to be significantly associated with disease susceptibility, severity, high viral load and increased concentration of CRP. Serum levels of endothelial markers viz. Ang2, VEGF, acute phase protein - Apo A1 and hepatic markers viz. AST and ALT can predict dengue disease severity at early stage of infection with high sensitivity and specificity with severity cut values of Ang2: 1129 pg/ml (AUC: 0.97), VEGF: 526 pg/ml (AUC: 0.99), Apo A1: 23.18 (AUC: 0.93), AST: 227.3 IU/L (AUC: 0.99) and ALT: 205 IU/L (AUC: 0.99), respectively. Quercetin significantly minimized DENV copy number, vascular leakage, concentration of inflammatory cytokines viz. TNFα and IL-6, intra cellular ROS response and histopathological hepatic alterations in DENV infected Balb/C mice compared to dengue-infected mice and thus, reflected it's therapeutic and immunomodulatory potential against dengue virus.

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Dengue (DENV) is one of the important re-emerging arboviral infections which infecting 50-100 million people across the globe every single year including India, which is considered to be dengue endemic region (1-3). The disease is caused by dengue viruses, belonging to *Flavivirus* family and transmitted principally by the *Aedes aegypti* mosquito. Other mosquito species such as *Ae. albopictus* and *Ae. polynesiensis* are also capable of transmitting dengue virus infection, but do so less effectively (2-3). However, epidemiology of DENV infection in Indian subcontinents has been found to be very much complex and also it frequently changed in each outbreak in terms of prevalent serotypes, affected geographical locations and disease severity (4-5). There are four distinct, but closely related dengue virus serotypes (DENV-1 to 4), with common genetic organization of 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (6). The structural genes encode proteins necessary for human cell surface attachment, fusion, virus entry, whereas non-structural genes encode enzymes responsible for viral replication into host cells (6). DENV genome is primarily recognized by host endosomally localized TLR3 of fibroblast, TLR7 and TLR8 of macrophages, dendritic and peripheral mononuclear cells, which leads to antiviral inflammatory cytokine production by triggering TLR3/TRIF/TRAF3 or TLR7or8/MyD88/TRAF6 or RIG1/MDA5 mediated pathways (7) DENV infection has a wide range of clinical manifestation ranging from self limiting simple dengue fever to life threatening disease complications viz. dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (8). WHO has divided course of DENV infection into three phases viz. febrile, critical and recovery. Febrile phase (0-3 days) involves high fever, joint & muscle pain, vomiting, headache and rash. In some patients disease proceeds to critical phase (0-6 days), characterized by increased capillary permeability and leakage leading to depletion of fluid from circulation and decreased blood supply to vital organs.

Recovery phase (≥7 days) occurs next, with resorption of leaked fluid into bloodstream (8). Though most DENV infected patients only suffer from mild febrile illness of dengue fever, only 5-10% patient experience severe life threatening dengue infection viz. DHF and DSS were categorized as severe dengue by WHO. Patient-mortality rate was reported between 10-20% in case of DHF patients and 40% among DSS patients (9). Dengue infection is confirmed in patient-sera by detecting presence of DENV NS1 antigen or anti-DENV-IgM antibody in ELISA or detecting viral genome by quantitative real time PCR (10-12). Pathophysiology of severe dengue development is poorly understood. Several factors viz. secondary dengue infection, circulating viral serotypes, viral-titre, host immune response have played crucial role in disease progression (8, 13). Currently, no effective biomarker is available to detect severe dengue development among infected patients. Moreover, neither specific antiviral agent exists for treatment of dengue infection nor any licensed WHO pre-qualified vaccine against dengue is available for clinical use till date. Today effective measures for vector control procedures are the sole weapon against rapid spread of dengue virus infection. Thus there is a pressing need to develop and evaluate novel alternative strategies to treat this viral infection and also to identify prognostic biomarkers for severe dengue development, for better clinical management of infected patients.

1.1. Geographical Landscape of DENV Infection:

DENV is the most fast spreading viral disease with increasing geographic expansion and incident rate (8) (Figure 1.1). More than 70% of the global population lives in Southeast Asia and Western-Pacific region bear around 75% of the total dengue cases. Dengue global pandemic begun in Southeast Asia after World War II and had rapidly increased in past 15 years (14). Being a South Asian country, many parts of India is considered as dengue endemic (4). According to WHO, dengue case fatality rates in Southeast Asia approximately 1%, but in India, reported case-fatality rates is 3-5% for DENV infection

(8). In India, first incidence of dengue was recorded in Madras (Chennai) in 1780 and first virologically proved dengue outbreak occurred in Calcutta (Kolkata) and Eastern Coast of India in 1963-1964 (15-17). Thereafter, it spread towards north and caused outbreaks in Delhi in 1967 and Kanpur in 1968 (17-18). At the same time it also spread in southern part of the country and gradually extended to whole country (19-20) (Figure 1.2). Since 1956, all four dengue serotypes have been reported in different parts of the country (21).

In Kolkata first recorded DHF occurred in 1963-64. Dengue epidemic occurred in Kolkata, in 2003, 2004, 2005 & 2006 by DENV-3; in 2007, it was DENV-2; in 2008, DENV-2 and in 2010, it was mainly due to DENV-2 and few cases by DENV-3 (22-23). Generally large dengue and DHF outbreaks took place in between the month of August – November and in 2005 it spread out in all districts of West Bengal (22). In past recent outbreaks incidence rate of dengue virus infection is climbing up rapidly in West Bengal (23-25).

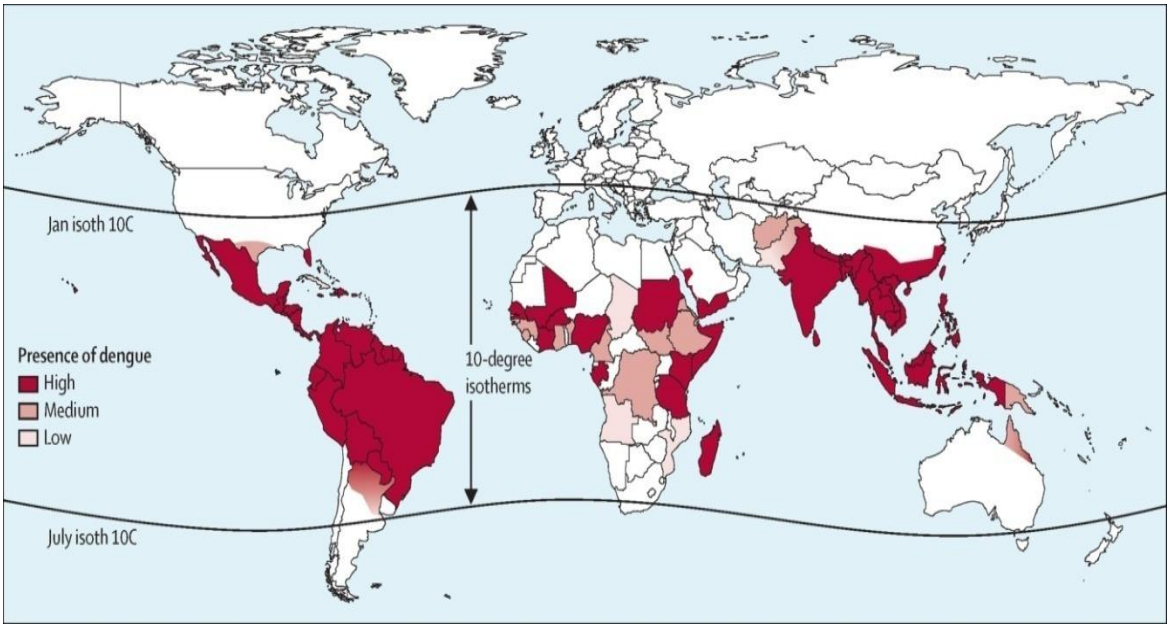
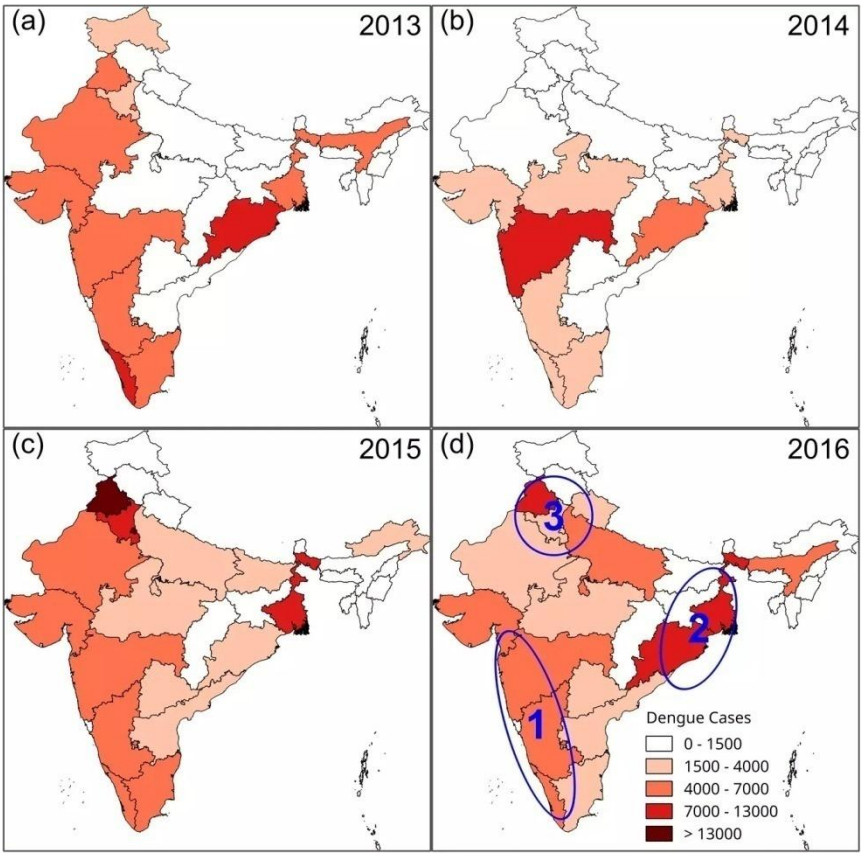


Figure 1.1: Geographical distribution of dengue virus infection (figure courtesy ref. 26).



Areas which consistently impacted by dengue fever are outlined in blue

Figure 1.2: Variations in annual dengue cases from 2013 to 2016 in different states of India (figure courtesy ref. 26).

1.2. Viral Transmission:

Primarily dengue virus is transmitted from a human to human through day biting female *Aedes* mosquitoes, mainly *Aedes aegypti*. Other sub species of *Aedes* mosquitoes such as *Ae. albopictus* and *Ae. polynesiensis* are also capable of transmitting the virus, but do so less efficiently (2-3) (Figure 1.3). Adult female *Aedes* mosquitoes prefer to feed on humans during dawn (2–3 h after sunrise) and during sunset, phenomenon known as "two peaks of biting activity" (28). During virimic phase viruses circulate in peripheral blood of the host (human) after transmission. When a female mosquito bites that person becomes infected with dengue virus and transmitted to another person by bite, known as human cycle (1). Whereas, dengue virus transmitted from lower primates to humans via

sylvatic cycle which, subsequently spread among human (29). Other unusual modes of transmission are vertical transmission (mosquito to mosquito) and non-vector mode of dengue virus transmission (mother to fetus, transfusion-related transmission, transplantation-related transmission and needle-stick-related transmission) (30). Often, female *Ae. aegypti* mosquitoes feed on several persons and rapidly accelerating dengue virus transmission. Transmission of DENV by *Aedes* mosquitoes followed sylvatic and human cycle in Africa and Asia (31). Transmission cycle of DENV is shown in Figure 1.4.

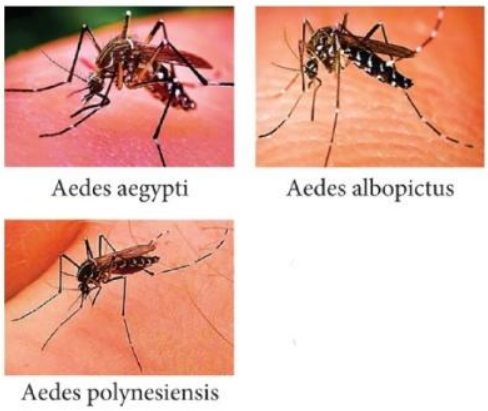


Figure 1.3: Representative image of different types of *Aedes* mosquitoes (figure courtesy ref. 29).

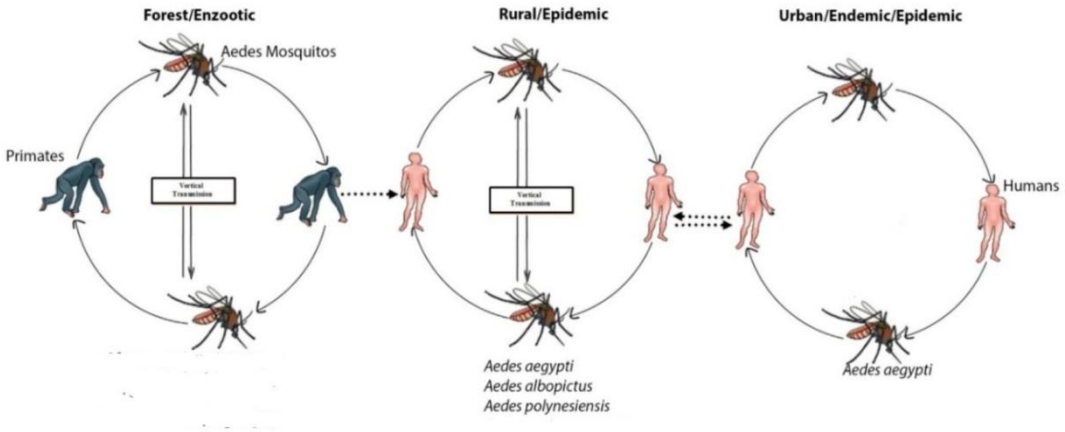


Figure 1.4: Different types of dengue virus transmission cycle (figure courtesy ref. 29)

1.3. DENV Genome and Viral Classification:

Dengue fever virus (DENV) is a single stranded RNA virus of the *Flaviviridae* family; with distinct serotypes of dengue virus (DENV 1-4) (Table 1.1) (32). It is an enveloped virus with a single-stranded positive sense 11-kb RNA genome. Genome encodes a single open reading frame and can be translated into 3 structural proteins, that is, the core (C), premembrane/membrane (prM/M), and envelope (E) protein, and 7 non-structural proteins, that is, NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5 and short non-coding regions on both the 5' and 3' ends [33, 34] (Figure 1.5). DENV E protein, found on viral surface, is important in initial attachment of virus to host cell (34). Several human cell surface molecules which interact with E protein have been shown to be important factors mediating attachment and viral entry. Surface of the mature virus particle is composed of 180 envelope (E) glycoprotein molecules and an equal number of membrane (M) protein molecules that assemble at endoplasmic reticulum-derived membranes (35, 36). E glycoprotein's ectodomains are arranged in a herringbone pattern on the surface of lipid membrane which facilitates binding of the virus to host cells (37, 38). Each E monomer consists of three domains: DI, DII and DIII (39). C-terminal portion of E protein consists of stem and membrane anchor regions. DENV prM (membrane) protein, which is important in formation and maturation of viral particle, consists of seven antiparallel β-strands stabilized by three disulfide bonds (40). Mature DENV virion's glycoprotein shell consists of 180 copies each of E and M proteins. Immature virion starts out with E and prM proteins forming 90 heterodimers that give a spiky exterior to the viral particle. DENV NS1 protein is first non structural protein consist of a 352-amino-acid polypeptide with a molecular weight of 46-55 kDa and works as a scaffolding protein that anchors replication complex to endoplasmic reticulum (ER) membrane (48). Next important non-structural part of DENV genome is

NS2B-NS3 protein. Immature C protein, which is present in cytoplasmic side of endoplasmic reticulum, is cleaved by viral NS2B-NS3 protein to form mature C protein (40). Dengue NS3 is a serine protease, as well as an RNA helicase and RTPase/NTPase. Dengue virus NS3 is a multifunctional protein consist of 618 amino acids which functions both as a serine protease as well as an RNA helicase and RTPase/NTPase (41). Protease domain consists of six β-strands which set into two β-barrels formed by residues 1–180 of the protein. The important amino acid residues, essential for it catalytic activity, also known as catalytic triad (His-51, Asp-75 and Ser-135) and is found between these two β-barrels (41-42). Catalytic activity is dependent on the presence of a 43 amino acid segment of the NS2B (residues 67–80) (40) cofactor. Rest of the NS3 residues of 180–618 amino acids form the three sub-domains of the DENV helicase which is composed of six-stranded parallel β-sheet surrounded by four α-helices (43). DENV NS3 helicase domain is consists of three sub-domains, sub-domains I and II, and sub-domain III that is composed of four α-helices surrounded by three shorter α-helices and two antiparallel β-strands (44). DENV NS4A is a nonstructural protein involved in altering cell membrane curvature and induction of autophagy (45-46). Apart from its membrane alteration property, NS4A is also helps in scaffolding viral replication complex and goes through oligomerization (47). Most important and largest non-structural part of DENV genome is DENV NS5 protein which is a 900-residue long peptide with a N-terminal end (residues 1–296) methyltransferase domain and a C-terminal end (residues 320–900) RNA-dependent RNA polymerase (RdRp) domain. Methyltransferase domain consists of an α/β/β sandwich flanked by N- and C-terminal sub-domains (40, 49). DENV methyltransferase enzyme (MTase) protects the viral genome by RNA capping and facilitating polyprotein translation (49). Whereas, RNA-dependent RNA polymerase (RdRp) that replicates the viral RNA (49).

Table 1.1: Classification of dengue virus

Virus classification	
Group	Group IV [(+) ssRNA]
Order	Unassigned
Family	*Flaviviridae*
Genus	*Flavivirus*
Species	*Dengue virus*

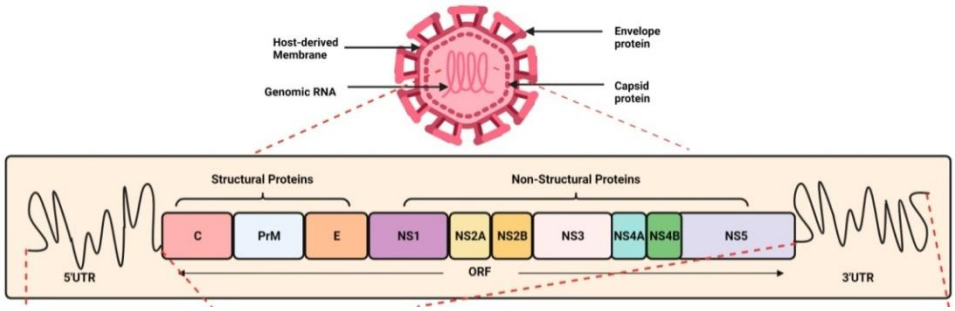


Figure 1.5: Schematic structural organization of Dengue virus genome (figure courtesy ref. 50).

1.4. Clinical Manifestation:

Clinical symptoms of patients usually begin 4 to 6 days after dengue infection and the course of illness is divided three different phases viz. febrile (0-3 days), critical (4-6 days) and recovery (≥7 days) (Figure 1.6) (8). Children often experience symptoms similar to those of common cold and gastroenteritis (vomiting and diarrhea) and have a greater risk of severe complications, though initial symptoms are generally mild but include high fever (51-53). WHO has classified dengue infection in two groups: simple and severe dengue, depending on clinical magnitude of symptoms (8). Simple dengue is sometime asymptomatic and often characterized with sudden-onset of fever, headache (typically located behind the eyes), muscle and joint pains and rash. Alternative name for dengue, "breakbone fever", comes from associated muscle and joint pains (54-55) (Figure 1.7). In 2009, World Health Organization (WHO) has categorized non-

severe/simple dengue cases in two groups, with warning signs and without warning signs, for better clinical manegment (Figure 1.8). Severe dengue (DHF/DSS) is characterized by acute onset of high fever, hemorrhagic manifestations, including positive tourniquet test, thrombocytopenia, rise in haematocrit, liver enlargement, severe organ impairment and shock, manifested by tachycardia, poor tissue perfusion with weak pulse and narrowed pulse pressure or hypotension with presence of cold, clammy skin and/or restlessness (8).

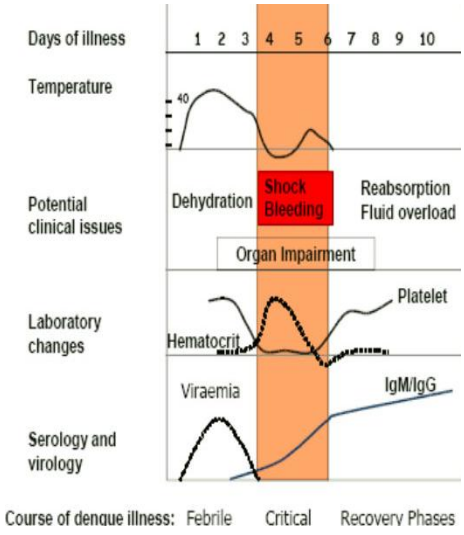


Figure 1.6: The course time line of dengue illness (figure courtesy ref. 8).

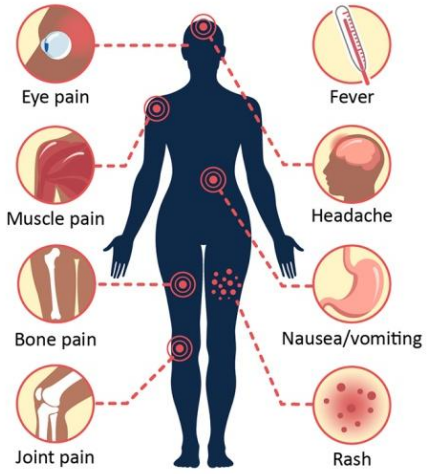


Figure 1.7: Schematic representation of classical dengue symptoms (figure courtesy ref. 56)

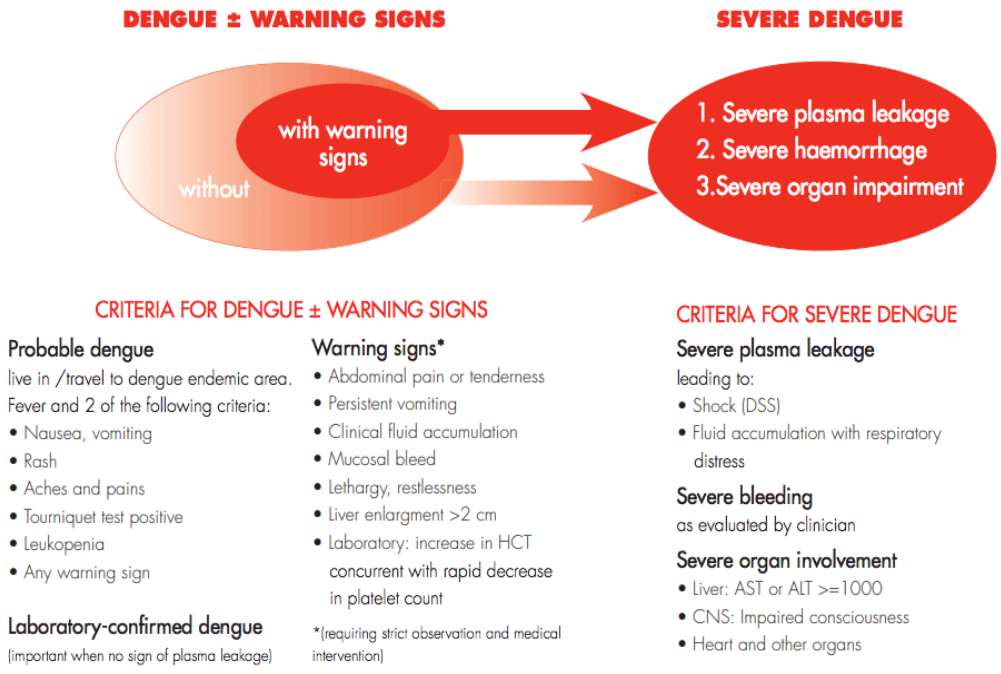


Figure 1.8: WHO Suggested dengue case classification and levels of severity (figure courtesy ref. 8)

1.5. Detection and Laboratory Diagnosis:

Laboratory confirmation of dengue infection is crucial as the broad spectrum of clinical presentations, ranging from mild febrile illness to several severe syndromes, can make accurate diagnosis difficult (57, 58). Serological assays are most commonly used for diagnosis of dengue infection as they are relatively inexpensive and easy to perform. Detection of dengue specific anti-dengue IgM or IgG antibodies in patient-sera is the standard for serologically confirming dengue infection (1) (Figure 1.9). Anti-dengue IgG antibody ELISA (enzyme-linked immunosorbent assay) is crucial to predict secondary dengue infection, which is a major factor in developing disease severity. Human IgM and IgG antibodies against dengue infection usually start developing after 5-7 days and 14 after infections, respectively (59). But, dengue NS1 protein is detectable by ELISA as early as on first day of fever and can be found up to 9 days in

serum (60) (Figure 1.9). Given all these advantages, dengue NS1-based ELISAs may be an important diagnostic tool for those acute samples in which IgM is not detectable and thus, viral NS1 antigen might be useful in early detection and high NS1 titre is an important prognostic parameter for severe dengue infection. Many nucleic acid amplification based tests have been developed for diagnosis of dengue infection in its early stage. Real-time RT-PCR assay is a one-step assay that allows viral titre to be quantified in approximately 1.5 hours (57). One advantage of this assay is its ability to determine viral titre early in dengue illness. (61). Scientific reports have suggested that dengue titre <10,000copies/ml was associated with mild illness whereas; viral titre ≥10,000 copies/ml was reported to be associated with WHO-classified warning signs (24).

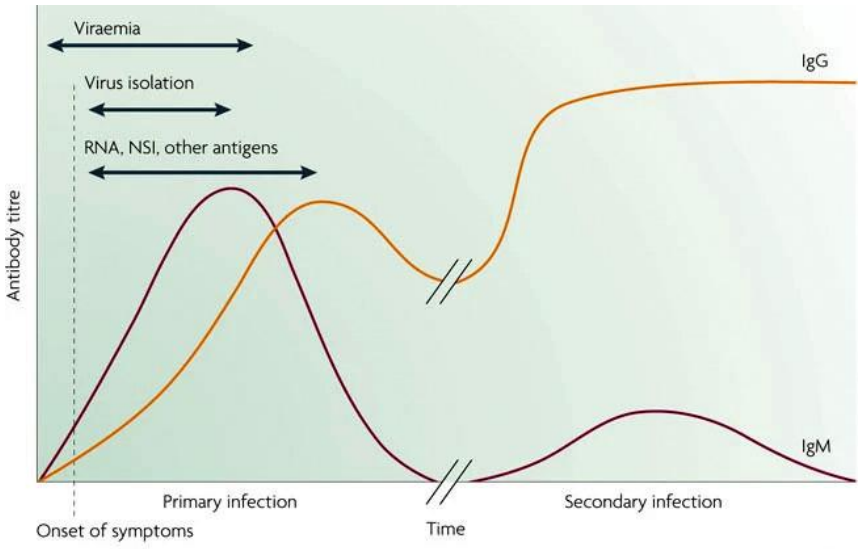
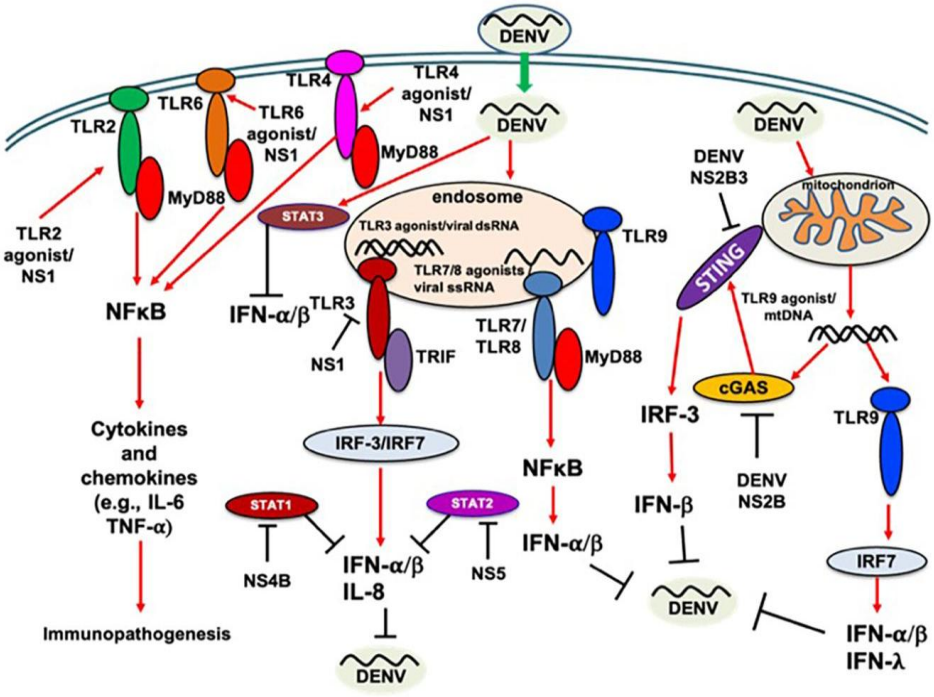


Figure 1.9: Major diagnostic parameters for dengue infection (figure courtesy ref. 58).

1.6. Host Innate Immune Response:

Pathophysiology of dengue virus infection is a complex mechanism. DENV enters host cells by receptor-mediated endocytosis, and its RNA is released to the cytosol for translation and replication (62). Host's early antiviral response against dengue virus is

primarily initiated by recognition of viral genomic RNA by endosomally-localized human TLRs: viz. TLR 3, 7, 8 and cytoplasmic RIG like receptors (RLRs) as first line of defense against single strand RNA virus (63). TLR3, TLR7 and TLR8 (TLR7 subfamily) are important for recognizing DENV RNA in the endosome (62-64) (Figure 1.10). TLR7 senses ssRNA with G- and U-rich sequences, whereas TLR3 recognizes DENV replication derived dsRNA (65-66). At the downstream of these processes, IRFs 3/7, AP1 and NFkβ are activated followed by production of interferon and inflammatory cytokines (63). Thus, induction of rapid innate immune response occurs, characterized by production of antiviral interferons: IFNα, IFNβ, TNFα and cytokines: NFkβ, TNFR-1, IL-1b, IL-2, IL-12 (63). Both TLR3 and TLR7 mediates a virus-specific humoral immune response for DENV clearance (67).



Red arrow indicates the induction/activation of components of TLR signaling by DENV; black line indicates the inhibition or cleavage or degradation of the host immune components/response or inhibition of DENV replication, as appropriate.

Figure 1.10: Schematic diagram of human TLRs signaling activated against dengue DENV infection (figure courtesy ref. 68).

1.7. Pathophysiology and Clinical Markers of Dengue Disease Severity

Dengue patients may progress through three clinical phases viz. febrile, critical, and recovery phases (8). Various forms of severe -manifestations may unfold only as the disease progresses through the critical phase (8). Patho-physiology of DHF development is poorly understood. Hepatic injury and disruption of endothelial permeability are major characteristics for DHF and DSS (68-69) (Figure 1.11). According to WHO, bleeding and increased vascular permeability usually occurs at defervescence stage due to induction in proinflammatory immune modulators leading to coagulation abnormality and vasculopathy (70-72). Severe vascular leakage leads to hypoxic injury of liver and results in hepatic dysfunction (73). According to WHO, high aspartate aminotransferase (AST) or alanine aminotransferase (ALT) concentrations of ≥1000 units/l and AST/ALT ratio >2 is considered as a criteria for severe-dengue (8, 74). Studies have shown that antibody dependent enhancement (ADE) due to secondary dengue infection, high viral load and activation of immune system are also coupled with DHF development (24, 72, 75-77). Currently, there are no reliable clinical or laboratory indicators (markers) that can accurately predict development of DHF, which is necessary for better clinical manegment. Based on the severe dengue infection pathophysiology these markers might include [i] transcriptomic signatures, [ii] anti-DENV antibodies, [iii] inflammatory and endothelial activation factors and [iv] metabolic and apoptotic mediators (Figure 1.12).

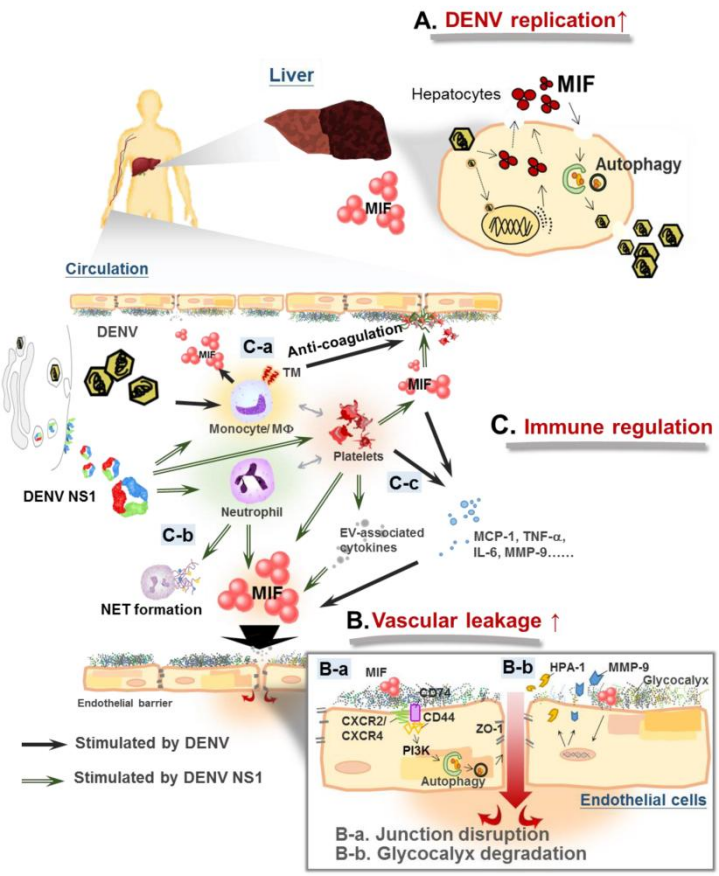


Figure 1.11: Schematic representation of severe dengue pathophysiology (figure courtesy ref. 78).

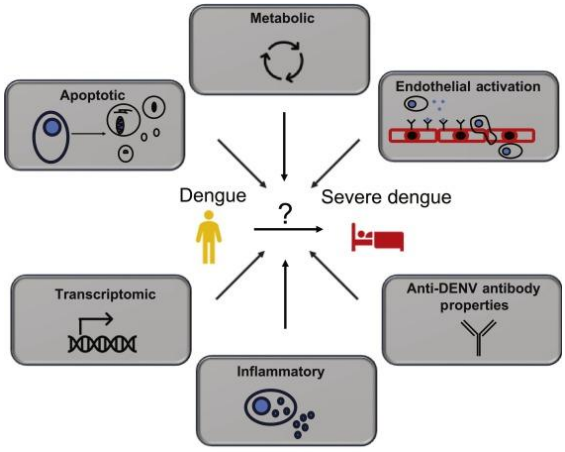


Figure 1.12: Schematic illustration of different categories of biomarkers candidates showing potential promise for prediction of progression to severe dengue (figure courtesy ref. 79).

1.8. Effect of quercetin, a bioactive natural compound against dengue virus:

Dengue virus infection displayed a variety of clinical spectrum (8). Compared to simple-dengue, severe-dengue infection is marked by significantly increased release of pro-inflammatory cytokines, viz. IL-6, TNF-α and augmented oxidative stress generating reactive oxygen species (ROS) which leads to increased permeability and hemorrhage (80, 81) Currently dengue infection is listed among category-A priority pathogen as declared by National Institute of Health, USA (82). Till date, there is lack of effective antiviral drugs against DENV and absence of a suitable animal model that can represent complexity of dengue pathogenesis has contributed to lack of understanding of the disease (83, 84). Only vaccine available in the market is CYD-TDV (chimeric yellow fever virus-dengue virus tetravalent dengue vaccine) (Sanofi Pasteur, France) but it has been reported to increase the threat of severe dengue in individuals, especially among children who have not been infected previously (85, 86). There are several dengue vaccine candidates currently under process of clinical development, but none have been successful to effectively treat dengue infection (87-91). Currently, Treatments are mainly symptomatic and prevention measures are taken in vector control manegment (8). Dengue patients are treated only for symptoms viz. muscle aches and pains and fever by supportive care such as, fever reducers and pain-killers and responsive fluid management (8). Thus, natural plant derived compounds with antiviral activity remain an important source for development of new antiviral drugs because of their low side-effects and their high accessibility in nature (92). Numerous phytochemicals and various flavonoids are reported to have antiviral activity against dengue virus (93, 94). Flavonoids are low molecular weight phenolic compound found widely in different kinds of plants. Quercetin is one such natural compound of

flavonol group, approved by the U. S. Food and Drug Administration (FDA; National drug code numbers are 65,448-3085, 65,448-3005), known for its antioxidant, antiviral and anti-inflammatory properties that can be found mainly in plants, black tea and fruits such as apple, berries, grapes, onions, tomato etc (95, 96). Due to its potential benefits on human health, quercetin has become a natural ingredient of great interest in pharmaceutical industries (96). Its biological activities have been largely attributed to its active phenolic hydroxyl groups and double bonds. Quercetin was also reported to inhibit Herpes Simplex and Influenza viral entry; it inhibited Japanese Encephalitis and Hepatitis C viral replication (97-100). Also, quercetin inhibited reverse transcriptase, proteases and viral capsid proteins of HIV, HCV and influenza viruses, respectively (101). Since, it has been reported to exhibit antiviral property against RNA viruses, it might also effective against dengue virus.

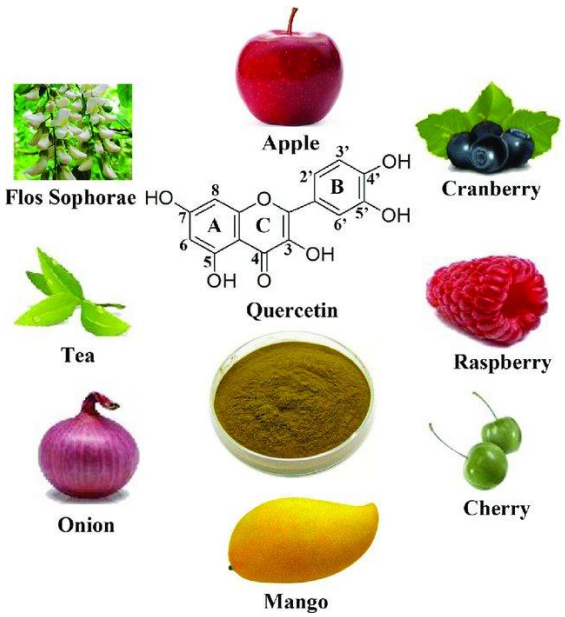


Figure 1.13: Chemical structure and natural sources of quercetin (figure courtesy ref. 102).

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GENERAL OBJECTIVES

2. GENERAL OBJECTIVES

1. Determination of Dengue virus (DENV) infection among symptomatic Eastern Indian patients by detecting anti-DENV-IgM antibody and viral genome.

2. Determination of infecting DENV serotypes.

3. Partial sequence analysis of DENV envelope (E) region.

4. Identification of differential genetic makeup of innate immune response: Toll Like Receptor (TLR)-pathway molecules (TLR3, TLR7 and TLR8) and C-reactive protein (CRP) between WHO classified dengue patients (with warning signs, without warning signs & severe) and healthy individuals.

5. Comparison of different serum protein markers concentration viz. angiopoietin-2 (ANG2), vascular endothelial growth factor (VEGF), fibrinogen (FB), C reactive protein (CRP), serum amyloid A2 (SAA2), serum amyloid P (SAP) and apolipoprotein A1(ApoA-1) between above mentioned groups.

6. Determination of anti-viral effects of Quercetin against DENV.

Chapter – 1

Determination of Dengue virus (DENV) infection among symptomatic Eastern Indian patients by detecting anti-DENV-IgM antibody, viral genome and Determination of infecting DENV serotypes

3. Chapter – 1

1. Determination of Dengue virus (DENV) infection among symptomatic Eastern Indian patients by detecting anti-DENV-IgM antibody and viral genome.

2. Determination of infecting DENV serotypes

- **Publications**

The scientific work mentioned in this chapter has been published in the following peer reviewed journal:

- **Mukherjee S**, Dutta SK, Sengupta S, Tripathi A*. Evidence of dengue and chikungunya virus co-infection and circulation of multiple dengue serotypes in a recent Indian outbreak. ***European Journal Clinical Microbiology & Infectious Disease***. 2017 Nov; 36(11):2273-2279. doi: 10.1007/s10096-017-3061-1. Epub 2017 Jul 29. PMID: 28756561.

3.1. REVIEW OF LITERATURE

Dengue virus (DENV) belongs to *Flavivirius* family, and exists in four antigenically distinct serotypes (DENV-1 to DENV-4) causing self-limiting febrile illness, with symptoms ranging from simple to severe dengue (1). Based on clinical manifestation World Health Organization (WHO) had categorized DENV infected patients in to three group viz. dengue with warning signs, without warning signs and severe dengue. WHO has estimated that despite of being significantly under-reported from developing nations, about 40% of the world's populations are at risk from dengue, of which Southeast Asia region contributing 52% of the global disease burden (2). Moreover, India has contributed to 34% of total global dengue incidence annually and emerged as major contributor for global dengue burden (3). In India, first incidence of dengue was recorded in Madras (currently Chennai) in 1780 and first virologically proved dengue outbreak occurred in Calcutta (currently Kolkata) and Eastern Coast of India in 1963-1964 (4-6). After that, it spread towards north and caused outbreaks in Delhi in 1967 and Kanpur in 1968 (6-7). It also spread in southern part of the country and gradually within whole country at the same time (8-9). Several dengue outbreaks were reported from eastern coast of India (10-15). Being a dengue endemic county all four dengue serotypes have been reported in different parts of the country; with predominant dengue serotypes keep changing every year (16). Previous scientific studies had described different DENV serotypes were associated with wide range of clinical characteristics (17). Earlier studies have highlighted that DENV-2 might lead to more severe disease outcome, whereas DENV-1 was coupled with milder illness (18-19). High viral titers were associated with severe disease outcome, which have been suggested to be necessary for plasma leakage, a hallmark of DHF (15, 20-23).

First virologically proved dengue outbreak occurred in Calcutta (currently Kolkata) and Eastern Coast of India in 1963-1964. Since 2005, Kolkata has witnessed several massive dengue outbreaks with severe outcome (24-29). Large outbreaks of DENV were reported from various parts of West Bengal during 2014-2017, affecting 73,071 people and death of 109 dengue patients (30). Clinicopathological landscape of dengue patients in West Bengal is getting complicated since last one decade in terms of case fatality, circulating serotypes and co-infection with chikungunya virus. Massive outbreaks were reported in 2016 which mainly affected Kolkata and its adjoining districts mainly North-24-parganas, Howrah and Hooghly (31-32). Due to such concurrent multiple outbreaks, all four dengue serotypes were circulating in West Bengal and serotype predominance kept changing in every outbreaks (31-34). In Kolkata, common circulating serotypes were DENV-1, 2 and 4 from 2008 to 2010, after which DENV-3 infections arose leading to massive dengue outbreak in 2012 (28). Scientific research also suggested that DENV-1 was the most predominant serotype in 2016 outbreak which was entirely replaced by DENV-2 during 2017-2021 outbreaks (31-34). Concurrent multiple dengue serotypic infection among patients resulted from such co-circulation, which led to complicated clinical outcome (20, 35). Recent studies from northern West Bengal have also reported co-infection with multiple dengue serotypes (36). Infection with one serotype can provide lifelong immunity to that serotype but not against all other dengue serotypes (2). Thus, primary infection can activate immune responses against that particular dengue serotype, but heterotypic infection by multiple serotypes results in antibody-dependent enhancement (ADE) thereby increasing disease severity (37). A longitudinal surveillance study by A.K. Hati among symptomatic patients in Kolkata during 2005-2007 reported 65.3% rate of secondary dengue infection, among which 10% developed DHF with 8.5% case-fatality (26). Co-circulation of multiple dengue

serotypes among Vietnamese patients was reported to enhance secondary dengue infection rate (38). According to WHO, viral titer played a crucial role in determining disease severity and it was reported that viral titer of severe dengue patients was 1-2 logs higher than that of milder infection (2). Similarly, previous studies have also showed significant association between high viral load and severe clinical manifestations as well as development of WHO-defined warning signs (29, 39). Moreover, Pal *et al*. also reported that viral load <10,000 copies/ml and viral load ≥10,000 copies/ml could be considered as low viral load (LVL) and high viral load (HVL), respectively and this HVL group was associated with severe clinical manifestations viz. vomiting, abdominal pain, leukopenia and clinical fluid accumulation (29). Among all severe clinical signs, clinical spectrum of hepatic dysfunction, thrombocytopenia and bleeding was quite evident during past outbreaks in West Bengal (34, 39-41). Previous reports also demonstrated that younger age groups (10-30years) were more susceptible to dengue virus infection (26, 28). Moreover, from 2010 onwards situation has become more complicated due to dengue and chikungunya co-infection. Taraphdar *et al*. had reported more severe clinical manifestations among co-infected patients compared to mono-infected patients (27).

Serological detection methods, viz. dengue NS1 and anti-dengue IgM ELISA were previously considered as gold standards for detection of dengue infection. Anti-dengue IgM ELISA could detect IgM antibodies among 50% of patients from 3-5 days after symptomatic onset. But, according to WHO, some patients could develop severe complications at critical stage: 4-6 days of symptomatic onset (2). Hence, early detection of dengue virus during initial stage of infection is urgently necessary for better clinical manegment (2). ELISA based dengue NS1 antigen detection and RT-PCR techniques to detect viral RNA are more efficient in terms of early detection of infection

(42-45). More importantly, dengue qRT-PCR based detection is more accurate as it detects viral RNA copy number in patient sera (46-48).

Thus, in Chapter 1 dengue infection among symptomatic Eastern Indian patients were investigated by RT-PCR, dengue NS1 and anti-dengue IgM ELISA and correlation between circulating serotypes, viral copy number and biochemical and haematological parameters of patient-sera with development of WHO-classified dengue severity symptoms was made.

3.2. MATERIAL AND METHODS:

3.2.1. Inclusion Criteria:

Acute febrile patients with history of any of the following symptoms: headache, bodyache/myalgia, joint pain/arthralgia, rash, with or without hemorrhagic manifestation from all age-groups and both sexes were selected as per WHO criteria.

3.2.2. Exclusion Criteria:

Patients with hematological malignancies, chronic liver disease, diabetes mellitus and renal diseases were excluded in this study.

3.2.3. Collection of Patients' Blood:

After obtaining institutional ethical committee approval, 5ml of blood samples were collected from 600 symptomatic febrile patients visiting outpatient department Calcutta School of Tropical Medicine, Kolkata, West Bengal, India from January 2014 to December 2016. All patients exhibited fever and any of the following symptoms: aches and pain, nausea, rash, vomiting, abdominal-pain, joint-swelling, bleeding, retro-orbital pain. After monitoring clinical symptoms in presence of in-house clinicians, patients

were categorized into three groups according to WHO 2009 classification: (i) dengue patients without warning signs, (ii) With warning signs, (iii) Severe dengue. Blood was collected in non-EDTA vials and serum was separated from patient-blood by density gradient centrifugation, aliquoted and stored at -20^{0}C for serological testing and at -80^{0}C freezer for molecular diagnostics. To avoid repeated freeze thawing, samples were aliquoted in multiple tubes.

3.2.4. Extraction of Viral RNA:

Viral RNA was extracted from 140µl of serum by using QIAamp Viral RNA Mini Kit according to manufacturer's protocol (Qiagen, Hylden, Germany). Briefly, 560µl of AVL buffer (lysis), containing carrier RNA was mixed with 140µl of serum by pulse vortexing for 15 seconds. The mixture was then incubated at room temperature (15-25^{o}C) for 10 minutes, followed by brief centrifugation. Then 560µl of ethanol (96 to 100%) was added to the above mixture and then mixed by pulse vortexing for 15 seconds. Carefully, 630µl solution from the above mixture containing AVL buffer, ethanol and serum was transferred to a QIAamp mini Columns along with collecting tubes. The columns were then centrifuged at 8000rpm for 1 minute. After centrifugation, the collecting tube containing filtrate was discarded and QIAamp column was then placed into fresh collecting tube. This process was repeated until all the mixture containing AVL buffer, ethanol and serum passed through respective columns. Buffer AW1 was then added to the QIAamp column and was centrifuged at 8000rpm for 1 minute; again, the filtrate was removed and column was placed into new collection tube. Then, 500µl of AW2 buffer was added to each column, which was subjected to centrifugation at 14000 rpm for 3 minutes. The column was then placed in new 1.5ml centrifuge tube, and 60µl of AVE buffer was added into each column. The column was

incubated at room temperature (15-28^{o}C) for 1 minute, followed by centrifugation at 8000rpm for 1 minute. Finally the elute containing viral RNA was aliquoted in small 0.2 ml micro centrifuge tubes and stored at -80^{o}C until use.

3.2.5. Quantification of Viral RNA:

Viral RNA was quantified using Cary 100 Scan UV-Visible Spectrophotometer (Varian, Inc., Palo Alto, CA, USA). Nucleases free water was used as a solvent to suspend the nucleic acids; 1ml of each diluted sample was placed in a quartz cuvette. The spectrophotometer was set zero using same solvent in which nucleic acid was dissolved as blank. The OD_{260}/OD_{280} ratio was calculated to check nucleic acid purity. Pure DNA has an OD_{260}/OD_{280} ratio of ~1.8; pure RNA has an OD_{260}/OD_{280} ratio of ~2.0. Low ratios could be caused by protein or phenol contamination.

Final concentration of RNA was determined using the following formula:

$$\text{RNA concentration} = OD_{260} \times 40\ \mu g/ml \times \text{dilution factor.}$$

3.2.6. Detection of Dengue Viral Genome and Determination of Viral Load by Using Quantitative Real Time (qRT-PCR):

Presence of DENV genome and their viral load among infected patient's serum was determined by real-time qRT-PCR using OneStep qRT-PCR MasterMix and other components of DENV Genesig kit (Primer Design Ltd., UK) according to manufacturer's protocol. Briefly, 5µl RNA extracted from patient serum was mixed with 10µl oasigTM OneStep 2x qRT-PCR Mastermix and 1µl DENV primer/probe mix. Final volume was adjusted by adding 4µl RNAse/DNAse free water. The mix was then transferred to well of Applied Biosystems® MicroAmp® Fast Optical 96-Well Reaction Plate (Thermo Fisher Scientific, USA). Each sample was loaded in triplicate.

Real time PCR was performed on ABI 7500 Fast instrument. Viral RNA amplification took place in four major steps: Reverse transcription at 55°C for 10 minutes; Enzyme activation at 95°C for 2 minutes; denaturation at 95°C for 10 second and Data collection at 60°C for 1 minute (both denaturation and data collection steps were repeated for 50 times). Final fluorescence was detected in FAM channel for all samples including control template. A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of positive control template (cloned DENV 3' untranslated region, provided within the kit) (Figure 3.1A and B). This kit is 100% specific for DENV and its minimum detection limit is 2copies/ml. Copy number of each virus in patient serum was calculated from corresponding Ct values using following formula (Table 3.1).

[Genome copies in PCR reaction (from standard positive control template)/Template volume used in PCR (in µl)] x [Elute volume (extracted volume of nucleic acid in µl)/ Sample volume (extracted blood in ml)]

(A) **(B)**

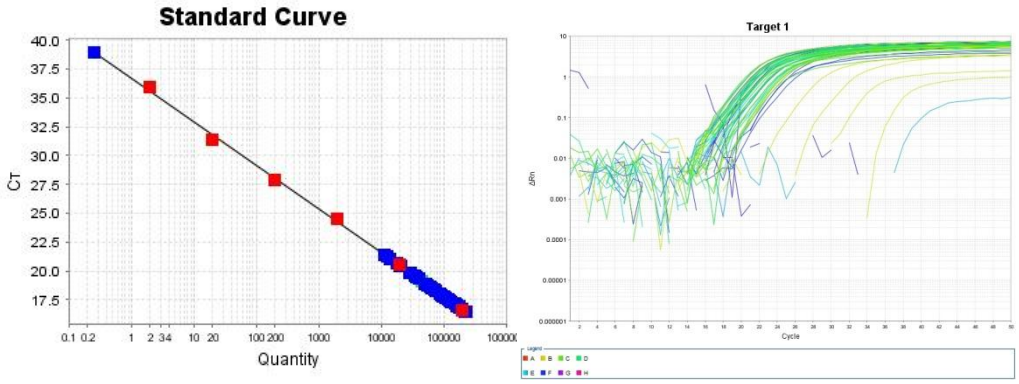

Figure 3.1: (A) Representative Standard curve for DENV copy number determination. (B) Representative amplification plot of DENV qRT-PCR.

Table 3.1: C_T values of 10-fold serially diluted DENV positive control template.

Sr. No.	Standards	Quantity (copies/5µl)	C_T Values
1.	Standard 1	$2X10^5$	15.25
2.	Standard 2	$2X10^4$	20.38
3.	Standard 3	$2X10^3$	23.89
4.	Standard 4	$2X10^2$	27.87
5.	Standard 5	20	31.19
6.	Standard 6	2	35.26

3.2.7. Determination of Dengue Virus Serotypes:

DENV serotypes of PCR+ samples were determined by real-time RT-PCR using One Step qRT-PCR Master Mix and other components of DENV serotyping Genesig kit (PrimerDesign Ltd., UK), according to manufacturer's protocol. Reactions were performed in ABI 7500 Fast instrument using serotype specific DENV controls present within kit. Briefly, 5µl of each of DENV serotype specific positive control (DENV-1 to 4) RNA template and RNA extracted from patient serum was mixed with 10µl Oasig™ OneStep 2x qRT-PCR Mastermix and 1µl DENV primer/probe mix. Final volume was adjusted by adding 4µl RNAse/DNAse free water. The mix was transferred to well of Applied Biosystems® MicroAmp® Fast Optical 96-Well Reaction Plate (Thermo Fisher Scientific, USA). Real time PCR was performed on ABI 7500 Fast instrument. Amplification took place in four major steps: Reverse transcription at 55°C for 10 minutes; Enzyme activation at 95°C for 2 minutes; denaturation at 95°C for 10 second and Data collection at 60°C for 1 minute (both the denaturation and data collection steps were repeated for 50 times). Fluorescence data were collected through the FAM, VIC,

Cy5 and ROX channels for DENV-1, DENV-2, DENV-3 and DENV-4 serotypes, respectively (Figure 3.2).

(A) **(B)**

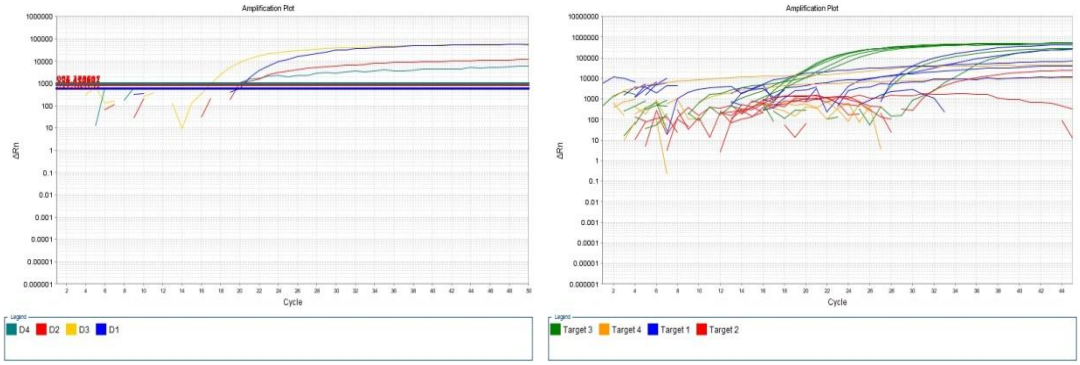

Figure 3.2: Representative amplification plot of DENV serotyping qRT-PCR (A) positive control template and (B) tested samples.

3.2.8. Detection of Dengue-NS1 Antigen:

Serum of patient suffering from fever for less than five days was subjected to serological diagnosis for presence of Dengue-NS1 antigen using DENGUE NS1 Ag MICROLISA kit (J. Mitra, New Delhi, India), according to manufacturer's instructions. Briefly 10µl of patient serum was diluted 100 times using sample diluent buffer. Then 100µl diluted serum was added to each well coated with dengue NS1 antigen and the plate was incubated at 37°C for 30 minutes. After incubation, the plate was washed twice with 1x wash buffer (provided with the kit) to remove unbounded materials. Plate was then incubated with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrates containing hydrogen peroxide for 10 minutes at room temperature. Finally, color forming reaction was stopped by adding stop solution (Figure 3.3). After stopping the reaction, absorbance of each well was read using microplate reader (Bio-Rad, California, USA) at

450nm wavelength. Result was interpreted as negative or positive according to the assay manual.

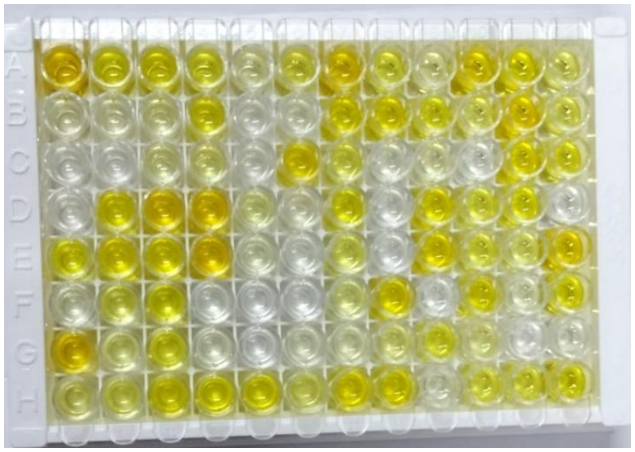

Figure 3.3: Representative image of dengue NS1 ELISA plate.

3.2.9. Determination of Anti-Dengue-IgM And Anti-Dengue-IgG:

Patient-serum was subjected to serological diagnosis for presence of anti-dengue-IgM and anti-dengue-IgG antibodies, using anti-dengue-IgM MAC-ELISA (National Institute of Virology, India) and Panbio dengue IgG Capture ELISA kit (Panbio, Austrelia), according to manufacturer's instructions. Briefly, 10µl of each of patient serum was diluted 100 times using sample diluent buffer. Then 100µl diluted serum was added to each well coated with anti-human IgM and IgG antibodies, respectively and the plate was incubated at 37°C for 30 minutes. After incubation, the plate was washed twice with 1x wash buffer (provided with the kit) to remove unbounded materials. Plate was then incubated with TMB substrate containing hydrogen peroxide for 10 minutes at room temperature. Finally color forming reaction was stopped by adding stop solution (Figure 3.4). After stopping the reaction, absorbance of each well was read using microplate reader (Bio-Rad, California, USA) at 450nm wavelength. Result was interpreted as negative or positive according to the assay manual.

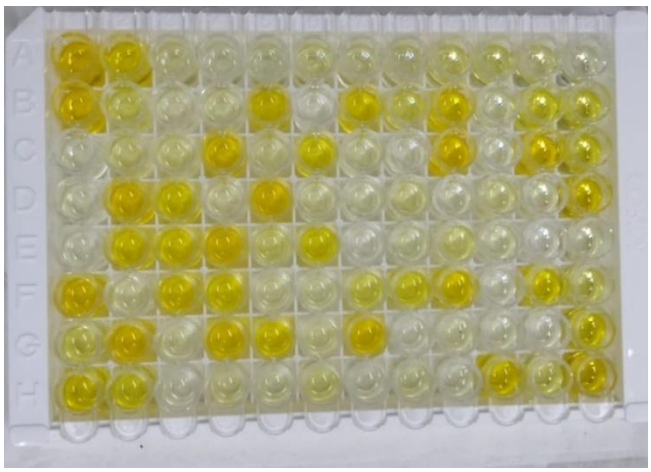

Figure 3.4: Representative image of anti-dengue IgM ELISA plate.

3.2.10. Statistical Analysis:

2x2 contingency Chi-square and multivariant t-test were performed to find out association between frequency of DENV infection, serotypes and viral load with development of WHO categorized clinical manifestations using GraphPad Prism[9] software (GraphPad Software, San Diego, California USA). Probability values of $p<0.05$ was considered as statistically significant.

3.3. RESULTS:

In this present study, prevalence of DENV infection has been analyzed among 600 symptomatic patients visiting Calcutta School of Tropical Medicine (CSTM) from different districts of West Bengal, Eastern India, during January 2014 to December 2016. Dengue incidence rate and demographic scenario were depicted in Table 1.

Table 3.2: Demographic scenario of dengue infection among the study population (2014-2016).

		Total symptomatic patients (n=600)	
		Dengue infected patients (n=309)	**Dengue uninfected patients (n=291)**
Age (Years)	Range	5-72	12-67
	Mean	36.06±7.2	39.3±8.01
Sex	Male	60.19% (186)	56.70% (165)
	Female	39.80% (123)	43.29% (126)
Course of illness	Febrile phase (0-3 days)	26.21% (81)	24.74% (72)
	Critical phase (4-6 days)	24.59% (76)	32.98% (96)
	Recovery phase (≥7 days)	49.19% (152)	42.26% (123)
Diagnostic parameters	Dengue NS1	60.19% (186)	NA
	Anti-dengue IgM	48.54% (150)	
	Anti-dengue IgG	20.38% (63)	
	qRT-PCR	67.31% (208)	
Type of dengue infection	Primary	79.61% (246)	
	Secondary	20.38% (63)	
Sample classification according to WHO 2009 guideline	Severe	9.70% (30)	
	With warning signs	47.57% (147)	
	Without warning signs	42.71% (132)	
Viral load (n=208)	High viral load (HVL: ≥10000 copies/ml)	61.05% (127)	
	Low viral load (LVL: <10000 copies/ml)	38.94% (81)	

Among 600 symptomatic patients 51.5% (n=309) were found to be dengue positive by quantitative real-time PCR (qRT-PCR), dengue NS1, anti-dengue IgM and IgG ELISA (Table 1). Three deaths were observed with case fatality rate <1%, (3/309) of which all three patients were suffering from severe dengue and one had dengue-chikungunya co-

infection and meningitis. Among the patients visiting CSTM, maximum dengue cases were reported from Kolkata (n=90) followed by North-24 parganas (N 24 pgns) (n=78), South-24 parganas (S 24 pgns) (n=57), Howrah (n=30) and Hooghly (n=16) (Figure 1A). Number of dengue infected cases started rising from July onwards and reached to maximum in September (n=74) and October (n=95), every year during the study period (Figure 1B). Age of all dengue infected patients ranged between 9-72 years with mean age 36.06±7.2 years (mean±SEM). The qRT-PCR was most effective in detecting dengue infection among 67.31% (208) cases, followed by dengue NS1 (60.19%), anti-dengue IgM (48.54%) and anti-dengue IgG (20.38%) ELISA (Table 1). The qRT-PCR was also found to be more efficient in detecting DENV genome within febrile phase (0-3 days) of infection, whereas, anti-dengue IgM ELISA was more effective in late stage of infection (Figure 2). Categorization of dengue positive cases, according to WHO 2009 classification, revealed 9.70% (30/309), 47.57% (147) and 42.71% (132) cases to be having severe dengue, dengue with warning signs and without warning signs, respectively. Phase-wise distribution of patients after symptomatic onset showed that 26.21% (86), 24.59% (76) and 49.19% (152) dengue patients were within febrile, critical and recovery phases, respectively. Among 309 dengue patients, 79.61% (246) and 20.38% (63) were primary and secondary dengue infection, respectively (Table 1).

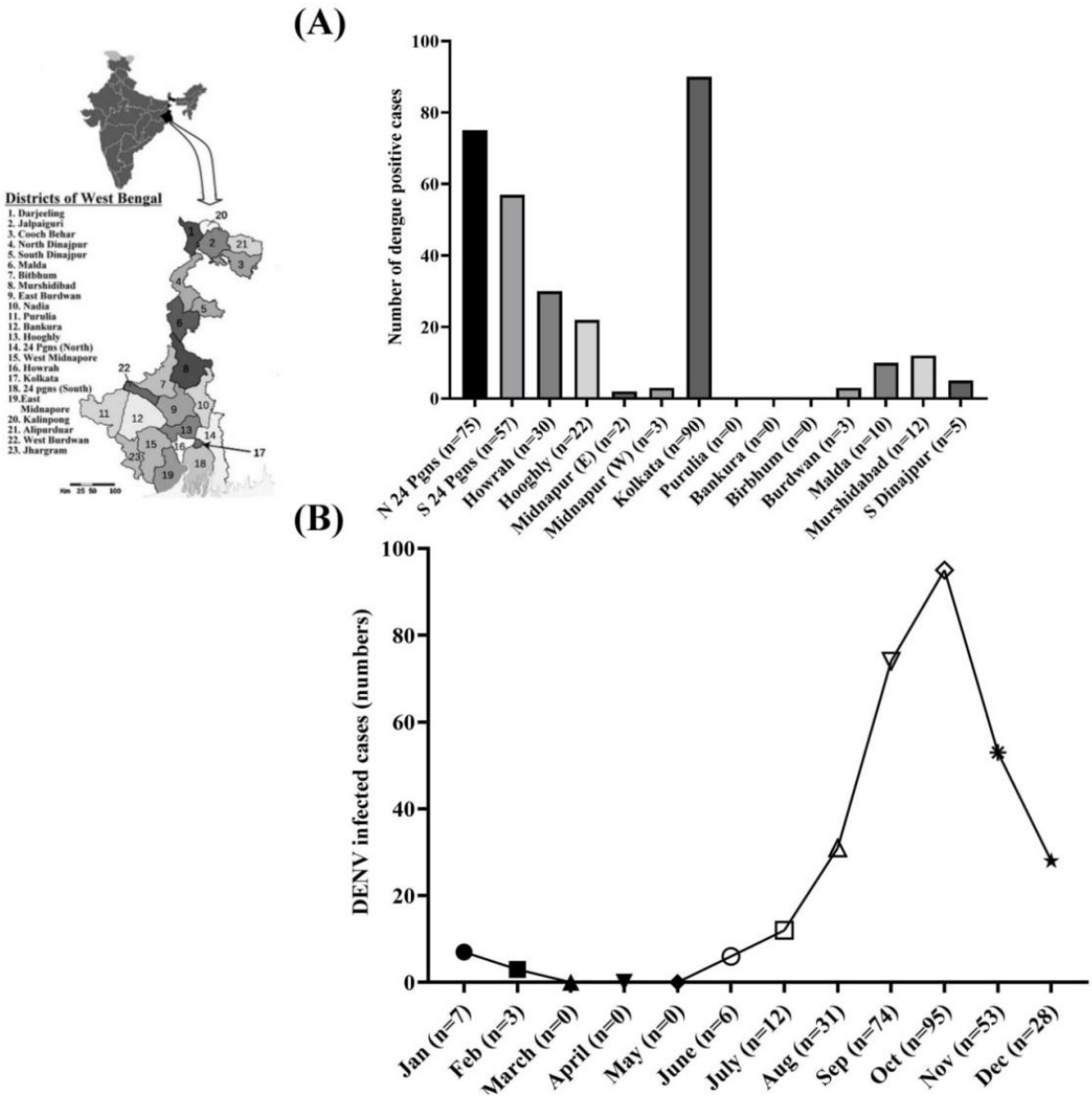

Figure 3.5: (A) District-wise distribution of dengue cases, (B) month-wise number of dengue infected cases.

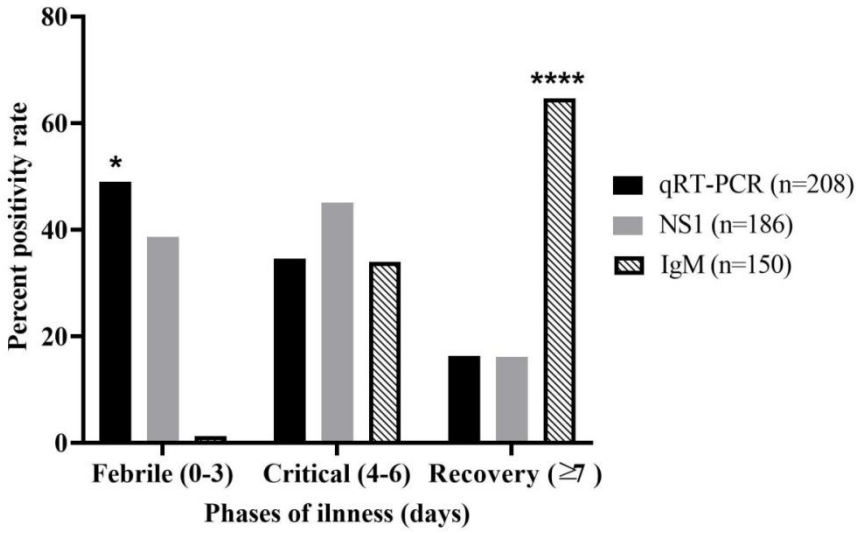

Figure 3.6: Detection efficiency of diagnostic tools among different phases of dengue infection.

Among PCR positive dengue patients, 61.05% (n=127) were having HVL and rest 38.94% (n=81) patients were with LVL (Table 1).

Age-wise comparison analysis among dengue infected patients revealed infection rate was highest among 0-20 years (31.39%) and lowest among elder age group (>61 years: 11.32%) (Figure 3A). Age-wise analysis among severe dengue, with and without warning signs patients showed younger age group patients (0-20 years) (p=0.01) were significantly more susceptible to severe form of dengue infection (Figure 3B). Same trend was also observed among patients with warning signs but was not statistically significant. Whereas, majority of dengue patients without warning signs belonged to 21-40 years age group (Figure 3A). All four serotypes were found during study period, among which 58.65% (122/208) were mono serotypic infection and 41.34% (86/208) were having multiple dengue serotypes. Among patients with single serotype infection, D2 (50.44%) was most predominant serotype followed by D4 (32.74%), D3 (17.69%) and D1 (7.07%) (Figure 3C). Whereas, among patients with multiple serotypic

infection, D2-D4 (45.97%) combination was most prevalent, followed by D2-D3 (31.03%), D1-D4 (12.64%) and D1-D2 (9.19%) (Figure 3D).

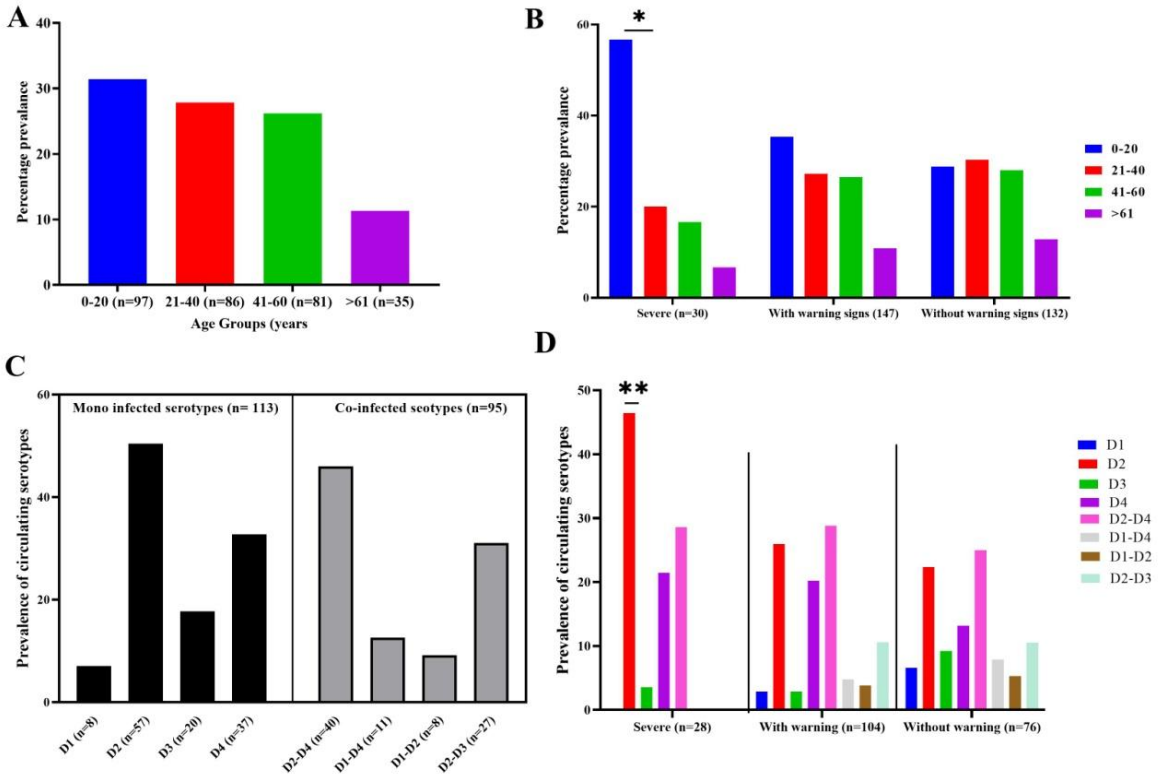

Figure 3.7: (A) Age-wise distribution of total dengue infected cases (B) Age-wise distribution among different WHO classified groups of dengue patients (C) distribution of dengue single and multiple serotypes (c) distribution of all serotypes among WHO classified dengue patients.

Clinicopathological characteristics of dengue infected patients were depicted in Table 2. Among acute stage patients, 86.66% were having severe infection; followed by 59.86% with warning signs and 32.57% without warning signs. Majority of severe (89.28%) and with warning signs (65.38%) dengue patients were harbouring dengue with HVL (p=0.00014), with mean viral load of 696698±67203 copies/ml and 71819±27823 copies/ml, respectively compared to those without warning signs (HVL: 44.73%, mean

viral load: 10023±4183 copies/ml) (Table 2). Among PCR positive dengue cases (n=208), severe and WHO–defined warning signs viz. retro-orbital pain (p=0.02), bleeding (p=0.0001), thrombocytopenia (p=0.0004), leucopoenia (p=0.0001), fluid accumulation (p=0.0001), hepatic dysfunction (p=0.0003), abdominal pain (p=0.0001) and vomiting (p=0.0001) were markedly prevalent among patients with HVL (n=127) compared to those with LVL (n=81) (Figure 4). Clinical signs such as retro-orbital pain (p=0.024) and hepatic dysfunction (p<0.0001) were significantly more common among severe dengue patients compared to those without warning signs. Clinical symptoms, viz. abdominal pain, vomiting, bleeding, fluid accumulation, thrombocytopenia and leukopenia were exclusively present only among severe dengue patients and those with warning signs. Abdominal pain (p<0.0001) and vomiting (p<0.0001) were considerably higher among both severe dengue patients and those with warning signs compared to patients without warning signs (Table2). Bleeding (p<0.0001), clinical fluid accumulation (p<0.0001), thrombocytopenia (p=0.01) and leucopoenia (p=0.02) were significantly higher among severe dengue patients compared to those with and without warning signs. Haematological analysis also showed notable decrease in platelets (p<0.0001) and WBC (p=0.02) count among severe dengue cases. Biochemical analysis revealed liver enzymes such as AST and ALT were significantly increased (p<0.0001) among severe dengue patients.

Table 3.3: Clinicopathological landscapes of infected patient (2014-16).

		Patients with severe dengue (n=30)	Patients with Warning signs symptoms (n=147)	Patients without Warning signs symptoms (n=132)	*p*-value
Sample classification	Acute phase (1-5 days) (n=207)	26	88	43	**<0.001[a]**
	Late phase (≥6 days) (n=102)	4	59	89	
Viral titer (n=208)	High viral load (HVL: ≥10000 copies/ml) (n=127)	**25**	**68**	34	**0.00014[a]**
	Low viral load (LVL: <10000 copies/ml) (n=81)	3	36	42	
	Mean viral load (copies/ml)	696698±67203	71819±27823	10023±4183	<0.0001[b]
Clinical symptoms	Fever	100% (30)	100% (147)	100% (132)	ns
	Nausea	36.6% (11)	40.13% (59)	28.03% (37)	ns
	Rash	46.66% (14)	38.09% (56)	28.03% (37)	ns
	Myalgia	63.33% (19)	69.38% (102)	71.21% (94)	ns
	Arthralgia	40% (12)	42.17 (62)	35.60% (47)	ns
	Headache	50% (15)	61.22% (90)	41.66% (55)	ns
	Retro-orbital pain	56.66% (17)	19.72% (29)	3.03% (4)	0.024[b]
	Abdominal Pain	80.00% (24)	60.54% (89)	0%	<0.0001[b]
	Vomiting	63.33% (19)	62.58% (92)	0%	<0.0001[b]
	Bleeding	86.66% (26)	4.76% (7)	0%	<0.0001[b]
	Hepatic dysfunctions	90.00% (27)	30.61% (45)	5.30% (7)	<0.0001[b]
	Fluid accumulation	60.00% (18)	13.60% (20)	0%	<0.0001[b]
	Thrombo-cytopenia	76.66% (23)	51.18% (65)	0%	0.01[b]
	Leukopenia	63.33% (19)	39.37% (50)	0%	0.02[b]
Haematological parameters	Haemoglobin (gm/dL)	14.39±0.48	14.16±0.33	14.83±0.29	ns
	Haematocrit (%)	57.93±7.23	44.61±3.78	42.3±3.87	ns
	Platelets (Pt) count ($x10^3mm^3$)	104.15±13.67	159.37±5.91	172.73±2.44	<0.0001[b]
	WBC count ($x10^3mm^3$)	4.19±0.19	5.894±0.092	6.32±0.388	0.02[b]
Biochemical parameters	AST	829.21±194.34	185.89±86.67	81.65±28.54	<0.0001[b]
	ALT	757.81±203.26	168.3±92.76	72.81±21.29	<0.0001[b]

[a]: two tailed contingency chi-square, [b]: multivariant t- test and ns: no significant

$p<0.05$ was considered as statistically significant.

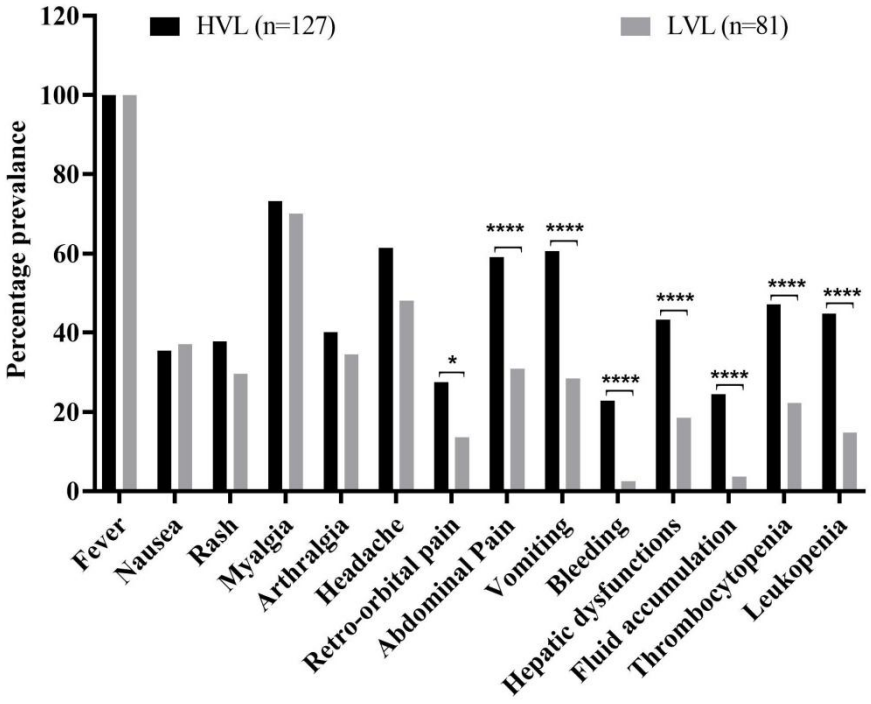

Figure 3.8: Comparative analysis of clinical symptoms between HVL and LVL groups.

3.4. DISCUSSION

This is one of the major cross-sectional study conducted in Eastern India with detailed analysis of various clinical outcomes among three WHO-classified dengue patients and circulating serotypes during 2014-2016. Both molecular (qRT-PCR) and serological assays (dengue NS1, anti-dengue IgM and anti-dengue IgG ELISA) were employed throughout the study for laboratory confirmation of dengue infection among West Bengal based eastern Indian patient population. Moreover, this is the first study from eastern India to evaluate importance DENV copy number in patient serum for disuse severity.

The qRT-PCR method could detect higher percentage of dengue infection among febrile stage patients compared to NS1 and anti-dengue IgM ELISA, indicating greater efficacy of molecular diagnostics compare to serological methods during early phase of

infection. Earlier scientific work also documented such efficiency of RT-PCR techniques in detection of dengue infection (45-48). Maximum dengue cases were reported in the months of September and October, which were mainly post-monsoon season in India and among younger patients of 0-20 year age group (31.39%). Percent prevalence of younger age group (0-20 years) patients was highest among severe dengue cases (46.66%), followed by those with (36.05%) and without (23.62%) warning signs. AK. Hati and Padhi *et al*. also found such association in their study, where maximum dengue cases were reported during September and October and dengue infection rate was highest among of 0-20 years age group (26, 49). Reason behind increasing dengue incidence rate in September-October is post-monsoon climates provide ample breeding habitats for Ae. Aegypti thereby, leading to high vector densities (50). Similar association of severe clinical manifestation among younger age-group patients was also found in previous studies from north India, Bangladesh and Vietnam (51-54). In the present study, dengue infection prevalence was highest in Kolkata and Kolkata adjoining districts, which was similar to that of previous reports from West Bengal (24, 31 and 33). Common clinical symptoms of dengue patients were fever, myalgia, arthralgia, retro-orbital pain, nausea, rash and headaches. Hallmark severe clinical symptoms viz. bleeding, fluid accumulation, thrombocytopenia, leucopoenia, hepatic dysfunction and increased liver enzymes were significantly prevalent among severe dengue cases. Previous clinical findings from this region also supported such association of severe clinical symptoms among DHF and DSS (severe dengue) patients (55-57). In severe dengue, plasma leakage is evident by detection of clinical fluid accumulation in terms ascites and plural effusion (2). The mechanism behind such phenomenon is massive T-cell activation during severe dengue infection, producing pro-inflammatory cytokines that begin the breakdown of glycocalyx layer to allow plasma to reach

intracellular junction and induce vascular leakage (2, 58-59). Previous study suggested that leukopenia may be caused by DENV induced damage of myeloid progenitor cells and thrombocytopenia occurs due to increased immune mediated destruction and suppression of bone marrow resulting from virus-antibody complex formation and DENV induced complement activation (60-62). In this study we found significantly high levels of liver enzymes viz. AST and ALT among severe dengue patients which might be either a consequence of direct viral toxicity or DENV induced immunologic injury of liver (63-64).

In this study we found that dengue patients with HVL had significantly increased severe clinical signs than that of patients with LVL, which emphasized that viral copy number might play an important role in development of severe disease outcome. Previously, Pal *et al*. and Pathak *et al*. also found such association between high viral load with severe clinical manifestations (29, 65). All four dengue serotypes were found during this study period with both monotypic and multiple serotypic dengue infection. Among monotypic infection, D2 was most prevalent, also found in maximum numbers of severe dengue patients. D2-D4 serotypic combination was most prevalent among multiple serotypic infections. Such predominance of D2 was also previously reported from West Bengal (31-34). Earlier studies have also revealed that D2 might be associated with severe disease outcome (18-19).

As both Dengue and Chikungunya infection have overlapping clinical symptoms and transmitted by the same vectors, all patients of this study were tested for chikungunya by RT-PCR and anti-chikungunya IgM ELISA, of which 37 were chikungunya positive and 75 were positive both dengue-chikungunya co-infections and were eliminated from the study.

3.5. CONCLUSIONS:

In conclusion, this study provides evidence of widespread distribution of dengue infection and co-circulation of both mono and multiple dengue serotypes among eastern Indian patients. This study also highlights serotype specific differences in clinical manifestations and importance of viral load in triaging severe dengue outcome beforehand for early and better clinical management of infected patients.

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Chapter – 2

Partial sequence analysis of DENV envelope (E) region.

4. CHAPTER – 2

1. Partial sequence analysis of DENV envelope (E) region.

- **Publications:**

The scientific work mentioned in this chapter has been communicated for publishing in peer reviewed journal:

Phylogenetic analysis of partial pre-membrane and envelope sequences of dengue viruses circulating in India. (Communicated)

4.1. REVIEW OF LITERATURE:

Dengue virus (DENV) infection is currently becoming one of the major public health concerns in dengue endemic regions of tropical and sub-tropical countries of Southeast Asia, including India (1-2). Dengue viral infection is clinically classified into mild and self-limiting dengue fever (DF) to severe dengue (dengue hemorrhagic fever: DHF and dengue shock syndrome: DSS) which may lead to potentially fatal complications (1, 4). DENV is a single stranded RNA virus belongs to the *Flavivirius* family with four genetically different serotypes (those having up to 70% homology among each other) which are further classified into various genotype (4-7). DENV genome comprises of three structural genes (capsid, membrane and envelope) and 7 non-structural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (8). The degree of a dengue outbreak is influenced by a shift in the circulating dengue virus serotypes/genotypes, pre-existing immunity pattern of a given population in relation to circulating genotypes/serotypes (9-10). Genomic shifts among circulating serotypes/genotypes have resulted in major outbreaks/epidemics in dengue endemic countries like India, Columbia, Malaysia and China (11-13). Burden of dengue in India had been shown to be grossly under-reported with clinically diagnosed cases being more than 200 times the reported number between 2006 and 2012 (14-15). Repeated dengue epidemics in endemic and hyperendemic regions of all over India, resulting in, co-circulation of multiple serotype with multiple genotypes, which eventually contributing to the complex mechanisms involved with pathogenesis in dengue (16-17). After its first report in Kolkata in 1963, dengue has become endemic in most of the Indian cities including eastern Indian states like West Bengal and Odisha (18-26). Being an RNA virus, DENV genome mounts up mutations

because of the error prone viral RNA polymerase which occurring throughout the viral genome eventually become a part of the genome and aid in the emergence of new genotypes/serotypes which harbor appreciable genetic diversity (10). Antibody against a particular serotype may provoke antibody dependent enhancement (ADE) when exposed to subsequent heterologus serotypic infection that can lead to DHF/DSS (27). DENV outbreaks took place when such new serotypes/genotypes emerge after an interval of time, in a particular region. Furthermore, inside same genotype, newer strains can either cluster into different clades or form distinct clades. These mutated viruses might lead to altered virulence and in due course influence disease patterns and transmission (10).

Hence, a continuous molecular monitoring of DENV genomes in a given region is necessary for better understanding of clinical spectrum associated with different strains. In the light of above observations, we performed phylogenetic and partial sequence analyses of DENV sequences representing all the four serotypes circulating West Bengal, an Eastern Indian states of India during 2016.

4.2. MATERIAL AND METHODS

4.2.1. Ethics Statement:

All experiments performed in this study, including collection of patient's blood as well as that of healthy individuals were done accordingly ethical standards of Clinical Research Ethical Committee, Calcutta School of Tropical Medicine, Kolkata (CREC-STM/53 dated 26.09.2013), following 1964 Helsinki Declaration and its later amendments. Written consents were received from patients and healthy individuals prior to participating in this study.

4.2.2. Detection of anti-Dengue-IgM & IgG Antibodies and Dengue-NS1 Antigen:

Suspected patients' sera were subjected to serological diagnosis for presence of dengue-NS1 antigen and anti-dengue-IgM, using IgM MAC ELISA kit (National Institute of Virology, India; sensitivity: 96% and specificity: 87%) and dengue-NS1 ELISA kit (Panbio, Austrelia; sensitivity: 77.7% and specificity: 93.6%), respectively, according to manufacturer's instructions as described in **Chapter-1**. Patient-serum was subjected to serological diagnosis for presence of anti-dengue-IgG antibody using Panbio dengue IgG capture ELISA kit (80.9% sensitivity, 87.1% specificity).

4.2.3. Extraction of Viral RNA and Determination of DENV Load:

Viral-RNA was extracted from 140µL of patients' sera, using QIAamp Viral RNA Mini Kit, according to manufacturer's protocol (Qiagen, Courtaboeuf, France) as described in **Chapter-1**. Presence of DENV genome and viral-load among infected patients' serum was determined by qRT-PCR (Taqman assay) in 20µl, using 8µl viral-RNA, OneStep qRT-PCR MasterMix and other components of DENV Genesig kit according to manufacturer's protocol (Primer Design Ltd., UK) as described in **Chapter-1**. Real time PCR was performed on ABI Prism 7500 Fast instrument. A standard-curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided in the kit). All samples were run in triplicate and DENV RNA copy number in patients' serum was calculated from their corresponding Ct values. Viral-RNA titer ≥10,000 copies/ml and <10,000 copies/ml was considered as high viral-load and low viral-load, respectively.

4.2.4. Determination of DENV Serotypes:

Dengue serotypes of PCR positive samples were determined by real-time RT-PCR in 20µl reaction mixture, containing 8µl viral RNA, One Step qRT-PCR Master-Mix and other components of DENV serotyping Genesig kit, according to manufacturer's protocol (PrimerDesign Ltd., UK) as described in **Chapter-1**. Reactions were carried out in ABI 7500 Fast instrument using serotype specific DENV controls present within the kit.

4.2.5. cDNA Conversion:

DENV RNA with Ct<30 were selected for sequencing. cDNA was synthesized in VERITI 96-well thermal cycler (Applied Biosystems, USA) using 3µg of DNase treated RNA and RevertAid First Strand cDNA Synthesis Kit, according to manufacturer's protocol (Thermo Fisher Scientific, USA). Briefly, 10x DNase-I buffer and enzyme DNase I, were added to the RNA template (3µg), further nuclease-free water was used to make the final volume up to 10µl. The mixture was then incubated at 37^{o}C for 30 minutes. After incubation, 1µl of 50mM EDTA was added to the mixture followed by incubation at 65°C for 10 minutes. Next, 1µl of random primer was added to the DNase treated RNA and incubated at 65°C for 5 minutes. To the above mixture, 4µL 5x Reaction Buffer, 1µL RiboLock RNase Inhibitor (20 U/µL), 2µL 10mM dNTP mix, and 1µL RevertAid M-MuLV RT (200 U/µL) were added. All the components were then mixed gently and centrifuged briefly. The mixture was then incubated at 25°C for 5 minutes, 42°C for 60 minutes and at 70°C for 5 minutes respectively in VERITI 96-well thermal cycler (Applied Biosystems, USA) and further the tube containing cDNA was stored at -20^{o}C for future use.

4.2.6. Primer Design and PCR Amplification of DENV Envelope Protein Partial Sequence:

The E protein gene sequences available in GenBank database of all dengue serotypes (DENV1, 2, 4: 1485bp and DENV3: 1479bp) circulating in India in between 2007-2016 were retrieved aligned in ClustalW software. Based on sequence clustal alignment two set of primer pairs for each serotype were designed using Primer3 software to amplify a segment of E region (Figure 4.1) (Table 4.1). PCR reaction was performed using prepared cDNA, in 20µl volume, using 1xPCR buffer (Fermentas, USA), 1mM of each dNTP, 1unit of Taq DNA polymerase (Fermentas, USA), 1.5mM $MgCl_2$ and 20p.moles of previously mentioned primers. Reaction mixture was then subjected to 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 52-55^{o}C for 1 min, and extension at 72^{o}C for 10 min. PCR reactions were carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA) and PCR products were electrophoresed on 2% agarose gel at 5 V/cm for an appropriate time period. The products were then visualized for 500-600bp DENV E specific PCR product was visualized after staining with ethidium bromide and compared using standard molecular weight markers (Figure 4.2). PCR product concentrations were measured in BIORAD XR+ Gel Documentation system (BIORAD, California, USA).

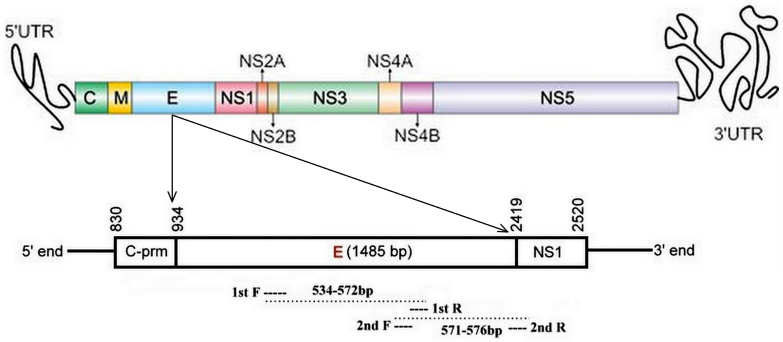

Figure 4.1: Representative illustration of DENV E region overlapping primer construction.

Table 4.1: List of primers pairs used for sequencing

Sr. No.	Serotype	5'-Primer-3'	PCR product Size
1.	DENV-2 set 1	F- CAGGAAAACATGGYAAGG R- ATGGAGARCCRTCYCCTTC	534bp
2.	DENV-2 Set 2	F- AGCAGARACACAACATGG R- CCARCTCACAACRCAACCAC	574bp
3.	DENV-3 set 1	F- CAACACCARGTGGGAAAT R- CCCCTTTGTACTCRACCTTA	538bp
4.	DENV-3 Set 2	F- AGTCTCAGAAACGCAGCA R- TAYGACACACCCCATGTC	571bp
5.	DENV-4 set 1	F- CACCCATGCDGTRGGAAA R- CTATGGGRACTTTRCAYGG	572bp
6.	DENV-4 Set 2	F- CACAGCATGGRACRACAG R- CTYTCCCAYTCCATGACA	576bp

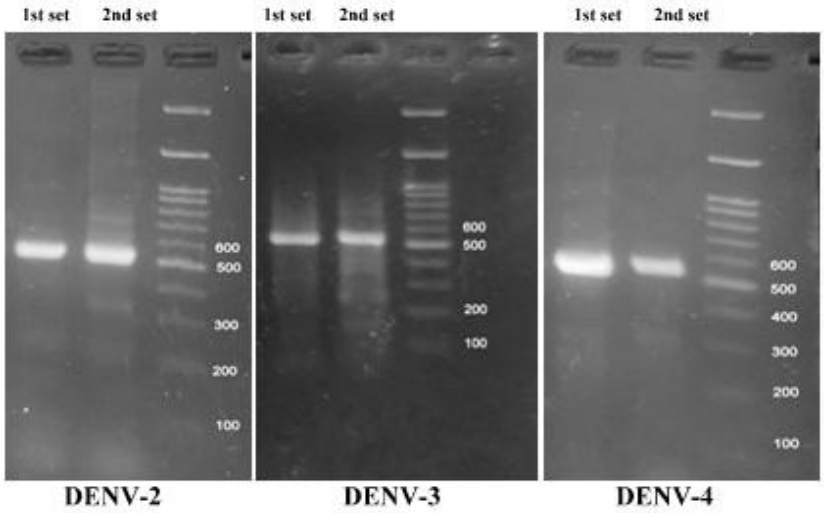

Figure 4.2: Representative image of PCR amplified regions of DENV E regions

4.2.7. Sequencing and Phylogenic Analysis:

The PCR products were then purified using PCR purification kit (QIAquick PCR Purification Kit, Valencia, CA, USA) according to manufacturers instruction. Briefly, 10µl PCR product was mixed with 50µl of buffer PB. The mixture was then added to QIAquick column placed in collecting tubes. The column was then centrifuged at 13000rpm for 1 minute. After centrifugation, the collecting tube containing the filtrate was discarded and the QIAquick column was placed into fresh collecting tube. 700µl of buffer PE was added to the QIAquick column and was centrifuged at 13000rpm for 1 minute. After centrifugation, the collecting tube containing the filtrate was discarded and the QIAquick column was then placed into fresh 1.5ml collecting tube. 50µl of EB buffer was added to the QIAquick column placed in fresh 1.5ml collecting tube. The column was then centrifuged at 13000rpm for 1 minute. Finally, the elute containing purified PCR product was stored at -20°C. The purified PCR products was then used for sequencing using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (ABI-Perkin Elmer, Nordrhein-Westfalen, Germany) according to manufacturer's instructions. Briefly, 2µl of purified PCR product was mixed with 4µl BigDye™ Terminator 3.1 Ready Reaction Mix, 2µl 5X Sequencing Buffer, 3.2µM of forward primer for respective DENV serotypes and 11µl of RNase/DNase-free water. The sequencing reaction was carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA). Reactions were amplified through 25 cycles according to the following conditions: initial denaturation at 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 minutes, followed by indefinite hold at 4°C. To each tube containing mixture, 5µl of 125mM EDTA and 60µl

of 100% ethanol was added and mixed gently. The mixture was then incubated at 15-28°C for 15 minutes in dark. The tubes containing mixture was then centrifuged at 12000rpm for 1minute. The supernatant was discarded and the pellet was further washed with 70% ethanol, by centrifugation at 10000rpm for 10 minutes. The pellet was then air dried at room temperature and was resuspended in 10µl Hi-Di™ Formamide. The resuspended pellet was then transferred to the 96 wells Plate base (catalog #4317237, Thermo Fisher Scientific, USA). The plate containing resuspended pellet was placed in ABI-Prism 3100 Avant Genetic Analyser (ABI-Perkin Elmer, Nordrhein-Westfalen, Germany) for sequencing. All amplicons were sequenced on both forward and reverse strands. The quality of base calling was checked by PHRED score analysis using ABI sequence analysis software V.5.1.1.

Raw sequences were subjected to similarity search using BLAST. The obtained partial nucleotide sequences of DENV E genes were submitted to the GenBank and were compared with those reported in GenBank database from other geographic regions. Phylogenetic analysis of partial nucleotide sequences of DENV E gene sequences obtained in this study were performed by using MEGA 5.2.2 (28). For construction of phylogenetic trees, the Maximum Likelihood and the Kimura two-parameter distance model were utilized. Statistical support for the nodes was assessed by bootstrapping with 1000 replicates.

4.3. RESULTS:

Sequencing was performed for RT-PCR amplified DENV E region of 20 dengue virus isolated from patient-sera selected according to their C_T values (C_T<30). The partial

nucleotide sequence of E gene of three dengue serotypes viz. DENV-2, DENV-3 and DENV-4 was determined in the present study, which was submitted to NCBI GenBank database. We identified more than one case each of DENV-2 genotype Cosmopolitan, DENV-3 genotype-III and DENV-4 genotype-I after sequencing among analysed patients. Since, all patients with DENV 1 infection demonstrated high C_T values (>30), DENV 1 E region could not be sequenced. Detailed clinical history of the patients sequenced for DENV E region has been listed in Table 4.2 with their NCBI GenBank accession numbers (Figure 4.3). Among analysed sequenced one DENV-2 cosmopolitan genotype, one DENV-4 genotype-I was belonging to severe dengue and rest were from WHO-classified with warning signs patients. Only one DENV-2 cosmopolitan genotype with NCBI GenBank accession number OQ933234.1 was having secondary dengue infection.

Table 4.2: List of dengue positive samples with GenBank accession number.

Sr. No.	Serotypes	NCBI Accession number	Viral load (copies/ml)	Type of infection	Disease category
1.	Dengue 2	OQ933234.1	8.67 $X10^5$	secondary	Severe
2.	Dengue 2	OQ970554.1	3.1 $X10^5$	Primary	With warning signs
3.	Dengue 2	OQ970583.1	1.89 $X10^5$	Primary	With warning signs
4.	Dengue 2	OQ970584.1	3.9 $X10^5$	Primary	Severe
5.	Dengue 3	OQ970586.1	5.6$X10^4$	Primary	With warning signs
6.	Dengue 3	OQ970640.1	4,5$X10^4$	Primary	With warning signs
7.	Dengue 3	OQ996974.1	3.89$X10^4$	Primary	With warning signs
8.	Dengue 4	OQ976898.1	8.89$X10^4$	Primary	With warning signs
9.	Dengue 4	1OQ998329.1	2.1 $X10^5$	Primary	Severe
10.	Dengue 4	OQ976903.1	7.287$X10^4$	Primary	With warning signs

(A)

Dengue virus type 2 isolate D2201 polyprotein, envelope protein E region, (POLY) gene, partial cds

GenBank: OQ970554.1

FASTA Graphics

Go to:

```
LOCUS       OQ970554                939 bp    RNA     linear   VRL 17-MAY-2023
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            region, (POLY) gene, partial cds.
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VERSION     OQ970554.1
KEYWORDS    .
SOURCE      Dengue virus type 2
  ORGANISM  Dengue virus type 2
            Viruses; Riboviria; Orthornavirae; Kitrinoviricota; Flasuviricetes;
            Amarillovirales; Flaviviridae; Flavivirus.
REFERENCE   1  (bases 1 to 939)
  AUTHORS   Tripathi,A. and Mukherjee,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-MAY-2023) Biochemistry and Medical Biotechnology,
            Calcutta School of Tropical Medicine, Calcutta School of Tropical
            Medicine, 108 c.r. avenue, Kolkata, West Bengal 700073, India
COMMENT     ##Assembly-Data-START##
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//
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(B)

Dengue virus type 3 isolate D2217 polyprotein, envelope protein E region, (POLY) gene, partial cds

GenBank: OQ970586.1

FASTA Graphics

Go to:

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  ORGANISM  Dengue virus type 3
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            Amarillovirales; Flaviviridae; Flavivirus.
REFERENCE   1  (bases 1 to 972)
  AUTHORS   Tripathi,A. and Mukherjee,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-MAY-2023) Biochemistry and Medical Biotechnology,
            Calcutta School of Tropical Medicine, Calcutta School of Tropical
            Medicine, 108 c.r. avenue, Kolkata, West Bengal 700073, India
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(C)

Dengue virus type 4 isolate D2226 polyprotein, envelope protein E region, (POLY) gene, partial cds

GenBank: OQ976898.1

FASTA Graphics

Go to:

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            Amarillovirales; Flaviviridae; Flavivirus.
REFERENCE   1  (bases 1 to 992)
  AUTHORS   Tripathi,A. and Mukherjee,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-MAY-2023) Biochemistry and Medical Biotechnology,
            Calcutta School of Tropical Medicine, Calcutta School of Tropical
            Medicine, 108 c.r. avenue, Kolkata, West Bengal 700073, India
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//
```

Figure 4.3: Representative photographs of DENV E region partial sequences of (A) dengue serotype 2 (B) dengue serotype 3 and (D) dengue serotype 4 submitted to the GenBank database of National Institute of Health, USA.

4.3.1. Phylogenetic Analysis:

Phylogenetic analysis revealed circulation of cosmopolitan genotype of DENV 2 serotype among Eastern Indian patients. These genotypes were closely related to strains previously reported from Indian, Bangladeshi and Chinese patients with 95.8%- 98.2% (mean 97.15%) nucleotide identity (Figure 4.4).

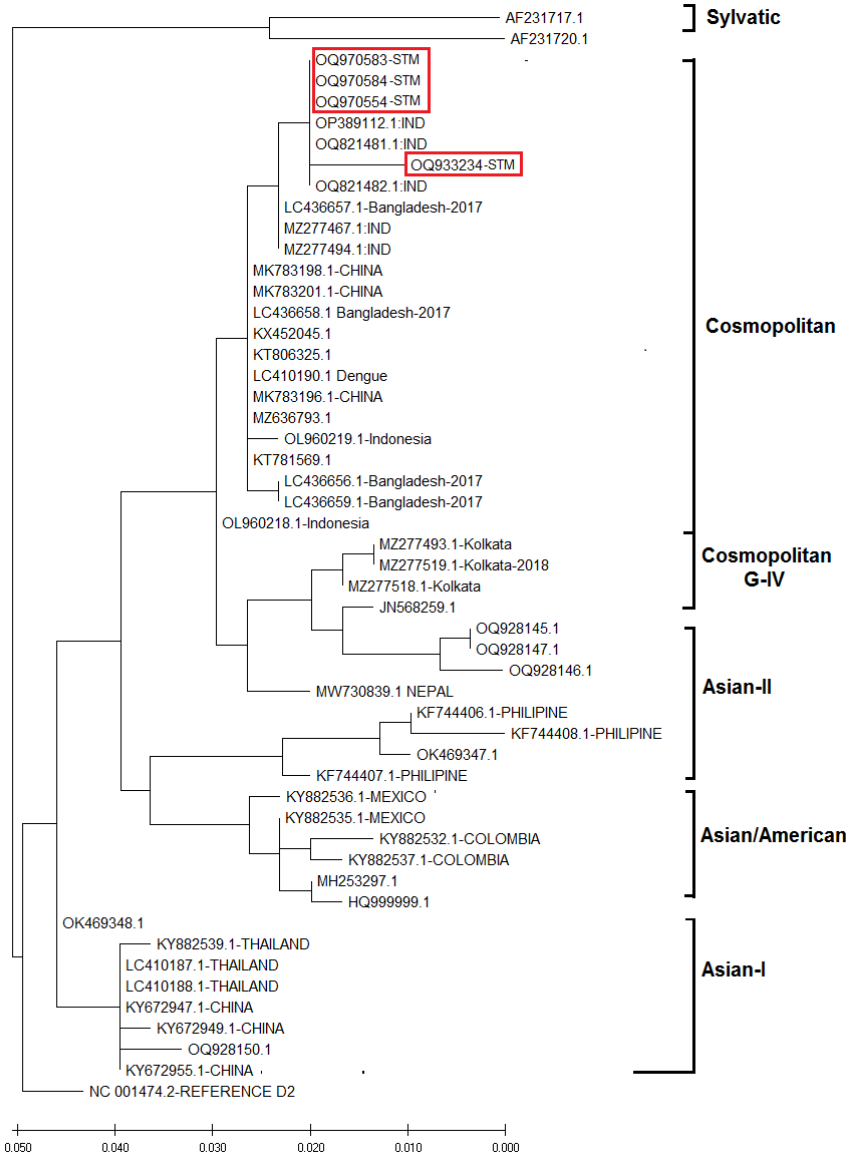


Figure 4.4: Maximum Likelihood Phylogenetic tree of DENV-2 isolates. Isolates sequenced in the study are marked by red colored square. Numbers on nodes indicate bootstrap support generated by 1000 replicates.

Comparing with global DENV-3 sequences, sequenced DENV-3 isolates of this study exhibited 97.27% sequence similarity with previously reported strains from New Delhi, Maharashtra and Gujarat regions of India with mean sequence divergence values of 3.75%. Whereas, phylogenetic analysis of DENV-3 revealed that the circulating DENV-3 serotypes belonged to genotype-III and shared two different clades under the same genotype (Figure 4.5).

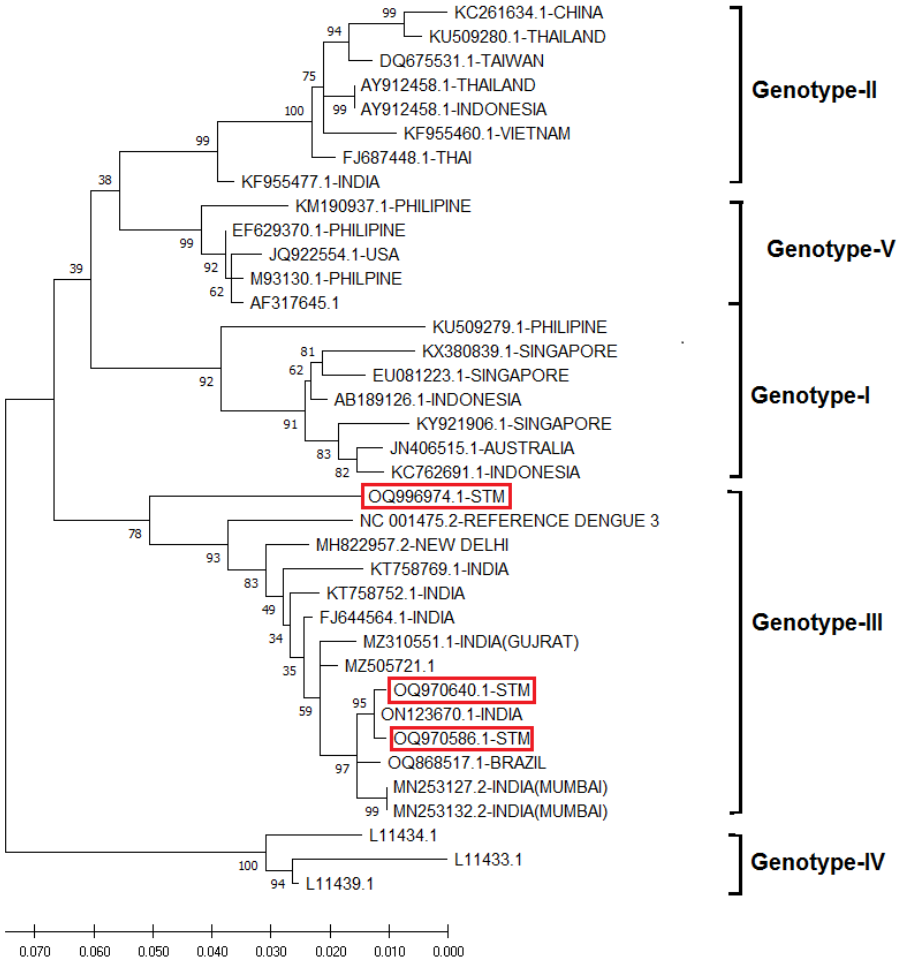


Figure 4.5: Maximum Likelihood Phylogenetic tree of DENV-3 isolates. Isolates sequenced in the study are marked by red colored square. Numbers on nodes indicate bootstrap support generated by 1000 replicates.

Comparing with global DENV 4 sequences retrieved from NCBI nucleotide database, sequenced DENV 4 isolates of this study exhibited nearly 99% sequence similarity with previously reported strains from western part of India viz. Maharashtra, Rajasthan and Punjab with a mean divergence rate of 2.7% (figure 4.6).

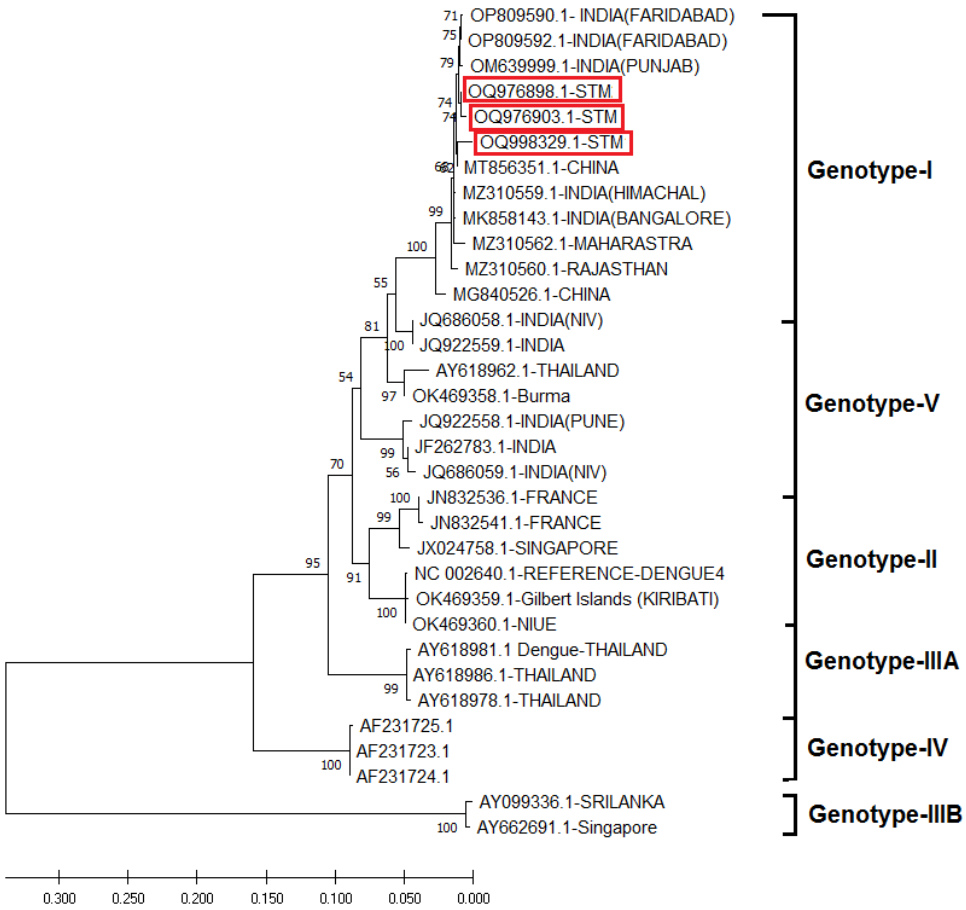
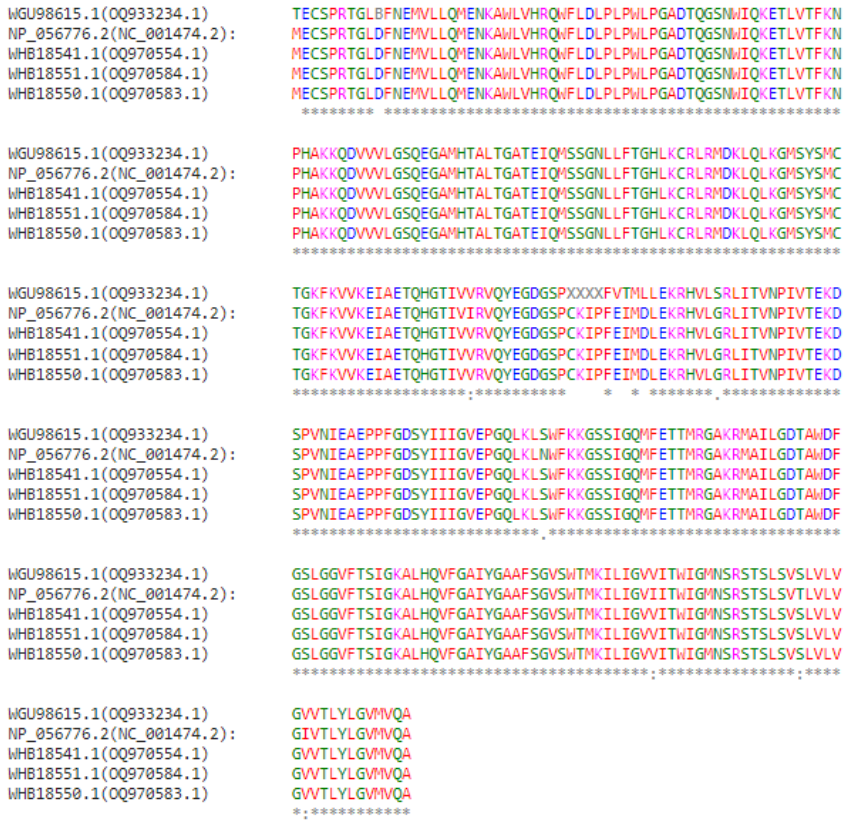


Figure 4.6: Maximum Likelihood Phylogenetic tree of DENV-4 isolates. Isolates sequenced in the study are marked by red colored square. Numbers on nodes indicate bootstrap support generated by 1000 replicates.

4.3.2. Nucleotide Sequence and Amino Acid Alignment Analysis:

Partial nucleotide sequences of the E genes of three dengue virus serotypes determined in the present study were submitted to NCBI GenBank database. Out of 1485bp of DENV 2 and DENV 4 E region, 939bp (nt546-1485) and 963bp (nt522-1485) were

sequenced, respectively. For DENV 3, 972bp (nt 507-1479) of total 1479bp of E region was sequenced. These sequences were compared and aligned with previously reported NCBI reference sequences for respective serotypes (NC_001474 for DENV2, NC_001475 for DENV3 and NC_002640 for DENV 4). The alignment found amino acid changes in 10 different positions of DENV2 (Figure 4.7), in 11 different positions of DENV3 (Figure 4.8) and in 23 different positions of DENV 4 submitted sequences (Figure 4.9). But none of these amino acid substitutions were situated in the crucial residues (N153, K291, K295, T405, F429 and F436) of the DENV E protein, required for its attachment with host cell receptors.



* Indicated Identical Amino Acids; '.' Indicate postulated deletation; blank space indicated one or more residues omitted from the alighment.

Figure 4.7: Alignment of E region partial amino acid sequences of DENV-2 NCBI submitted isolates with DENV-2 reference strain (NC_001474.2). NCBI

accession ids were given under bracket with their respective proteins ids.

* Indicated Identical Amino Acids; '.' Indicate postulated deletation; blank space indicated one or more residues omitted from the alighment.

Figure 4.8: Alignment of E region partial amino acid sequences of DENV-3 NCBI submitted isolates with DENV-2 reference strain (NC_001475.2). NCBI accession ids were given under bracket with their respective proteins ids.

* Indicated Identical Amino Acids; '.' Indicate postulated deletation; blank space indicated one or more residues omitted from the alighment

Figure 4.9: Alignment of E region partial amino acid sequences of DENV-4 NCBI submitted isolates with DENV-2 reference strain (NC_002640). NCBI accession ids were given under bracket with their respective proteins ids.

4.4. DISCUSSION

First virologically proved dengue outbreak occurred in Calcutta (currently Kolkata) and Eastern Coast of India in 1963-1964. Since 2005, Kolkata has witnessed several massive dengue outbreaks with severe outcome (19-24). Large outbreaks of DENV were reported from various parts of West Bengal during 2014-2017, affecting 73,071 people

and death of 109 dengue patients (29). But till date, no study has been performed to analyse sequences, genotypes and phylogeny of dengue serotypes infecting patients of this region. This is the first study to identify the dengue virus subtype/genotype and lineages infecting eastern Indian patient population, using partial sequencing of E region. Previously, E-NS1 junction, C-prM and E region were commonly used for DENV genotyping; phylogenetic tree based on sequence of any of these regions have the same topology across genotypes (30-32).

Due to highest mutation rate among Flavivirus group, dengue serotypes are divided into different genotypes and lineages (33-35). DENV2 represent six different genotypes including Asian-I, Asian-II, cosmopolitan, Asian/American and sylvatic genotype. In this study, all sequenced DENV-2 isolates belonged to Cosmopolitan genotype which have a wide geographic distribution and has been previously reported from northern part of India viz. Delhi, Luckhnow and Gwalior (36, 37). DENV-2 Cosmopolitan genotypes have circulated in Asia for past 55 years and were very much restricted to the courtiers of south Asia. Earlier studies from India have confirmed circulation of American genotype in India from 1956 to 1980s and then from 1974 onwards, strains clustered mostly under cosmopolitan genotype (38-39). Recent studies found circulation and micro-evolution of more than one lineage of DENV-2 Cosmopolitan genotype, which might contribute to dominant emergence of DENV-2 in various states of India (40)

In this study partial sequencing of DENV E region revealed that all circulating DENV-3 isolates belonged to genotype-III. This genotype-III of DENV-3 has been previously reported to cause major dengue epidemics in several parts of Asia, Africa and the United States, and also has been implicated to have potential to cause international

dengue pandemic (41, 42). In India, genotype III isolates of DENV-3 have been previously identified to cause major outbreaks in Delhi and Gwalior (43-46). It is important to note that DENV-3 isolates mentioned in this study form a distinct clade within genotype-III and it would be desirable to assess role of this clade in disease severity.

In this study period, DENV-4 emerged as second leading serotype after DENV-2 and was also found to be associated with severe disease outcome. DENV-4 had been genetically classified into five genotypes, genotype-I to genotype-V; genotype-II has been further sub-divided into two groups viz. Genotype-IIA and IIB (47). In the current study, circulating DENV-4 isolates were genetically classified as genotype-I, which has been previously reported from other Indian states viz. Delhi, Maharashtra, Telangana and Andhra Pradesh (D4 imp). In India, first reported DENV-4 circulating genotype was of genotype-V from Tamil Nadu in 1968 (48). In India a strain (JQ922559, 1978) with an unknown origin was reported to be the ancestor for DENV-4 genotype-I (48). Co-infection with multiple serotypes poses risk of emergence of recombinant virus strains that could have distinct properties (14).

In the current study, multiple amino acid substitutions within DENV E region were reported among sequenced isolates of DENV-2, DENV-3 and DENV-4, which differed from their respective NCBI reference sequences. However, none of these amino acid substitutions were situated in the crucial residues (N153, K291, K295, T405, F429 and F436) of DENV E protein, required for its attachment with host cell receptors. Detailed analyses are required to understand the importance of these amino acid substitutions in disease pathogenesis.

4.5. CONCLUSIONS:

Present study is the first from eastern Indian region to sequence and phylogenetically analyse DENV-2, DENV-3 and DENV-4, which revealed that the circulating strains ~~were~~ belonged to cosmopolitan genotype, genotype-III and genotype-I, respectively. This warrant of getting the complete picture of all circulating DENV genotypes and any shift in dominance of these genotypes that might help to determine the epidemiological and evolutionary pattern of Dengue outbreaks in eastern India.

4.6. REFERENCES (CHAPTER 2):

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CHAPTER – 3

Identification of differential genetic makeup of innate immune response: Toll Like Receptor (TLR)-pathway molecules (TLR3, TLR7 and TLR8) between WHO classified dengue patients (with warning signs, without warning signs & severe) and healthy individuals.

5. Chapter – 3

1. Identification of differential genetic makeup of innate immune response: Toll Like Receptor (TLR)-pathway molecules (TLR3, TLR7, TLR8 and MyD88) between WHO classified dengue patients (with warning signs, without warning signs & severe) and healthy individuals.

- **Publication:**

The scientific work mentioned in this chapter has been published in the following peer reviewed journal:

- **Mukherjee S**, Tripathi A*. Contribution of Toll like receptor polymorphisms to dengue susceptibility and clinical outcome among eastern Indian patients. **Immunobiology**. 2019; 224(6): 774-785. doi: 10.1016/j.imbio.2019.08.009. Epub 2019 Aug 25. PMID: 31481269.

5.1. REVIEW OF LITERATURE:

DENV infection has wide range of clinical symptoms ranging from self limiting febrile illness/simple dengue to life threatening severe complications viz. dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (1). Only 1% of simple dengue patients might develop severe dengue (DHF/DSS) with 10% to 20% case fatality (1). World Health Organization (WHO) has categorized the large group of non-severe dengue patients into two subgroups- patients with and without warning signs; the earlier subgroup being at higher risk of developing severe dengue (1).

Reasons behind such disparity in clinical outcome among DENV infected individuals remain inadequately understood, host immuno-genetic makeup might play vital role in determining disease pathogenesis (12-13). Several studies has been reported that host innate immune response plays an essential part during early stages of DENV infection in both priming protection as well as disease induction through pattern recognition receptors (PRRs) (14-15). Toll-like receptors (TLRs), one of the most important category of PRRs, abundant on surface of monocytes/macrophages and dendritic cells (DCs) are the key sensors to primarily recognise any viral genomic RNA within patient-body and triggers antiviral response by type–I interferon signalling (16-17). TLR3 recognizes viral double stranded RNA genome (produced during replication cycle of several viruses), resulting in production of type-I interferons (18). Role of TLR3 during DENV2 infection was first documented by Tsai et al. by using in-vitro infection model (19). Similar to, TLR3, TLR7 and TLR8 are part of TLR7 subfamily, located inside endosome and acts to recognize viral single stranded RNA (18, 20). Sun et al. elucidated involvement of TLR7-dependent signaling pathways in DENV recognition (21). Another study highlighted TLR7 and TLR8 induced robust activation and production of

TNF-α in dengue infection among children (22). Thus, host innate immune responses towards dengue disease pathogenesis might be influenced by functional and genetic variations in TLR genes viz. single nucleotide polymorphisms (SNPs), which thereby might affect disease susceptibility and pathogenesis.

TLR3 is located in chromosome number 4, whereas, TLR7 and 8 are located on X chromosome (23-24). Several polymorphisms of TLR genes viz. rs3775290 (TLR3); rs179008, rs179010, rs5741880, rs3853839 (TLR7); rs3764879, rs3764880 and rs5744080 (TLR8) have been reported to be associated with hepatitis-B (HBV), hepatitis-C (HCV), chikungunya, dengue, HIV and Crimean–Congo Hemorrhagic fever virus infection among French, German, Indian, Spanish, Brazilian, Chinese, Japanese, Polish, Moroccan and Turkish populations (25-35). Genetic locations of these polymorphisms have been depicted in Figure 5.1.

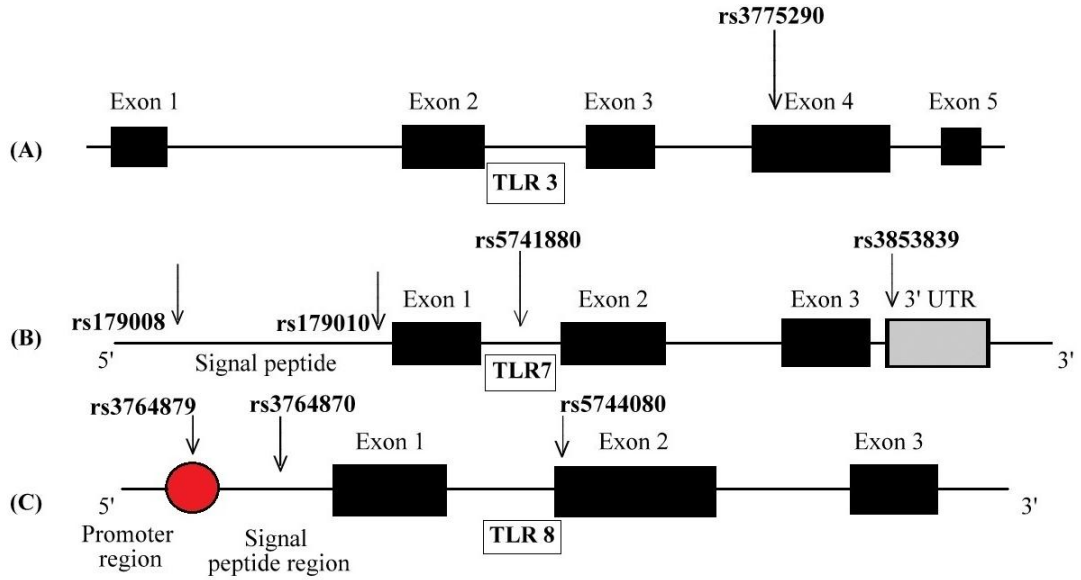


Figure 5.1: Schematic representation of genomic location of (A) TLR 3 polymorphism viz. 3775290, (B) TLR 7 polymorphisms viz. rs179008, rs179010, rs5741880 and rs3853839 (C) TLR 8 polymorphisms viz. rs3764879, rs3764880 and rs5744080.

Variations in genotype of rs3764879 within TLR-8 gene have been predicted to modulate patient's immune responses during HCV infection (32). The G allele and A/G genotype of rs3764880 has been related to clearance of HCV infection and protection from progression of HIV infection (28, 36). All these studies indicated importance of TLR polymorphisms towards viral disease infectivity and disease pathogenesis among different ethnic human populations.

Since DENV is an RNA virus, it is plausible that functional variations within TLRs might alter recognition of viral RNA, thereby affecting post-infection clinical outcome. But limited studies were done from eastern India and as well as other dengue endemic regions of India to find out the role of human TLR polymorphisms in DENV susceptibility and disease severity and all these previous data indicated the importance of host genetics of Eastern Indian patients towards developing dengue disease susceptibility/severity. Thus, in this present study, attempts have been made to understand contribution of TLR3, 7 and 8 polymorphisms towards developing dengue susceptibility and disease severity among infected Eastern Indian patients.

5.2. MATERIALS AND METHODS:

5.2.1. Ethics Statement:

All procedures performed in this study involving collection of blood from human participants (symptomatic patients as well as healthy controls) were in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013) and with that of 1964 Helsinki Declaration

and its later amendments. Written consents were received from patients and healthy control individuals prior to participation in this study.

5.2.2. Patients and Healthy Controls:

Among 309 dengue patients mentioned in **Chapter-1**, confirmed by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, 201 patients of 2014-2015 were included in Chapter-3 for determining role of patient's genetic makeup of TLR3, 7, 8 genes in determining disease susceptibility and severity. According to WHO classification, dengue patients were classified as (i) with and (ii) without warning signs. To carry out age-matched case control study, blood from 157 healthy unrelated individuals of same ethnicity, who neither had any signs and history of other infections nor were detected with dengue infection by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, were collected from the same community. Blood was collected from all age groups and both sexes.

5.2.3. SNP Selection and Genotyping:

SNPs in the TLR genes, TLR3 (rs3775290), TLR7 (rs179008, rs5741880, rs179010, rs3853839) and TLR8 (rs5744080, rs3764879, rs3764880) were selected based on their minor allele frequencies (MAF) and previously reported associations with other viral infections (25-34). SNP genotyping was carried out by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based procedure. Briefly, genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Genomic DNA was extracted from peripheral blood samples using

QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, 200µl of whole blood was mixed with 20µl QIAGEN Protease followed by addition of 200µl buffer AL. The tube was then incubated at 56^{o}C for 10 minutes. To each tube, 200µl of 96-100% ethanol was added. Lysate was then transferred to their respective QIAamp Mini spin columns. The columns containing lysate were centrifuged at 8000rpm for 1 minute and the filtrate was discarded. The columns were then washed using 500µl buffer AW1 and were placed in fresh 1.5ml micro-centrifuge tube. To each column 150µl buffer AE was added and incubated at room temperature (15-28^{o}C) for 1minutes. Then the columns were centrifuged at 8000 rpm for 1 minute. Elute containing DNA was stored at -20^{o}C for future use.

Based on sequences available in GenBank database, seven set of primer pairs were designed using Primer3 software, to amplify rs3775290 polymorphic region of TLR3; rs179008, rs5741880, rs179010, rs3853839 polymorphic regions of TLR7 and rs5744080, rs3764879, rs3764880 polymorphisms of TLR8 (Table 5.1). PCR reaction was performed in 20µl volume, using 1xPCR buffer (Fermentas, USA), 1mM of each dNTP, 1unit of Taq DNA polymerase (Fermentas, USA), 1.5mM $MgCl_2$ and 20p.moles of previously mentioned primers. PCR reactions were carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA) at desired annealing temperature for 30 cycles and PCR products were electrophoresed on 2% agarose gel at 5 V/cm for an appropriate time period. Respective PCR products were digested with TaqI (Himedia, India), Bsh1285I, ER1381, MvaI, Eco130I, TaiI, Hin1II (Fermentas, USA) and Hpy188I (NEB, UK) accordingly, as mentioned in Table 5.1 and digested products were electrophoresed in 2.5% agarose gel, followed by its visualization under UV trans-

illuminator. Different RFLP patterns were validated by sequencing of the respective PCR products using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in an ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA).

5.2.4. Statistical Analysis:

Association of any particular genotype of previously mentioned SNPs with dengue disease susceptibility and specific dengue symptoms (WHO-defined with/without warning signs) was calculated by using SPSS software version 22. Allele and genotype frequencies were compared between different study groups using Pearson's Chi square test. For genotypic associations, p-values, odds ratio (OR) & minor allele frequencies (MAF) were calculated. A p-value of <0.05 was considered statistically significant. For controls, Hardy-Weinberg equilibrium was analyzed for eight polymorphisms with program Haploview (37). The LDlink 3.0 program of National Institute of Health (NIH) was used for Linkage disequilibrium (LD) measurements (D′) by using south Asian population data, present within 1000 genome project of Genome Wide Association Studies (GWAS), the largest public catalogue of human variation and genotype data (38). Functional effects of these SNPs were predicted using SNPinfo web server and for non-synonymous effect of amino acid changes in case of non-synonymous polymorphisms was predicted by using SIFT, Polyphen2, MutationTaster and by G23D servers.

Table 5.1: List of primer sets and restriction enzymes used in PCR-RFLP.

Sr. No	Gene name with SNP Id	5'-Primer-3'	PCR product Size	Restriction Enzymes and digestion temperature	Restriction digestion pattern
1.	TLR3 rs3775290	F-GGAGCACCTTAACATGGA R-GACCAAGGCAAAGGAGTT	367bp	TaqI 65^{o} C	C : 367 bp T: 275 bp + 92 bp
2.	TLR7 rs5741880	F- CCTACTCAAGTACAAAGGGG R- TCCAGTCTCATGGTCACT	193bp	Hpy188I 37^{o} C	G : 193 bp T : 128 bp + 65 bp
3.	TLR7 rs179010	F-CCCAGTGTCTGTCATGCTAA R-CTGGCTCTTGTCTTGGATTG	335bp	BstNI 37^{o} C	T : 335 bp C : 190 bp + 145 bp
4.	TLR7 rs179008	F-CTTCTACCCTCTCGAAAGC R-TAGGAAACCATCTAGCCCC	342bp	ApoI 37^{o} C	A : 188 bp + 92 bp + 62 bp T : 188 bp + 154 bp
5.	TLR7 rs3853839	F-ACCAATTGCTTCCGTGTC R-TTTCTTCTCCCATCCTCCAG	515bp	Sty I 37^{o} C	G : 514 bp C : 97 bp + 418 bp
6.	TLR8 rs3764879	F-GTGTGTGTCTGATTTGGG R-TAGGCTCACACCATTTGC	386bp	MaeII 37^{o} C	G : 386 bp C : 161 bp + 225 bp
7.	TLR8 rs3764880	F-GTGTGTGTCTGATTTGGG R-TAGGCTCACACCATTTGC	386bp	NiaIII 65^{o} C	A :137 bp + 156 bp + 93 bp G : 136 bp + 250 bp
8.	TLR8 rs5744080	F- GTTACCCCAAATACCCTCTG R-AAGCACCACCATCACAAG	416bp	MaeII 65^{o} C	C : 237 bp + 179 bp T : 416 bp

5.3. RESULTS:

In this case-control study, genetic polymorphisms of TLR genes was investigated among 201 dengue infected patients, collected during 2014-2016 dengue outbreaks in eastern India along with age-sex matched 157 healthy control volunteers from the same region (Table 5.2). The male to female ratio of dengue patients was 1:1.27, whereas that of the control group was 1:1.01. Mean age of dengue patients was 32.01 ± 14.7 years (range: 5-70 years), whereas that of control was 36.2 ± 11.8 years (range: 18–63 years). At the time of blood collection, major symptoms among dengue patients were myalgia (78.64%), arthralgia (41.25%), headache (32.33%), rash (26.86%), nausea (25.37%),

persistent vomiting (23.88%), abdominal pain (19.90%) joint swelling (11.94%), bleeding (6.96%) and retro-orbital pain (6.46%). Among 201 dengue patients, 126 were positive by qRT-PCR, 144 by anti-dengue-IgM and 63 by dengue NS1 ELISA. Among PCR positive dengue patients, Dengue serotype 2 was most prevalent, followed by serotype 4 (Table 5.2). Among dengue infected patients, 30.84% (62/201) exhibited WHO-classified Warning signs, whereas, 69.16% (139/201) were without any Warning signs. As only 1 patient was found to have severe dengue and was incorporated within patients with WHO-classified warning signs group.

5.3.1. Functional Prediction for TLR3, 7 & 8 Polymorphisms:

According to SNPinfo web server, two polymorphisms: rs179010 and rs3764879, might affect transcription factor binding sites of TLR7 and 8 genes, respectively (Table 5.3). The rs3764880 polymorphism might affect splicing activity of TLR8 due to its location near exon-intron junction. TLR7-rs179008 (gly11leu) and TLR8-rs3764880 (met1val) were predicted to be non-synonymous polymorphisms. The rs3764880 was also predicted to be damaging in nature by SIFT server. Genotype distributions of the seven SNPs for control population were in Hardy-Weinberg equilibrium at $p>0.05$, except one SNP of TLR7 (rs3853839), which violated Hardy-Weinberg equilibrium. Careful examination of raw genotyping results revealed no detectable genotyping error. As this genotype distribution might arise by chance or could reflect a significant association with dengue disease symptoms, results for this SNP were not excluded from study. The pair-wise LD measuring D′ for seven TLR7 and TLR8 SNPs indicated that TLR8 polymorphisms, rs3764879 and rs3764880 were in complete linkage disequilibrium, with R^2: 0.969, D´: 1.0 and p-value: <0.01. (Figure 5.2).

Table 5.2: (A) Demographic characteristics of patients and controls (B) distribution of circulating serotypes.

		Dengue (+) patients [n = 201]	Control (Healthy individuals) [n = 157]
Age-group (years)		32.01 ± 14.7 (range: 5-70)	36.23 +11.8 (range: 18–63)
Sex	Male	113	78
	Female	78	79
Diagnostic parameters	qRT-PCR	144	0
	Anti-dengue IgM	126	0
	Dengue NS1	63	0
WHO classified symptoms	With warning signs	62 (30.84%)	NA
	Without warning signs	139 (69.16%)	
Clinical Symptoms	Rash	54 (26.86%)	NA
	Nausea	51 (25.37%)	
	Myalgia	124 (61.69%)	
	Arthralgia	83 (41.29%)	
	Joint swelling	24 (11.94%)	
	Headache	65 (32.33%)	
	Persistent vomiting	48 (23.88%)	
	Abdominal pain	40 (19.9%)	
	Bleeding	14 (6.96%)	
	Retro-orbital pain	13 (6.46%)	
(b) Distribution of dengue serotypes (n = 121 [§])			
Dengue 1	**Dengue 2**	**Dengue 3**	**Dengue 4**
15 (12.29%)	59 (48.36%)	9 (7.37%)	38 (31.14%)

[§]Out of 126 PCR positive dengue patients, serotyping could be performed among 121 samples due unavailability of sera of remaining 4 patients.

NA= not applicable

Table 5.3: (A) Genetic characterization of TLR polymorphisms and (B) predicted effect of non-synonymous TLR polymorphisms on protein structure.

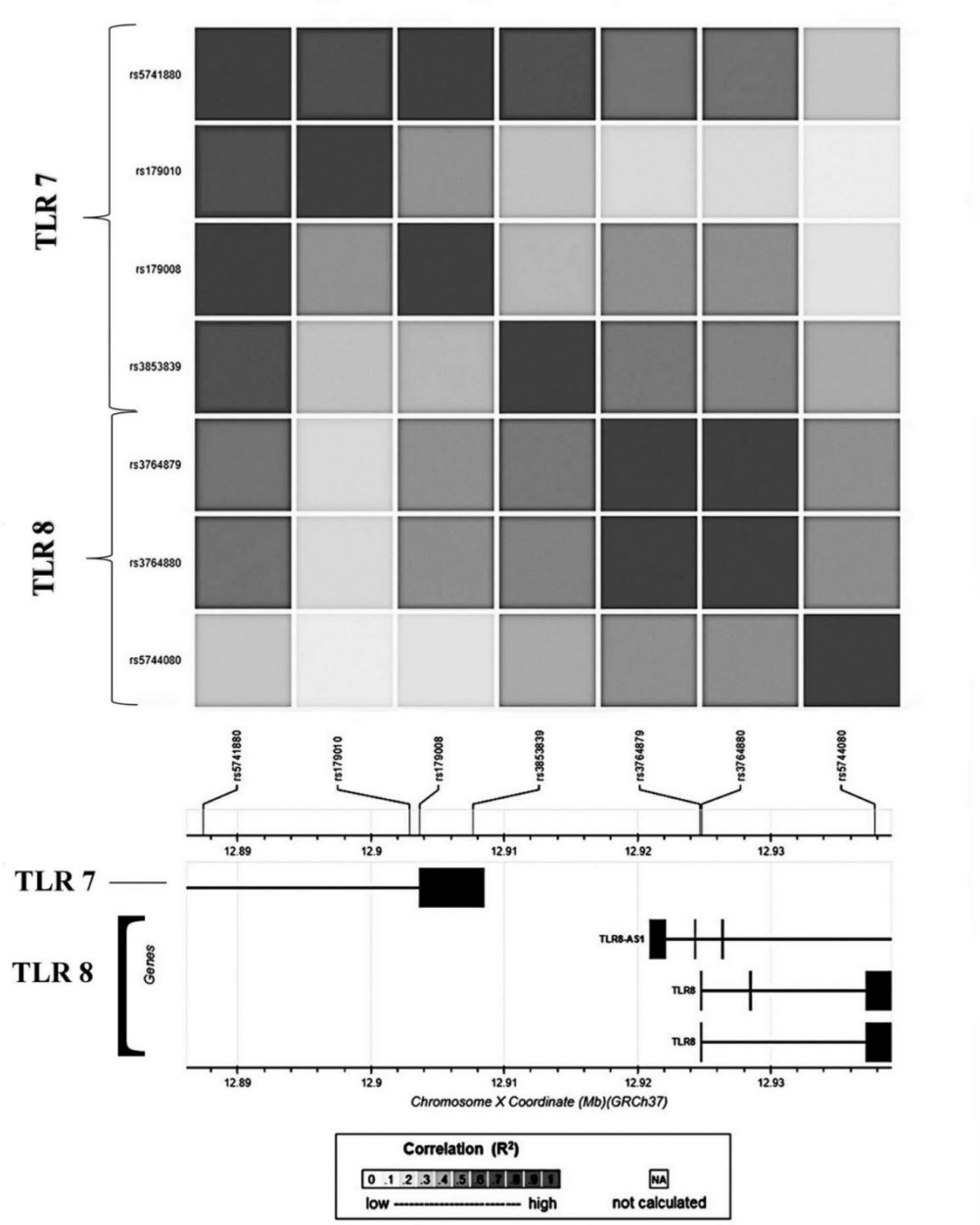
(A)

Gene name and SNP ref. No.		Chromosomal position	Alleles	Transcription factor binding site	Affects splicing regulation	Non-synonymous polymorphism
TLR3	rs3775290	Chr. 4 187241211	T/C	___	___	___
TLR7	rs5741880	Chr. X 12797337	G/T	___	___	___
	rs179010	Chr. X 12812806	C/T	Yes	___	___
	rs179008	Chr. X 12813580	A/T	___	___	Yes
	rs3853839	Chr. X 12817579	C/G	___	___	___
TLR8	rs3764880	Chr. X 12834747	A/G	___	ESE†/ESS‡ and abolishes domain	Yes
	rs5744080	Chr. X 12834618	C/T	___	___	___
	rs3764879	Chr. X 12847725	C/G	Yes	___	___

(b)

Gene name and SNP ref. No.	Amino acid change	Effects				
		SIFT prediction	Polyphen2 prediction	Mutation Taster prediction	FATHMM prediction	Meta SVM prediction
TLR7 rs179008	gly11leu	Tolerated	Benign	Neutral	Tolerated	Tolerated
TLR8 rs3764880	met1val	Damaging	Benign	Polymorphism	Tolerated	Tolerated

†ESE = Exonic splicing enhancer, ‡ESS = Exonic splicing silencer



rs_number	rs5741880	rs179010	rs179008	rs3853839	rs3764879	rs3764880	rs5744080
rs5741880	1.0	0.115	0.015	0.093	0.078	0.074	0.017
rs179010	0.115	1.0	0.042	0.163	0.088	0.078	0.016
rs179008	0.015	0.042	1.0	0.031	0.049	0.051	0.009
rs3853839	0.093	0.163	0.031	1.0	0.489	0.465	0.245
rs3764879	0.078	0.088	0.049	0.489	1.0	0.969	0.308
rs3764880	0.074	0.078	0.051	0.465	0.969	1.0	0.322
rs5744080	0.017	0.016	0.009	0.245	0.308	0.322	1.0

Dark color indicated strong linkage disequilibrium between SNPs. Pairwise LD (R2) is shown for each combination of SNPs in table form.

Figure 5.2: Pairwise linkage disequilibrium pattern of seven SNPs in TLR7 & TLR8 genes from South Asian BEB data from 1000 genome project.

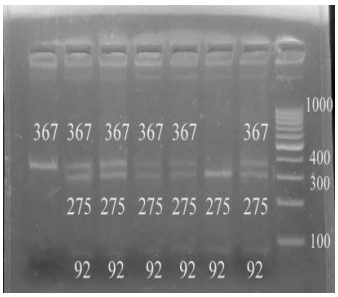
5.3.2. Genotypic Association of the Polymorphisms with Dengue Disease Susceptibility:

Polymorphic variants were confirmed by restriction digestion pattern followed by sequencing. Representative images of PCR-RFLP and sequence chromatogram were illustrated in Figure 5.3 and Figure 5.4.

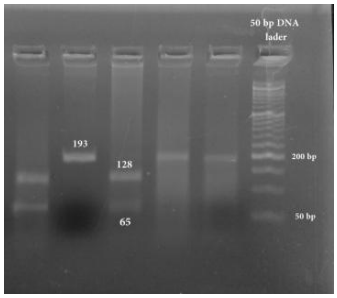
Systematic analysis revealed that CC genotype of TLR3-rs3775290 was significantly positively associated with dengue disease susceptibility [OR = 4.15; 95% CI 1.86 - 9.24; p = 0.0002] (Table 5.4). Distribution of CC genotype was more than three-fold higher among patient population compared to control group. The rs3775290 C-allele was found to be significantly associated with dengue disease susceptibility. Similarly, TT and CC genotypes of TLR7-rs179008 and TLR7-rs3853839 respectively, were found to be significantly associated with dengue disease susceptibility [rs179008: OR = 2.89, 95% CI 1.20 – 6.94, p = 0.01; rs3853839: OR = 2.01, 95% CI 1.23 – 3.25, p = 0.004]. Compared to healthy individuals, frequency of TT genotype of TLR7-rs179008 was more than double among dengue infected patients. The T-allele and C-allele of each of TLR7-rs179008 and TLR7-rs3853839 were found to be significantly associated with the disease. GT genotype of TLR7-rs5741880 and CT genotype of TLR7-rs179010 were significantly related with decreased risk of dengue infection.

In case of TLR8 gene, AA genotype of TLR8-rs3764880 and CC genotype of TLR8-rs5744080 demonstrated significant association with dengue disease susceptibility [rs3764880: OR = 2.58, 95% CI 1.32 – 5.07, p = 0.004 and rs5744080: OR = 2.49, 95% CI 1.30 – 3.36, p = 0.001] (Table 5.4). The G-allele of TLR8-rs3764880 and C-allele of TLR8-rs5744080 exhibited significant association with dengue infectivity. Frequency of TLR8-rs3764880-AG genotype markedly decreased among diseased population

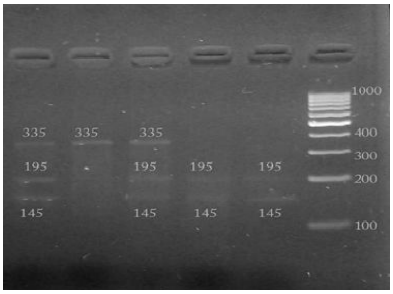
compared to healthy individuals, indicating significant association of this genotype with lower risk of dengue infection.



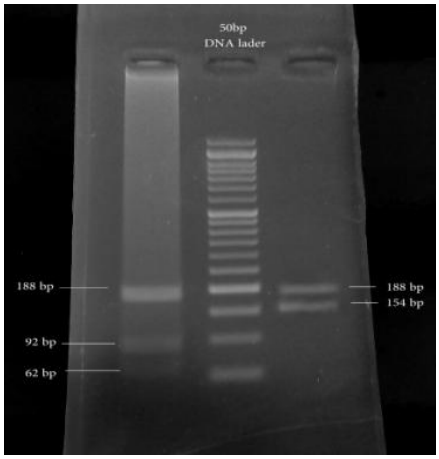
TLR 3 (rs3775290)



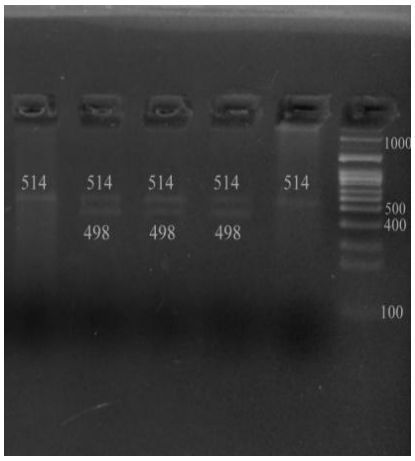
TLR 7 (rs5741880)



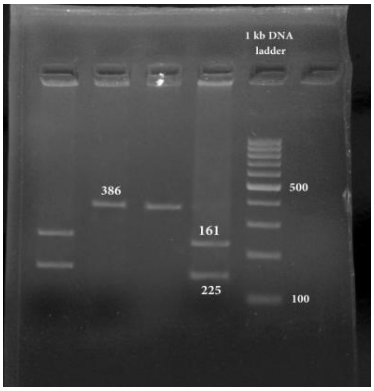
TLR 7 (rs179010)



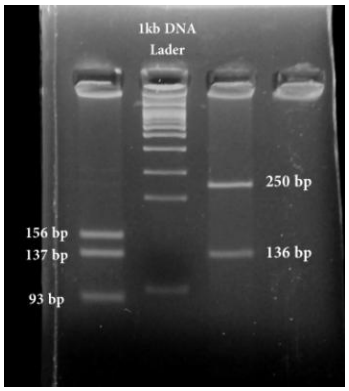
TLR 7 (rs179008)



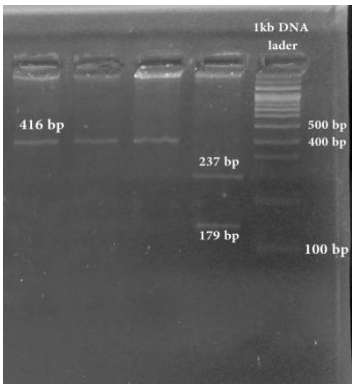
TLR 7 (rs3853 839)



TLR 8 (rs3764879)



TLR 8 (rs3764880)



TLR 8 (rs5744080)

Figure 5.3: Resolution of PCR-RFLP products of TLR 3, 7 and 8 after agarose gel electrophoresis.

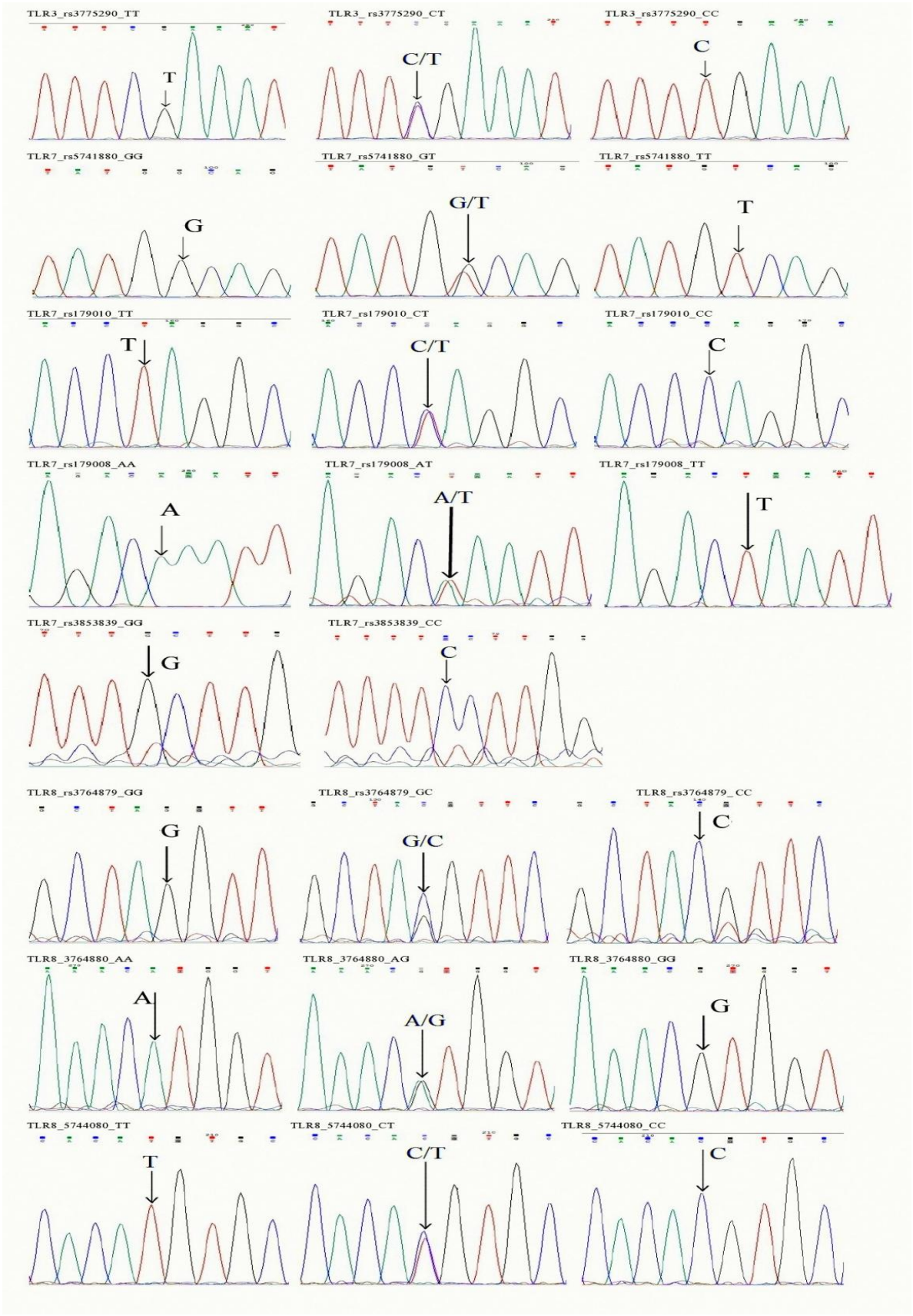


Figure 5.4: Chromatogram of DNA sequencing of each TLR (TLR 3, 7, 8) SNPs.

Table 5.4: Genotypic and allelic distribution of TLR3, 7 and 8 polymorphisms among Dengue patients and healthy controls.

SNP Ref. No. Chromosomlocation	Genotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
		n=157	**n=192 NA=9**				
	CC	5.09	18.22	4.15	**0.0002***		
	TT	33.75	31.77	0.91	0.69		
	CT	61.14	50	Ref			
	C allele	35.66	43.22	2.13	**0.04***	0.35	0.43
TLR 3 rs3775290	T allele	64.33	56.77	0.728			
	MALE	**n=78**	**n=113**				
Chr.4	CC	6.41	21.23	3.93	**0.005**		
	TT	28.20	28.31	1	0.98		
	CT	65.30	50.44	Ref			
	FEMALE	**n=79**	**n=79**				
	CC	3.9	13.92	4.09	**0.02***		
	TT	39.24	36.70	0.89	0.74		
	CT	56.96	49.36	Ref			
		n=157	**n=196 NA=5**				
	TT	12.73	13.26	1.04	**0.**88		
	GT	5.61	11.46	0.45	**0.046***		
	GG	75.79	81.12	Ref			
	G allele	81.52	83.92	1.18	0.40	0.18	0.16
TLR 7 rs5741880	T allele	18.47	16.08	0.84			
	MALE	**n=78**	**n=109**				
Chr. X	T	25.64	20.18	0.73	0.37		
	G	74.35	79.81	Ref			
	FEMALE	**n=79**	**n=87**				
	TT	0	4.59	3.90	0.19		
	GT	22.27	12.64	0.49	0.08		
	GG	77.21	82.75	Ref			
		n=157	**n=196 NA=5**				
	CC	30.57	34.18	0.98	0.47		
	C T	17.83	9.69	1.01	**0.02***		
	TT	51.5	56.12	Ref			
	C allele	39.49	39.03	1.07	0.90	0.39	0.39
TLR 7 rs179010	T allele	60.50	60.96	0.93			

SNP Ref. No. Chromosomlocation	Genotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
	MALE	**n=78**	**n=112**				
	C	48.71	44.64	0.84	0.73		
Chr. X	T	51.28	55.35	Ref			
	FEMALE	**n= 79**	**n=84**				
	CC	12.6	20.23	1.75	0.19		
	TC	35.4	22.61	0.53	0.07		
	TT	51.8	57.14	Ref			
		n=157	**n=193**				
TLR 7 rs179008			**NA=8**				
	TT	4.45	11.91	2.89	**0.01***		
	AT	3.82	0	0.12	**0.02***		
Chr. X	AA	91.71	88.08	Ref			
	T allele	6.36	11.91	1. 98	**0.01***	0.06	0.12
	A allele	93.6	88.01	0.50			
	MALE	**n=78**	**n=108**				
	T	3.84	12.96	3.72	**0.03***		
	A	96.15	87.03	Ref			
	FEMALE	**n=79**	**n=85**				
	TT	5.06	10.58	2.24	0.19		
	AT	7.59	0	0.13	**0.03***		
	AA	87.34	89.41	Ref			
		n=157	**n=175**				
			NA=26				
	CC	22.29	35.42	2.01	**0.004***		
	GC	0	0	-	-		
	GG	77.7	64.57	Ref			
	C allele	22.29	35.42	1.91	**0.0002***	0.22	0.35
TLR 7 rs3853839	G allele	77.07	64.57	0.52			
	MALE	**n=78**	**n=100**				
	C	19.24	37.00	2.46	**0.009***		
Chr. X	G	80.76	63.00	Ref			
	FEMALE	**n=79**	**n=75**				
	CC	25.3	33.33	1.47	0.27		
	GC	0	0	-	-		
	GG	74.6	66.66	Ref			
		n=157	**n=196**				
			NA=5				
	CC	22.29	27.55	1.32	0.25		
	GC	9.55	8.16	0.84	0.64		
	GG	68.15	64.28	Ref			
	C allele	27.07	31.63	1.24	0.18	0.27	0.31

SNP Ref. No. Chromosomlocation	Genotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
TLR8 rs3764879	G allele	72.92	68.36	0.80			
	MALE	**n=78**	**n=110**				
	C	28.20	38.18	1.57	0.15		
Chr. X	G	71.79	61.81	Ref			
	FEMALE	**n=79**	**n=86**				
	CC	16.4	13.95	0.82	0.65		
	GC	18.9	18.60	0.97	0.94		
	GG	64.5	67.44	Ref			
		n=157	**n=190 NA=11**				
	AA	8.28	18.94	2.58	**0.004***		
	AG	23.56	1.05	0.03	**0.<0001***		
	GG	68.15	80.00	Ref			
	A allele	20.06	19.47	0.57		0.20	0.19
TLR8 rs3764880	G allele	79.93	80.52	1.73	**0.001***		
	MALE	**n=78**	**n=111**				
	A	8.97	23.42	3.10	**0.009***		
Chr. X	G	91.02	76.57	Ref			
	FEMALE	**n=79**	**n=79**				
	AA	7.59	12.65	1.76	0.29		
	AG	46.83	2.53	0.02	**<0.0001***		
	GG	45.56	84.81	Ref			
		n=157	**n=191 NA=10**				
	CC	12.73	26.70	2.49	**0.001***		
	CT	14.64	13.08	0.87	0.67		
	TT	72.61	60.20	Ref			
	C allele	20.06	33.24	1.98	**0.0001***	0.20	0.33
	T allele	79.93	66.75	0.50			
TLR8 rs5744080	**MALE**	**n=78**	**n=103**				
	C	12.82	38.83	1.84	0.06		
	T	74.35	61.16	Ref			
Chr. X	**FEMALE**	**n=79**	**n=88**				
	CC	0	12.50	11.57	**0.004***		
	CT	29.11	28.40	0.96	0.92		
	TT	70.88	59.09	Ref			

*$p<0.05$ at 95% CI was considered as statistically significant., "ref"= reference genotype, "NA"= Could not be PCR-amplified.

As TLR7 and 8 genes are located on X chromosome, any gender biasness of these polymorphisms among DENV patients needed to be analyzed. Being Located in X chromosome males represent hemizygous, whereas, females represent homozygous genotypes. Frequency of hemizygous T genotype of rs179008 of TLR 7 and C genotype polymorphisms rs3853839 and rs5744080 of TLR7 and TLR8 were significantly higher among DENV male patients than that of control males (Table 5.4A). TLR7-rs3764880-AG and TLR8-rs179008-AT homozygous genotypes were present in significantly higher frequency among healthy female controls compared to female dengue patients. The TLR8-rs5744080-CC genotype was more prevalent among female dengue infected patients.

5.3.3. Genotypic Association of Polymorphism-Combinations with Disease Susceptibility and Severity:

Various genotypes of SNPs of TLR7 and TLR8 that were significantly associated with dengue infection were further analyzed to find out their combined effect on enhancement of dengue disease susceptibility (Table 5.5). Statistical analysis revealed that combination of CC/C-CC/C of TLR7-rs3853839-TLR8-rs5744080 [OR = 6.59, 95% CI 0.81 – 53.25, p = 0.042] and TT/T-AA/A of TLR7-rs179008-TLR8-rs3764880 [OR = 7.31, 95% CI 0.91 – 58.34, p = 0.028] were significantly higher among patient population compared to healthy control group.

Table 5.5: Association of genotype combinations of different TLR7 and TLR8 polymorphisms with (A) Dengue disease susceptibility and (B) development of WHO classified warning signs among patients.

(A)

SNP Combinations	Frequencies (%)		Odds Ratio	*p* value at 95% C.I.
	Control (N = 157)	Dengue infected (N = 201)		
rs5741880-rs3853839 TT/T-CC/C	0	0.49% (1)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs179008 TT/T- TT/T	0	0.49% (1)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs3764880 TT/T-AA/A	0	0.49% (1)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs5744080 TT/T-CC/C	0	0.49% (1)	0.79 [0.04 – 12.80]	0.87
rs3853839-rs179008 CC/C- TT/T	0	2.98% (6)	4.89 [0.58- 41.05]	0.10
rs3853839-rs3764880 CC/C-AA/A	4	5.97% (12)	2.42 [0.76 – 7.68]	0.11
rs3853839-rs5744080 CC/C- CC/C	0	3.98% (8)	6.59 [0.81 – 53.25]	**0.042***
rs179008-rs3764880 TT/T -AA/A	1	4.47% (9)	7.31 [0.91 – 58.34]	**0.028***
rs179008-rs5744080 TT/T-CC/C	0	1.99% (4)	3.22 [0.35 - 29.17]	0.27
rs3764880-rs5744080 AA/A-CC/C	0	2.48% (5)	4.80 [0.57 – 40.33]	0.11

*$p<0.05$ at 95% CI was considered as statistically significant.

(B)

SNPs Combination	Frequency (%) Symptomatic Dengue patients (n = 201)	Frequency of Symptoms (%)	
		With warning signs	Without warning signs
rs3853839-rs5744080 CC/C- CC/C	3.98% (8)	37.5% (3)	62.5% (5)
rs179008-rs3764880 TT/T-AA/A	4.47% (9)	**100% (9)**	0

5.3.4. Correlation between SNP-Genotypes with Clinical Symptoms and WHO-Defined Warning Signs:

To determine the correlation between different SNP-genotypes with dengue disease symptoms, Pearson's Chi square test was performed (Table 5.6). The CC genotype of TLR3-rs3775290 and TT/T of TLR7-rs5741880 exhibited significant association with development of rash among infected patients compared to reference genotypes of respective polymorphisms. CT genotype of TLR7-rs179010 and AA/A of TLR8-rs3764880 exhibited significant association with development of myalgia among dengue infected patients. Only CC/C of TLR7-rs179010 was significantly correlated with arthralgic manifestation of the infected patients. The TT/T and GT of TLR7-rs5741880, CC/C of TLR7-rs179010, TT/T genotype of TLR7-rs179008 and AA/A of TLR8-rs3764880 demonstrated significant correlation with development of headache among patients. The CC genotype of TLR3-rs3775290 and CC/C of TLR7-rs179010, and TLR8-rs5744080; TT/T genotype of TLR7-rs5741880 and TLR7-rs179008 and AA/A genotype of TLR8-rs3764880 demonstrated significant association with persistent vomiting of dengue patients. The TT/T of TLR7-rs179008 and AA/A of TLR8-rs3764880 were strongly associated with development of abdominal pain among dengue infected patients. The CC genotype of TLR3-rs3775290 and TT/T of TLR7-rs179008 and AA/A of TLR8-rs3764880 exhibited significant association with haemorrhagic

manifestation of dengue patients. The TT/T of TLR7-rs5741880, TLR7-rs179008 and AA/A of TLR8-rs3764880 were significantly related with development of retro-orbital pain among the patients. Overall, TT/T of TLR7-rs179008 and AA/A of TLR8-rs3764880 exhibited significant association with WHO-defined dengue warning signs viz. persistent vomiting, abdominal pain and hemorrhagic manifestation.

Table 5.6: Association of TLR polymorphisms with development of different clinical symptoms among Dengue patients.

Gene and SNP Ref. No.	Genotype (female)/ Haplotype (male)	Statistical parameter	Symptoms (%)									
			Nausea	Rash	Myalgia	Arthralgia	Joint Swelling	Retro orbital pain	Headache	WHO-defined dengue warning signs		
										Persistent Vomiting	Abdominal pain	Haemorrhage
TLR3 rs3775290	CC	n/N (%)	28.57	45.71	68.57	45.71	14.3	14.3	40	40	31.42	14.3
		p value	0.65	**0.007**	0.52	0.67	0.31	0.08	0.34	**0.01**	0.06	**0.04**
		O.R.	0.82	3.007	1.30	1.17	1.83	3.03	1.46	2.70	2.29	3.88
	CT (ref)	n/N (%)	30.20	26.04	62.5	41.66	12.5	5.20	31.25	13.54	16.66	4.16
		p value	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	TT	n/N (%)	13.11	16.39	59.01	39.34	6.55	4.91	29.50	21.31	19.67	8.19
		p value	**0.01**	0.15	0.66	0.77	0.68	0.93	0.81	0.90	0.63	0.28
		O.R.	0.34	0.55	0.86	0.90	0.77	0.94	0.92	0.95	1.22	2.05
TLR 7 rs5741880	TT/T	n/N (%)	12.5	75	87.5	75	0	25	62.5	50	37.5	12.5
		p value	0.34	**0.001**	0.24	0.06	-	**0.03**	**0.03**	**0.04**	0.19	0.55
		O.R.	0.37	9.23	3.30	4.33	-	5.55	4.20	3.96	2.58	1.92
	GG/G (ref)	n/N (%)	27.67	24.52	56.60	40.88	12.57	5.66	27.04	20.75	18.86	6.91
		p value	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	GT	n/N (%)	13.79	24.13	79.31	34.48	10.34	6.89	48.27	31.03	20.68	6.89
		p value	0.11	0.96	0.21	0.51	0.73	0.79	**0.02**	0.22	0.81	0.99
		O.R.	0.41	0.97	1.81	0.76	0.80	1.23	2.51	1.71	1.12	0.99
TLR 7 rs17901	CC/C	n/N (%)	29.26	41.46	65.85	58.53	9.75	7.31	53.65	34.14	21.95	9.75
		p value	0.33	0.12	0.29	**0.01**	0.61	0.66	**0.003**	**0.03**	0.60	0.47
		O.R.	1.48	1.78	1.49	2.57	0.74	1.38	2.95	2.33	1.26	1.59

Gene and SNP Ref. No.	Genotype (female)/ Haplotype (male)	Statistical parameter	Symptoms (%)									
			Nausea	Rash	Myalgia	Arthralgia	Joint Swelling	Retro orbital pain	Headache	WHO-defined dengue warning signs		
										Persistent Vomiting	Abdominal pain	Haemorrhage
	TT/T	n/N (%)	21.81	25.45	56.36	35.45	12.72	5.45	28.18	18.18	18.18	6.36
	(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	CT	n/N (%)	35.55	17.77	84.44	28.88	8.88	8.88	28.88	26.66	24.44	6.66
		p value	0.07	0.30	**<0.01**	0.66	0.49	0.42	0.92	0.23	0.23	0.94
		O.R.	1.97	0.63	4.208	0.84	0.66	1.69	1.05	1.63	1.63	1.05
TLR 7 rs3853839	CC/C	n/N (%)	27.41	27.41	53.22	43.54	8.06	4.83	30.64	25.80	16.12	4.83
		p value	0.43	0.90	0.11	0.80	0.71	0.71	0.52	0.67	0.58	0.71
		O.R.	1.32	1.04	0.59	1.08	0.81	0.77	0.80	1.16	0.79	0.81
	GG/G	n/N (%)	22.12	26.54	65.48	41.59	9.73	6.19	35.39	23.01	19.46	6.19
	(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
TLR 7 rs179008	TT/T	n/N (%)	28.57	23.80	71.42	47.61	14.28	19.04	61.90	76.19	71.42	42.85
		p value	0.73	0.40	0.26	0.50	0.72	**0.01**	**0.003**	**<0.01**	**<0.01**	**<0.01**
		O.R.	0.83	1.51	1.81	1.35	1.26	4.26	4.33	14.55	14.70	25.05
	AA/A	n/N (%)	25.00	27.32	62.20	40.11	11.62	5.23	30	18.02	14.53	2.90
	(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
TLR 8 rs3764880	AA/A	n/N (%)	38.88	33.33	86.11	38.88	8.33	19.44	55.55	66.66	61.11	19.44
		p value	0.051	0.23	**0.001**	0.65	0.68	**0.0003**	**0.001**	**<0.01**	**<0.01**	**0.007**
		O.R.	2.12	1.61	4.63	1.19	0.77	6.81	3.27	10.66	11.69	5.00
	GG/G	n/N (%)	23.02	23.68	57.23	39.47	12.50	3.94	27.63	15.78	11.84	4.60
	(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	GA	n/N (%)	50	0	50	100	0	0	0	0	0	0
		p value	0.37	NA	0.83	0.08	NA	NA	NA	NA	NA	NA
		O.R.	3.34	NA	0.74	7.64	NA	NA	NA	NA	NA	NA
TLR 8 rs5744080	CC/C	n/N (%)	30.43	43.47	65.21	60.86	17.39	13.04	43.47	52.17	34.78	8.69
		p value	0.47	0.06	0.81	0.09	0.41	0.24	0.47	**0.01**	0.29	0.89
		O.R.	1.42	2.39	1.11	2.17	1.65	2.31	1.38	3.15	1.65	0.90
	TT/T	n/N (%)	23.47	24.34	62.60	41.73	11.30	6.08	35.65	25.21	24.34	9.56
	(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Gene and SNP Ref. No.	Genotype (female)/ Haplotype (male)	Statistical parameter	Symptoms (%)									
			Nausea	Rash	Myalgia	Arthralgia	Joint Swelling	Retro orbital pain	Headache	WHO-defined dengue warning signs		
										Persistent Vomiting	Abdominal pain	Haemorrhage
	TC	n/N (%)	26.41	22.640.80	58.49	28.30	11.32	5.66	22.64	9.43	5.66	1.88
		p value	0.68	0.90	0.61	0.09	0.99	0.91	0.09	**0.01**	0.03	0.07
		O.R.	1.17		0.84	0.55	1.001	0.92	0.52	0.301	0.18	0.18
TLR 8 rs3764879	CC/C	n/N (%)	36.36	27.27	57.57	48.48	12.12	6.06	33.33	30.30	18.18	9.09
		p value	0.14	0.97	0.33	0.45	0.92	0.82	0.72	0.33	0.83	0.58
		O.R	1.82	1.01	0.67	1.33	0.94	0.83	1.15	1.52	0.89	1.47
	GG/G	n/N (%)	23.80	26.98	66.66	41.26	12.69	7.14	30.15	22.22	19.84	6.34
	(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		O.R	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	GC	n/N (%)	21.62	27.02	48.64	35.13	8.10	5.40	40.54	27.02	24.32	8.10
		p value	0.78	0.99	**0.04**	0.50	0.44	0.71	0.23	0.54	0.55	0.70
		O.R	0.88	1.002	0.47	0.77	0.60	0.74	1.57	1.29	1.29	1.30

*$p<0.05$ at 95% CI was considered as statistically significant. NA = Not applicable, "ref" = reference genotype.

Association of particular genotypes with presence or absence of any WHO-classified warning signs among dengue infected patients were analyzed (Figure 5.5). The TLR3-rs3775290-CC genotype, TLR7-rs179008-TT/T, TLR8-rs3764880-AA/A and TLR8-rs5744080-CC/C were found to be present in significantly higher frequencies among dengue patients exhibiting WHO-defined warning signs; whereas, TLR7-rs5741880-GG/G, TLR7-rs179010-TT/T, TLR7-rs179008-AA/A, TLR8-rs3764880-GG/G and TLR8-rs5744080-CT genotypes, were considerably more prevalent among patients exhibiting no warning signs. Further, prevalence of specific genotypic combinations of significantly associated polymorphisms were analyzed among dengue patients with/without warning signs (Table 5.4B). The TT/T-AA/A combination of TLR7-rs179008-TLR8-rs3764880 was predominantly present among patients with warning signs.

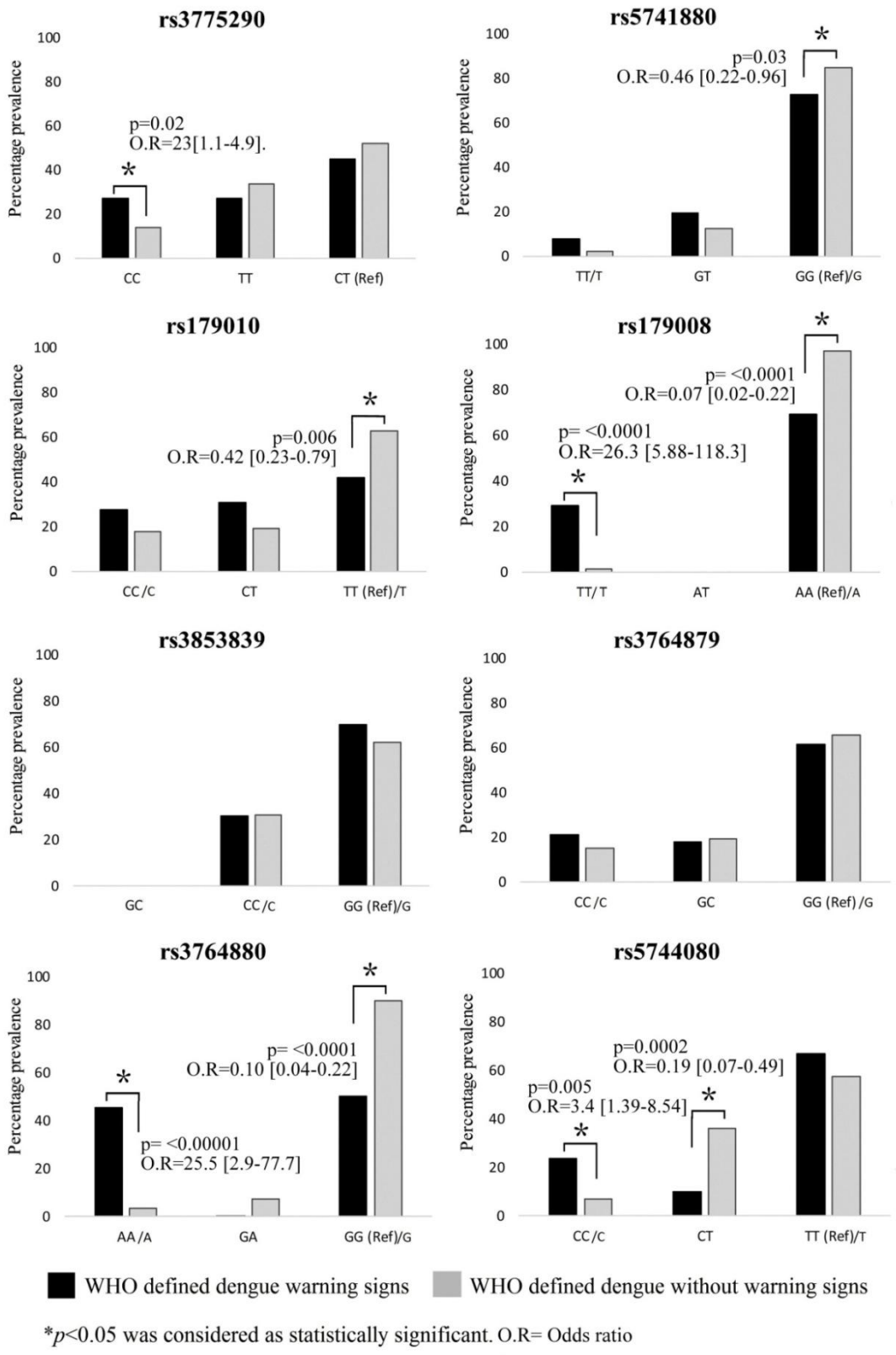


Figure 5.5: Genotypic distribution pattern of the TLR polymorphisms with development of WHO-classified with/without warning signs among dengue patients.

5.4. DISCUSSION:

Though TLR3, 7, 8 has long been known to be the first line of defense in innate immune recognition of dengue viral RNA genome, there has been very limited study regarding role of TLR polymorphisms in determining dengue disease susceptibility. Present study clearly depicted the importance of various polymorphisms of TLR3, 7, 8 genes in determining dengue disease pathogenicity among Eastern Indian patient population.

Both TLR3 and TLR7 are important for blocking dengue virus replication and elicit cellular antiviral response within the host. CC genotype of TLR3-rs3775290 was significantly associated with dengue disease susceptibility among Eastern Indian patients, with three fold higher prevalence of this genotype among diseased population. This genotype of rs3775290 has also been previously detected to be higher among active chronic HBV patients of Turkish origin (25). Involvement of this polymorphism has also been implicated among Tunisian chronic HCV patients and intrauterine transmissibility of HBV among Chinese patients (26, 39).

TLR7 plays a key role in increasing B-cell sensitivity to RNA-containing autoantigens in development of systemic autoimmunity (32). Patients with TT genotypes of TLR7-rs179008 and CC genotypes of TLR7-rs3853839 demonstrated significant association with dengue infectivity within study population. The TLR7-rs179008 polymorphism has been previously correlated with increased risk of disease progression among HCV mediated liver disease among Moroccan population and with post-bronchiolitis lung function deficiency of Finnish patients (28, 40). Similar to the current study, GC genotype of TLR7-rs3853839 was significantly higher among chikungunya virus infected Indian patients (29). This TLR7 polymorphism has also been previously

associated with protection against HCV persistence and susceptibility to enterovirus-71 mediated Hand foot and mouth disease among Chinese population (41-42).

The AA and CC genotypes of TLR8-rs3764880 and TLR8-5744080 respectively were significantly associated with dengue infection. This A-allele of TLR8-rs3764880 was previously strongly associated with advanced liver disease among HCV infected Moroccan patients (28). Similar to this study, frequency of G-allele of rs3764880 was found to be significantly higher among pulmonary tuberculosis patients of Pakistani origin (43). On the contrary, this allele conferred significant protective effect against progression of disease among HIV positive patients of German origin (36). The genotypic distribution of TLR8-rs5744080 polymorphism has been significantly different between osteoarthritis patients and healthy individuals of Japan (44).

Further, the present study demonstrated significantly increased risk of dengue disease susceptibility with presence of two genotypic combinations of these polymorphisms. Contribution of such genotypic combinations of polymorphisms of JAK1 and several cytokines towards increased risk of Dengue haemorrhagic fever (DHF) development has been previously reported (30, 45). Involvement of various genotypic combinations of TLR polymorphisms towards various disease phenotypes has also been previously demonstrated in ulcerative colitis, Crohn's disease and severe malarial anaemia (Meena et al., 2015; Munde et al., 2012).

The TLR7-rs179008-TT/T, TLR8-rs5744080-CC/C and TLR8-rs3764880-AA/A could be associated with risk of developing WHO-classified warning signs among dengue infected patients. Interestingly, reference genotypes of TLR7-rs5741880, TLR7-rs179010, TLR7-rs179008, TLR8-rs3764880 polymorphisms were significantly more prevalent among patients without any warning signs - thus these genotypes seemed to

impart protection against development of warning signs among infected individuals. Presence of TT/T of TLR7-rs179008 and AA/A of TLR8-rs3764880 among dengue patients significantly correlated with development of WHO-classified warning signs viz. persistent vomiting, abdominal pain and haemorrhage, as observed in this study. The genotypic combination TT/T of rs179008 and AA/A of rs3764880 was significantly prevalent among patients with warning signs. Contribution of such genotypic combination of various SNPs towards increased risk of DHF development has been previously demonstrated among several polymorphisms of JAK1, interleukin-10 and interferon gamma (45, 48).

Though this study dealt with only association of specific genotypes of host innate immunity genes with development of dengue disease severity, previous studies has also demonstrated development of severe dengue due to several viral factors also, viz. viral load, dengue virus serotype etc. (1, 49-50).

5.5. CONCLUSIONS:

In conclusion, the present study indicated increased risk of dengue disease susceptibility among individuals with specific genetic variants of majority of the analyzed TLR3, TLR7 and TLR8 polymorphisms – indicating importance of TLR polymorphisms in dengue disease pathogenesis. Increased risk of dengue infectivity was also predicted among certain genotypic combinations of these polymorphisms. Moreover, specific genotype(s) of certain polymorphism(s), alone or in combination, were associated with development of WHO-classified warning signs, which might act as potential prognostic biomarkers for predicting disease severity among dengue infected patients.

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CHAPTER – 4

Identification of differential genetic makeup of innate immunity and acute phase reactant viz. C-Reactive protein between WHO classified dengue patients (severe, with warning signs & without warning signs) and healthy individuals.

6. Chapter – 4

1. Identification of differential genetic makeup of innate immunity and acute phase reactant viz. C-Reactive protein between WHO classified dengue patients (severe, with warning signs & without warning signs) and healthy individuals.

- **Publication:**

The scientific work mentioned in this chapter has been published in the following peer reviewed journal:

- **Mukherjee S**, Tripathi A*. Association of serum C-reactive protein level and polymorphisms with susceptibility to dengue infection and severe clinical outcome among eastern Indian patients. ***Medical Microbiology and Immunology***. 2020 Oct; 209(5): 631-640. doi: 10.1007/s00430-020-00690-2. Epub 2020 Jul 27. PMID: 32720219.

6.1. REVIEW OF LITERATURE:

Dengue infection results in a wide range of clinical spectrum, viz. classical simple dengue fever to life threatening severe dengue (DHF/DSS) (1). Pathophysiology of developing severe dengue is still unclear. Several viral factors and host immuno-genetic makeup might be crucial in dengue disease pathogenicity (1-4). Acute phase response (APR) against infections is the early defence mechanism of the host (5). C-reactive protein (CRP), an acute phase protein, genetically located in chromosome 1q23.2 region, which has been previously used as inflammatory and infectious biomarker in clinical set up (6-7). Involvement of inflammatory response during dengue infection is well established and liver is the main organ involved in DENV infection (8-11). CRP is synthesised from liver immediately within six hour of inflammatory onset induced by DENV (12). CRP level might play important role to distinguish between viral and bacterial infections to assess severity of illness as well as to differentiate between dengue and other febrile illness (13, 14). CRP acts as a scavenger molecule, generally binds to toxic molecules released from damaged tissues and removes them from circulation and activates complement system to opsonise microorganisms (15). Besides this, CRP mediated activation of complement and interaction with Fcγ receptors also helps to link innate and adaptive immune system (16-18). DENV infections have left specific imprints in human genome which evolved into single nucleotide polymorphisms (SNPs), involved in protection/susceptibility to dengue and these SNPs in immune response genes have also been previously implicated in severe dengue development (19-21). CRP promoter region has three cis-acting polymorphisms that contribute to variance in their CRP level, viz. rs3091244, rs3093059 and rs3093062 (22-23) (Figure 6.1). CRPrs3091244 is a functionally important tri-allelic SNP at position -

390 relative to start codon and rs3093062 which is an intronic microsatellite at position -409 are associated with CRP levels (24).

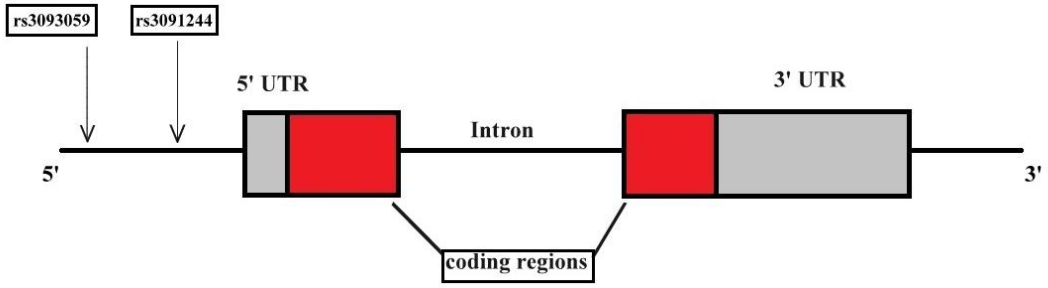


Figure 6.1: Schematic illustration of the C-reactive protein gene representing the location of polymorphic variants rs3093059 and rs3091244.

Severe dengue development has been associated with haemorrhagic manifestation, high viral load and hepatic dysfunction; CC genotype of rs3091244 and TT genotype of rs3093059 of CRP have been previously implicated in haemorrhagic manifestation of stroke (25-26). Polymorphic variants of CRP rs3093059 and rs3091244 were previously associated with altered viral load, CRP levels and hepatic inflammation among HBV and HCV carriers of Brazil and China (27-29). Similarly, specific genotypes of CRP might also affect development of increased serum CRP levels among dengue patients (12). Differential CRP level has been previously implicated between simple DF and severe dengue patients (12, 30). As CRP plays an important role in innate immunity as an early defence system against infections, similar to other innate immunity genes (viz. TLR3, -7, -8, CTLA-4, DC-SIGN, CLEC5A, NOD2), polymorphisms of CRP might also affect susceptibility towards dengue infection. Recent studies also suggest that CRP acts through FcγR and has a sequence homology and high affinity to FcγR(FcγRIIa) binding regions of IgG (31-32). DHF/DSS is attributed to antibody-dependent enhancement (ADE) which is mediated by human FcγRIIa (33). But till date, no in

depth investigation has been done to recognize the effect of genotypic distribution of CRP polymorphisms on CRP level and dengue viral load among infected patients, which in turn might affect dengue disease severity.

Being a dengue endemic county, most Indian states have been classified as having constant jeopardy of dengue transmission (33). Dengue disease burden in India is being inadequately analysed and a study reported that actual number of dengue cases in the country were 282 times the number reported by national vector-borne disease control programme (34). Meta analysis have demonstrated that in India dengue case-fatality ratio (CFR) is 2.6% with 95% CI 2·0–3·4, which was higher than CFR (1.14%) of dengue patients worldwide (33-34). Hence, identification of an early acute phase predictive marker for dengue disease severity could help clinicians for better clinical management.

In this chapter, we aimed to investigate the contribution of CRP level and CRP gene polymorphisms towards development of dengue disease susceptibility and severity among infected Eastern Indian patients.

6.2. MATERIALS AND METHODS

6.2.1. Ethics Statement:

All experiments performed in this study, including collection of blood from patients as well as healthy controls were done according to ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013), which followed 1964 Helsinki Declaration and its later amendments. Prior participating in this study written consents were received from patients and healthy control groups.

6.2.2. Study Population and Collection of Patients' Blood:

Among 309 dengue patients mentioned in **Chapter-1**, confirmed by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, 206 patients of 2015-2016 were included in Chapter-4 for determining role of patient's genetic makeup of CRP gene in determining disease susceptibility and severity. To carry out age-matched case control study, bloods from 102 healthy unrelated individuals of same ethnicity, who neither had any signs and history of other infections nor were detected with dengue infection by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, were collected from the same community. These symptomatic patients and age-matched healthy controls were Bengali population from same geographical location of Eastern India, whose ethnicity is similar to that of Bengali Bangladeshis (BEB) of south Asian population (SAS), present within 1000 genome project of Genome Wide Association Studies (GWAS), the largest public catalogue of human variation and genotype data.

6.2.3. Categorization of Patients according to WHO (2009) Classification:

Clinical symptoms of dengue patients were also monitored after next 10 days (symptoms monitored during first and subsequent visit to hospital) and patients were categorized into three groups: severe, with and without warning signs, according to WHO-classification [1].

6.2.4. Extraction of Viral RNA and Determination of DENV Load:

Viral RNA was extracted from 140µL of patients' sera, using the QIAamp Viral RNA Mini Kit according to manufacturer's protocol (Qiagen, Courtaboeuf, France), as described in **Chapter-1**. Presence of DENV genome and viral load among infected patients' sera was determined by quantitative real-time RT-PCR (Taqman assay) in total volume of 20µl using 8µl viral RNA, OneStepqRT-PCR MasterMix and other components of DENV Genesig kit (Primer Design Ltd., UK) according to

manufacturer's protocol. Real time PCR was performed on ABI Prism 7500 Fast instrument. A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided within the kit). DENV RNA copy number in patient's serum was calculated from corresponding Ct values. Each sample was loaded in triplicate. Viral RNA titer ≥10,000 copies/ml and <10,000 copies/ml was considered as high viral load and low viral load, respectively.

6.2.5. Detection of Anti-Dengue-IgM & IgG Antibody and Dengue-NS1 Antigen:

Suspected patient's serum was subjected to serological diagnosis for presence of anti-dengue-IgM (96% sensitivity, 87% specificity), using dengue IgM MAC ELISA Kit and (National Institute of Virology, Pune, India), according to manufacturer's instructions, as described in **Chapter-1**. Dengue patient's serum was subjected to serological diagnosis for presence of dengue-NS1 antigen and anti-dengue-IgG antibody using Panbio dengue IgG capture ELISA kit (80.9% sensitivity, 87.1% specificity) and Panbio dengue early NS1 ELISA Kits (Panbio, Australia), respectively.

6.2.6. Determination of DENV Serotypes:

Dengue serotypes of PCR positive samples was determined by real-time RT-PCR in of 20µl reaction mix, containing 8µl viral RNA, One Step qRT-PCR Master Mix and other components of DENV serotyping Genesig kit (PrimerDesign Ltd., UK), according to manufacturer's protocol, as described in **Chapter-1**. Reactions were carried out in ABI 7500 Fast instrument using serotype specific DENV controls present within the kit.

6.2.7. Quantification of CRP:

CRP level in patient's serum was estimated once for each of the symptomatic febrile patients belonging to acute (within day1-3 of symptomatic onset), critical (4-6 days) and late (≥7 days) phase of infection. Quantification CRP in serum was done by immunoturbidimetry method using AUTOSPAN turbi gold kit (SPAN diagnostics,

India), according to manufacturer's instructions. Briefly, 3µl of patient serum/calibrator was mixed with 500µl ten times diluted latex reagent (containing latex particle coated with anti-human CRP antibodies) and its absorbance was measured at 550nm wave length after 10 seconds (A1) and 120 seconds (A2). Serum concentration (mg/L) of CRP was measured using the following formula:

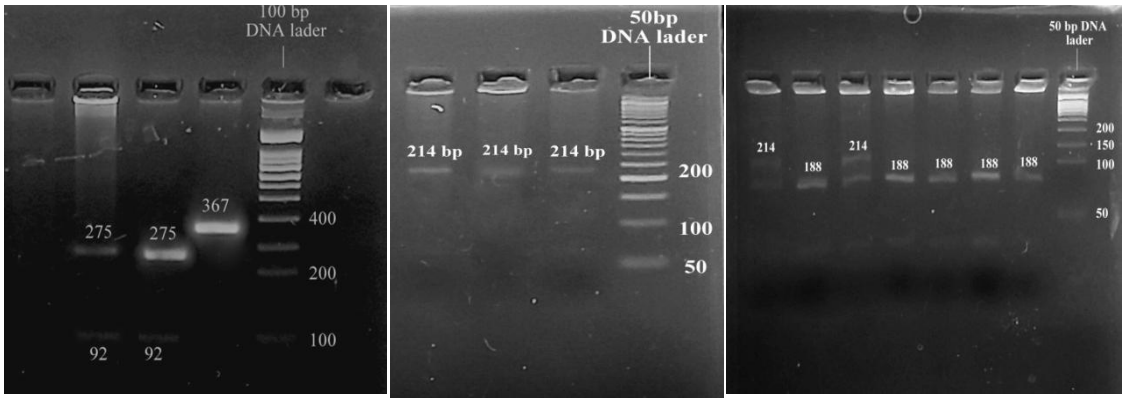
$$\text{Serum concentration of CRP (mg/L)} = \frac{(A2 - A1)\ \text{Serum sample}}{(A2 - A1)\ \text{Calibrator}} \times \text{concentration of calibrator}$$

6.2.8. SNP genotyping:

SNP genotyping was carried out using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based procedure. Briefly, genomic DNA was extracted from blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Based on sequences available in GenBank database, three primer pairs were designed using Primer3 software, to amplify rs3093059 and rs3091244 polymorphic regions of CRP. PCR reaction was carried out in 20µl volume, using 1xPCR buffer (Fermentas, USA), 1mM of each dNTP, 1unit of Taq DNA polymerase (Phusion High-Fidelity DNA Polymerase, Fermentas, USA), 1.5mM $MgCl_2$ and 20p.moles of previously mentioned primers. PCR reaction was carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA) at desired annealing temperature for 30 cycles and PCR products were electrophoresed on 2% agarose gel at 5 V/cm for an appropriate time period. Respective PCR products were digested with Tas I, Bfa I (Fermentas, USA) and Taq I (Himedia, India), separately as mentioned in Table 6.1 and digested products were visualized on 2.5% agarose gel (Figure 6.2). Different RFLP patterns were validated by sequencing of the respective PCR products using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in an ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA).

Table 6.1: List of primers and restriction enzymes used in PCR RFLP.

Gene	SNP Id	5'-Primer-3'	PCR Size	Restriction enzymes and digestion temperatures	Digestion pattern
CRP	rs3093059	F-TATCCTGACTCCTGCCTG R-CCCATCTATGAGTGAGAACACG	367bp	TasI 65° C	C: 367bp T: 275bp + 92bp
	rs3091244	F- AATGTGTCCATGGCTCTG R- AATGGGAAATGGTAACATATTAATC	214bp	Bfa I 37° C	C/T: 214bp A: 187bp + 27bp
		F- AATGTGTCCATGGCTCTG R- AATGGGAAATGGTAACATATTAATC	214bp	TaqI 65° C	A/T : 214bp C: 188 bp + 26 bp



rs3093059 with Tas I **rs3091244 with Bfa I** **rs3091244 with Taq I**

Figure 6.2: Representative image of PCR-RFLP products of rs3093059 and rs3091244 after digestion with TasI, BfaI and TaqI restriction enzymes, respectively.

6.2.9. Statistical Analysis:

Association of any particular genotype of respective SNPs with dengue disease susceptibility, CRP level, viral load and specific dengue symptoms (WHO-defined with/without warning signs) was calculated by using SPSS version 22 and GraphPad Prism[8] software (Los Angeles, CA, USA). Allele and genotype frequencies were compared between different study groups using Pearson's Chi square test and multivariant analysis were performed by using one way ANOVA. Mean and median

values of viral load and CRP level in each cohort were compared using t-test. For genotypic associations, *p*-values, odds ratio (OR) & minor allele frequency (MAF) were calculated. A *p*-value of <0.05 was considered statistically significant. For controls, Hardy-Weinberg equilibrium was analyzed for two polymorphisms with Haploview programme.

6.3. RESULTS:

Blood was obtained from age-sex matched 102 healthy control volunteers with same ethnicity from same region and without any past dengue encounter, symptoms and history of other infections or dengue infection as tested by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, to carry out case control study. Detailed clinic-pathological history of 206 dengue patients has been depicted in Table 6.2. Male to female ratio of dengue infected patients was 1.14:1 (110:96), whereas that of control group was 1.04: 1 (52:50) (Table 1). Mean age of dengue patients was 32.01±14.7 years (range: 7-72 years), whereas that of control was 36.2 ±11.8 years (range: 20–68 years). Among dengue infected patients, 14.56% (30/206) and 30.09% (62/206) were exhibited severe signs and WHO-defined warning signs, whereas 55.33% (114/206) were without any warning signs. Among PCR positive dengue patients, 56.14% (32/57) were within HVL group, whereas remaining 43.85% (25/57) were LVL.

During acute phase of infection (within day 1-3 of symptomatic onset), mean CRP level was significantly higher among dengue patients compared to those with other febrile illness (49.84mg/L vs 22.86mg/L; p=0.001) (Figure 6.3A). CRP concentration was also significantly higher among dengue patients with severe dengue (mean±SEM: 67.41±7.65mg/L, p<0.0001) and WHO-defined warning signs (mean±SEM: 45.71±2.91mg/L, p<0.0001) compared to that of patients without warning signs (mean±SEM: 9.96±0.88mg/L) (Figure 6.3B). Markedly higher CRP concentration was

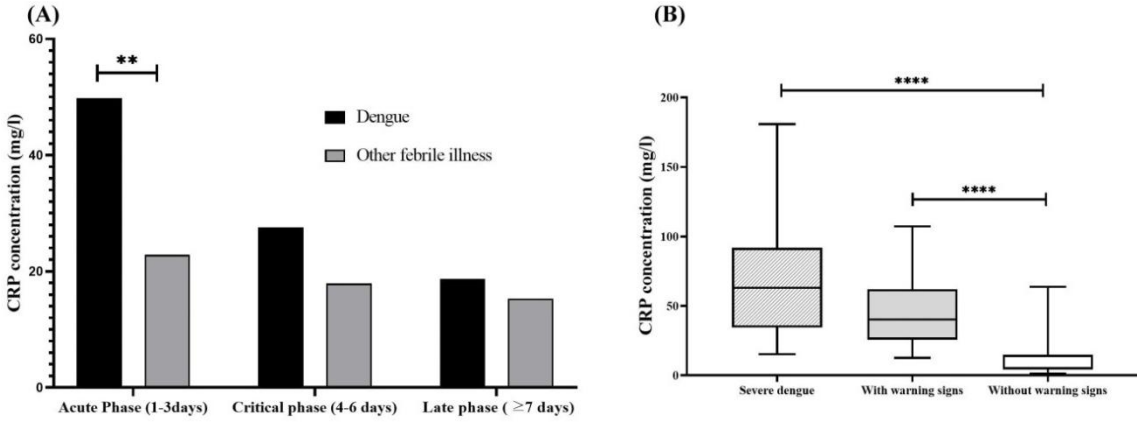
observed among anti-DENV IgG positive patients (31.20±7.34mg/L, p=0.0029) compared to negative ones (15.72±1.61mg/L) (Fig. 6.4A). CRP level was also significantly higher among DENV qRT-PCR positive (37.68±3.44mg/L, p=0.019) and DENV NS1 positive patients (29.48±4.5.6mg/L, p=0.0007) compared to DENV qRT-PCR negative (26.11±3.21mg/L) and DENV NS1 negative patients (15.67±1.51mg/L), respectively (Figure 6.4C, 6.3D).

Table 6.2: Demographic scenario and clinic-pathological history of the study population.

		Symptomatic Patients (n=348)		**Healthy Controls (n=102)**
		Dengue (n=206)	**[#]Other febrile illness (n=142)**	
Mean age (in years)		32.01±14.7	34.07±15.3	36.2 ±11.8
Sex	Male	53.39% (110)	52.02% (77)	50.98% (52)
	Female	46.60% (96)	47.98% (71)	49.02% (50)
Course of illness	Acute phase	33.49% (69)	25.35% (36)	NA
	Critical phase	53.88% (111)	57.04% (81)	
	Late phase	12.62% (26)	17.6% (25)	
Sample Classification according to WHO	Severe dengue	14.56% (30)	0	
	With warning signs	30.09% (62)	21.13% (30)	
	Without warning signs	55.33% (114)	78.87% (112)	
Diagnostic parameters	Anti-Dengue IgM	70.38% (145)	0	
	Dengue NS1	35.43% (73)	0	
	qRT-PCR	27.66% (57)	0	
Type of Infection	Primary Dengue	94.17% (194)		
	Secondary Dengue	5.83% (12)		
		qRT PCR confirmed Dengue (n=57)		
Vital titer	High viral load (HVL: ≥10000 copies/ml)	56.14% (32)	NA	
	Low viral load (LVL: <10000 copies/ml)	43.85% (25)		
Circulating Serotypes	Dengue 1	21.05% (12)		
	Dengue 2	50.87% (29)		
	Dengue 3	8.77% (5)		
	Dengue 4	19.29% (11)		

[#]Other febrile illness mostly comprised of viral infections viz. chikungunya, influenza and other arboviruses.

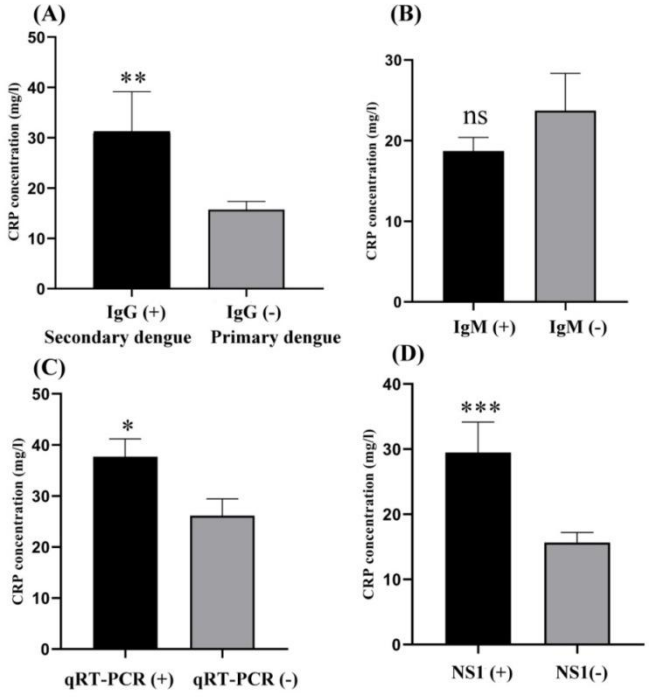
[‡]No bleeding/haemorrhagic and severe clinical manifestation was observed among other febrile illness group.



Two tailed *p*-value <0.05 was considered as statistically significant.

* p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

Figure 6.3: Differential serum CRP concentration (A) among patients with dengue infection and with other febrile illness in different phases of infection (B) among dengue patients with and without WHO-defined warning symptoms.



†Two tailed p value <0.05 considred as statistically significant.

‡ns = non significant.

Figure 6.4: Serum CRP levels among (A) secondary and primary dengue infected patients, (B) dengue IgM positive and IgM negative (C) dengue qRT-PCR positive and negative (D) dengue NS1 positive and negative patients.

Dengue patients with HVL demonstrated significantly higher CRP concentration compared to LVL patients (median: HVL: 37.82mg/L, range: 4.08-91.94mg/L, LVL: 15.76mg/L, range: 2.41-77.98mg/L; p=0.03) (Figure 6.5A). Among circulating serotypes, CRP level of DENV4 (39.14±6.37mg/L) and DENV2 (36.25±5.11mg/L) infected patients was markedly higher than that of DENV1 (14.49±3.03mg/L) and DENV3 (5.87±1.98mg/L) infection (Figure 6.5B).

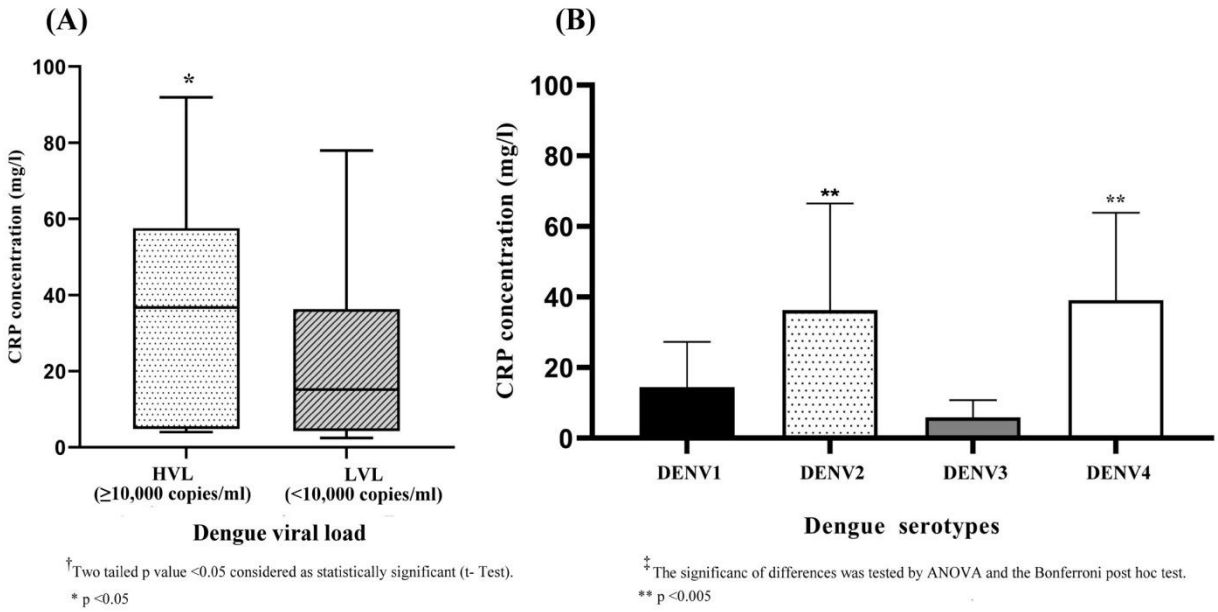


Figure 6.5: Serum CRP level among dengue patients (A) with High Viral Load and Low Viral Load (B) with infected serotypes.

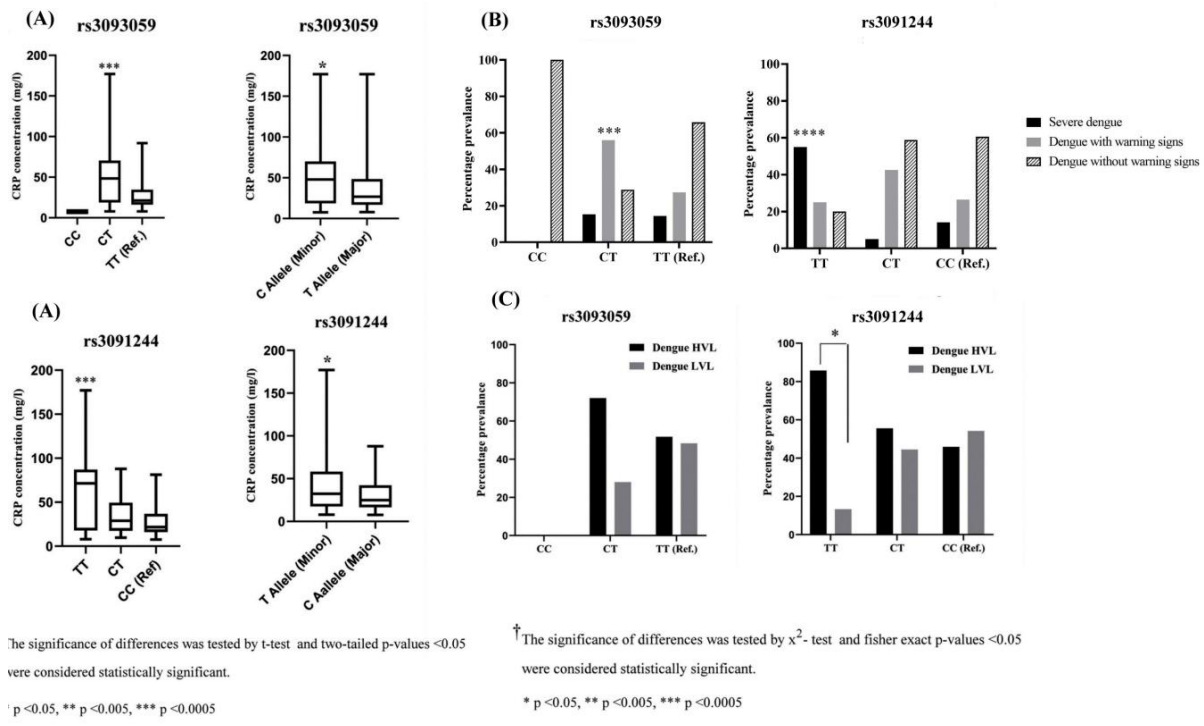
Genotypic distribution of rs3091244 indicated significantly higher prevalence of TT genotype among dengue patients compared to that of age matched healthy controls [OR = 3.54; 95% CI 1.02 – 12.23; p = 0.03] (Table 6.3). Similarly, CT genotype [OR = 1.92; 95% CI 1.03 – 3.55; p = 0.04] and C allele [OR =1.84; 95% CI 1.05 – 3.21; p = 0.03] of rs3093059 was markedly predominant among CRP positive dengue patients.

Table 6.3: Genotypic and allelic distribution of CRP polymorphisms among Dengue patients and healthy controls.

Sr. No	SNP Ref No. And Chromosome no.	Genotype and allele distribution	Healthy Controls (%) N = 102	Dengue infected patients N = 206	*p* value at 95% CI	Odds Ratio
1	rs3093059 chr1	CC	2	1	0.25	0.24 [0.02-2.72]
		CT	21	59	0.16	1.54 [0.87-2.79]
		TT	79	146	Ref	
		C allele	25	61	0.45	1.24 [0.75-2.04]
		T allele	179	351		
2	rs3091244 chr1	TT	3	20	**0.03***	3.54 [1.02-12.23]
		TC	39	80	0.45	1.21 [0.74-1.99]
		CC	60	106	Ref	
		T allele	45	120	0.06	1.45 [0.97-2.15]
		C allele	159	292		
		AC	0	0	NA	NA
		AA	0	0	NA	NA

*p<0.05 at 95% CI was considered as statistically significant.
"ref"= reference genotype, "NA"= Not applicable.

Dengue patients having CT genotype [p=0.0001] and C allele [p=0.01] of rs3093059 and TT genotype [p=0.0001] and T allele [p=0.01] of rs3091244 exhibited higher CRP concentration in serum compared to patients harboring other genotypes of the polymorphisms (Figure 6.6A). CT genotype of rs3093059 [p= <0.006] and TT genotype of rs3091244 [p<0.0001] were significantly more prevalent among severe dengue and WHO-defined warning signs patients, respectively compared to those without any such signs (Figure 6.6B). Prevalence of TT genotype of rs3091244 was also significantly higher among dengue HVL patients [p=0.01] (Figure 6.6C).



The significance of differences was tested by t-test and two-tailed p-values <0.05 were considered statistically significant.

p <0.05, ** p <0.005, *** p <0.0005

†The significance of differences was tested by x^2- test and fisher exact p-values <0.05 were considered statistically significant.

* p <0.05, ** p <0.005, *** p <0.0005

Figure 6.6: Genotypic distribution pattern of polymorphic variants of rs303059 and rs3091244 among dengue patients (a) with serum CRP levels (b) with and without WHO-defined warning signs (c) High Viral Load and Low Viral Load.

6.4. DISCUSSION:

CRP, an acute phase protein, is generally considered as non-specific inflammatory biomarker, which is elevated during microbial infections. But, very limited study has been performed regarding the role of CRP in dengue disease pathogenesis.

Present study depicted importance of specific genotypes of CRP polymorphisms in determining CRP concentration in dengue patients' blood and contribution of CRP in development of WHO-defined warning signs among Eastern Indian patient population. CRP level increased significantly among dengue patients within acute phase of infection and among patients with qRT-PCR/NS1 antigen positivity, high viral load, secondary

infection and infections with DENV4 & DENV2 serotypes. Similar to this study, higher concentration of CRP was previously reported among Taiwanese and Indonesian dengue patients within 1-3 days of symptomatic onset; previous report from 8 countries across Asia and Latin America suggested that CRP levels during first 3 days of illness, could be a useful biomarker for early dengue risk prediction and might assist differentiating dengue from other febrile illnesses (14). Significantly higher CRP level was also detected among severe dengue patients (DHF/DSS) of Indian, Taiwanese and Venezuelan origin and among Venezuelan patients with secondary dengue infection (12, 35-37). Due to higher prevalence of viral particles viz. NS1 antigen, viral RNA (high viral titter) during acute phase of infection, CRP level might have increased significantly to activate complement system and to elicit cellular antiviral response for opsonization of the pathogen (15). Similar to our findings, previous records also confirmed elevated CRP level to be associated to disease severity, secondary infection and DENV2 and DENV4 serotypes and among Venezuelan dengue patients (35). Higher CRP levels indicated more serious tissue damage resulting from greater inflammation, which is often associated with poor disease outcome (38-39). Also, in this study, mortality was reported in one dengue-NS1 positive acute phase (3 day) patient with abnormally high CRP level of 177.14mg/ml.

In the present study, higher concentration of CRP was also found among dengue patients with CT genotype & C-allele of rs3093059 and TT genotype & T-allele of rs3091244. Comparable to this result, increased concentration of CRP was previously detected among Taiwanese patients with C-allele of rs3093059 and T-allele of rs3091244 (40, 41). Though specific genotypes of rs3093059 and rs3091244 were previously associated with hemorrhagic stroke, ischemic stroke, colorectal cancer, HBV-related hepato-cellular carcinoma among Han-Chinese, American-Indian,

Taiwanese and Chinese population, this the first study to investigate role of these CRP polymorphisms for development of dengue disease susceptibility and WHO-defined warning signs among dengue patients (25, 29, 42-45). In this study, patients with TT genotype of rs3091244 and CT genotype of rs3093059 were significantly more susceptible to dengue infection and development of WHO-defined warning sign. Furthermore, TT genotype of rs3091244 was more prevalent among dengue patients with high viral load. Contribution of such genotypic distribution of various SNPs towards increased risk of dengue disease severity and development of warning signs has been previously demonstrated among several polymorphisms of immune system genes viz. JAK1, interleukin-10, interferon gamma and TLRs [19-20]. However, results of this study have to be validated among larger number of dengue patients with severity symptoms (DHF/DSS), which is the limitation of this study. Further, in addition to CRP, levels of several other acute phase proteins, pro-inflammatory cytokine and endothelial markers are also altered during disease pathogenesis which might act as better marker for severe dengue development.

6.5. CONCLUSION:

This is the first study to highlight implication of specific genotypes of CRP polymorphisms viz. rs3091244-TT and rs3093059-CT in determining CRP concentration in patients' blood and role of CRP in development of severe and WHO-defined warning signs among Eastern Indian dengue patients. Moreover, significantly increased CRP level among acute phase dengue patients might act as potential biomarkers for predicting disease severity.

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CHAPTER – 5

Comparison of different serum protein markers concentration (ApoA-1, ANG2, VEGF, SAA2 CRP, FB, SAP etc.) between WHO classified dengue patients (severe, with warning signs & without warning signs) and healthy individuals

7. Chapter – 5

2. Comparison of different serum protein markers concentration (ApoA-1, ANG2, VEGF, SAA2, CRP, FB, SAP etc.) between WHO classified dengue patients (severe, with warning signs & without warning signs) and healthy individuals.

- **Publication**

The scientific work mentioned in this chapter has been published in the following peer reviewed journal:

- **Mukherjee S**, Saha B, Tripathi A*. Clinical significance of differential serum-signatures for early prediction of severe dengue among Eastern Indian patients. ***Clinical and Experimental Immunology***. 2022 May 13; 208(1): 72-82. doi: 10.1093/cei/uxac018. PMID: 35348620; PMCID: PMC9113256.

7.1. REVIEW OF LITERATURE:

Dengue-virus (DENV) infection comprise of a wide clinical spectrum, from simple dengue fever to severe dengue (dengue hemorrhagic fever: DHF; dengue shock syndrome: DSS) which can be life threatening (1). According to World Health Organisation (WHO) simple dengue patients with warning-signs are at higher risk of developing severe-dengue disease compared to patients without warning-signs (1, 3). Dengue patients may progress through three clinical phases viz. febrile, critical and recovery-phases. Febrile-phase is characterized by sudden onset of fever, myalgia, arthralgia, headache, nausea and vomiting; critical-phase is characterized by sudden drop of temperature to 37.5º–38ºC, increase in capillary permeability, increasing haematocrit, leukopenia, thrombocytopenia and plasma leakage; whereas recovery-phase is characterized by decreased in gastrointestinal symptoms, stabilization in hemodynamic status and rashes (1). During early febrile-phase, it is often not possible to predict clinically whether a dengue patient will progress to severe disease. Various forms of severe-manifestations may unfold only as the disease progresses through critical-phase.

Pathophysiology of severe-dengue development is poorly understood. Several factors viz. secondary dengue infection, circulating serotypes, viral titer, host immune response have played crucial role in disease progression (1, 4).Mainly three organ systems of infected patients are primarily affected in DHF/DSS: immune-system, liver and vascular-endothelium (4). Hepatic injury and disruption of endothelial permeability are major differential symptoms for DHF and DSS (5-6). Acute-phase-response (APR) is human's first line of defense against any infection, with altered production of acute-phase-proteins (APPs) mainly by hepatic-cells - comprising of large heterogeneous

group of proteins (7-8). During the time of infection, levels of certain APPs viz. CRP, SAP, SAA2, ApoA1 are elevated and these proteins are referred to as "positive" acute-phase reactants (8). These also act as pattern recognition molecule to trigger host antimicrobial defense mechanisms upon infection, by activating complement system and opsonising pathogen (7-9). Hence, these APPs can be crucial as diagnostic tool in many infectious diseases including viral infections. Previously, significance of CRP, SAP, SAA2, ApoA1 in host defense mechanism against several viral infections like HIV, HCV and Influenza virus has been reported (8-15). But their beneficial role as prognostic biomarker to dengue disease severity has not been well studied. Characteristic hallmark of severe dengue is increased vascular permeability, coagulation abnormalities and fibrolysis, which has been an active area of research (1, 16). Angiogenic proteins viz. Angiopoietin1 (Ang1) and Angiopoietin2 (Ang2) which act in maintaining vascular function and vascular integrity; Ang2 is synthesized in endothelial cells and is a potent inducer of vascular permeability (16-18). Another important angiogenic protein is vascular endothelial growth factor (VEGF) that promotes growth, proliferation and migration of endothelial cell (17-18). A regulated activation of coagulation system is a part of host-defence against infectious agents; but viral activation of endothelium may result in alterations in coagulation and fibrinolytic systems by degrading fibrinogen directly and prompting secondary activation of pro-coagulant homeostatic mechanisms (19-20). Previous studies have demonstrated importance of these endothelial and coagulation factors viz. Ang-2, VEGF and fibrinogen in other viral hemorrhagic fever viz. Crimean-Congo virus, Hanta virus and Ebola virus, as potent clinical-markers (21-25). Hence, investigation of clinical alteration of these factors among acute phase dengue patients and association of platelet degradation might be crucial for early prediction of severe dengue. Hepatic-dysfunction

due to reduced perfusion associated hypoxic injury, as a consequence of a severe vascular-leakage, is another crucial feature of severe-dengue and according to WHO, aspartate aminotransferase (AST) or alanine aminotransferase (ALT) concentration ≥1000 units/liter can be considered as a criteria for severe dengue infection (1, 26-27). Studies have suggested that serum aminotransferases, including ALT and AST are crucial and promising in diagnosis and assessment of viral liver diseases (28). Identification of liver function kinetics associated with severe dengue infection is also very important. Involvement of inflammasome activation during DENV infection, is well established which fuels dengue severity (29). Multiple baseline metabolic parameters viz. serum lipid profile and lipoproteins can exacerbate inflammatory response in severe dengue infection (30). Previous studies have demonstrated importance of these lipoproteins viz. high density lipoprotein (HDL) in exerting a pleiotropic effect on the immune system, such as reduction of inflammation and apoptosis (31, 32)

Being a dengue endemic county, most Indian states are classified as having constant risk of dengue transmission and more precisely, the situation has worsened in eastern India in past few years (33). Hence, availability of dependable markers that could accurately predict severe dengue during early stages of infection might be helpful in triaging patients for better clinical management.

This study deals with evaluation of serum levels of acute phase serum proteins (SAP, SAA2, CRP and Apo A1), endothelial-markers (Ang2 and VEGF), coagulation factor (fibrinogen) and hepatic-markers (AST and ALT) among WHO-classified dengue patients, which can be used as disease severity marker(s) in dengue-endemic areas during early stage of infection.

7.2. MATERIALS AND METHODS:

7.2.1. Ethics Statement:

All experiments performed in this study, including collection of patient's blood as well as that of healthy individuals were done accordingly ethical standards of Clinical Research Ethical Committee, Calcutta School of Tropical Medicine, Kolkata (CREC-STM/53 dated 26.09.2013), following 1964 Helsinki Declaration and its later amendments. Written consents were received from patients and healthy individuals prior to participating in this study.

7.2.2. Collection of Patients' Blood:

Among 309 dengue patients mentioned in **Chapter-1**, confirmed by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, 87 patients from 2016 outbreak were included in **Chapter-5**. To carry out age-matched case control study, blood was collected from 25 healthy unrelated individuals within same community and of same ethnicities, who neither had any signs and history of other infections nor were detected with dengue infection by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR. Sera were separated from blood by density gradient centrifugation and aliquots were stored at -20^0C and -80^0C freezer.

7.2.3. Case Definition:

Clinical symptoms were monitored by clinicians after next 10 days (symptoms monitored during first and subsequent visit to hospital) and patients were categorized into three groups: dengue patients without warning signs (DwoWS), with warning signs (DwWS) and severe-dengue (SD), according to WHO 2009 classification.

7.2.4. Extraction of Viral RNA and Determination of DENV Load:

Viral-RNA was extracted from 140µL of patients' sera, using QIAamp Viral RNA Mini Kit, according to manufacturer's protocol (Qiagen, Courtaboeuf, France) as described in **Chapter-1**. Presence of DENV genome and viral-load among infected patients' serum was determined by qRT-PCR (Taqman assay) in 20µl, using 8µl viral-RNA, OneStep qRT-PCR MasterMix and other components of DENV Genesig kit according to manufacturer's protocol (Primer Design Ltd., UK) as described in **Chapter-1**. Real time PCR was performed on ABI Prism 7500 Fast instrument. A standard-curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided in the kit). All samples were run in triplicate and DENV RNA copy number in patients' serum was calculated from their corresponding Ct values. Viral-RNA titer ≥10,000 copies/ml and <10,000 copies/ml was considered as high viral-load and low viral-load, respectively.

7.2.5. Detection of Anti-Dengue-IgM & IgG Antibodies and Dengue-NS1 Antigen:

Suspected patients' sera were subjected to serological diagnosis for presence of dengue-NS1 antigen and anti-dengue-IgM, using IgM MAC ELISA kit (National Institute of Virology, India; sensitivity: 96% and specificity: 87%) and dengue-NS1 ELISA kit (Panbio, Austrelia; sensitivity: 77.7% and specificity: 93.6%), respectively, according to manufacturer's instructions as described in **Chapter-1**. Patient-serum was subjected to serological diagnosis for presence of anti-dengue-IgG antibody using Panbio dengue IgG capture ELISA kit (80.9% sensitivity, 87.1% specificity).

7.2.6. Determination of DENV Serotypes:

Dengue serotypes of PCR positive samples were determined by real-time RT-PCR in 20µl reaction mixture, containing 8µl viral RNA, One Step qRT-PCR Master-Mix and other components of DENV serotyping Genesig kit, according to manufacturer's protocol (PrimerDesign Ltd., UK) as described in **Chapter-1**. Reactions were carried out in ABI 7500 Fast instrument using serotype specific DENV controls present within the kit.

7.2.7. Quantification of Serum Protein, Endothelial Factors and Coagulation Factor:

Serum levels of SAA, SAP, Ang2, VEGF and Fibrinogen among three groups of dengue patients were determined by sandwich ELISA kits (USCN LifeScience, USA), according to manufactures' instructions. Briefly, the given standard with the kit and patient samples were serially diluted according to the manufacturer's instructions. 100µl of diluted standards and patient samples were added to the respective wells. 100µl of biotinylated antibody reagent was then added to each well. Then the reaction-plate was mixed well by tapping gently and incubated for 1 hours at 37°C. The unbound components of the wells were removed by washing the wells four times using wash buffer provided in the kit. Then 100µl 1x Streptavidin-HRP solution was added to each well and incubated for 30 minutes at 37°C. Again the wells were washed four times using wash buffer to remove unbounded component. Further the wells were incubated with 100µl Stabilized Chromogen solution for 30 minutes at room temperature in dark for color development. Once the color was developed, the reaction was stopped by adding 100µl stop solution provided in the kit. The optical density was read at wavelength 450nm using iMark microplate absorbance reader (BIORAD, USA).

Standard-curve was drawn using GraphPad prism software and levels of each protein were analyzed.

7.2.8. Quantification of CRP:

Quantification of CRP was done by immunoturbidimetry method using AUTOSPAN turbi gold kit (SPAN diagnostics, India), according to manufacturer's instructions. Briefly, 3µl of patient serum/calibrator was mixed with 500µl ten times diluted latex reagent (containing latex particle coated with anti-human CRP antibodies) and its absorbance at 550nm wavelength was measured after 10 seconds (A1) and 120 seconds (A2). Serum concentration (mg/L) of CRP was measured using following formula:

$$\text{Serum concentration of CRP (mg/L)} = \frac{(A2 - A1)\ \text{Serum sample}}{(A2 - A1)\ \text{Calibrator}} \times \text{concentration of calibrator}$$

7.2.9. Quantification of Biochemical and Hematological Parameters:

Biochemical analysis of blood samples, viz. AST, ALT, ALP, bilirubin (Blr) and albumin was done by using a standard auto-analyzer (ERBA model No. EM360). In addition, white blood cell counts, hemoglobin (Hb), and platelet count (Pt) were measured using an automatic cell counter (SYSMEX No -KX100). All tests were performed according to manufacturers' instruction.

7.2.10. Determination of Serum Levels of Apo A1:

Quantification of serum Apo A1 level was carried out using SDS-PAGE followed by western-blotting. Each serum sample was diluted and sample solution (50µg/well) was loaded to 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (90V/24mA, 4hrs). Transfer tank was used for transferring the proteins to polyvinylidene fluoride (PVDF) membrane (30V/90mA, 18hours). To block non-

specific site, PVDF membrane was blocked with 5% BSA and then incubated with diluted primary-antibody (apoA-I:sc-376818, Santa Cruz Biotechnology, USA) (1:2000) for overnight. After successive washing, PVDF membrane was incubated with HRP conjugated secondary-antibody (m-IgGκ BP-HRP:sc-516102, Santa Cruz Biotechnology, USA) (1:20000) for an hour. After proper washing, immune-reactive bands were visualized after addition of Electro-Chemi-Luminescence reagent (Thermo Fisher scientific, USA), which was auto-radiographed. Quantification of Apo A1 in auto-radiographs was carried out through densitometric scanning by ImageJ software (National Institutes of Health, USA).

7.2.11. Protein Network Analysis:

Human gene symbols for studied serum-proteins uploaded to web-based STRING[11.5] tool to construct protein-protein interaction network and only experimentally validated interactions with a combined score >0.4 were selected as significant (34).

7.2.12. Statistical Analysis:

Statistical analyses were done using GraphPad Prism[9] software. Statistical-correlation of levels of serum-markers with presence of WHO-defined dengue severity symptoms during different phases of illness were analyzed using Pearson's correlation matrix and multi-variant analysis was performed by using ANOVA (with bonferroni correction). Mean value with ±SEM of serum-marker levels in each cohort was compared using t-test and $p<0.05$ was considered to be statistically significant. Cut-off value of each marker(s) was determined. Sensitivity and specificity of serum-markers associated with severity was analyzed by receiver operating characteristic curve (ROC). Principal-component-analysis (PCA) followed by hierarchical correlation cluster analysis was used to reduce the number of predictive markers. Principle components (PCs) with

eigenvalues higher than or equal to 1.0 were selected. Serum-marker levels of patients and healthy controls were clustered to obtain a heatmap.

7.3. RESULTS:

In this current study, importance of serum-level of APPs, endothelial, coagulation and hepatic-factors during dengue disease pathogenesis of Eastern Indian patients was evaluated. Out of 168 symptomatic patients, 87 (51.78%) were found to be dengue infected as confirmed by qRT-PCR, anti-dengue IgM and dengue NS1 ELISA among which 10.34% (9/87) were severe dengue, 48.27% (42/87) exhibited WHO-defined warning-signs, whereas 41.37% (36/87) were without any warning-signs. 13.79% (12/87) were found to be of secondary-dengue infection by anti-dengue IgG ELISA. Detailed clinicopathological history of patients has been depicted in Table 7.1.

Table 7.1: Demographic setting and clinicopathological parameters of the study population

			Healthy Controls (n=25)	**Dengue (n=87)**			***p*-value**			
Age			29.2±6.90	25.08±12.70			0.318			
Sex	Male		60% (15)	52.87% (46)						
	Female		40% (10)	47.12% (41)			0.74			
Diagnostic parameters	Dengue NS1antigen						50.57% (44)			
	qRT-PCR						57.47% (50)			
	Anti-Dengue IgM antibody						60.91% (53)			
	Anti-Dengue IgG antibody						13.79% (12)			
			Healthy Controls (n=25)	Patients with severe dengue (SD) (n=9)	Patients with Warning signs (DwWS) (n=42)	Patients without Warning signs (DwoWS) (n=36)	*p*-value[†]	Primary dengue infection (n=75)	Secondary dengue infection (n=12)	*p*-value[‡]
Infection type	Primary Dengue (n=75)		NA	8% (6)	45.33% (34)	**46.66% (35)**	**0.02**[a]	NA		NA
	Secondary Dengue (n=12)		NA	**25% (3)**	66.66% (8**)**	8.33% (1)		NA		
Sample classification	Febrile phase (1-3days) (n=33)		NA	4	19	10	NA	24	9	NA
	Critical phase (4-6 days) (n=21)		NA	5	12	4		20	1	
	Recovery phase (>7 days) (n=33)		NA	0	11	22		31	2	
Circulating			D1 (n=5)	0	20% (1)	**80% (4)**	**0.01**[a]	100% (5)	0	ns

			Healthy Controls (n=25)	**Dengue (n=87)**		***p*-value**			
serotypes (n=50)	Single serotype (n=38)	D2 (n=24)	25% (6)	**66.66% (16)**	8.33% (2)		70.83% (17)	29.16% (7)	
		D3 (n=4)	0% (0)	**75% (3)**	25% (1)		100% (4)	0	
		D4 (n=5)	20% (1)	**80% (4)**	0% (0)		100% (5)	0	
	Multiple serotype (n=12)	D1-D3 (n=1)	0% (0)	0% (0)	100% (1)	0.054	100% (1)	0	ns
		D2-D3 (n=3)	0% (0)	0% (0)	100% (3)		100% (3)	0	
		D2-D4 (n=7)	28.57% (2)	71.42% (5)	0% (0)		71.42% (5)	28.57% (2)	
		D1-D2 (n=1)	0% (0)	100% (1)	0% (0)		100% (1)	0	
Viral titter (n=50)	High viral load (HVL: ≥10000 copies/ml) (n=36)	NA	**88.88% (8)**	**76.66% (23)**	45.45% (5)	0.07	75% (27)	**25% (9)**	0.24
	Low viral load (LVL: <10000 copies/ml) (n=14)	NA	11.11% (1)	30.43% (7)	54.54% (6)		92.8% (13)	7.14% (1)	
	Mean viral load (copies/ml)	NA	**260678±62273**	**58448±19009**	10426±1149	**<0.0001**[b]	36785±11804	**240595±56058**	**0.005**[b]
Clinical symptoms	Fever	NA	100% (9)	100% (42)	100% (36)	ns	100% (75)	100% (12)	ns
	Nausea	NA	33.3% (3)	40.47% (17)	22.22% (8)	ns	29.33% (22)	50% (6)	ns
	Rash	NA	**77.77% (7)**	38.09% (16)	25% (9)	**0.005**[a]	37.33% (28)	33.33%(4)	ns
	Myalgia	NA	66.6% (6)	78.57% (33)	91.66% (33)	ns	80%(60)	83.33% (10)	ns
	Arthralgia	NA	44.44% (4)	47.61% (20)	33.33% (12)	ns	41.33% (31)	41.66% (5)	ns
	Joint Swelling	NA	22.22% (2)	11.90% (5)	8.33% (3)	ns	12% (9)	25% (3)	ns
	Headache	NA	44.44% (4)	61.90% (26)	40.62% (13)	ns	49.33 (37)	50% (6)	ns
	Retro-orbital pain	NA	**55.55% (5)**	16.66% (7)	2.77% (1)	**0.024**[a]	12% (9)	**33.33% (4)**	**0.02**[a]

		Healthy Controls (n=25)		Dengue (n=87)		*p*-value			
	Abdominal Pain	NA	**77.77% (7)**	**66.66% (28)**	0%	**<0.0001**[a]	33.33% (25)	**83.33% (10)**	**0.002**[a]
	Vomiting	NA	**66.66% (6)**	**64.28% (27)**	0%	**<0.0001**[a]	32% (24)	**75% (9)**	**0.008**[a]
	Bleeding	NA	**100% (9)**	0%	0%	**<0.0001**[a]	6.66% (5)	**33.33% (4)**	**0.01**[a]
	Hepatic dysfunctions	NA	**88.8% (8)**	23.80% (10)	2.77% (1)	**<0.0001**[a]	14.66% (11)	**58.33% (7)**	**0.002**[a]
	Fluid accumulation	NA	**44.44% (4)**	11.11% (4)	0%	**<0.0001**[a]	5.33% (4)	**33.33% (4)**	**0.01**[a]
Haematological parameters	Haemoglobin (gm/dL)	14.42±0.52	13.95±0.46	14.09±0.36	14.183±0.26	ns	14.32±0.48	14.08±0.31	ns
	Platelets (Pt) count ($x10^3mm^3$)	166.02±6.06	**112.02±12.86**	160.73±5.09	167.7±2.68	**<0.0001**[b]	153.93±8.71	138.01±9.35	0.24
	WBC count ($x10^3mm^3$)	6.03±0.143	**4.25±0.198**	5.94±0.098	6.06±0.379	**0.02**[b]	5.97±0.329	**4.41±0.12**	**0.001**[b]
Biochemical parameters	ALP (IU/L)	50.52±1.85	**111.7±8.21**	**100.3±4.45**	53.33±1.05	**<0.0001**[b] **<0.0001**[b]	75.39±3.28	**117.41±8.74**	**0.0001** [b]
	Bilirubin (BR) (mg/dL)	0.478±0.066	**0.83±0.099**	0.44±0.034	0.412±0.02	**0.0001**[b]	0.48±0.069	0.81±.12	**ns**
	Albumin (Alb) (gm/dL)	4.45±0.17	**2.89±0.18**	4.24±0.089	4.49±0.057	**<0.0001**[b]	4.39±0.078	**3.86±0.21**	**0.01**[b]
Lipid Profile	HDL (mg/dL)	68.412±2.51	**86.85±3.351**	70.19±1.305	68.74±1.322	**<0.0001**[b]	71.16±1.89	**87.96±4.12**	**0.001**[b]
	LDL (mg/dL)	114.74±3.08	**88.98±4.610**	110.6±2.555	113.2±2.91	**<0.0001**[b]	**114.35**±1.92	**86.29**±4.39	**0.0001**[b]
	Triglyceride (TG) (mg/dL)	143.91±2.58	**180.8±5.23**	**168.8±3.16**	153.8±2.09	**<0.0001**[b] **0.003**	167.24±1.88	176.65±5.97	**ns**
	Total Cholesterol (TC) (mg/dL)	184.84±2.21	182.83±2.14	189.6±1.407	191.5±1.766	Ns	188.32±1.09	184.29±2.41	ns

[a]Contingency chi-square test (fisher's exact), [b]Paired t-test, NA: Not applicable.

[*]*p*<0.05 at 95% CI was considered as statistically significant, ns: non-significant

[†]*p*-value was calculated with respect to patients without warning signs.

[‡] *p*-value was calculated in between primary and secondary dengue cases.

Among dengue infected patients, male to female ratio was 1.12:1 (46:41), whereas that of control group was 1.5: 1 (15:10). Mean age of dengue patients was 25.08±12.70 years (range: 15-61 years), whereas that of control was 29.2±6.90 years (range: 22–57 years). Interestingly, co-circulation of both single serotype and co-infection by multiple dengue serotypes was observed in this study, among which D2 (61.53%) was most prevalent mono-infected dengue serotype followed by D4 (15.38%), D1 (12.82%) and D3 (10.25%); D2-D4 (63.63%) combination was the most predominant co-infecting serotypes followed by D2-D3 (18.18%), D1-D2 (18.18%) and D1-D3 (9.09%). Among PCR positive dengue patients (n=50), approximately 72% (36/50) had viral-load of ≥10000 copies/ml of sera (high-viral-load), whereas remaining 28% (14/50) harbored <10000 copies/ml viral-load (low-viral-load: LVL). Majority of SD patients (8/9) had high viral-load with average viral-titter of 2,60,678±62273 copies/ml, which is significantly higher than that of both DwWS (58,448±19009 copies/ml) and DwoWS (10,426±1725 copies/ml) ($p<0.0001$ at 95% CI) patients. Whereas, 70.58% (24/34) of patients with WHO-defined DwWS and 57.14% (4/7) DwoWS were in HVL group (Table 1). Several clinical, haematological and biochemical parameters had shown significant alteration among SD patients. Clinical symptoms viz. rash, retro-orbital-pain, bleeding, hepatic-dysfunction and fluid accumulation were significantly higher among SD patients. Leukopenia ($p=0.02$, 95% CI) and thrombocytopenia ($p<0.0001$, 95% CI) was significantly more prevalent among SD patients (WBC: $4.25\pm0.198 \times 10^3 mm^3$, Pt: $112.02\pm12.86 \times 10^3 mm^3$) than that of DwWS (WBC: $5.94\pm0.098 \times 10^3 mm^3$, Pt: $160.73\pm5.09 \times 10^3 mm^3$) and DwoWS (WBC: $6.06\pm0.379 \times 10^3 mm^3$, Pt: $167.7\pm2.68 \times 10^3 mm^3$) patients. Similarly, serum ALP was elevated significantly among SD patients

(ALP: 111.7±8.21 IU/L, p<0.0001 at 95% CI) than that of DwoWS patients (ALP: 53.33±1.05). On the other hand, significantly increased bilirubin (0.83±0.099 mg/DL, p=0.0001) and decreased albumin (2.89±0.18mg/DL, p<0.0001) levels were exclusively observed among SD patients.

Concentrations of both vascular-endothelial-markers viz. Ang2 and VEGF were significantly high among SD (Ang2: 1728±140.9pg/ml, *p*-value<0.0001; VEGF: 644.1±22.81pg/ml, *p*-value<0.0001) and DwWS patients (Ang2: 849.9±36.12pg/ml, *p*-value=0.003; VEGF: 304±17.58pg/ml, *p*-value=0.0005) than that of DwoWS (Figure 7.1A). But, significantly lower concentration of coagulation factor, fibrinogen was detected only among SD patients (43612±7093ng/ml, *p*-value<0.0001) compared to DwoWS (63381±1758ng/ml) patients (62256±1486ng/ml). Among APPs, levels of CRP (*p*-value<0.0001), SAA2 (*p*-value<0.0001) and Apo-A1 (*p*-value<0.0001) increased extensively among SD patients (CRP: 66.14±5.71mg/ml, SAA2: 10063±824.6pg/ml, ApoA1: 43.9±4.8) and considerably increased among DwWS patients (CRP: 32.1±4.7mg/ml, SAA2: 8949±618.4pg/ml, ApoA1: 22.8±1.8) compared to DwoWS patients (CRP: 10.71±2.02mg/ml, SAA2: 5211±277.5pg/ml, ApoA1: 16.76±0.89) (Figure 7.2). However, SAP level didn't show any significant alternation among studied patient-groups. Both hepatic-markers viz. AST and ALT elevated markedly among SD (AST: 390.6±19.25, *p*-value<0.0001; ALT: 236.5±8.39IU/L, *p*-value<0.0001) and DwWS patients (AST: 113.3±13.61IU/L, *p*-value<0.0001, ALT: 84.06±8.45IU/L, *p*-value<0.0001) compared to DwoWS patients (AST: 46.79±2.24IU/L, ALT: 41.9±1.45IU/L). AST/ALT ratio indicating hepatic-dysfunction had been significantly elevated among SD (1.64±0.08, *p*-value<0.0001) and DwWS (1.3±0.03, *p*-value=0.001)

patients compared to DwoWS patients (1.1±0.028). A cut-off value of dengue disease severity was analyzed by ROC curve to differentiate SD fever from less severe forms of this disease for identification of markers significantly altered among SD patients compared to non-severe forms. Severity cut-off values were as follows: Ang2≥1129pg/ml (sensitivity-87.50%, specificity-90%, diagnostic *p*-value<0.0001), Fibrinogen<53092 ng/ml (sensitivity-62.50%, specificity-90%, diagnostic *p*-value=0.007), VEGF>526pg/ml (sensitivity-100%, specificity-98.57%, diagnostic *p*-value<0.0001), CRP>46.76mg/ml (sensitivity-87.50%, specificity-87.14%, diagnostic *p*-value=0.0001), SAA2>720.3pg/ml (sensitivity-87.50%, specificity-87.14%, diagnostic *p*-value<0.01), ApoA1>23.18 percent (sensitivity-100%, specificity-75%, diagnostic *p*-value<0.001), AST>227.3IU/L (sensitivity-100%, specificity-95.71%, diagnostic *p*-value<0.0001), ALT>205.2IU/L (sensitivity-100%, specificity 95.7%, diagnostic *p*-value<0.0001) and AST/ALT ratio>1.45 (sensitivity-87.50%, specificity-87.14%, diagnostic *p*-value=0.0001) to predict SD (Figure 1B).

During febrile-phase of infection, endothelial-markers viz. Ang2 (*p*<0.0001), VEGF (*p*<0.0001), coagulation-marker fibrinogen (*p*=0.01), acute-phase glycoprotein ApoA1 (*p*<0.0001) and all hepatic-markers viz. AST (*p*<0.0001), ALT (*p*<0.0001) and AST/ALT ratio (*p*<0.0001) had shown noticeable strong significant alteration among SD patients compared to DwWS and DwoWS (Figure 7.3). But, CRP (*p*=0.0008) and SAA2 (*p*=0.0017) levels elevated significantly during critical-phase of illness among SD group.

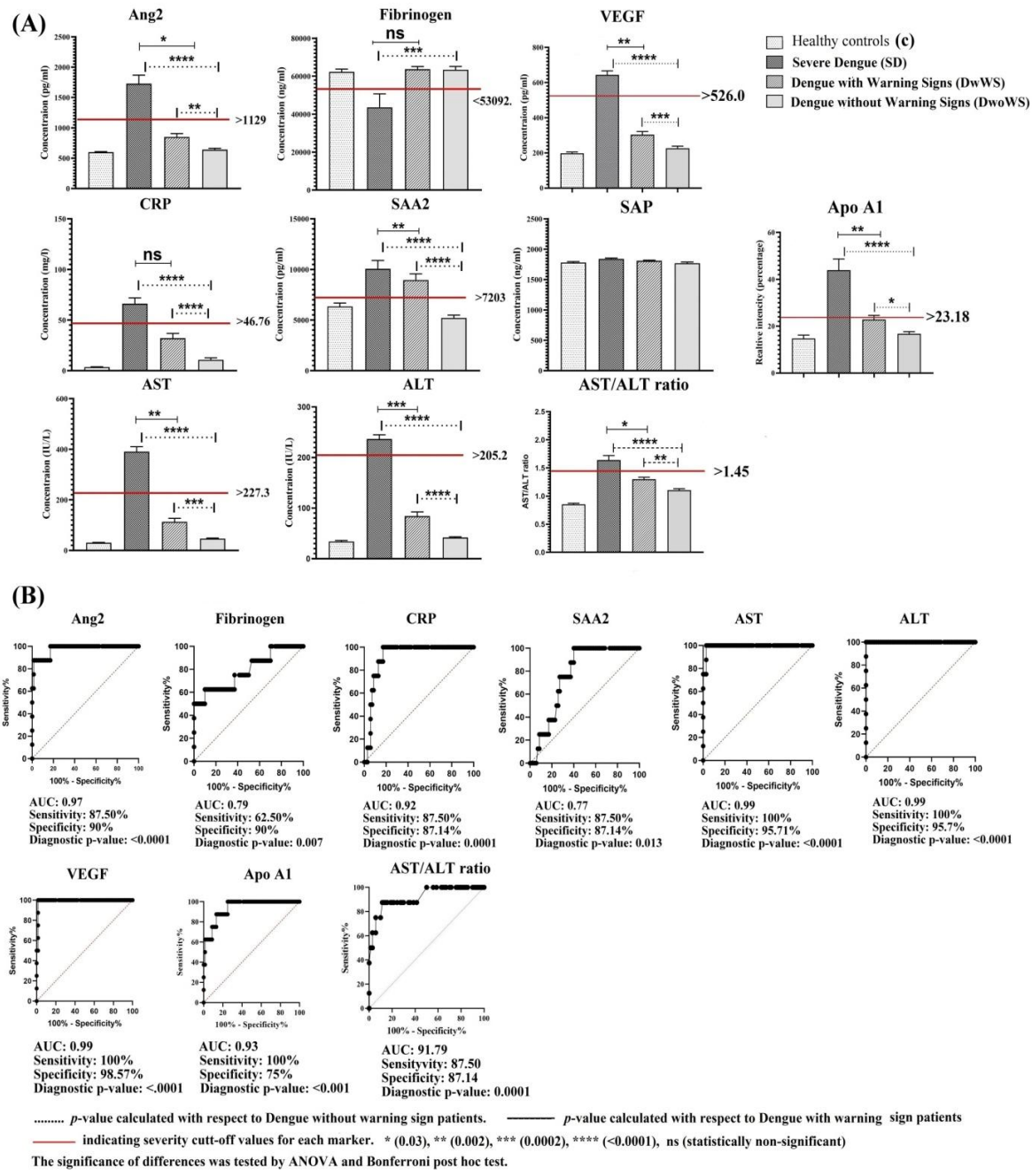


Figure 7.1: (A) Differential concentration of serum-markers among WHO-classified dengue samples and healthy controls. (B) ROC curve analysis of significantly altered markers.

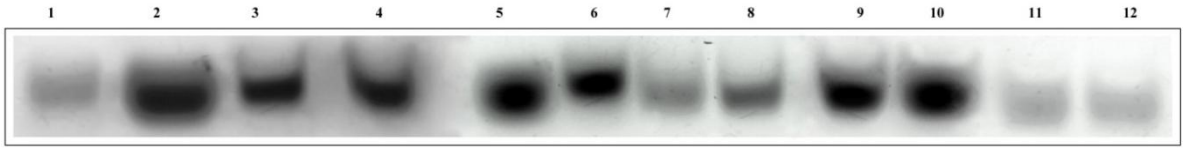
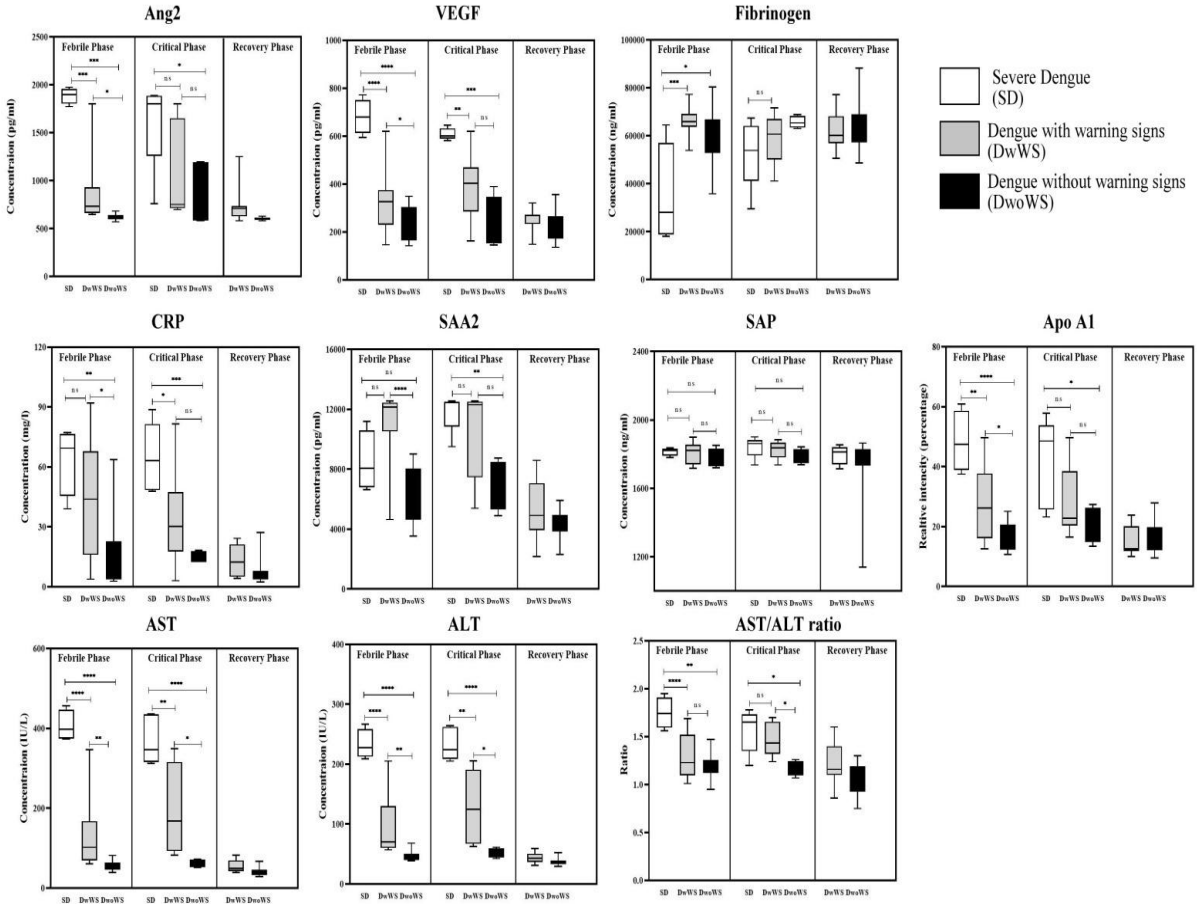


Figure 7.2: Representative photograph of western blot analysis of serum Apolipoprotein-A1 (ApoA1) among dengue patients and healthy controls (lane 1: healthy control, lane 2: severe dengue, lane 3: dengue with warning signs symptoms and lane 4: dengue without warning signs symptoms, lane 5-6: high-viral-load (HVL) group, lane 7-8: low-viral-load (LVL) group, lane 9-10: secondary dengue, lane 11-12: primary dengue.)



Two-tailed *p*-value <0.05 was considered as statistically significant. *: 0.03, **: 0.002, ***: 0.0002, ****: <0.0001 and ns: non-significant.

Figure 7.3: Comprehensive analysis of serum-markers at different stages of dengue infection.

Significantly higher levels of Ang2 (HVL: 1126±101.4pg/ml, LVL: 746.8±81.04, *p*=0.005), VEGF (HVL: 422.6±30.44pg/ml, LVL: 279.2±37.01, *p*=0.005), CRP (HVL: 47.2±5.8mg/ml, LVL: 21.23±5.78mg/ml, *p*=0.003), SAA2 (HVL: 9880±606.9pg/ml, LVL: 7677±867.7pg/ml, *p*=0.04), ApoA1 (HVL: 30.91±2.7, LVL: 21.64±2.2, *p*=0.01) and AST (HVL: 191.2±27.04IU/L, LVL: 104.6±28.43IU/L, *p*=0.03) were observed among HVL-group of patients (Figure 7.4.). However, levels of fibrinogen, SAP, ALT, AST/ALT ratio didn't significantly alter with respect to viral-titer.

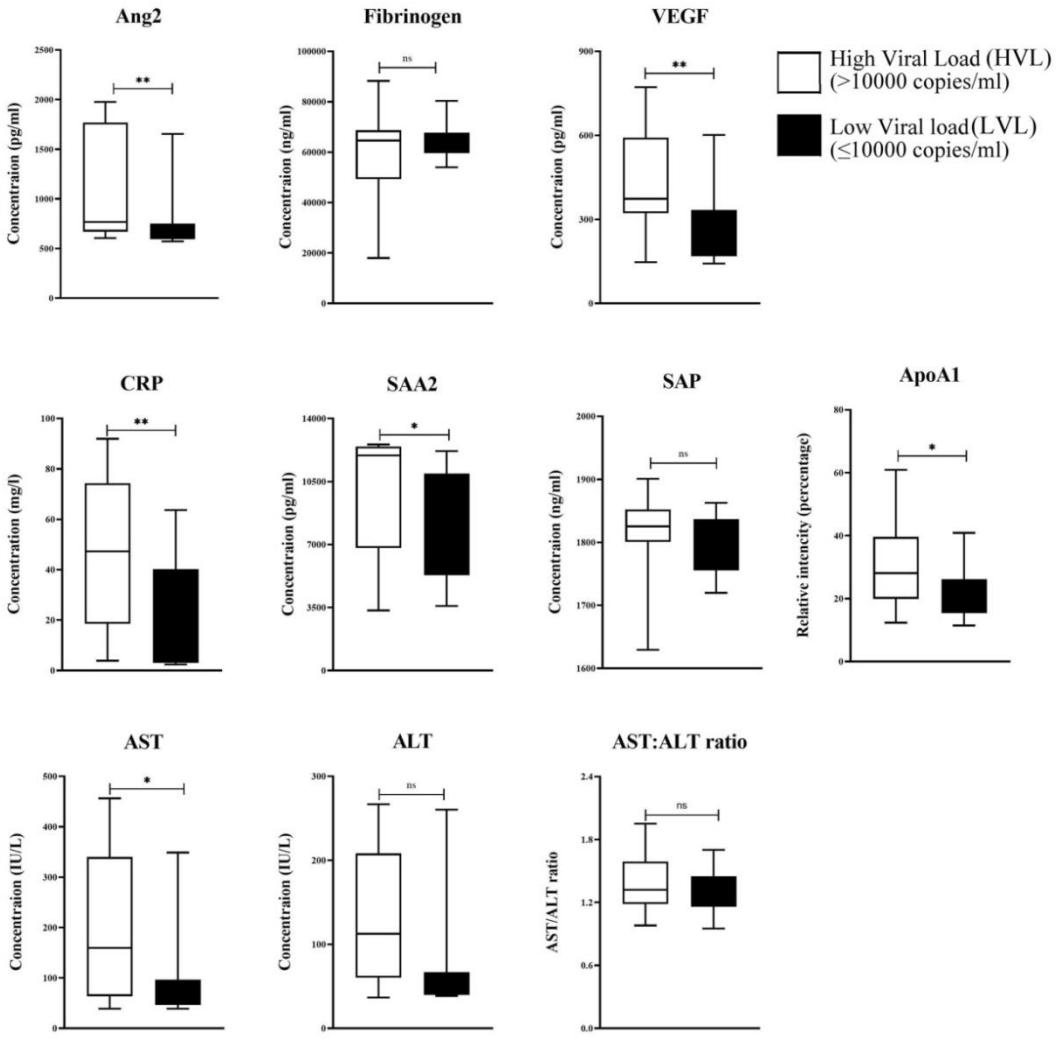
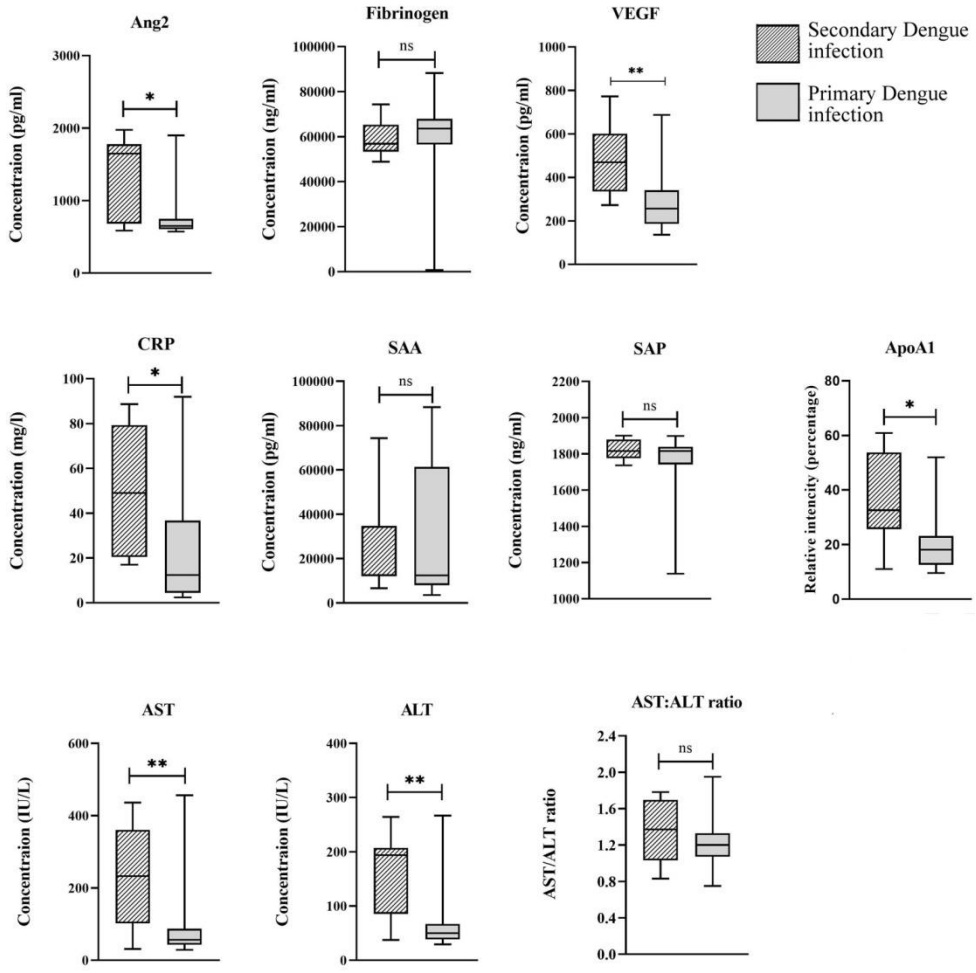


Figure 7.4: Comparison of serum-markers between high and low viral-load dengue patients.

Levels of studied markers were also analyzed among primary and secondary-infections (Figure 7.5.). Significantly increased serum-concentration of Ang2 (secondary: 1277±196.3pg/ml, primary: 802.3±43.4 pg/ml, p=0.04), VEGF (secondary: 478.4±53.36pg/ml, primary: 280.3±15.83, p=0.005), CRP (secondary: 49.4±9.4mg/ml, primary: 23.05±2.98, p=0.02), ApoA1 (secondary: 37.31±5.49, primary: 20.04±1.15, p=0.01), AST (secondary: 248.5±48.9IU/L, primary: 94.43±11.96IU/L, p=0.001) and ALT (secondary: 157±25.90IU/L, primary: 70.23±6.96IU/L, p=0.009) were observed among secondary-dengue patients compared to primary-infections.



Two tailed p-value <0.05 was considered as statistically significant (paired t-test with Welch correction)

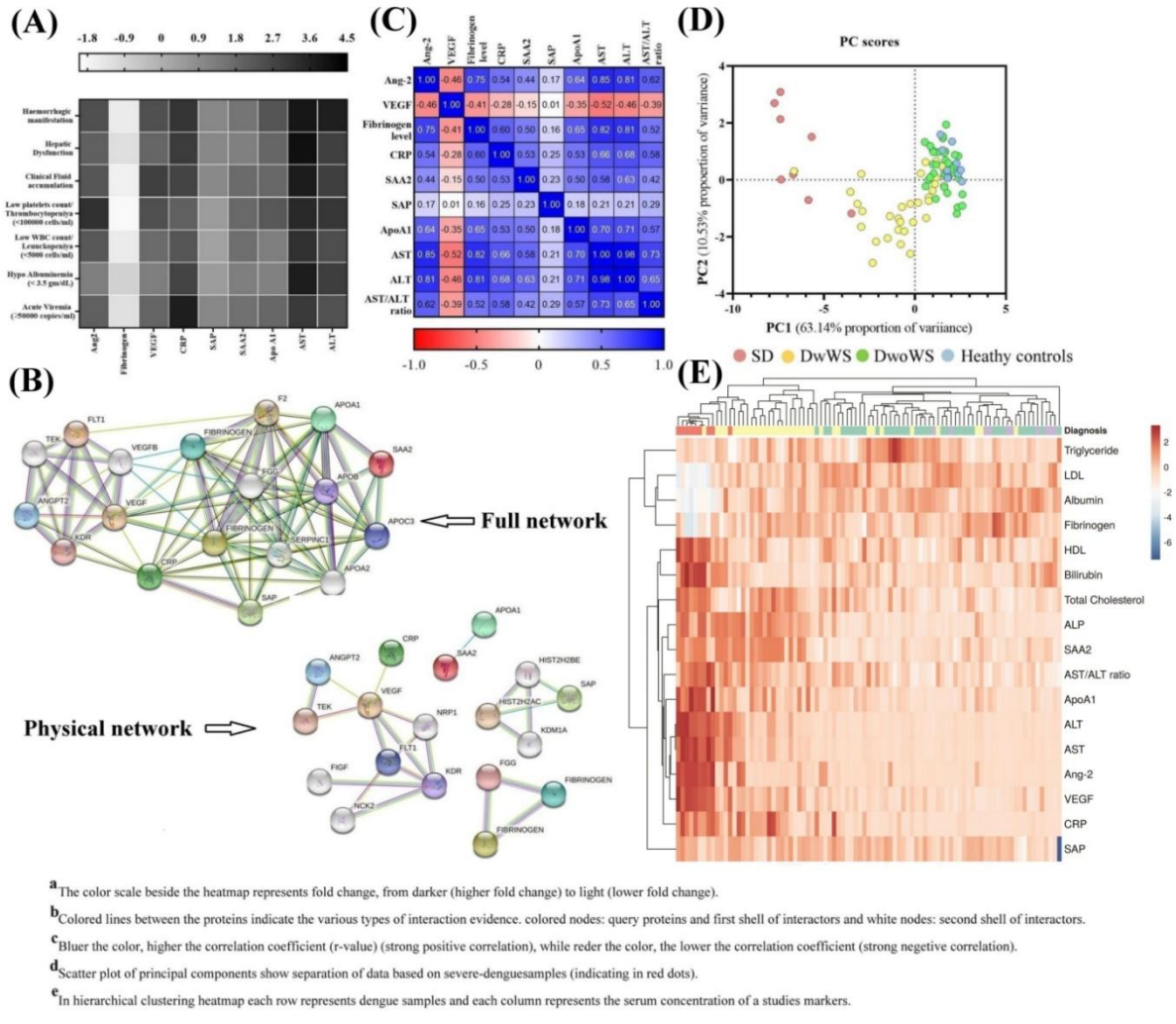
*** (0.03), ** (0.002), *** (0.0002), **** (<0.0001), ns (Statistically non-significant)**

Figure 7.5: Differential concentration of serum-markers between secondary and primary dengue infection patients.

Heatmap analysis of studied markers with various clinicopathological manifestations contributing to dengue disease severity revealed that Ang2, VEGF increased more than 2-fold and fibrinogen decreased 1.8-fold among patients with haemorrhagic manifestation, clinical-fluid accumulation and low platelets count (<100000cells/ml) (Figure 7.6A). Whereas, CRP level was remarkably elevated among patients with haemorrhage (3.13-fold), acute viremia (3.72-fold) (>50000copies/ml), hepatic-dysfunction (2.9-fold) and clinical-fluid accumulation (2.76-fold). AST and ALT concentrations were significantly increased among patients with haemorrhage (AST: 3.9-fold, ALT: 3.7-fold), hepatic-dysfunction (AST: 4.08-fold, ALT: 2.86-fold), thrombocytopenia (AST: 3.4-fold, ALT: 3.08-fold) and hypo-albuminemia (AST: 3.75-fold, ALT: 2.90-fold). Figure 7.6.B depicted protein network generated by studied proteins using STRING database. Functional network analysis revealed Ang2, VEGF & CRP to be functionally and physically connected and SAA2 & ApoA1 to be functioning together (PPI enrichment *p*-value=1.1e-11). Signaling pathway analysis revealed Complement and coagulation cascades, HIF-1signalling and platelet-activation to be primarily affected during severe dengue pathogenesis– thereby affecting plasminogen-activation, blood-vessel endothelial cell migration and fibrinolysis. Correlation analyses of all studied markers also validated this connectivity by a strong positive correlation between Ang2, VEGF and CRP (Figure 7.6.C). Fibrinogen was negatively correlated with Ang2 and VEGF. Both hepatic-markers, AST & ALT had shown positive correlation with CRP, SAA2 and ApoA1.

PCA identified PC1 (63.14%) and PC2 (10.53%) to have highest proportion of variance. Hence, considering components 1 and 2, PCA could distinguish between SD and DwWS/DwoWS groups, with DwoWS group overlapping with healthy individuals (Figure 7.6D). Hierarchical-clustering and heatmap analysis of all serum-markers

among SD, DwWS, DwoWS and healthy groups, screened on the basis of fold change, almost segregated SD group from the rest, with VEGF, Ang2, ApoA1, AST and ALT clearly distinguishing SD group (Figure 7.6.E). However, further studies are required to validate correlated markers and to explore underlying functional mechanisms.



[a] The color scale beside the heatmap represents fold change, from darker (higher fold change) to light (lower fold change).

[b] Colored lines between the proteins indicate the various types of interaction evidence. colored nodes: query proteins and first shell of interactors and white nodes: second shell of interactors.

[c] Bluer the color, higher the correlation coefficient (r-value) (strong positive correlation), while reder the color, the lower the correlation coefficient (strong negetive correlation).

[d] Scatter plot of principal components show separation of data based on severe-denguesamples (indicating in red dots).

[e] In hierarchical clustering heatmap each row represents dengue samples and each column represents the serum concentration of a studies markers.

Figure 7.6: (A) Heatmap analysis of differential fold change of serum-markers with clinicopathological parameters (B) protein-protein network analysis of studied serum proteins (C) Pearson's correlation analysis among studied serum-markers (D) variance among different group of dengue patients according to PCA analysis (E) hierarchical clustering heatmap of studied markers among different group of dengue patients.

7.4. DISCUSSION:

Among various forms of dengue disease, severe-dengue is considered life-threatening with case-fatality rate of 2.6% among infected Indian patients and 10-20% patient mortality rate among DHF and 40% among DSS patients (2). Hence, early clinical prediction of severe-dengue cases will be helpful in better patient-management. Till date there are no reliable and specific biomarkers for early diagnosis and prognosis of severity development among infected patients. This is the first detailed comprehensive molecular analysis of serum-markers from eastern-Indian dengue patients with molecules related to acute phase of infection (CRP, SAA2, SAP, ApoA1), vascular-endothelium (Ang2, VEGF), coagulation (fibrinogen) and hepatic injury (AST, ALT and AST:ALT ratio) for early identification of severe cases.

In this study, Ang2 and VEGF concentrations significantly increased among SD patients compared to both DwWS and DwoWS. Whereas, fibrinogen level decreased among SD patients compared to DwoWS. These were more clearly evident during febrile-phase (0-3 days) of infection, implicating their importance to differentiate severe cases at early stage of disease. Higher Ang2 and VEGF levels were previously reported among Srilankan and Indian severe-dengue patients, respectively during 9^{th} day and significantly lower fibrinogen among Vietnamese severe-dengue patients during 2^{nd} day of symptomatic onset (20, 35-36).

As hepatic-cells were mainly affected in severe-dengue, APPs, produced from hepatic cells provided first line of defense against such viral infection (7). Similar to this study, significant elevation of CRP, SAA2 and ApoA1 levels were previously reported among severe-dengue patients of Singaporean and Taiwanese origin (37-38). Liver involvement in dengue infections ranges from mild subclinical biochemical changes to severe liver

disease. AST:ALT ratio was considered as a superior marker for various types of liver injuries than alteration in AST or ALT alone (39). This is the first study to find significantly higher AST:ALT ratio of ≥1.7 among patients with SD during first 3 days of symptomatic onset. Similar to this study, significant elevation of liver enzymes were reported among severe-dengue patients from Srilanka, Brazil and Taiwan (40-41).

Higher levels of APPs (CRP, SAA2, ApoA1), endothelial markers (Ang2, VEGF) and hepatic-marker, AST were reported among HVL patients compared to LVL. HVL has been previously associated with SD infection; presence of higher number of viral particles might have significantly triggered APR thereby activating complement system to elicit cellular antiviral response (1, 7). Such immune activation might have contributed to endothelial-dysfunction and vascular-leakage, which could induce hypoxic injury with hepatic-dysfunction (16, 26-27). CRP, ApoA1, AST, ALT, Ang2 and VEGF were significantly elevated among secondary dengue patients compared to those with primary infections. Previous studies have also indicated implication of immune-complexes in mounting compliment activation, vascular permeability and coagulopathy to be significantly associated secondary-infection (42).

Decreasing fibrinogen concentration of coagulation cascade among severe-dengue patients might have activated complement system and platelet-activation, thereby liberating vasoactive peptides (anaphylatoxins) and increasing permeability of blood capillaries (43-44). On the other hand, increase in Ang2 and VEGF levels might have altered HIF-1 signaling, which could have increased dengue replication in this group of patients (VL: 98769-488956 copies/ml) (45). This relation was further evident in overlapping of severe-dengue patient with DwWS group in PCA (Figure 7.6.D), demonstrating lowest viral load (VL: 8867 copies/ml) and Ang2, VEGF levels among

SD group. Similarly, DwWS patients overlapped with SD group in PCA exhibited relatively higher VL and highest Ang2 and VEGF level of this group. Thus, this study had important clinical significance for better understanding of severe-dengue development among infected patients. Until now, clinically applicable biomarker(s) for severe-dengue are lacking. Therefore, exploring serum-proteomic analysis of dengue patients during first three days of symptomatic onset might be a powerful platform for discovery of novel, specific and more reliable biomarkers for severe dengue diagnosis and prognosis.

7.5. CONCLUSION:

We conclude that serum levels of VEGF, Ang2, ApoA1, AST and ALT may be useful as potential and reliable biomarkers for predicting dengue disease severity during early stage of infection.

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CHAPTER – 6

Determination of anti-viral effects of Quercetin against dengue virus.

8. Chapter – 6

1. Determination of anti-viral effects of Quercetin against dengue virus.

- **Publication**

The scientific work mentioned in this chapter has been communicated for publication in peer reviewed journal:

Evaluation of antiviral, therapeutic, immunomodulatory effect of quercetin against dengue virus infected Balb/C mice. (Communicated)

8.1. REVIEW OF LITERATURE:

Dengue virus (DENV) infection is considered to be among one of the most concerning global health issues among 112 dengue endemic countries across the globe which causes an array of clinical manifestations ranging from simple-dengue fever to severe fatal clinical manifestation (1). Compared to simple-dengue, severe-dengue infection is marked by significantly increased release of pro-inflammatory cytokines, viz. IL-6, TNF-α and augmented oxidative stress generating reactive oxygen species (ROS) which leads to increased permeability and hemorrhage (2-3). World health organization (WHO) has linked increased hepatic and renal impairment with increase in dengue disease severity (1). Currently dengue infection is listed among category-A priority pathogen as declared by National Institute of Health, USA (4).

Till date, there is lack of effective antiviral drugs against DENV and absence of a suitable animal model that can represent complexity of dengue pathogenesis has contributed to lack of understanding of the disease (5-6). Only vaccine available in the market is CYD-TDV (chimeric yellow fever virus-dengue virus tetravalent dengue vaccine) (Sanofi Pasteur, France) but it has been reported to increase the threat of severe dengue in individuals, especially among children who have not been infected previously (7-8). There are several dengue vaccine candidates currently under process of clinical development, but none have been successful to effectively treat dengue infection (9-13). At present, dengue patients are treated only for symptoms viz. muscle aches and pains and fever by supportive care such as, fever reducers and pain-killers and responsive fluid management (1). Natural plant derived compounds remain an important source for development of new antiviral drugs because of their low side-effects and their high

accessibility in nature (14). Numerous phytochemicals and various flavonoids are reported to have antiviral activity against dengue virus (15-16). Quercetin is one such natural compound of flavonol group, approved by the U. S. Food and Drug Administration (FDA; National drug code numbers are 65,448-3085, 65,448-3005), known for its antioxidant, antiviral and anti-inflammatory properties that can be found mainly in plants, black tea and fruits such as apple, berries, grapes, onions, tomato etc (17-18). Due to its potential benefits on human health, quercetin has become a natural ingredient of great interest in pharmaceutical industries (18). Its biological activities have been largely attributed to its active phenolic hydroxyl groups and double bonds. Quercetin was also reported to inhibit Herpes Simplex and Influenza viral entry in green monkey and canine kidney cells; it inhibited Japanese Encephalitis and Hepatitis C viral replication in Vero and Huh-7 cells (19-22). Also, quercetin inhibited reverse transcriptase, proteases and viral capsid proteins of HIV, HCV and influenza viruses, respectively (23). Major challenge in development of potent antiviral therapeutic drug against dengue virus is absence of suitable experimental animal model that reflects the pathological features and clinical symptoms, as seen in humans (24). AG129 and Balb/C mice strains were reported to sustain DENV replication and develop a robust immune response without febrile manifestation (24-25). However, infection of these mice strains with mouse-adapted DENV resulted in demonstration of signs for severe disease viz. liver damage and vascular-leak syndrome, similar to humans (25).

This is the first study aimed to investigate prophylactic, antiviral, therapeutic and immunomodulatory potential of quercetin against mouse-adapted DENV within Balb/C mice model.

8.2. MATERIALS AND METHODS

8.2.1. Chemicals and Reagents:

Quercetin and DMSO were purchased from Sigma-Aldrich (Missouri, USA**)** and Sissco Research Laboratories Pvt. Ltd (India), respectively. All used reagents were of analytical grade.

8.2.2. Animal Ethical and Study Design:

All mice related experiments were performed on 6-8 weeks (12-15g) old Balb/c mice. Detailed flowchart of the work flow has been represented in Figure 8.4. These experimental protocols were approved by Institutional Animal Ethics Committee (Ref. no. ARECSTM/071 dated 19/1/2018). Animals were maintained at customary pathogen free environmental conditions (23°C – 25°C, 12 h/12h light/dark cycle) with standard pelleted diet and water ad libitum. All animals were habituated to laboratory atmosphere for a week before study proceeded.

8.2.3. Determination of Toxicological and Behavioral Effects of Quercetin

8.2.3.1. Acute Toxicity and Dose Calculation:

Acute toxicity study was evaluated as per OECD guidelines (OECD 425). Briefly, both male and female Balb/c mice were divided into five groups, each comprising 4 animals. Group-I received normal saline and represented as normal control group (NC); Group-II received 0.5% DMSO as vehicle control (VC); Group-III received high dose (HD), Group-IV received mid dose (MD) and Group-V received low dose (LD) of quercetin once daily for 14 days. Precise observational studies, which included changes in

physical appearance and behavior, were conducted. Body weight lose/gain was also monitored on regular basis.

Quercetin doses used for toxicity study were derived from its LD_{50} values present within PubChem database, prepared in 0.5% DMSO for oral and intraperitoneal (ip) administration (Figure 8.1). Dose equivalent to LD_{50} was considered as HD; followed by two-fold serial dilutions to get MD and LD, respectively. For oral: $HD_{quercetin}$: 160mg/kg/day, $MD_{quercetin}$: 80mg/kg/day, $LD_{quercetin}$: 40mg/kg/day and for ip: $HD_{quercetin}$: 16mg/kg/day, $MD_{quercetin}$: 8mg/kg/day, $LD_{quercetin}$: 4mg/kg/day were selected for acute toxicity study. Doses were calculated according to body weights of experimental animals.

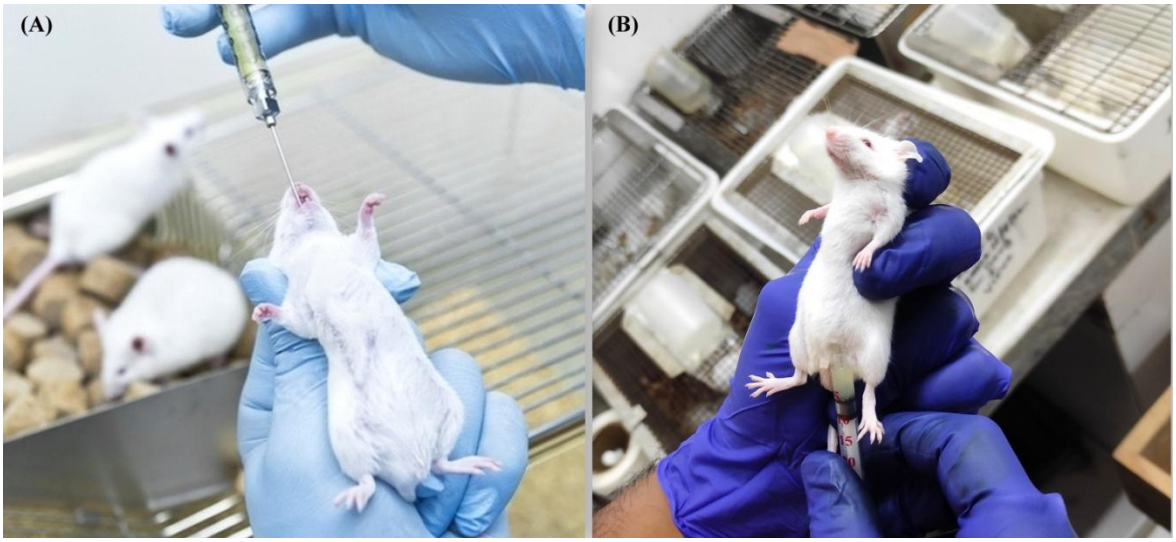


Figure 8.1: Representative pictures of oral and i.p. administration of quercetin to Balb/C mice.

8.2.3.2. Behavioral Studies:

Effect of quercetin on behavioral changes of mice, viz. anxiety and depression, was evaluated after 14th day of initial quercetin administration, which was scored by an independent observer, blinded about animal groups and drug treatments.

To determine any anxiety-like behavioral phenotype upon quercetin treatment, mice were food-deprived overnight after quercetin dosage after 14th day and placed in the middle of a novel open field square (45x45 cm) chamber and allowed to freely explore for 6 minutes. Movement was recorded and tracked with video camera and ToxTrac software (26), respectively (Figure 8.2 and Figure 8.3). Points were given depending upon their natural behavioral attributes viz. searching, sniffing, rearing and grooming. To determine any depression-like behavioral change due to Quercetin-treatment, mice were suspended by tail for 6 minutes by attaching them to a horizontal bar with adhesive tape placed 2cm from tip of tail. Mice were considered immobile only when they hung passively and were completely motionless.

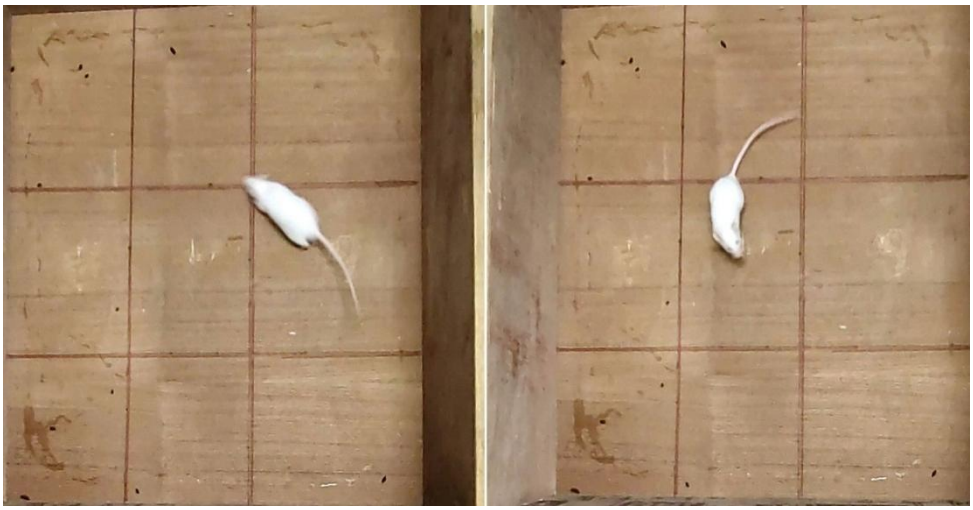


Figure 8.2: Representative picture of open field test for anxiety-like behavior.

Figure 8.3: Representative picture of tail suspension test for depression-like behavior.

8.2.4. Determination of Antiviral Activity of Quercetin:

8.2.4.1. Preparation Viral Stocks:

DENV-2 stock was prepared in suckling mouse brain. Newborn suckling mice were inoculated with approximately 10µl of high-titer ($3.69x10^5$copis/ml) DHF patient serum intrcerebraly and then sacrificed after symptomatic onset of paralysis and hunched back. After three blind passages, brain was aspirated and macerated with 7.5% bovine serum albumin solution in PBS and centrifuged at 12000rpm for 15 minutes; supernatant was stored at −80 °C until use, as mouse-adapted DENV stock.

8.2.4.2. Determination of 50% Lethal Infection Dose:

Twelve 6–8 weeks old Balb/C mice were divided into 3 groups with 4 in each group; Mice were intraperitoneally inoculated (0.4ml) with three dengue viral titers viz. $2 x10^5$, $1x10^5$ and $5x10^4$ copies/ml, respectively and observed for morbidity and mortality for 30

days post-infection (dpi). Mice were continuously monitored throughout the course of infection and those which exhibited overwhelming signs of severe disease or weight loss were included in survival curve analysis. To determine viral kinetics, mice-blood was collected from its facial vein at 1 day interval for 8 days. Morbidity scoring was done based on 1-6 scale, where, 1: healthy, 2: mild signs of lethargy, 3: lethargy with ruffled fur, 4: lethargy with ruffled fur & decreased mobility 5: lethargy with ruffled fur, decreased mobility & hunched posture, 6: moribund.

8.2.4.3. Treatments of Infected Balb/C Mice with Quercetin:

Twenty eight BALB/c mice, aged between 6-8 weeks were divided into seven groups; [1] Control-group: uninfected and untreated, [2] Infected-group: only DENV-2 infected, [3] Pre-treatment-group: treated with quercetin from 3 days before DENV-2 infection (to check prophylactic activity) [4] Co-treatment-group: treated with quercetin along with DENV-2 infection (to check virucidal activity), [5] Post-treatment-group A (Po-A): treated with quercetin 24hpi [6] Post-treatment-group B (Po-B): treated with quercetin 48hpi [7] Post-treatment-group C: treated with quercetin 72hpi of DENV-2 (to check therapeutic activity). Infection of mice was achieved by inoculating with 400µl of $5x10^4$ copies/ml DENV2 intraperitonealy, while non-toxic doses of quercetin were selected for oral and intraperitoneal administration and administrated once daily for 3 days. Mice were monitored daily for development of any clinical signs until completion of study and blood was collected from its facial vein at 1 day interval for 8 days pi, weights of mice were recorded before they were anaesthetized and sacrificed by cardiac puncture. Livers and kidneys were collected for histopathological analysis.

8.2.4.4. Viral RNA Isolation and Quantification of Viral Load:

Serum was obtained from blood by centrifugation and RNA was extracted by QIAamp viral extraction kit, as per manufacturer's instructions. Viral load of infected and treated mice sera was determined by qRT-PCR in 20µl, using 8µl viral-RNA, OneStep qRT-PCR MasterMix, and other components of DENV Genesig kit, according to manufacturer's protocol (Primer Design Ltd., UK). Real-time PCR was performed on ABI Prism 7500 Fast instrument. A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided within kit). DENV RNA copy number in mice sera was calculated from corresponding Ct values.

8.2.4.5. Quantification of Vascular Leakage:

0.2ml of Evans-blue solution (0.5% in PBS) was injected in tail vein of mouse by intravenous route. After two hours, mouse was euthanized and its liver was harvested and placed in pre-weighed tubes containing formamide for 24 hours. Presence of Evans-blue in formamide was detected by measuring its absorbance at 610nm to determine its concentration per gram tissue weight.

8.2.4.6. Measurement of Cytokine ConcentrationS

Concentration of cytokines viz. TNF-α and IL-6 in mice-sera was assayed by using sandwich ELISA kits (R&D Systems, Minneapolis, the USA), according to manufacturer's protocol. Results were represented as pg/ml. Absorbance was measured at 450nm and tests were done in triplicate. Standard curve was drawn in GraphPad

prism9 and concentration of each cytokine was determined from their respective OD values.

8.2.4.7. Measurement of Intracellular Ros in whole Blood:

ROS-sensitive fluorescent probe H_2DCFDA (Thermo Fisher Scientific, USA) were used to quantify ROS generation in mice whole blood and rate of H_2DCFDA oxidation was measured in F-2700 Fluorescence-Spectrophotometer (HITACHI, Japan). Briefly, blood samples were 10 times diluted with PBS and incubated with 25mM H_2O_2 for 4 hours in complete darkness. After H_2O_2 stimulation, samples were again incubated with 25mM H_2DCFDA for 30 minutes in dark. Fluorescence was measured in fluorescence-spectrophotometer with excitation and emission maxima of 488 and 530nm, respectively.

8.2.5. Haematological, Biochemical Parameters and Histology of Liver and Kidney:

Animal was anesthetized with isofluren inhalation after acute-toxicity (i.e. 15^{th} day) and anti-viral quercetin treatment (i.e. 8^{th} day) study; blood was collected by cardiac puncture for different haematological and biochemical studies. Total red blood cells (RBC), white blood cells (WBC), percent hemoglobin (Hb), haematocrit (HCT) and differential count (DC) were analyzed by Erba H360 auto analyzer (Erba, Italy). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea and creatinine were estimated using standard kits (Span Diagnostics, India).

After blood collection, animals were killed by cervical dislocation. Each selected organ was cut into small pieces and kept in paraformaldehyde (10% at room temperature) for

24 hours and processed for histological study. After fixation, tissues were dehydrated through increasing ethanol grade series (30%, 50%, 70%, 90% and 100%), then tissues were embedded in paraffin (Merck). Paraffin embedded dehydrated tissue sections were cut using a microtome (Leica Biosystems, Germany) and stained with hematoxylin and eosin. Sections were photographed using BX43-Olympus-Life science microscope coupled with Olympus DP21 stand-alone digital camera (Olympus, Tokyo, Japan).

8.2.6. Statistical Analysis:

Statistical analyses were done using GraphPad Prism[9] software. Sample size calculation was done by using Resource Equation approach to calculate minimum number of mice required for this study (27). To assess the effect of quercetin treatment on viral load, cytokines level, haematological and biochemical parameters of sera, multivariant analysis by ANOVA (with post-hoc correction) and paired t-tests were performed among each treatment group and vehicle-control. Data was expressed as mean±SEM (standard error mean) and p-value <0.05 was considered as statistically significant.

8.3. RESULTS:

A schematic representation of experiments to study toxicological and anti-viral effect of quercetin has been depicted in Figure 8.4. During period of quercetin treatment for 14 days, no death was observed among experimental mice. No sign of long-term abnormalities in physical and behavioral appearances was observed among mice receiving all doses of quercetin via i.p. route and those receiving low-dose (LD) and mid-dose (MD) of quercetin orally. Mice receiving high-dose (HD) of quercetin orally exhibited some signs of toxicity, viz. erected hair and body weakness, initially for first few days, which got normalized with time.

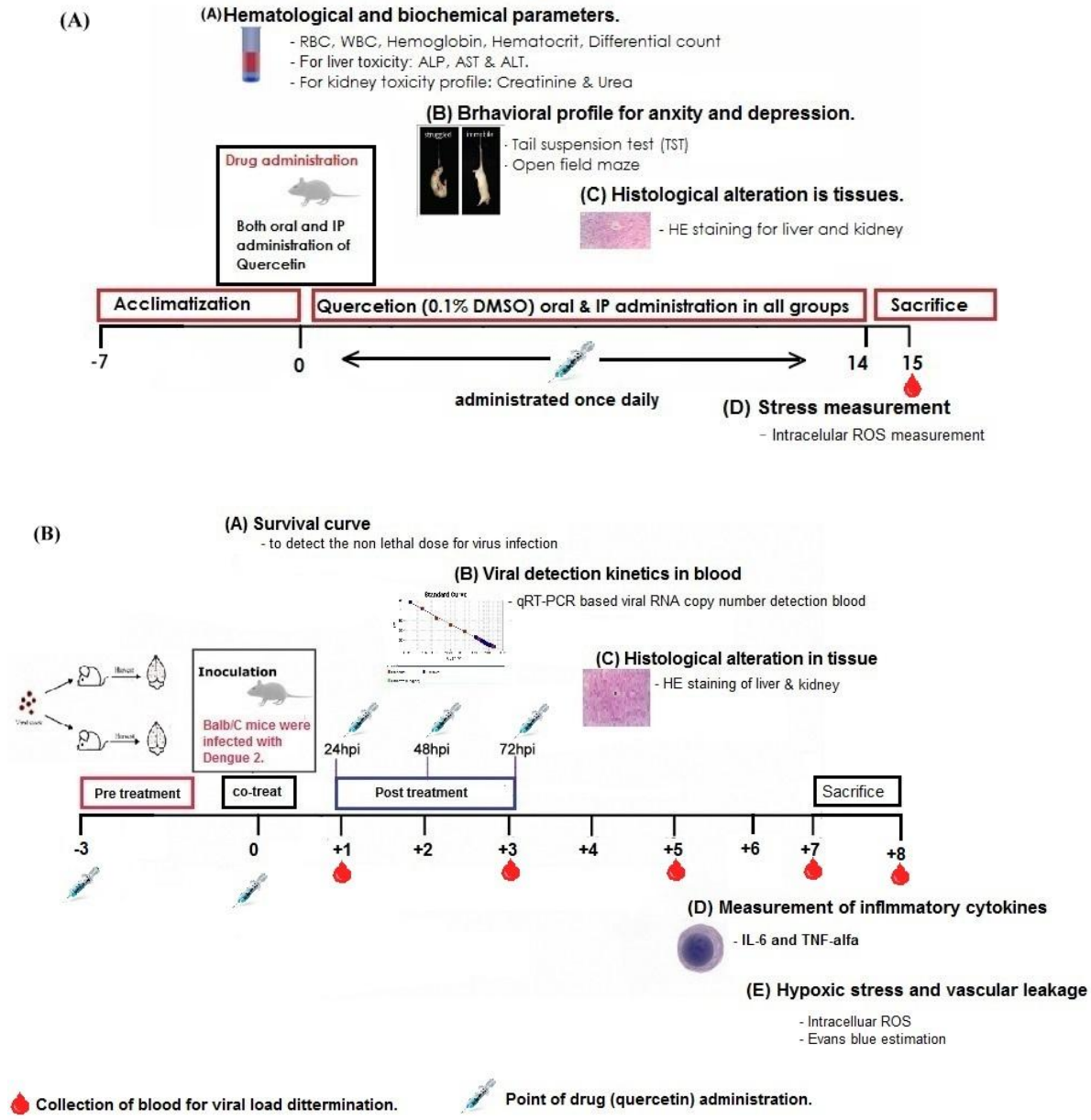


Figure 8.4: Schematic respresentation of treatment time line for (A) toxicological study and (B) antiviral study of quercetin on experimental animal.

8.3.1: Toxicological Profile of Quercetin:

Acute toxicity of quercetin on oral and i.p. administration to mice was assessed at behavioral, haematological, biochemical and histological levels. No significant change

in body weight was observed among treated-group of animals during the period of quercetin administration compared to VC (Table 8.1).

8.3.1.1. Effect of Quercetin on Mice Anxiety and Depression Like Behavior:

Open field maze test for determining changes in anxiety and locomotion by measuring crossing, sniffing, rearing, grooming and distance travelled revealed no significant change in level of anxiety and locomotor disability among both oral and i.p. quercetin treated groups compared to VC. However, significant decrease in despair based depression was observed among oral and i.p. administrated $MD_{quercetin}$ and $LD_{quercetin}$ mice in tail suspension test in terms of mobility (oral: One-way ANOVA F-value=58.25, $p<0.0001$; i.p.: F-value=76.40, $p<0.0001$), which indicated anti-depressive property of quercetin (Table 8.1). But no significant change was observed among $HD_{quercetin}$ group.

8.3.1.2. Effect of Quercetin on Mice Haematological and Biochemical Parameters:

Results of all haematological and biochemical parameters were depicted in Table 8.1. No noticeable changes in haematological parameters were observed among quercetin treated mice compared to their respective VC.

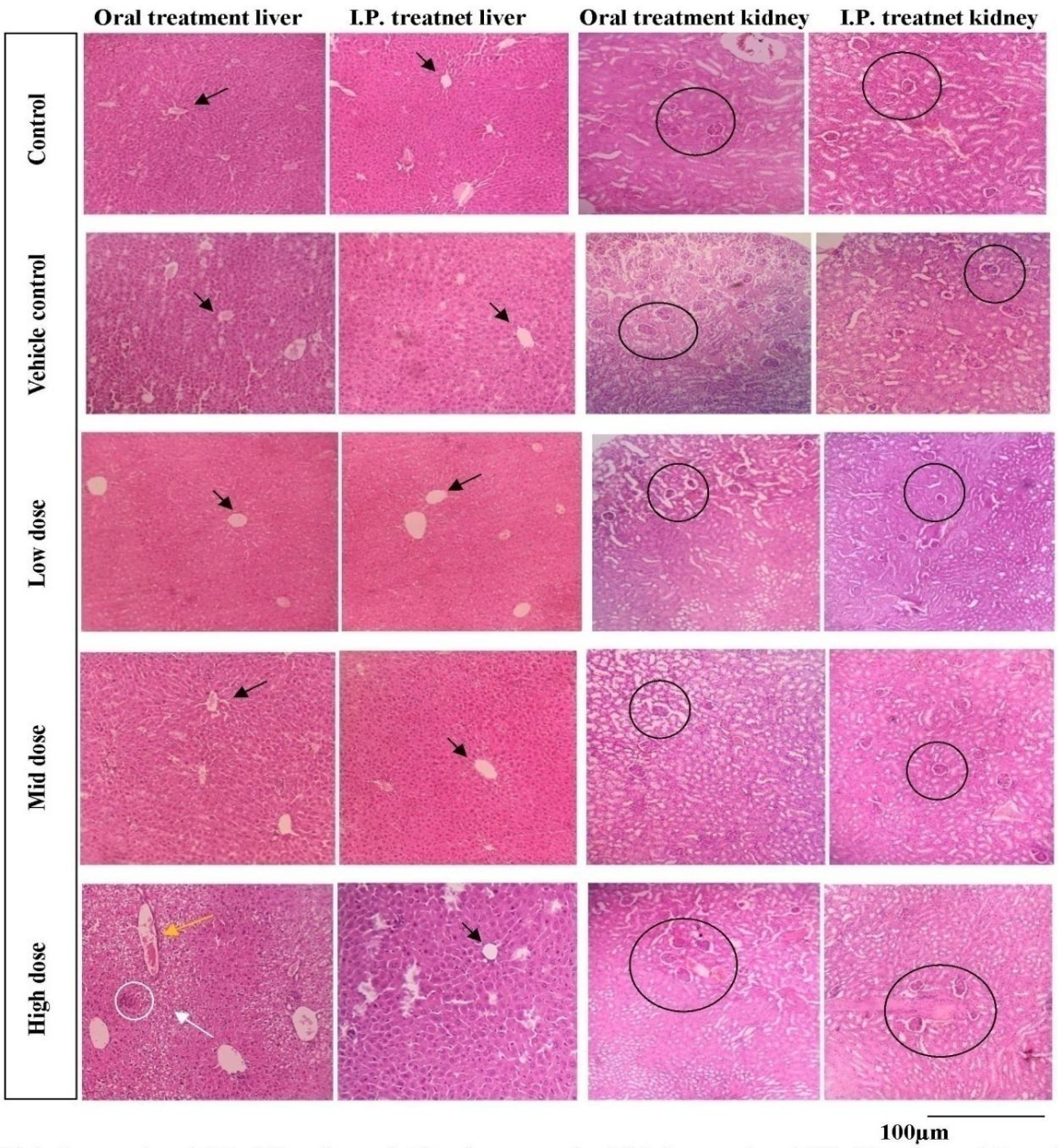
Significantly increased serum AST and ALP levels was observed among mice that received $HD_{quercetin}$ orally compared to VC (AST: F-value=14.95, $p=0.0005$; ALT: F-value=8.47, $p=0.0018$) and remained unchanged in $LD_{quercetin}$ and $MD_{quercetin}$ oral groups. But these hepatic and renal markers remained unchanged among mice receiving $LD_{quercetin}$, $MD_{quercetin}$ and $HD_{quercetin}$ orally and intraperitonealy, respectively. Significantly decreased intracellular ROS production (oral: F-value=8.799, $p=0.001$;

i.p.: F-value=29.65, p<0.0001) was observed among mice treated with $LD_{quercetin}$ and $MD_{quercetin}$ compared to VC (Table 8.1).

8.3.1.3. Effect of Quercetin on Mice Liver and Kidney Histopathology:

Histological analysis of mice liver revealed mild vascular congestion and inflammatory changes in mice treated with $HD_{quercetin}$ orally compared to VC and HC (Figure 8.5). Rest of other quercetin treated mice groups including VC and HC showed normal, well defined histological structures without any signs of vascular or inflammatory changes. Mice receiving $HD_{quercetin}$ orally exhibited glomerular atrophy within renal histology compared to VC and HC group; however, such degenerative phenomena was absent in mice receiving $HD_{quercetin}$ intraperitonealy (Figure 8.2). Normal histology of glomerulus and tubules was found within mice kidney tissue receiving $MD_{quercetin}$ and $LD_{quercetin}$ through oral and i.p. route (Figure 8.5).

Thus, all toxicological findings indicated oral treatment of $LD_{quercetin}$, $MD_{quercetin}$ and intraperitoneal administration of all doses were found to be non-toxic; hence, anti-viral effect of quercetin on dengue infected Balb/C mice was monitored using $MD_{quercetin}$ (80mg/kg) and $HD_{quercetin}$ (16mg/kg) orally and intraperitonealy, respectively.



High dose oral and I.P: 160mg/kg and 16mg/kg quercetin, Mid dose oral and I.P: 80mg/kg and 8 mg/kg quercetin, Low dose oral and I.P: 40mg/kg and 4 mg/kg quercetin, vehicle control: 0.5% DMSO and control: normal saline.
Photos were taken at 10x magnification.
High Black arrow indicated central vein with healthy hepatocytes around it. Yellow arrow indicated vascular congestion. White arrow indicated fatty changes (steatosis) in liver. White circle indicated immunological infiltrates.
Black circles indicated healthy glomerulus with Bowman's capsule.

Figure 8.5: Histological sections of Balb/C mice liver and kidney in acute toxicity test for oral I.P quercetin administration.

Table 8.1: Distribution of behavioral, haematological and biochemical parameters among control, vehicle control and quercetin administrated groups of Balb/C mice.

		Control	Oral administration				*p* value at 95% CI	IP administration				*p* value at 95% CI
			Vehicle Control	High Dose 160mg/KG	Mid Dose 80mg/KG	Low Dose 40mg/KG		Vehicle Control	High Dose 16mg/KG	Mid Dose 8mg/KG	Low Dose 4mg/KG	
Body weight (gm)	initial	12.72±0.42	13.12±0.24	12.8±0.27	12.88±0.21	13.20±0.18		13.02±0.187	12.88±0.39	13.05±0.19	12.93±0.4	
	final	15.39±0.33	15.74±0.31	15.33±0.39	15.56±0.27	15.9±0.23		15.61±0.28	15.49±0.27	15.69±0.212	15.66±0.51	
	change	2.67±0.018	2.62±0.021	2.51±0.015	2.68±0.027	2.7±0.025	ns	2.59±0.019	2.61±0.02	2.64±0.018	2.73±0.03	ns
Behavior												
Open field maze	Distance travel (mm)	27778±2344	28022±2454	28231±2387	28133±2238	28912±2469	ns	28145±2876	28987±2345	30006±3234	30039±3009	ns
	Score (mean)	22.6±0.88	22.3±1.20	23±1.15	24.3±0.88	26±0.58	ns	22±1.527	24.3±0.33	26±1.15	28.66.±1.15	ns
Tail suspension	Mobility time (min)	2.186±0.065	2.096±0.054	2.166±0.053	**2.9366±0.063**	**3.04±0.069**	**<0.001**	2.17±.086	2.183±0.057	**3.22±0.049**	**3.26±0.072**	**<0.0001**
Haematological Parameter												
Haemoglobin		13.84±0.5	14.21±0.8	14.1±0.78	13.9±0.67	14.3±0.87	ns	14.02±0.45	13.78±0.81	14.34±0.56	14.15±0.69	ns
RBC (10^6/µl)		8.61±0.82	8.72±0.87	8.84±0.89	8.79±0.51	8.87±0.75	ns	8.78±0.69	8.932±0.79	8.91±0.67	8.72±0.89	ns
WBC (10^3/µl)		5.69±0.8	5.76±0.86	5.89±0.93	5.86±0.81	5.77±0.78	ns	5.82±0.75	5.83±0.81	5.9±0.96	5.84±0.84	ns
HCT (%)		43.89±0.7	43. 3±0.79	43.66±0.87	45.66±0.9	44.66±0.77	ns	44.6±0.67	46.3±0.84	44.3±0.74	46.66±0.83	ns
Differential Count												
Gran	N	20.76±1.76	21.32±0.987	20.8±1.88	22.05±1.59	21.16±1.69	ns	21.02±1.63	20.12±1.59	20.54±1.8	21.05±1.78	ns

		Control	Oral administration				*p* value at 95% CI	IP administration				*p* value at 95% CI
			Vehicle Control	High Dose 160mg/KG	Mid Dose 80mg/KG	Low Dose 40mg/KG		Vehicle Control	High Dose 16mg/KG	Mid Dose 8mg/KG	Low Dose 4mg/KG	
	E	1.2±0.45	1.14±0.51	1.22±0.63	1.27±0.72	1.23±0.51	ns	1.07±0.68	1.34±0.66	1.29±0.77	1.18±0.63	ns
	B	-	-	-	-	-	na	-	-	-	-	na
Agranulocyte	L	75.25±2.57	75.06±2.65	75.74±2.37	74.67±2.7	75.3±2.34	ns	74.94±2.45	76.02±2.31	75.66	75.52±2.54	ns
	M	2.78±0.76	2.45±0.72	2.14±0.97	2.01±0.88	2.31±0.82	ns	2.97±0.68	2.52±0.71	2.51±0.79	2.25±0.78	ns
Biochemical Parameters for Liver												
AST		78.72±2.345	74.57±4.56	**112.45±9.45**	78.69±5.6	75.51±5.67	**0.0005**	75.9±7.86	81.08±17.81	74.94±5.67	73.98±4.89	ns
ALT		54.81±3.54	57.48±6.76	60.78±8.76	55.35±7.02	57.81±6.78	ns	56.46±5.87	57.69±10.81	58.26±9.15	55.41±6.84	ns
ALP		69.34±4.6	67.87±3.78	**105.18±7.65**	70.81±4.88	70.57±5.65	**0.0018**	68.67±4.21	69.39±6.11	67.27±4.9	70.94±±7.29	ns
Biochemical Parameters for Kidney												
Urea		21.76±2.12	22.53±2.65	26.88±5.76	22.12±4.41	21.34±3.21	ns	20.95±2.96	25.69±5.59	21.83±4.72	21.83±4.72	
Creatinine		0.79±0.05	0.81±0.07	0.84±0.078	0.86±0.06	0.823±0.045	ns	0.80±0.081	0.84±0.065	0.85±0.056	0.803±0.034	ns
Stress Measurement												
ROS production		112.76±6.7	128.57±8.34	**87.27±7.89**	**81.37±8.65**	**78.34±4.76**	**0.001**	124.36±5.26	**81.28±6.89**	**58.75±4.56**	**52.35±5.6**	**<0.0001**

Two tailed *p*-value <0.05 at 95% CI was considered as statistically significant.

8.3.2. Survival Curve for Sub-Lethal Viral Inoculums and Viral Kinetics:

Intraperitoneal infection of Balb/C mice using $2x10^5$ and $1x10^5$ copies/ml of mouse-adapted DENV stock led to 100% mortality with median survival time of 3 and 7 days, respectively. Whereas, those inoculated with 5 $x10^4$ copies/ml viral stock had mortality rate of 50% and remaining survived the course of infection with median survival time of 24 days (Figure 8.6A). Thus, $5x10^4$copies/ml was considered as LD50 value of viral inoculation. After infection, viral RNA level increased steadily in mice sera, which peaked at 5^{th} day pi, decreasing thereafter (Figure 8.6B).

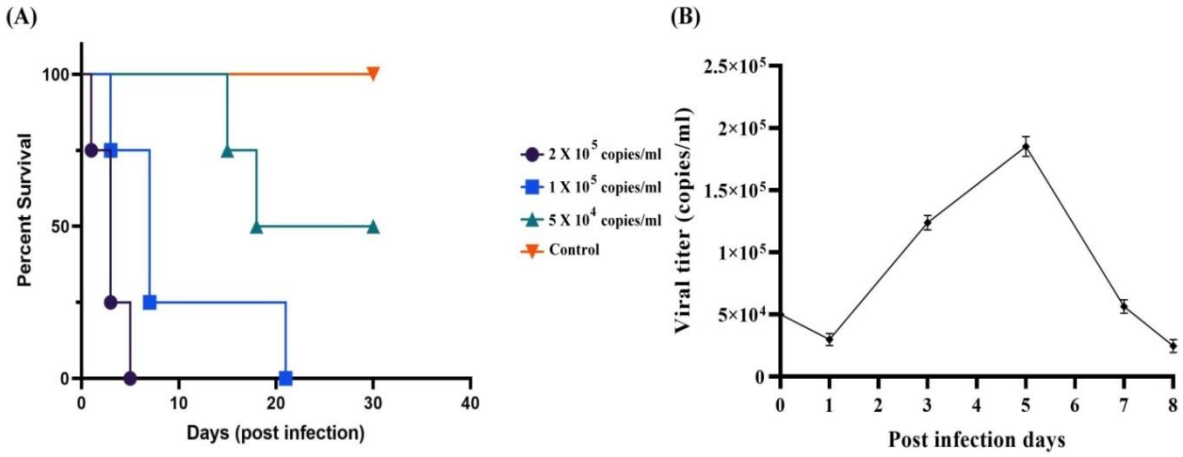


Figure 8.6: Determination of 50% lethal DENV2 dose for infecting Blab/C mice (A) Kaplan-Meier survival curves of mice infected with $2X10^5$ (*n* = 4), $1X10^5$ (*n* = 4) and $5X10^4$ (*n* = 4) copies/ml via the i.p. route (B) viral kinetics of the mice infected with 50% lethal dose ($5X10^4$ copies/ml).

8.3.3. Effect of Quercetin on DENV Infected Balb/C Mice:

Effect of quercetin against DENV infection was assessed in terms of reduction in DENV RNA copy number, severity scoring and alteration in haematological, biochemical, histological parameters and cytokine profiling among quercetin treated

groups with respect to infected and control groups. Significant decrease in mice body-weight was observed among infected group compared to treatment groups (p=0.032) (Table 8.2).

8.3.3.1. Effect of Quercetin on DENV RNA Copies:

DENV infection kinetics among quercetin pre-treatment, co-treatment and three post-treatment groups (group-A, group-B and group-C) were depicted in Figure 8.4A and 8.4B. Oral administration of quercetin significantly reduced viral copy-number from day3 onwards among co-treatment (day3: p=0.023, day5: p<0.0001, day7: p= 0.001) and post-treatment group-A (day3: p=0.04, day5: p=0.0002, day7: p= 0.003) with respect to infected-group (Figure 8.7A). However, significantly decreased viral titer was noted among post-treatment group-B from day5 onwards (day5: p<0.0001, day7: 0.021) compared to infected-group.

Interestingly, i.p. co-treatment group significantly reduced viral RNA copies from day1 (p=0.004) onwards throughout the course of treatment (day3: p=0.0023, day5 and 7: p<0.0001) (Figure 8.7.B). Whereas, i.p. pre-treatment (day3: p=0.008, day5 p=0.003 and 7: p<0.001), post-treatment group-A (day3: p=0.0041, day5 and 7: p<0.001) and group-B (day3: p=0.025, day5: p<0.001 7: p<0.001) exhibited significant viral copy number reduction from day3 onwards throughout the course of treatment.

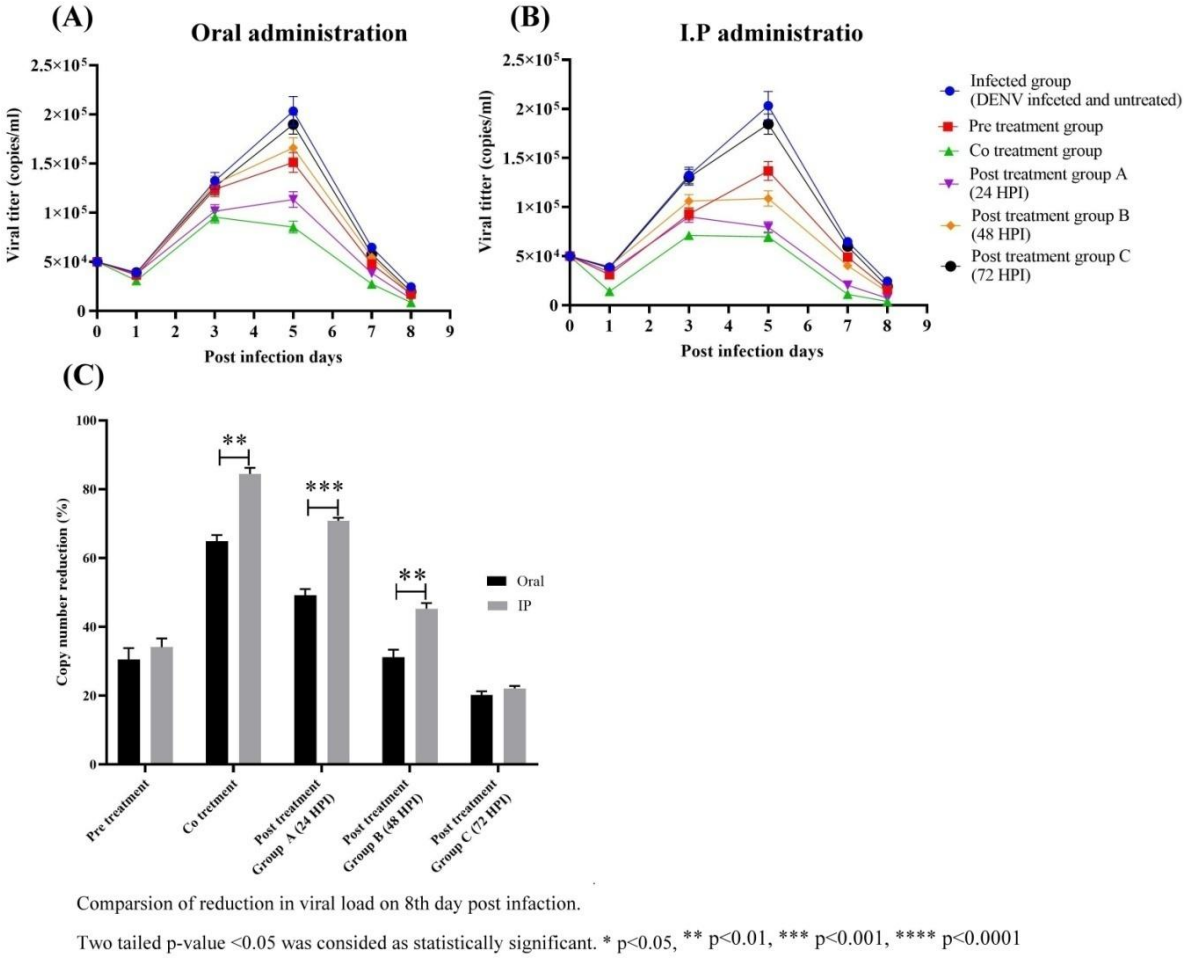
However, both oral and i.p. administration of quercetin among post-treatment group-C didn't show any significant reduction in viral titer throughout the course of study. Comparison of drug delivery route indicated i.p. administration of quercetin was more effective in significantly reducing higher copy-number of DENV among co-treatment (0.0015, paired t-test at 95% CI), post-treatment group-A (0.0004, t-test at 95% CI) and group-B (0. 0.0067, t-test at 95% CI) compared to its oral intake (Figure 8.7C).

Table 8.2: Effect of quercetin oral and I.P. administration on clinical signs, haematological and biochemical parameters among infected and treatment groups of Balb/C mice.

	Control	Dengue Infected	Oral administration					p value at 95% CI
			Pre-treatment	Co treatment	Post treatment group A	Post treatment group B	Post treatment group C	
Changes in body weight	↑0.72±0.17	**↓3.21±0.69**	↑1.57±0.15	↑1.67±0.2	↑1.59±0.18	↑1.61±0.22	**↓0.93±0.81**	**<0.0001**
Clinical signs score	1	**4.5±0.28**	1.74±0.48	1.5±0.28	1.75±0.47	2±0.4	**4±0.4**	**0.0001**
Haematological Parameter								
Haemoglobin	13.92±0.5	14.2±0.43	14.31±0.65	14.05±0.78	13.71±0.67	14.03±0.87	13.78±0.8	ns
RBC (10^6/µl)	8.96±0.82	9.12±0.57	8.78±0.7	8.64±0.49	9.01±0.62	8.87±0.43	9.21±0.82	ns
WBC (10^3/µl)	5.76±0.61	**11.64±0.92**	6.15±0.7	5.81±0.57	6.06±0.41	6.81±0.63	9.02±0.81	**0.03**
HCT (%)	42.58±0.54	**69.96±6.3**	47.61±3.21	43.02±3.04	44.72±3.45	49.32±3.78	58.16±5.14	**0.03-0.04**
Platelets (10^3/µl)	825.6±30.15	**562.3±45.96**	811±37.56	817.2±33.6	821.6±35.21	798.87±48.67	744.86±56.78	**0.003**
Liver Biochemical Parameters								
AST	77.72±5.35	**178.4±12.3**	87.69±5.6	81.1±7.67	85.08±10.81	84.34±5.67	**113.26±8.8**	**<0.0001**
ALT	54.81±4.5	**98.78±9.8**	58.35±7.02	56.8±6.8	57.24±6.1	60.26±7.8	**85.41±6.84**	**0.004**
ALP	68.34±4.8	**123.27±10.5**	70.81±4.88	72.7±5.58	78.3±7.05	76.27±4.98	**91.4±±7.2**	**<0.0001**
Renal Biochemical Parameters								
Urea	20.53±2.65	28.08±5.76	24.12±3.41	22.84±5.21	24.95±2.96	23.69±5.9	26.83±4.7	ns
Creatinine	0.81±0.056	**1.33±0.078**	0.9±0.07	0.83±0.055	0.85±0.071	0.88±0.065	0.85±0.056	**0.001**
	Control	**Dengue Infected**	**IP administration**					**p value**

	Control	Dengue Infected	Oral administration					p value at 95% CI
			Pre-treatment	**Co treatment**	**Post treatment group A**	**Post treatment group B**	**Post treatment group C**	
			Pre-treatment	**Co-treatment**	**Post-treatment 24 HPI**	**Post-treatment 48 HPI**	**Post-treatment 72 HPI**	**at 95% CI**
Changes in body weight	↑0.72±0.17	**↓3.21±0.69**	↑1.59±0.17	↑1.69±0.27	↑1.61±0.22	↑1.64±0.25	**↓0.87±0.63**	**<0.0001**
Clinical signs score	1	**4.5±0.28**	1.75±0.25	1.25±0.25	1.5±2.8	1.750±2.5	**3±0.4**	**<0.0001**
Haematological Parameter								
Haemoglobin	13.8±0.5	14.2±0.43	14.31±0.65	14.05±0.78	13.71±0.67	14.03±0.87	13.78±0.8	ns
RBC (10^6/µl)	8.96±0.82	9.12±0.57	8.78±0.7	8.64±0.49	9.01±0.62	8.87±0.43	9.21±0.82	ns
WBC (10^3/µl)	5.76±0.61	11.64±0.92	6.15±0.7	5.81±0.57	6.06±0.41	6.81±0.63	9.02±0.81	**0.03**
HCT (%)	43.58±0.54	**69.96±6.3**	47.61±3.21	43.02±3.04	44.72±3.45	49.32±3.78	58.16±5.14	**0.03-0.04**
Platelets (10^3/µl)	825.6±30.15	**562.3±45.96**	804±39.27	822.2±30.6	819.6±33.2	807.87±39.6	771.86±46.18	**0.0023**
Liver Biochemical Parameters								
AST	77.72±5.35	**178.4±12.3**	88.69±5.34	79.91±6.76	81.08±7.1	80.46±6.68	**108.21±6.89**	**<0.0001**
ALT	54.81±4.5	**98.78±9.8**	58.45±7.8	55. 1±6.78	58.89±10.81	58.26±6.15	**78.9±8.2**	**0.0021**
ALP	68.34±4.8	**123.27±10.5**	72.19±5.29	70.57±5.65	73.34±6.1	77.27±5.44	**88.4±±7.9**	**<0.0001**
Renal Biochemical Parameters								
Urea	20.53±2.65	28.08±5.76	22.29±4.19	21.34±3.21	22.5±2.4	25.69±5.59	27.2±4.2	ns
Creatinine	0.81±0.056	**1.33±0.078**	0.92±0.08	0.823±0.067	0.84±0.088	0.86±0.056	0.87±0.06	**<0.0001**

p-value was calculated with respect to infected group at 95% CI and control group used as reference. ↑= indicated increase and ↓= indicated decrease.



Comparsion of reduction in viral load on 8th day post infaction.

Two tailed p-value <0.05 was consided as statistically significant. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Figure 8.7: (A) Day wise comparison in dengue viral load in mice serum among infected and quercetin treatment groups. (B) Comparison between oral and I.P route of quercetin administration in reduction of viral copy number on 8th day post infection.

8.3.3.2. Effect of Quercetin on Weight Loss and Disease Severity of DENV Infected Balb/C Mice:

Significant weight loss was observed among infected-group (F=34.59, p=0.0001) and quercetin Post-treatment-group-C, both via oral (F=13.29, p=0.0023) and i.p (F=8.4, p=0.03) route compared to uninfected, untreated control-group (Table 8.2). However, no significant weight loss was observed among other treatment groups. Significant increase

in clinical-sign scores was observed among DENV infected-group compared to control-group (Table 8.2). However, treatment with quercetin could minimize this increase in clinical sign scores among pre-treatment, co-treatment, post-treatment group-A and group-B, up to that of control-group.

8.3.3.3. Effect of Quercetin on Haematological and Biochemical Parameters of DENV Infected Balb/C Mice:

Significant increase in WBC ($p=0.031$), HCT ($p=0.02$) and decrease in platelets ($p=0.001$) was observed among infected-group compared to control-group (Table 8.2). However, all these parameters of both orally and intraperitonealy quercetin treated groups were at par with control-group. Interestingly, slight increase in WBC, HCT and decrease in platelet numbers was observed among post-treatment group-C, but that change wasn't statistically significant.

Liver and renal function tests showed marked increase in liver enzyme levels: AST ($p<0.0001$), ALT ($P=0.004$), ALP ($P<0.001$) and creatinine ($P=0.001$), among infected-group compared to control-group. However, these parameters of orally and intraperitonealy treated quercetin groups were close to that of control-group, except post-treatment group-C (Table 8.2). In both oral and i.p. post-treated group-C, liver enzymes were significantly higher than that of control and other treatment-groups.

8.3.3.4. Effect of Quercetin on Histological Parameters of DENV Infected Balb/C Mice:

Micrographs showing differences between liver sections of control-mice and DENV infected-mice with or without quercetin treatments were depicted in Figure 8.5. Histopathological examination showed regular structured hepatic tissue of control-

group. Severe hepatic injuries like generalized steatosis (i.e., lipid accumulation within hepatocytes), dilated veins, venous congestion, signs of haemorrhage and inflammatory cell infiltration was noted among infected-group and post-treatment group-C compared to control-group (Figure 8.8). Whereas, hepatic injury was not observed among orally and intraperitonealy quercetin administrated pre-treatment, co-treatment, post treatment group-A and group-B.

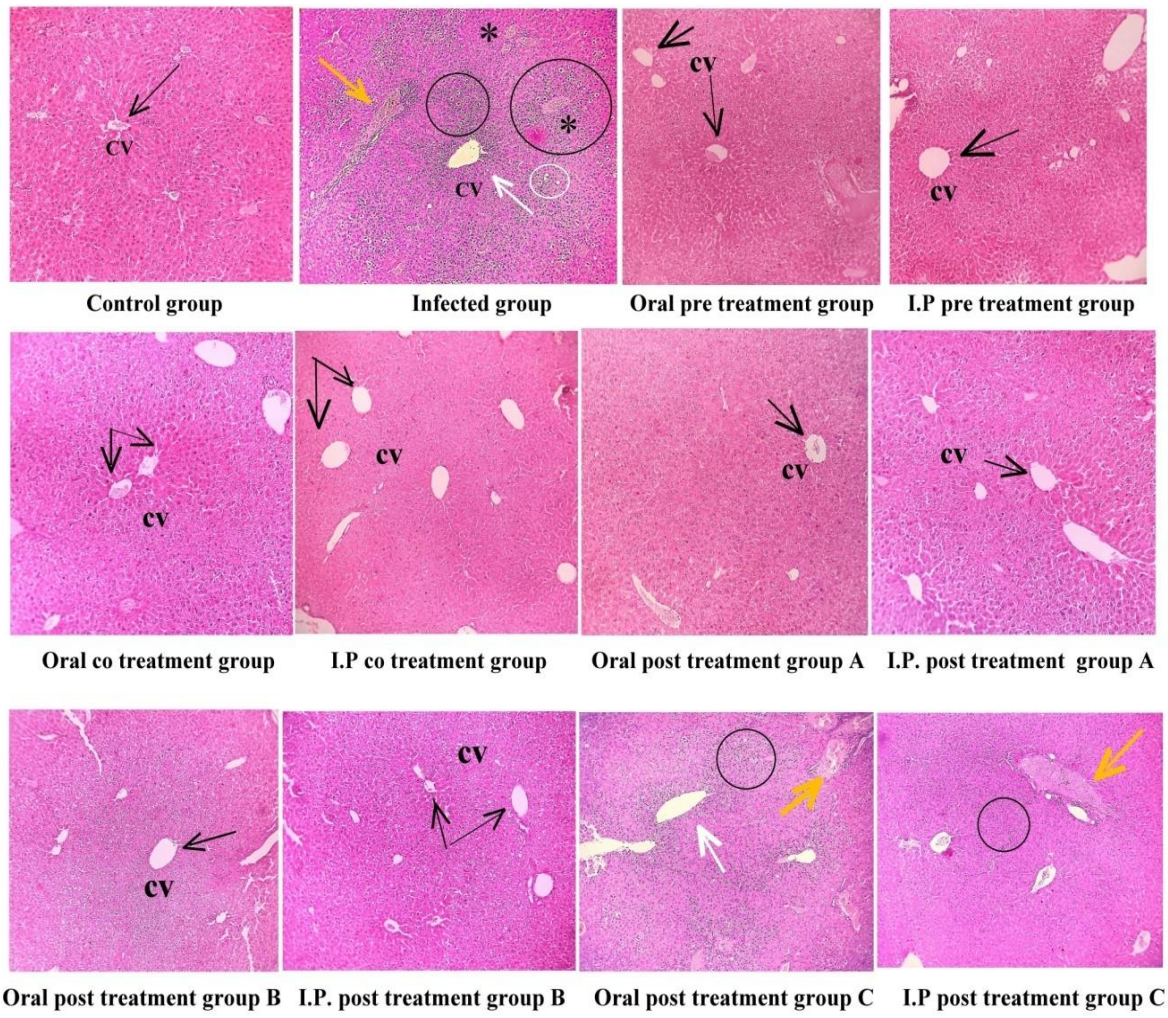
Renal histopathological examination showed atrophy of glomerulus and accumulation of inflammatory infiltrate among infected-group only, whereas, no such changes were observed in control-group and all quercetin treatment-groups (Figure 8.9).

8.3.3.5. Effect of Quercetin on Intracellular Cytokine Profile, ROS Production and Vascular Leakage in DENV Infected Balb/C Mice:

Effect of quercetin on DENV induced secretion of inflammatory cytokines, ROS production and vascular leakage were depicted in Figure 8.10. Serum levels of TNF-α and IL-6 significantly increased ($p<0.0001$) among infected-group compared to control mice. However, levels of these cytokines significantly reduced among quercetin pre-treatment, co-treatment and post-treatment group-A and group-B ($p<0.0001$), both via oral and i.p. route. Reduction in level of these cytokines was not significant for quercetin post-treatment group-C compared to infected mice, both orally and intraperitonealy (Figure 8.10A and 8.10B).

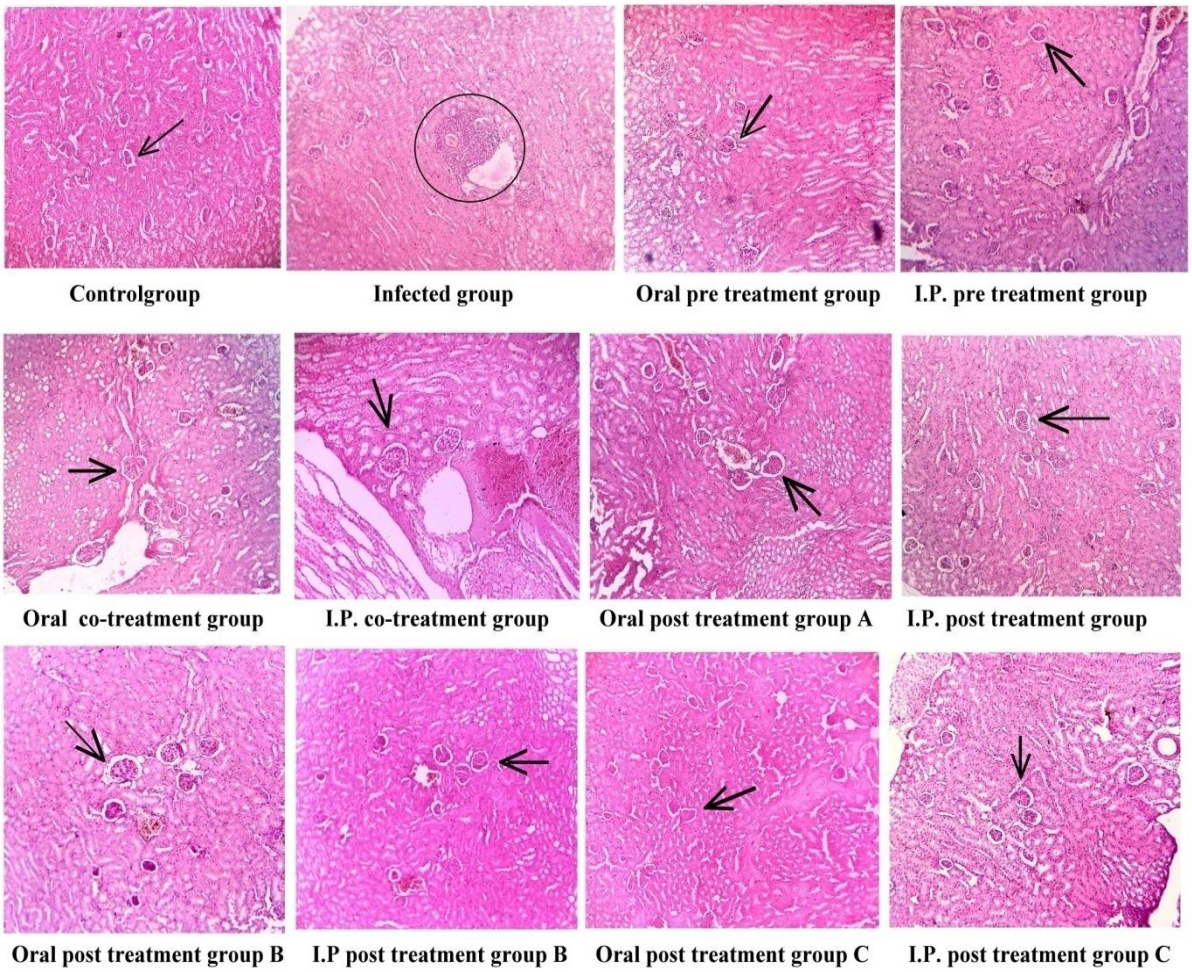
Intracellular ROS response was significantly increased among infected-group ($p<0.0001$) compared to control-group (Figure 8.10C). However, intracellular ROS production of pre-treatment, co-treatment and post-treatment group-A was comparable to that of control mice.

Vascular leakage was higher among DENV infected-group ($p<0.0001$), as evidenced by evans-blue measurement. However, no such vascular leakage was observed among quercetin treatment groups viz. pre-treatment, co-treatment, post-treatment group-A and group-B, both orally and intraperitonealy (Figure 8.10D).



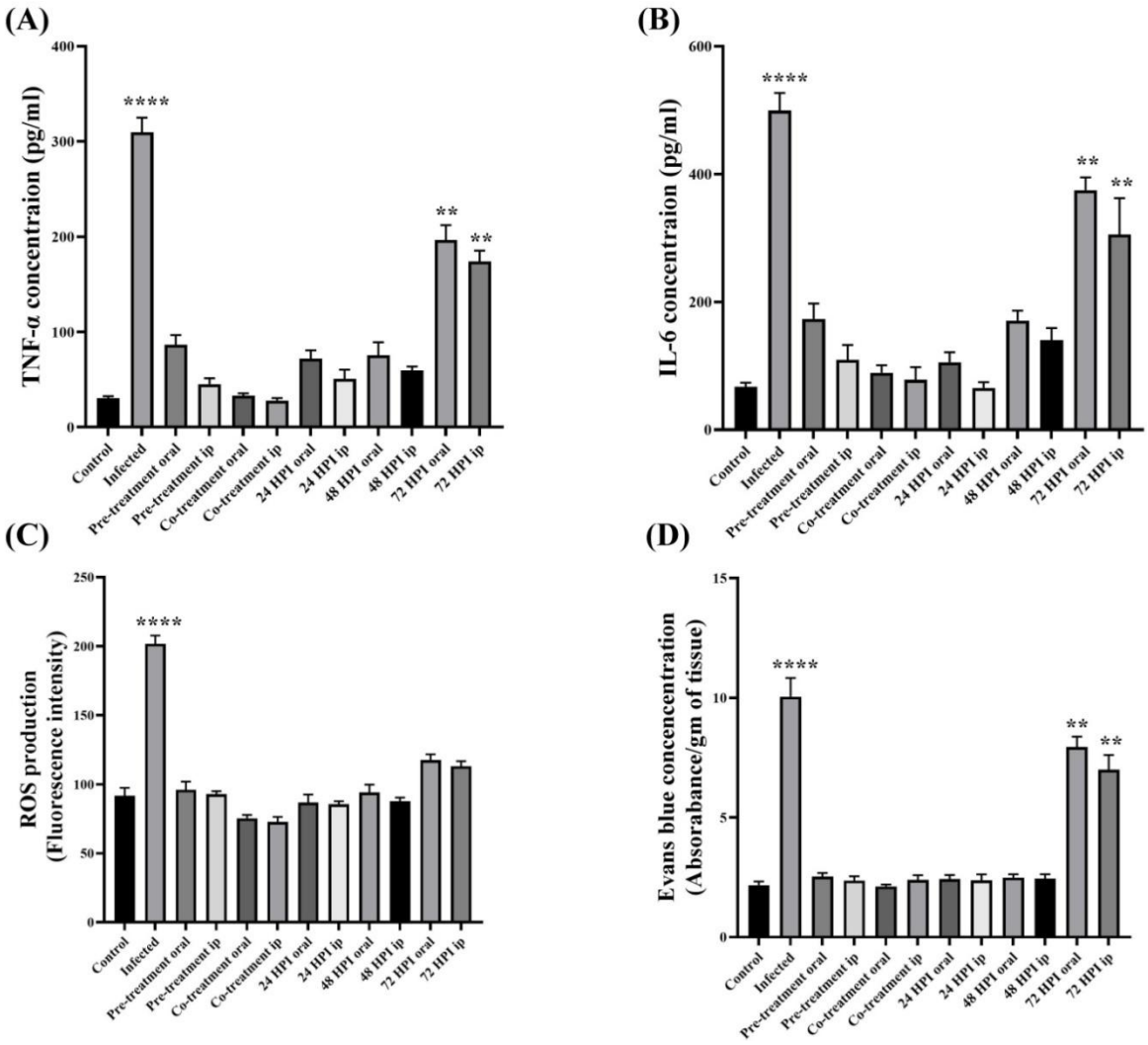
Other treatment groups showed normal helathy liver architecture like control except post treatment group C and infected group (DENV2 infected and untreated).
Infected group showed severe vascular congestion (marked with yellow arrow), dialed central vein (marked with white arrow). accumulation of inflamatory cells (marked with black circle) around cv (central vein), presence of steatosis (marked with white arrow) and haemorrhagic signs (marked with *).

Figure 8.8: Effect of oral and i.p. quercetin treatment on liver histopathology of dengue infected Balb/C mice:



No significance change was observed among treatment grpups. except infection group.
Inflamatory inflitate cells were observed only in infected group and was denonated in black circle.
Normal and healthy renal architecture was observed among treatment groups and healthy g;omerulus with bowman's capsule was indicated with black arrows.

Figure 8.9: Effect of oral and i.p. quercetin treatment on renal histopathology of dengue infected Balb/C mice:



Two tailed *p*-value < 0.05 was considered as statistically significant at 95% CI.

* *p*< 0.05, ** *p*<0.01, *** *p*<0.001 and **** *p*<0.0001

Figure 8.10: Measurement of (A) (B) pro inflammatory cytokine viz. TNF-α and IL-6 (C) intracellular ROS and (D) vascular leakage by evans blue estimation among control, dengue infected and dengue infected quercetin treatment groups mice.

8.4. DISCUSSION:

Development of new therapeutics against DENV is important to reduce its burden and for better clinical management. Biodiversity of the natural products provide new insights into antiviral therapy with effective prophylactic/therapeutic strategies against DENV infection. Though, quercetin has long been known to be a major bioactive compound of berries and berry-like fruits with antiviral activity, no investigations were done to analyze its antiviral (co-treatment), prophylactic (pre-treatment), therapeutic (post-treatment) immunomodulatory and hepato-protective effect in murine DENV infection model.

Current study is the first to demonstrate its above mentioned effects on DENV infected Balb/C mice by oral and ip route of administration. Mid-dose ($MD_{quercetin}$:80mg/kg) and low-dose ($LD_{quercetin}$:40mg/kg) of quercetin treatment orally were non-toxic for mice, whereas, its intraperitoneal administration of all doses up to 16mg/kg ($HD_{quercetin}$) was safe – as evident from changes in general behavior, biochemical and haematological parameters of mice. Moreover, behavioral analyses and intracellular ROS measurement revealed anti-depressive and antioxidant property of highest non-toxic oral and ip doses of quercetin (oral: 80mg/kg and ip: 16mg/kg) in murine model. This finding was consistent with previous studies on winstar rats and swiss-albino mice using 300mg/kg and 30mg/kg quercetin, respectively (24-25).

Mouse-adapted DENV strain used in this study supported robust DENV replication in murine model to elicit severe clinical manifestations (17). The study demonstrated that quercetin administration both orally and intraperitoneally reduced DENV copy-number in pre-treatment, co-treatment, post-treatment group-A (24hpi) and group-B (48hpi) infected mice sera, which indicated both virudical and therapeutic potential of quercetin.

Co-treatment group exhibited marked reduction in viral-load compared to other treatment groups; but treatment of mice with quercetin after 72 hpi (post-treatment group-C) was not sufficient to significantly reduce viral-titer. Comparative analysis between oral and ip administered groups showed ip treatment to be better in reducing viral titer than oral route. This might be due to faster adsorption of quercetin via ip route and metabolic degradation of quercetin during in-vivo oral administration (28). Beside its antiviral activity, previous studies also suggested that apart from its interaction with virus, quercetin might also modulate host innate-immune response (29-31). This study also highlighted significant reduction in DENV induce clinical morbidity and normalization of haematological parameters among quercetin co-treatment and post-treatment group-A compared to infected-group. This might be due to reduction in viremia level among treatment groups, thereby lowering morbidity (32). DENV infection reported to trigger innate-immune response by marked increase in pro-inflammatory cytokines and augmented oxidative stress generating ROS which lead to hepatic impairment and vascular leakage (2-3, 33). Studies also highlighted that infection with mouse adapted DENV reflected signs of these severe manifestations like human in murine model (25, 34). In the current study these severe clinical manifestations viz. increased WBC, haematocrit, thrombocytopenia, AST, ALT, ALP and creatinine were evident among infected group, which got normalized at par with control mice among quercetin treatment groups, except post-treatment group-C. The pathological mechanisms behind virus-associated liver and kidney injury are due to either immune complex deposition or immune reactions, resulting in accumulation of immunological infiltrate, severe coagulopathy, endothelial damage and increased vascular permeability (35-36). In this study, histopathological analysis also showed severe vassal congestions, hepatic damage and infiltration in liver and renal tissues

among infected and post-treatment group-C, compared to control and rest of the treatment groups, which might be due to hepato-protective and renal-protective effect of quercetin (37-38). Quercetin has been reported to down-regulate IL-6, NF-κB, IL-6, TNF-α and intracellular ROS production thereby, possibly reducing vascular permeability by augmenting vascular-endothelial-growth-factor (38-39). Present study also demonstrated anti-inflammatory, antioxidant and anti-vascular regression effect of quercetin among treatment-groups compared to infected-groups.

8.5. CONCLUSIONS:

Thus, the present study confirmed therapeutic, anti-viral, anti-inflammatory and immunomodulatory effect of quercetin against DENV infected Balb/C mice which might be helpful in managing severe clinical outcome among dengue patients by limiting the use of other non-steroidal anti-inflammatory drugs. But, further research needs to be validated the potentiality of quercetin against DENV infection among other viral serotypes.

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GENERAL CONCLUSION

9. GENERAL CONCLUSION

This work imparted a deep knowledge in understanding of dengue disease pathogenesis among eastern Indian patient population, identification of factors important for clinical management of severe dengue patients and evaluation of antiviral therapeutic potential of quercetin against dengue infection.

Chapter 1 confirmed emergence and massive spread of DENV with changing pattern in circulating viral serotypes in Kolkata and its adjoining states of West Bengal, part of eastern India during 2014-2016. This study also highlighted that impact of disease burden was highest in months of September and October and one fourth of all dengue patients were having secondary dengue infection. DENV 2 and DENV 4 were most prevalent serotypes among all four circulating serotypes in this region. This study also demonstrated that severe dengue patients had significantly high viral load with severe clinical manifestations viz. haemorrhage, clinical fluid accumulation, hepatic dysfunction, thrombocytopenia and leucopoenia compared to those with and without WHO-defined warning signs. During early stage of infection, qRT-PCR could detect dengue infection more efficiently than other conventional detection methods which might be helpful for better clinical management in triaging severe outcome.

In **Chapter 2** partial sequencing of DENV E region demonstrated circulation of cosmopolitan genotype, genotype-III and genotype-I of DENV 2, DENV 3 and DENV 4 serotypes, respectively with 10 amino acids substitution of DENV 2, 11 amino acids substitution of DENV 3 and 23 amino acids substitution of DENV 4 serotypes compared to their respective reference sequences (NC_001474 for DENV 2, NC_001475 for DENV 3 and NC_002640 of DENV4) available in NCBI GenBank database.

Chapter 3 and Chapter 4 elucidated the importance of host genetic makeup of innate immune genes viz. TLRs (TLR3, TLR7 and TLR8) and CRP as early defence mechanism and acute phase response against the pathogen (DENV). These chapters highlighted importance of certain polymorphic variants of these genes viz. TLR7_rs179008 AA genotype and TLR8_3764880 CC genotype and CRP_rs3093059 TT to be associated with dengue disease susceptibility and development of disease severity. Polymorphic variants of CRP-rs3093059-TT and CRP-rs3091244-CT genotypes were also associated with high viral copy number and increasing CRP concentration among dengue patients.

Chapter 5 elucidated diagnostic potential of serum proteomic and metabolic markers viz Ang2, VEGF, AST, ALT and ApoA1 during early stage of infection with high sensitivity and specificity to identify severe dengue development beforehand, which might be beneficial for clinical disease manegment. Results showed that angiogenic and endothelial markers viz, ANG2 and VEGF were associated with severe clinical manifestation such haemorrhage, thrombocytopenia and clinical fluid accumulation among infected patients. Whereas, AST and ALT and acute phase lipoproteins ApoA1 were associated with high viremia and hepatic dysfunction of the patients.

Chapter 6 explored and established anti-inflammatory, antiviral, therapeutic and immunomodulatory role of bioactive compound quercetin against dengue virus infection among in-vivo mice model. Co-treatment and post-treatment with non toxic oral (80mg/kg) and I.P (16mg/kg) doses of quercetin after 24 hours post infection showed significant reduction in dengue viral titer, inflammatory markers, intracellular ROS response and vascular leakage among infected mice. Thus, these findings might help physicians in managing severe dengue patients.

LIST OF PUBLICATIONS

DURING PH.D. TENURE

10. LIST OF PUBLICATIONS DURING PH.D. TENURE

1. **Mukherjee S**, Dutta SK, Sengupta S, Tripathi A. Evidence of dengue and chikungunya virus co-infection and circulation of multiple dengue serotypes in a recent Indian outbreak. Eur J Clin Microbiol Infect Dis. 2017 Nov; 36 (11): 2273-2279. doi: 10.1007/s10096-017-3061-1. Epub 2017 Jul 29. PMID: 28756561.

2. **Mukherjee S**, Tripathi A. Contribution of Toll like receptor polymorphisms to dengue susceptibility and clinical outcome among eastern Indian patients. Immunobiology. 2019 Nov; 224 (6): 774-785. doi: 10.1016/j.imbio.2019.08.009. Epub 2019 Aug 25. PMID: 31481269.

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Appendix: Publications

Eur J Clin Microbiol Infect Dis
DOI 10.1007/s10096-017-3061-1

ORIGINAL ARTICLE

Evidence of dengue and chikungunya virus co-infection and circulation of multiple dengue serotypes in a recent Indian outbreak

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Abstract In India, dengue endemic areas overlap with chikungunya-affected areas and both the viruses are transmitted by same vector, *Aedes aegypti* — thereby increasing opportunity of co-infection by both viruses. Present study was carried out to understand the DENV–CHIKV infection dynamics during recent outbreaks in eastern India (West Bengal state) and its implication on disease manifestations. Blood was collected from 326 symptomatic febrile patients. Patients' serum was subjected to serological diagnosis for presence of anti-dengue-IgM, anti-chikungunya-IgM antibodies and dengue-NS1 antigen by ELISA. Viral RNA was extracted, and presence of dengue virus (DENV) and chikungunya virus (CHIKV) genome, their viral load (VL), and serotype among infected patients' plasma was determined by real-time qRT-PCR. Statistical analysis was performed by using EPI INFO software. DENV and CHIKV were detected in 54% and 33% of symptomatic patients respectively, among whom 23% were harboring both viruses. WHO classified warning signs were detected among 64% DENV patients and 61% DENV–CHIKV double-infected patients. Patients with warning signs always had much higher DEN VL than those without warning signs. Hemorrhagic manifestation and abdominal pain was found in significantly higher frequency among patients with high dengue VL (>10,000 copies/ml). DENV2 was the most predominant serotype among monotypic dengue patients, whereas DENV2–DENV4 combination was most prevalent among patients infected with dual dengue serotypes. This study indicated that DENV–CHIKV double infection and high dengue VL contributed towards severe disease manifestations among infected patients. DENV2 and DENV2-DENV4 combination were the most prevalent serotype(s) found in current outbreak.

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Introduction

Dengue (DENV) and chikungunya (CHIKV) viral infections with potential fatal complications are reported from various parts of India every year [1]. India is endemic to all four serotypes of DENV — thereby causing a full spectrum of the disease. Multiple DENV serotypes in an outbreak have been reported from various parts of the country, viz. Delhi, Kolkata, Lucknow, Kerala [2–4]. But, the predominant dengue serotypes keep changing every year. For example, DENV-1, 2, and 4 serotypes were common circulating strains in Kolkata from 2008 to 2010, after which DENV-3 infections arose leading to a massive dengue outbreak in 2012 [5]. On the contrary, after a gap of three decades, CHIKV re-emerged in India during late 2005 after massive Indian Ocean islands outbreak, which eventually spread all over the country [6]. Thus, DENV-endemic areas overlap with CHIKV-affected areas in India. Although DENV and CHIKV belong to different families of togaviridae and flaviviridae respectively, both are transmitted by the same vector, i.e., *Aedes aegypti* in India [3]. Also, every year the transmission period of both viruses in India mostly appears to be during post-monsoon period (September–November) when climatic conditions become favorable for mosquito breeding [4]. Thus, frequency of concurrent infection by both viruses has remarkably increased in India since 2005. During the early acute febrile phase of

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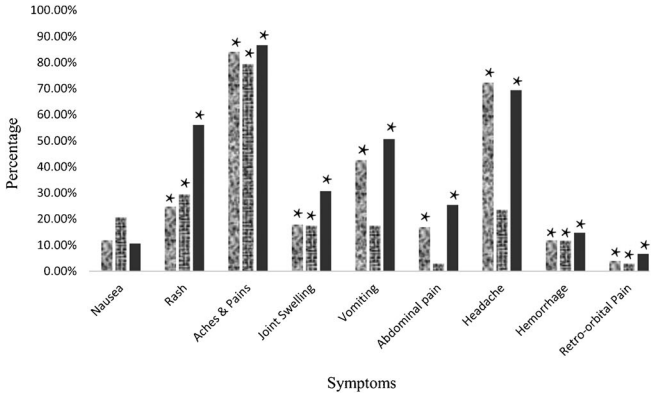

Symptoms	DENV (n = 101)			CHIKV (n = 34)			DEN-CHIK double virus (n = 75)		
	Occurrence in percentage (%)	*p*-value (contingency chi-square at 95% CI)	Odds ratio	Occurrence in percentage (%)	*p*-value (contingency chi-square at 95% CI)	Odds ratio	Occurrence in percentage (%)	*p*-value (contingency chi-square at 95% CI)	Odds ratio
Nausea	12 (11.88)	.899	1.05	7 (20.58)	.114	2.24	8 (10.66)	.94	1.03
Rash	25 (24.75)	.015	2.39	10 (29.41)	.015	3.03	42 (56.00)	<.0001	9.27
Aches & Pains	85 (84.15)	<.0001	7.79	27 (79.41)	<.0001	5.66	65 (86.66)	<.0001	9.54
Joint Swelling	18 (17.82)	.003	3.97	6 (17.40)	.018	3.92	23 (30.66)	<.0001	8.109
Vomiting	43 (42.57)	<.0001	5.4015	6 (17.40)	.40	1.562	38 (50.66)	<.0001	7.48
Abdominal pain	17 (16.83)	.005	3.71	1 (2.94)	.587	0.55	19 (25.33)	<.0001	6.22
Headache	73 (72.27)	<.0001	17.55	8 (23.52)	.131	2.07	52 (69.33)	<.0001	15.22
Hemorrhage	12 (11.88)	<.0006	15.505	4 (11.76)	.0001	15,33	11 (14.66)	.00012	19.76
Retro-orbital Pain	4 (3.96)	129	4.742	1 (2.94)	.352	3.48	5 (6.66)	.024	8.21

Fig. 1 Frequency of symptoms among DENV, CHIKV, and patients infected by both viruses

illness, both DENV and CHIKV infections have similar disease manifestations of high fever, myalgia, arthralgia, headache, nausea, rash, and vomiting. However, facial flushing, abdominal pain, and retro-orbital pain are frequently reported among dengue patients, whereas joint-swelling is more commonly found among chikungunya patients [4, 7].

However, dynamics of CHIKV and DENV infection during such period of multiple illnesses and its implication during clinical manifestation among patients remain poorly studied.

Material and methods

Inclusion criteria

Acute febrile patients with history of any of the following symptoms: headache, bodyache, myalgia, arthralgia, rash, with or without haemorrhagic manifestation from all age-groups and both sexes were selected, as per WHO criteria.

Exclusion criteria

Hematological malignancies, bleeding disorders, chronic liver disease, diabetes mellitus, renal diseases, etc.

Collection of patients' blood

After obtaining institutional ethical committee approval, 5 ml of blood samples were collected from 326 symptomatic febrile patients visiting Calcutta School of Tropical Medicine and Institute of Post Graduate Medical Education and Research and Medical College Hospital, Kolkata, West Bengal, India from July 2014 to October 2015. All the patients exhibited fever along with any of the following symptoms: aches and pain, nausea, rash, vomiting, abdominal pain, joint-swelling, bleeding, retro-orbital pain. Sera and plasma were separated from patient blood by density gradient centrifugation and stored at -80 °C in a freezer.

Extraction of viral RNA and determination of dengue and chikungunya VL

Viral RNA was extracted from 140 μl of plasma by using QIAamp Viral RNA Mini Kit according to manufacturer's protocol (Qiagen, Courtaboeuf, France) [8]. Presence of DENV and CHIKV genome and their VL among infected patients' plasma was determined by real-time qRT-PCR in total volume of 20 μl, using 8 μl viral RNA, OneStep qRT-PCR MasterMix, and other components of DENV and

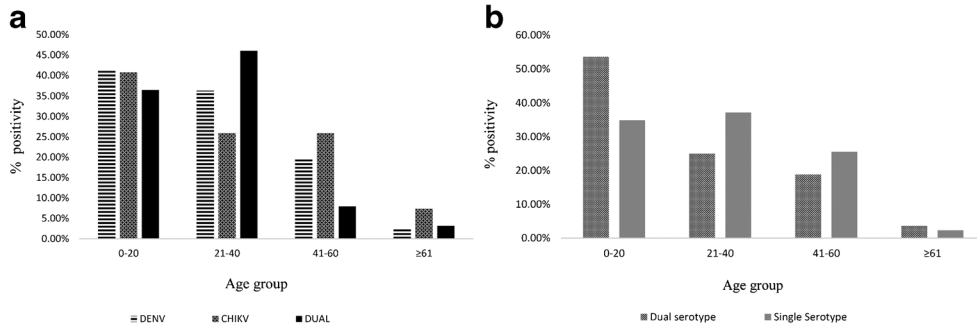

Fig. 2 Age-group wise distribution of (*A*) DENV, CHIKV, & DEN–CHIK (patients infected with both), and (*B*) DENV single and dual serotype-infected patients

Table 1 Dengue and chikungunya viral load and serotype distribution. A: variation of dengue and chikungunya viral load with age and WHO-defined warning signs. B: Dengue serotype distribution among Southern and Northern districts

A							
		Viral load					
		Dengue mono infected patients (n = 101)		Chikungunya mono infected patients (n = 34)		DEN–CHIK double-virus-infected patients (n = 75)	
						Dengue	Chikungunya
Age Group	Younger age group (<20)	205,810 copies/ml		44,107 copies/ml		215,148 copies/ml	113,263 copies/ml
	Elder age group (>20)	700,200 copies/ml		280,700 copies/ml		417,972 copies/ml	313,403 copies/ml
WHO symptoms	With warning signs	519,487 copies/ml		-----		464,980 copies/ml	149,018 copies/ml
	Without warning signs	16,284 copies/ml		-----		17,517 copies/ml	351,928 copies/ml
Diagnostic tools	PCR (+)	94% (95/101)		94% (32/34)		-----	-----
		HVL (58.4%)	LVL (41.6%)	HVL (18.5%)	LVL (81.5%)		
		616,166 copies/ml	2,888 copies/ml	471,808 copies/ml	3,216 copies/ml		
	IgM/NSI	5.94% (6/101)		5.88% (2/34)		-----	-----
B							
West Bengal	Dengue single serotype (n = 64)						
	D1	D2		D3		D4	
Southern Districts (n = 53)	3.12% (2/53)	56.60% (30/53)		5.66% (3/53)		33.96% (18/53)	
Northern Districts (n = 11)	18.18% (2/11)	27.27% (3/11)		9.09% (1/11)		45.45% (5/11)	
Districts	Dengue dual serotype (n = 54)						
	D2-D4	D1-D4		D1-D2		D2-D3	D3-D4
Southern Districts (n = 48)	79.16% (38/48)	8.33% (4/48)		0%		4.16% (2/48)	6.25% (3/48)
Northern Districts (n = 4)	100% (4/4)	0%		0%		0%	0%

*2 Samples are not included in this table as they were from other adjoining states

CHIKV Genesig kit (Primer Design Ltd., UK) respectively, according to the manufacturer's protocol [9, 10]. Real-time PCR was performed in an ABI 7500 instrument. A standard curve of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template. Copy number of each virus in patient serum was calculated from corresponding Ct values. Each sample was loaded in triplicate.

Determination of dengue virus serotypes

DENV serotype of PCR+ (positive) samples was determined by real-time RT-PCR in total volume of 20 μl containing 8 μl RNA, OneStep qRT-PCR MasterMix, and other components of DENV serotyping Genesig kit (PrimerDesign Ltd., UK), according to manufacturer's protocol. Reactions were performed in an ABI 7500 instrument using serotype-specific DENV controls present within the kit.

Detection of dengue-NS1 antigen

Sera of patients suffering from fever for less than 5 days were subjected to serological diagnosis for presence of dengue-NS1 antigen using NIV Pune dengue-NS1 ELISA Kits (National Institute of Virology, Pune, India) according to manufacturer's instructions [11]. Results were interpreted as negative or positive according to the assay manual.

Determination of anti-dengue-IgM and anti-chikungunya-IgM

Sera of patients were subjected to serological diagnosis for presence of anti-Dengue-IgM and anti-Chikungunya-IgM antibodies using NIV dengue and chikungunya MAC ELISA Kits (National Institute of Virology, India; dengue MAC-ELISA: 96% sensitivity, 87% specificity, and chikungunya MAC-ELISA: 87% sensitivity, 100% specificity) respectively, according to manufacturer's instructions [12, 13]. Results were interpreted as negative or positive according to the assay manual.

Statistical analyses

2 × 2 contingency Chi-square and odds-ratio analysis were performed to find any association between frequency of DENV, CHIKV, and DEN–CHIK double-virus infection with different clinical symptoms using CDC Epi-Info software (version 3, CDC, Atlanta, GA, USA). Statistical analysis of symptoms among DENV, CHIKV and DEN–CHIK double-virus-infected patients was calculated using DENV, CHIKV uninfected patients as control group. Probability values of $P < 0.05$ was considered as statistically significant. Odds-ratio values >1 indicated that a condition/event was more likely to occur in a particular disease group.

Results

Among the analyzed 326 symptomatic patients, DENV and/or CHIKV infection was detected in 64.41% [(101 + 75 + 34)/326 = 210/326] of them by qRT-PCR, dengue-NS1-ELISA, anti-dengue/chikungunya IgM-ELISA (Fig. 1). Infection burden for DENV [54%: (101 + 75)/326] was greater among infected patients than CHIKV [33%: (34 + 75)/326)]. Seventy-five dengue patients and 23 chikungunya patients were within acute stage of infection, whereas 53 acute stage patients were doubly infected with CHIKV–DENV. Such, concomitant infection by both viruses was observed in 23% (75/326) of patients.

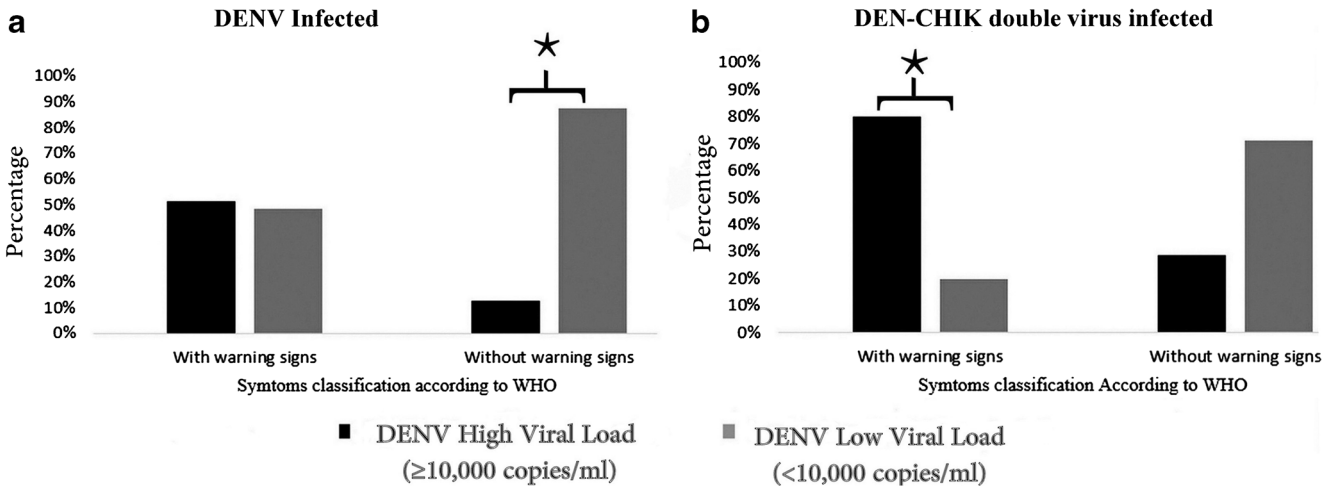

Fig. 3 Comparison of DENV load among (*A*) DENV-infected patients and (*B*) patients infected with both DENV and CHIKV with/without warning symptoms (WHO)

Frequency of DENV infection was highest among patients of the 0–20-year age group, and gradually decreased with increasing age (Fig. 2). Interestingly, dual DENV serotypes were detected among approximately half of these early age-group dengue patients (0–20 years). Also, frequency of dual dengue serotype infection gradually decreased with increasing age. However, frequency of single dengue serotype infection was approximately of equal frequency between the 0–20 and 21–40 year age groups, which also decreased in the higher age group. CHIKV infection was also highest in 0–20 years age group, though it was found to be of considerably higher frequency among middle age-group patients also. The highest number of patients found to be infected by both DENV and CHIKV was in the 21–40 year age group (Fig. 2).

Clinically, aches and pains, joint-swelling and haemorrhagic manifestations were significantly higher among DENV-, CHIKV-, and dual-virus-infected patients than that of uninfected symptomatic patients (Fig. 1). Compared to chikungunya patients; rash, vomiting, abdominal pain, and headache was significantly more prevalent among DENV- or DEN–CHIK dual-virus-infected patients. However, nausea was reported more frequently among CHIKV infected patients. Vomiting, abdominal-pain and headache was statistically more prevalent among DENV- and DEN–CHIK double-virus-infected patients. Compared to CHIKV/DENV uninfected symptomatic patients, retro-orbital pain was statistically more prevalent among DEN-CHIK double-virus-infected patients. Death was reported in only one patient infected with DEN-CHIK double viruses. Among DENV- (n = 101) and DEN-CHIK double-virus (n = 75)-infected patients, 65 and 46 exhibited WHO classified warning signs respectively, while the rest were without warning symptoms [14].

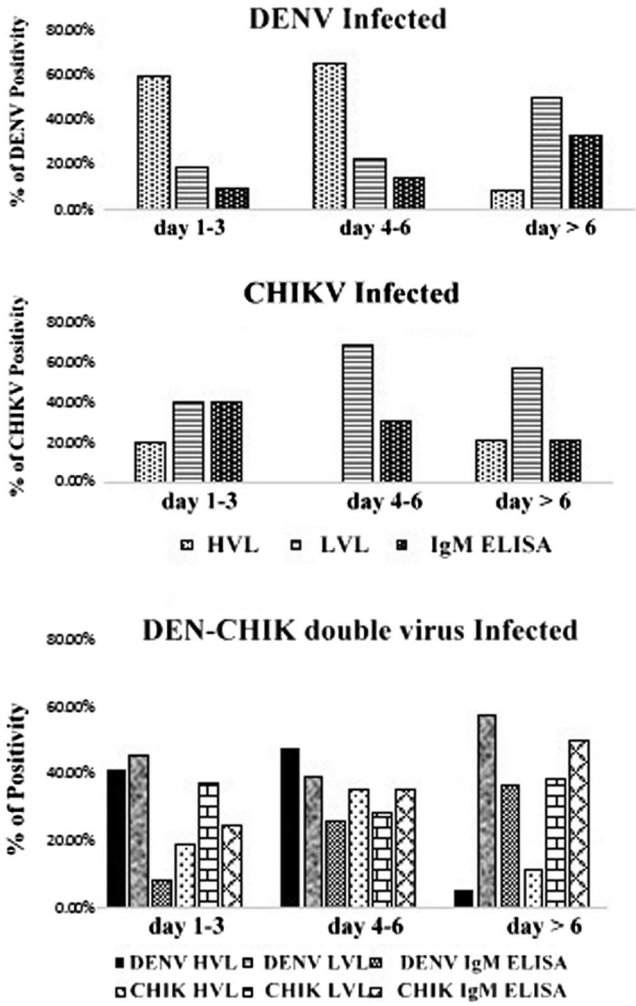

Fig. 4 Percentage of patients with high VL , low VL, & IgM according to days of infection

Within CHIKV-infected patients, rash and joint-swelling was significantly higher among the 21–40- and >40-year age groups, while abdominal pain was exclusively detected among the 0–20-year age group (Supplementary Fig. 1). Nausea and hemorrhagic manifestation progressively increased with increasing age of dengue patients, while retro-orbital pain was exclusively detected among the 0–20-year age group. Among DEN–CHIK double-virus-infected patients, rash and joint-swelling gradually increased with increasing age. Overall, symptoms such as rash, joint-swelling, vomiting, abdominal pain, hemorrhagic manifestation and retro-orbital pain were more prevalent among DEN–CHIK double-virus-infected patients of all age groups compared to patients infected with either DENV or CHIKV alone.

Ninety-four percent of both dengue and chikungunya patients were tested PCR+ (Table 1). Approximately 58% PCR+ dengue patients were in the high-VL group (HVL: ≥10,000 copies/ml), whereas the majority (81%) of PCR+ chikungunya patients were in the LVL-group (LVL: <10,000 copies/ml) [10, 11]. Average VL of mono-infected elder age group (>20 years) patients was higher in comparison to younger age group (0–20 years) patients. Average dengue VL was significantly higher among DENV and DEN–CHIK double-virus-infected patients exhibiting WHO-defined warning signs compared to similar patients without warning symptoms (Fig. 3). However, the reverse trend was noticed for CHIKV load among DEN–CHIK double-virus-infected patients. Average dengue VL was comparable among patients with/without anti-dengue-IgM, whereas mean chikungunya VL was lower among patients with anti-CHIK-IgM. Percentage of DENV-HVL among DENV and DEN–CHIK double-virus-infected individuals was higher during the viremic and critical phases of infection that gradually decreased among recovery phase patients, whereas that of DENV-LVL and anti-dengue-IgM patients followed the opposite trend (Fig. 4) [14]. Compared to the LVL group, hemorrhagic manifestation and headache was found in significantly higher frequency among HVL group dengue patients (Fig. 5). Nausea, rash, and joint-swelling were statistically more prevalent among HVL group chikungunya

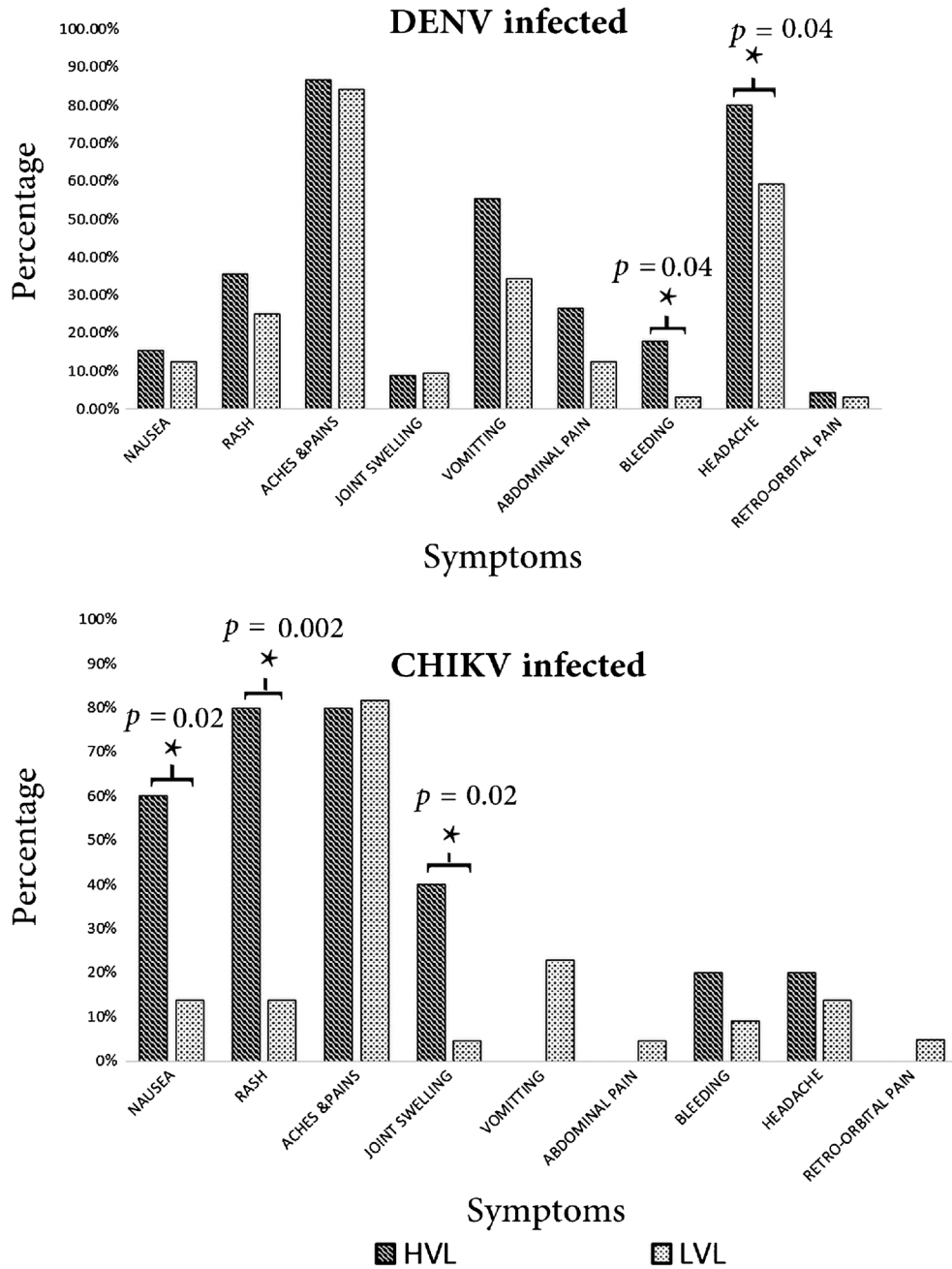

Fig. 5 Comparison of clinical symptoms between HVL and LVL groups among DENV- and CHIKV-infected patients

patients. Interestingly, the majority (80%) of the dengue HVL patients of DEN–CHIK double-virus group had CHIK LVL. A similar trend was observed for chikungunya HVL patients (80%) of this group.

One important aspect of this study was that out of 176 DENV-PCR+ dengue and DEN–CHIK double-virus-infected patients, dengue serotyping could be performed among 118 patients. Sixty-four patients were infected with single dengue serotype, whereas 54 patients were infected with multiple dengue serotypes (Table 1.). Categorization of patients according to their location indicated DENV2 infection was maximum among Southern districts patients whereas, DENV4 was highest among Northern districts. But, among multiple DENV serotype infected patients, DENV2–DENV4 combination was most prevalent in both Southern and Northern districts.

Discussion

Co-infection with DEN–CHIK double viruses and prevalence of more than one DENV serotypes among infected individuals indicated the complexity of arboviral outbreaks in India. DENV or DEN–CHIK double-virus-infected patients with

Dengue HVL exhibited WHO defined warning symptoms. DEN–CHIK double-virus-infected patients had more severe clinical manifestations than mono-infected patients. Dengue HVL patients were more prevalent during viremic and critical phase of disease [14]. Dengue infection was more prevalent among early age group patients, half of which were infected with DENV2–DENV4 combinations. CHIKV was found to infect patients of all age-groups.

Similarly to the findings of Hernández SI et al., chikungunya VL was found to be associated with absence of chikungunya-specific IgM antibodies in patient's blood. An important observation of this group was association of dengue HVL and chikungunya LVL among DEN–CHIK double-virus-infected patients showing WHO warning symptoms, and DEN-LVL & CHIK-HVL among patients without warning symptoms.

Dengue serotyping analysis indicated DENV2 and DENV4 were the major serotypes circulating in West Bengal, eastern India during 2014–2015. But difference in most prevalent DENV serotypes among monotypic patients of Southern and Northern districts indicated possibilities of two separate DENV outbreaks in these two regions. Greater prevalence of DEN–CHIK double-virus-infected patients in Southern districts indicated greater complexity of arboviral infection in this region.

Conclusion

In this study, we provide evidence of widespread distribution of dengue and chikungunya viruses, co-infection with both viruses and co-circulation of multiple dengue serotypes among eastern Indian patients. This highlights the complexity of the current arboviral outbreak scenario in India. Hence, realistic and effective control protocols are urgently needed for proper disease management.

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Compliance with ethical standards

Funding This study was partly funded by Department of Science and Technology, Government of West Bengal, India [615(sanc.)/ST/P/S& T/9G- 14/2010].

Conflict of interest The authors declare no conflicts of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with ethical standards at the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments.The study was approved by the Research Ethics Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013).

Informed consent Informed consent was obtained from all individual participants included in the study.

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Contribution of Toll like receptor polymorphisms to dengue susceptibility and clinical outcome among eastern Indian patients

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ABSTRACT

Dengue infection has been one of the major public health concerns in India causing simple dengue fever (DF) to severe dengue infection. In the present study, contribution of TLR3, 7 and 8 polymorphisms towards dengue disease susceptibility and severity among Eastern Indian patients was analysed. Genomic DNA was extracted from blood of 201 dengue infected patients and 157 healthy individuals, followed by genotyping of eight polymorphisms of TLR3 (rs3775290), TLR7 (rs5741880, rs3853839, rs179008 and rs179010) and TLR8 (rs3764879, rs3764880 and rs5744080) genes by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Functional analyses of the polymorphisms were predicted. Genotypic association of polymorphisms, alone and in combination, with dengue disease susceptibility and development of WHO-defined warning signs among patients was calculated by using SPSS software. TLR7-rs179008 & TLR8-rs3764880 were implicated to be non-synonymous polymorphisms. Specific genotypes of majority of the analysed TLR polymorphisms exhibited significant positive association with disease susceptibility. CC/C and AA/A of TLR7-rs179008 ($p < 0.0001$) and TLR8-rs3764880 ($p < 0.00001$) respectively were significantly associated with development of warning signs among dengue infected patients. Particular genotypic combinations of rs3853839-rs5744080 and rs179008-rs3764880 increased the risk of dengue infectivity, whereas, presence of last combination was more prevalent among dengue patients with warning signs. Thus these polymorphic variants of TLR3, 7 and 8 might act as potential prognostic biomarkers for predicting disease severity among dengue virus infected patients.

1. Introduction

Dengue infection has been one of the major public health concerns in India and other tropical countries worldwide, which is increasingly spreading in new geographical locations (Dengue, 2009). This infection is transmitted by infected *Aedes aegypti* vector, which results in diverse clinical outcomes ranging from simple febrile illness of dengue fever (DF) to severe dengue infection. World Health Organisation (WHO) has categorized the large group of non-severe dengue patients into two subgroups - patients without and with warning signs; the latter subgroup being at higher risk of developing severe dengue. Several studies from the Americas and South-east Asian countries have proposed presence of any warning signs among non-severe dengue cases to be strongly associated with severe dengue disease outcome (Leo et al., 2013; Horstick et al., 2015; Alexander et al., 2011).

Though the reasons behind such variations in clinical outcome among dengue virus (DENV) infected individuals remain poorly understood, host immunity and genetic predisposition might play crucial role in such differential disease pathogenesis (Yacoub et al., 2013; Chen et al., 2015). Host innate immune system receptors, mainly Toll-like receptors (TLRs) are the key sensors to primarily recognise any viral genomic RNA within patient-body. In general, endosomally-localized TLR3, TLR7, TLR8 and cytoplasmic RIG like receptors recognize viral RNA genome (Bustos-Arriaga et al., 2011). Thus, genetic variations in TLR genes viz. single nucleotide polymorphisms (SNPs) might influence the innate immune responses towards pathogenic challenges and affect the dengue disease susceptibility and clinical outcome among infected individuals. Several polymorphisms of TLR genes viz. rs3775290, rs179008, rs179010, rs5741880, rs3853839, rs3764879, rs3764880 and rs5744080 have been reported to be associated with hepatitis-B (HBV), hepatitis-C (HCV), chikungunya, dengue, HIV, Cytomegalo and Crimean–Congo Hemorrhagic fever virus infectivity among French, German, Spanish, Brazilian, Chinese, Japanese, Polish, Moroccan and Turkish populations (Dutta and Tripathi, 2017; Studzińska et al., 2017; Goktas et al., 2016; Huang et al., 2015; Fakhir et al., 2017; Valverde-Villegas et al., 2017; Askar et al., 2010; Zhu et al., 2017; Wang et al.,

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2014; Alagarasu et al., 2015a). Variations in genotype of rs3764879 within TLR-8 gene have been predicted to modulate patient's immune responses during HCV infection (Wang et al., 2014). The G allele and A/G genotype of rs3764880 has been related to clearance of HCV infection and protection from progression of HIV infection (Fakhir et al., 2017; Oh et al., 2008). All these studies indicated the importance of TLR polymorphisms towards viral disease infectivity and disease pathogenesis among different ethnic human populations.

First virologically proved dengue epidemic occurred in Kolkata and Eastern Coast of India in 1963–1964 and afterwards several massive outbreaks took place in Kolkata (West Bengal, Eastern India) (Gupta et al., 2012). Also recently, dengue infection has been detected among 54–61% febrile patients from several recent outbreaks in Eastern India during the last five years (Pal et al., 2014; Mukherjee et al., 2017).

All these previous data indicated the importance of host genetics of Eastern Indian patient population towards dengue disease susceptibility. In the present study, attempts have been made to understand the contribution of TLR3, 7 and 8 polymorphisms towards dengue disease susceptibility and development of disease severity among infected Eastern Indian patients.

2. Materials and methods

2.1. Ethical approval

All procedures performed in this study involving collection of blood from human participants as well as healthy controls were in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013) and with that of the 1964 Helsinki Declaration and its later amendments. Prior to participation in the study, written consents were obtained from patients and healthy control individuals.

2.2. Patients and healthy individuals

After obtaining institutional ethical committee approval, 5 ml of blood samples were collected from each of 425 symptomatic febrile patients (within 0–7 days of symptomatic onset), visiting Calcutta School of Tropical Medicine, Institute of Post Graduate Medical Education and Research and Medical College Hospital, Kolkata, West Bengal, India from July 2014 to October 2016. All patients exhibiting fever along with any of the following symptoms: headache, body ache, myalgia, arthralgia, rash, with or without haemorrhagic manifestation, were selected as per WHO criteria of dengue infection. Among those 425 symptomatic febrile patients, 201 (47.29%) were positive for dengue infection by anti-dengue-IgM ELISA/dengue-NS1ELISA/quantitative RT-PCR. Symptoms of dengue patients were also monitored after next 7 days (symptoms monitored during first and subsequent visit to hospital) and patients were categorised into two groups: with and without warning signs, according to WHO classification. Blood from healthy age-matched unrelated individuals of same ethnicity (n = 157), without any signs and history of other infections and without dengue infection as tested by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, were collected from the same community, to carry out case control study, as described previously (Dutta and Tripathi, 2017).

2.3. SNP selection and genotyping

SNPs in the TLR genes, TLR3 (rs3775290), TLR7 (rs179008, rs5741880, rs179010, rs3853839) and TLR8 (rs5744080, rs3764879, rs3764880) were selected based on their minor allele frequencies (MAF) and previously reported associations with other viral infections (Dutta and Tripathi, 2017; Studzińska et al., 2017; Goktas et al., 2016; Huang et al., 2015; Fakhir et al., 2017; Valverde-Villegas et al., 2017; Askar et al., 2010; Zhu et al., 2017; Wang et al., 2014; Alagarasu et al., 2015b). SNP genotyping was carried out by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based procedure. Briefly, genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Seven primer pairs were designed using GenBank database sequences and Primer3 software, to amplify rs3775290 polymorphic region of TLR3; rs179008, rs5741880, rs179010, rs3853839 polymorphisms of TLR7 and rs5744080, rs3764879, rs3764880 polymorphisms of TLR8 (Table S1). PCR was carried out in 20 µl reaction volume as mentioned previously (Dutta and Tripathi, 2017). The respective PCR products were digested with TaqI (Himedia, India), Bsh1285I, ER1381, MvaI, Eco130I, TaiI, Hin1II (Fermentas, USA) and Hpy188I (NEB, UK) as mentioned in Table S1, followed by agarose gel electrophoresis. Various RFLP band patterns were confirmed by sequencing the PCR products by using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA).

2.4. Statistical analysis

Association of any particular genotype of respective SNPs with dengue disease susceptibility and specific dengue symptoms (WHO-defined with/without warning signs) was calculated by using SPSS software version 22. Allele and genotype frequencies were compared between different study groups using Pearson's Chi square test. For genotypic associations, *p*-values, odds ratio (OR) & minor allele frequency (MAF) were calculated. A p-value of < 0.05 was considered statistically significant. For controls, Hardy-Weinberg equilibrium was analysed for eight polymorphisms with the programme Haploview (Barrett et al., 2005). The LDlink 3.0 program of National Institute of Health (NIH) was used for Linkage disequilibrium (LD) measurements (D′) by using south Asian population data, present within 1000 genome project of Genome Wide Association Studies (GWAS), the largest public catalogue of human variation and genotype data (Machiela and Chanock, 2015). Functions of the SNPs were predicted using SNPinfo web server and effect of amino acid changes in case of non-synonymous polymorphisms was predicted by using SIFT, Polyphen2, MutationTaster and by G23D softwares.

3. Results

In this case-control study, genetic polymorphism of TLR genes was investigated among 201 dengue infected patients, collected during 2014–2016 dengue outbreaks in eastern India along with age-sex matched 157 healthy control volunteers from the same region (Table S2). The male to female ratio of dengue patients was 1:1.27, whereas that of the control group was 1:1.01. Mean age of dengue patients was 32.01 ± 14.7 years (range: 5–70 years), whereas that of control was 36.2 ± 11.8 years (range: 18–63 years). Among 201 dengue patients, 126 were positive by qRT-PCR, 144 by anti-dengue-IgM and 63 by dengue NS1 ELISA. Among PCR positive dengue patients, Dengue serotype 2 was most prevalent, followed by serotype 4 (Table S2). At the time of blood collection, major symptoms among dengue patients were myalgia (61.69%), arthralgia (41.25%), headache (32.33%), rash (26.86%), nausea (25.37%), persistent vomiting (23.88%), abdominal pain (19.90%) joint swelling (11.94%), bleeding (6.96%) and retro-orbital pain (6.46%). Among these dengue infected patients, 30.84% (62/201) exhibited WHO classified Warning signs, whereas 69.16% (139/201) were without any Warning signs. DENV serotypic distribution among patients with and without warning signs was similar (Fig. S1).

3.1. Prediction for TLR3, 7 & 8 polymorphisms

According to SNPinfo web server, two polymorphisms (rs179010 and rs3764879) might affect the transcription factor binding sites of

Table 1
(a) Genetic characterization of TLR polymorphisms and (b) predicted effect of non-synonymous TLR polymorphisms on protein structure.

(A)

Gene	SNP ref. No.	Chromosomal Location	Position	Alleles	Transcription factor binding site	Affects splicing regulation	Non-synonymous polymorphism
TLR3	rs3775290	Chr. 4	187241211	T/C	–	–	–
TLR7	rs5741880	Chr. X	12797337	G/T	–	–	–
	rs179010	Chr. X	12812806	C/T	Yes	–	–
	rs179008	Chr. X	12813580	A/C	–	–	Yes
	rs3853839	Chr. X	12817579	C/G	–	–	–
TLR8	rs3764880	Chr. X	12834747	A/G	–	ESE[†]/ESS[‡] and abolishes domain	Yes
	rs5744080	Chr. X	12834618	C/T	–	–	–
	rs3764879	Chr. X	12847725	C/G	Yes	–	–

(b)

Gene	SNP ref. No.	Amino acid change	Effect				
			SIFT prediction	Polyphen2 prediction	MutationTaster prediction	FATHMM prediction	MetaSVM prediction
TLR7	rs179008	gly11leu	Tolerated	Benign	Neutral	Tolerated	Tolerated
TLR8	rs3764880	met1val	Damaging	Benign	Polymorphism	Tolerated	Tolerated

[†] ESE = Exonic splicing enhancer.
[‡] ESS = Exonic splicing silencer.

TLR7 and 8 genes respectively (Table 1). The rs3764880 polymorphism might affect the splicing activity of TLR8 due to its location near exon-intron junction. The TLR7-rs179008 (gly11leu) and TLR8-rs3764880 (met1val) were predicted to be non-synonymous polymorphisms. The rs3764880 was also predicted to be damaging by SIFT server. Except one SNP of TLR7 (rs3853839), remaining seven polymorphisms followed Hardy-Weinberg equilibrium at $p > 0.05$. However data of rs3853839 was incorporated in this study as its genotypic distribution might arise by chance or could reflect a significant association with dengue disease symptoms. The pair-wise LD measuring D′ for the seven TLR7 and TLR8 SNPs indicated that TLR8 polymorphisms, rs3764879 and rs3764880 were in complete linkage disequilibrium, with R^2: 0.969, D′: 1.0 and *p*-value: < 0.01 (Fig. 1).

3.2. Genotypic association of the polymorphisms with dengue disease susceptibility

Systematic analysis revealed that CC genotype of TLR3-rs3775290 was significantly positively associated with dengue disease susceptibility [OR = 4.15; 95% CI 1.86–9.24; p = 0.0002] (Table 2). The distribution of CC genotype was more than three-fold among patient population compared to control group. The rs3775290 C-allele was found to be significantly associated with dengue disease susceptibility. Similarly, CC genotypes of TLR7-rs179008 and TLR7-rs3853839, were found to be significantly associated with dengue disease susceptibility [rs179008: OR = 2.89, 95% CI 1.20–6.94, p = 0.01; rs3853839: OR = 2.01, 95% CI 1.23–3.25, p = 0.004]. Compared to healthy individuals, the frequency of CC genotype of TLR7-rs179008 was more than double among dengue infected patients. The C-allele of each of TLR7-rs179008 and TLR7-rs3853839 were found to be significantly associated with the disease. The GT genotype of TLR7-rs5741880 and CT genotype of TLR7-rs179010 were significantly related with decreased risk of dengue infection.

In case of TLR8 gene, AA genotype of TLR8-rs3764880 and CC genotype of TLR8-rs5744080 demonstrated significant association with dengue disease susceptibility [rs3764880: OR = 2.58, 95% CI 1.32–5.07, p = 0.004 and rs5744080: OR = 2.49, 95% CI 1.30–3.36, p = 0.001] (Table 2). The G-allele of TLR8-rs3764880 and C-allele of TLR8-rs5744080 exhibited significant association with dengue infectivity. Frequency of TLR8-rs3764880-AG genotype markedly decreased in disease population compared to healthy individuals, indicating significant association of this genotype with lower risk of dengue infection.

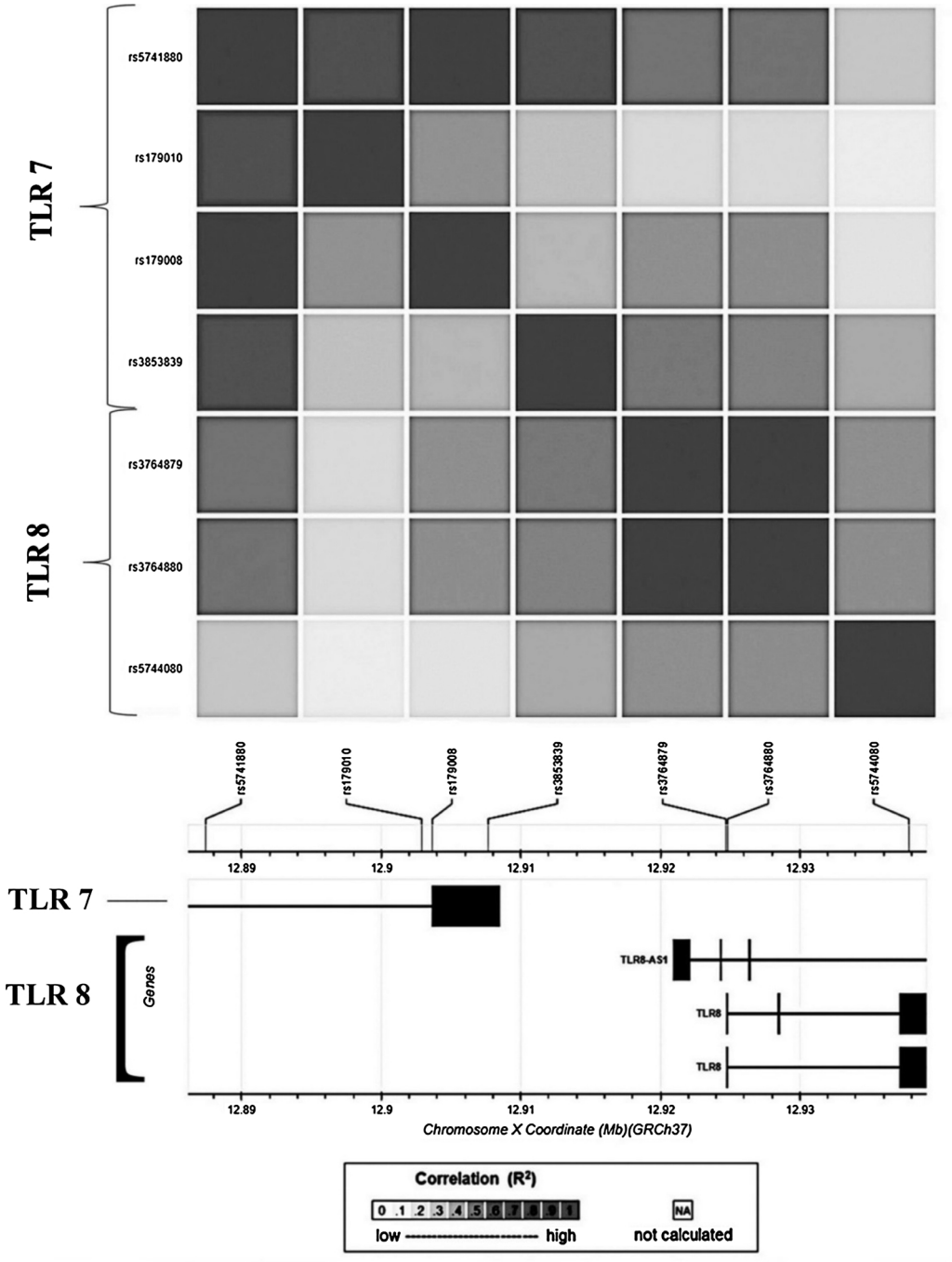
As TLR7 and 8 genes are located on X chromosome, any gender biasness of these SNPs among DENV patients needed to be analysed. The frequency of C genotype of rs179008 and rs3853839 polymorphisms of TLR7 and rs5744080 polymorphisms of TLR8 was significantly higher among DENV male patients than that of the control males (Table 2). The TLR7-rs3764880-AG and TLR8-rs179008-AC genotypes were present in significantly higher frequency among healthy female controls compared to female dengue patients. The TLR8-rs5744080-CC genotype was more prevalent among female dengue infected patients.

3.3. Genotypic association of polymorphism-combinations with disease susceptibility and severity

Various genotypes of SNPs of TLR7 and TLR8 that significantly associate with dengue infection were further analysed to find out their combined effect on enhancement of dengue disease susceptibility (Table 3a). Statistical analysis revealed that combination of CC/C-CC/C of TLR7-rs3853839-TLR8-rs5744080 [OR = 6.59, 95% CI 0.81–53.25, p = 0.042] and CC/C-AA/A of TLR7-rs179008-TLR8-rs3764880 [OR = 7.31, 95% CI 0.91–58.34, p = 0.028] were significantly higher among patient population compared to healthy control group.

3.4. Correlation between SNP-genotypes with clinical symptoms and WHO-defined warning signs

To determine the correlation between different SNP-genotypes with dengue disease symptoms, Pearson's Chi square test was performed (Table 4). The CC genotype of TLR3-rs3775290 and TT/T of TLR7-rs5741880 exhibited significant association with development of rash among infected patients compared to reference genotypes of respective polymorphisms. The CT genotype of TLR7-rs179010 and AA/A of TLR8-rs3764880 exhibited significant association with development of myalgia among dengue infected patients. Only CC/C of TLR7-rs179010 was significantly correlated with arthralgic manifestation of the patients. The TT/T and GT of TLR7-rs5741880, CC/C of both TLR7-rs179010 and TLR7-rs179008 and AA/A of TLR8-rs3764880 demonstrated significant correlation with development of headache among patients. The CC genotype of TLR3-rs3775290 and CC/C of TLR7-rs179010, TLR7-rs179008 and TLR8-rs5744080, TT/T of TLR7-rs5741880 and AA/A of TLR8-rs3764880 demonstrated significant association with persistent vomiting of dengue patients. The CC/C of



rs_number	rs5741880	rs179010	rs179008	rs3853839	rs3764879	rs3764880	rs5744080
rs5741880	1.0	0.115	0.015	0.093	0.078	0.074	0.017
rs179010	0.115	1.0	0.042	0.163	0.088	0.078	0.016
rs179008	0.015	0.042	1.0	0.031	0.049	0.051	0.009
rs3853839	0.093	0.163	0.031	1.0	0.489	0.465	0.245
rs3764879	0.078	0.088	0.049	0.489	1.0	0.969	0.308
rs3764880	0.074	0.078	0.051	0.465	0.969	1.0	0.322
rs5744080	0.017	0.016	0.009	0.245	0.308	0.322	1.0

Fig. 1. Pairwise linkage disequilibrium pattern of seven SNPs in TLR7 & TLR8 genes from South Asian BEB data from 1000 genome project.
Legends: Genomic location of these genetic polymorphisms on chromosome X. Dark colour indicated strong linkage disequilibrium between SNPs. Pairwise LD (R2) is shown for each combination of SNPs in table form.

TLR7-rs179008 and AA/A of TLR8-rs3764880 were strongly associated with development of abdominal pain among dengue infected patients. The CC genotype of TLR3-rs3775290 and CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 exhibited significant association with haemorrhagic manifestation of dengue patients. The TT/T of TLR7-rs5741880, CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 were significantly related with development of retro-orbital pain among the patients. Overall, the CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 exhibited significant association with WHO-defined dengue warning signs viz. persistent vomiting, abdominal pain and

Table 2
Genotypic and allelic distribution of TLR3, 7 and 8 polymorphisms among Dengue patients and healthy controls.

SNP Ref. No.	Chromosomal Location	Genotype/ Haplotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
TLR 3 rs3775290	Chr.4		n = 157	n = 192 NA = 9				
		CC	5.09	18.22	4.15 [1.86 - 9.24]	0.0002*		
		TT	33.75	31.77	0.91 [0.58 - 1.43]	0.69		
		CT	61.14	50	ref			
		C allele	35.66	43.22	2.13 [1.58 – 2.86]	0.04*	0.35	0.43
		T allele	64.33	56.77	0.728 [0.53 - 0.98]			
		MALE	n = 78	n = 113				
		CC	6.41	21.23	3.93 [1.43-10.83]	0.005		
		TT	28.20	28.31	1 [0.59-1.90]	0.98		
		CT	65.30	50.44	ref			
		FEMALE	n = 79	n = 79				
		CC	3.9	13.92	4.09 [1.09-15.30]	0.02*		
		TT	39.24	36.70	0.89 [0.47-1.70]	0.74		
		CT	56.96	49.36	ref			
TLR 7 rs5741880	Chr. X		n = 157	n = 196 NA = 5				
		TT	12.73	13.26	1.04 [0.56 – 1.95]	0.88		
		GT	11.46	5.61	0.45 [0.21 – 1.00]	0.046*		
		GG	75.79	81.12	ref			
		G allele	81.52	83.92	1.18 [0.79 – 1.75]	0.40	0.18	0.16
		T allele	18.47	16.08	0.84 [0.57 – 1.25]			
		MALE	n = 78	n = 109				
		T	25.64	20.18	0.73 [0.36-1.46]	0.37		
		G	74.35	79.81	ref			
		FEMALE	n = 79	n = 87				
		TT	0	4.59	3.90 [0.42-35.67]	0.19		
		GT	22.27	12.64	0.49 [0.21-1.11]	0.08		
		GG	77.21	82.75	ref			
TLR 7 rs179010	Chr. X		n = 157	n = 196 NA = 5				
		CC	30.57	34.18	0.98 [0.72 – 1.32]	0.47		
		C T	17.83	9.69	1.01 [0.75 – 1.38]	0.02*		
		TT	51.5	56.12	ref			
		C allele	39.49	39.03	1.07 [0.77 – 1.47]	0.90	0.39	0.39
		T allele	60.50	60.96	0.93 [0.67 – 1.28]			
		MALE	n = 78	n = 112				
		C	48.71	44.64	0.84 [0.47-1.51]	0.73		
		T	51.28	55.35	ref			
		FEMALE	n = 79	n = 84				
		CC	12.6	20.23	1.75 [0.74-4.09]	0.19		
		TC	35.4	22.61	0.53 [0.26-1.05]	0.07		
		TT	51.8	57.14	ref			

(*continued on next page*)

Table 2 (*continued*)

SNP Ref. No.	Chromosomal Location	Genotype/ Haplotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
TLR 7 rs179008	Chr. X		n = 157	n = 193 NA = 8				
		CC	4.45	11.91	2.89 [1.20 – 6.94]	0.01*		
		AC	3.82	0	0.12 [0.01 – 1.08]	0.02*		
		AA	91.71	88.08	ref			
		C allele	6.36	11.91	1. 98 [1.15 – 3.49]	0.01*	0.06	0.12
		A allele	93.6	88.01	0.50 [0.29 – 0.86]			
		MALE	n = 78	n = 108				
		C	3.84	12.96	3.72 [1.03-13.43]	0.03*		
		A	96.15	87.03	ref			
		FEMALE	n = 79	n = 85				
		CC	5.06	10.58	2.24 [0. 65-7.52]	0.19		
		AC	7.59	0	0.13 [0.01-1.18]	0.03*		
		AA	87.34	89.41	ref			
TLR 7 rs3853839	Chr. X		n = 157	n = 175 NA = 26				
		CC	22.29	35.42	2.01 [1.23 – 3.25]	0.004*		
		GC	0	0	–	–		
		GG	77.7	64.57	ref			
		C allele	22.29	35.42	1.91 [1.35 – 2.69]	0.0002*	0.22	0.35
		G allele	77.07	64.57	0.52 [0.37 – 0.73]			
		MALE	n = 78	n = 100				
		C	19.24	37.00	2.46 [1.12-4.93]	0.009*		
		G	80.76	63.00	ref			
		FEMALE	n = 79	n = 75				
		CC	25.3	33.33	1.47 [0.73-2.96]	0.27		
		GC	0	0	–	–		
		GG	74.6	66.66	ref			
TLR8 rs3764879	Chr. X		n = 157	n = 196 NA = 5				
		CC	22.29	27.55	1.32 [0.81 – 2.16]	0.25		
		GC	9.55	8.16	0.84 [0.40 – 1.76]	0.64		
		GG	68.15	64.28	ref			
		C allele	27.07	31.63	1.24 [0.89 – 1.72]	0.18	0.27	0.31
		G allele	72.92	68.36	0.80 [0.57 – 1.11]			
		MALE	n = 78	n = 110				
		C	28.20	38.18	1.57 [0.84-3.18]	0.15		
		G	71.79	61.81	ref			
		FEMALE	n = 79	n = 86				
		CC	16.4	13.95	0.82 [0.35-1.92]	0.65		
		GC	18.9	18.60	0.97 [0.44-2.13]	0.94		
		GG	64.5	67.44	ref			

(*continued on next page*)

Table 2 (*continued*)

SNP Ref. No.	Chromosomal Location	Genotype/ Haplotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
TLR8 rs3764880	Chr. X		n = 157	n = 190 NA = 11				
		AA	8.28	18.94	2.58 [1.32 – 5.07]	0.004*		
		AG	23.56	1.05	0.03 [0.008 – 0.14]	0. < 0001*		
		GG	68.15	80.00	ref			
		A allele	20.06	19.47	0.57 [0.40 – 0.81]		0.20	0.19
		G allele	79.93	80.52	1.73 [1.22 – 2.45]	0.001*		
		MALE	n = 78	n = 111				
		A	8.97	23.42	3.10 [1.21-7.57]	0.009*		
		G	91.02	76.57	ref			
		FEMALE	n = 79	n = 79				
		AA	7.59	12.65	1.76 [0.60-5.11]	0.29		
		AG	46.83	2.53	0.02 [0.006-0.12]	< 0.0001*		
		GG	45.56	84.81	ref			
TLR8 rs5744080	Chr. X		n = 157	n = 191 NA = 10				
		CC	12.73	26.70	2.49 [1.30 – 3.36]	0.001*		
		CT	14.64	13.08	0.87 [0.47 – 1.61]	0.67		
		TT	72.61	60.20	ref			
		C allele	20.06	33.24	1.98 [1.40 – 2.81]	0.0001*	0.20	0.33
		T allele	79.93	66.75	0.50 [0.35 – 0.71]			
		MALE	n = 78	n = 103				
		C	12.82	38.83	1.84 [0.96-3.50]	0.06		
		T	74.35	61.16	ref			
		FEMALE	n = 79	n = 88				
		CC	0	12.50	11.57 [1.45-91.77]	0.004*		
		CT	29.11	28.40	0.96 [0.49-1.89]	0.92		
		TT	70.88	59.09	ref			

"ref"= reference genotype, "NA"= Could not be PCR-amplified.

* $p < 0.05$ at 95% CI was considered as statistically significant.

Table 3
Association of genotype combinations of different TLR7 and TLR8 polymorphisms with (a) Dengue disease susceptibility and (b) development of WHO classified warning signs among patients.

(a)

SNP combinations	Frequencies (%)		Odds Ratio	*p* value at 95% C.I.
	Control (N = 157)	Dengue infected (N = 201)		
rs5741880-rs3853839 TT/T-CC/C	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs179008 TT/T- CC/C	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs3764880 TT/T-AA/A	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs5744080 TT/T-CC/C	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs3853839-rs179008 CC/C- CC/C	0	6 (2.98)	4.89 [0.58- 41.05]	0.10
rs3853839-rs3764880 CC/C-AA/A	4	12 (5.97)	2.42 [0.76 – 7.68]	0.11
rs3853839-rs5744080 CC/C- CC/C	0	8 (3.98)	6.59 [0.81 – 53.25]	**0.042***
rs179008-rs3764880 CC/C-AA/A	1	9 (4.47)	7.31 [0.91 – 58.34]	**0.028***
rs179008-rs5744080 CC/C-CC/C	0	4 (1.99)	3.22 [0.35 - 29.17]	0.27
rs3764880-rs5744080 AA/A-CC/C	0	5 (2.48)	4.80 [0.57 – 40.33]	0.11

(b)

SNPs Combination	Frequency (%) Symptomatic Dengue patients (n = 201)	Frequency of Symptoms (%)	
		With WHO-defined warning signs	Without warning signs
rs3853839-rs5744080 CC/C- CC/C	8(3.98)	3 (37.5)	5 (62.5)
rs179008-rs3764880 CC/C-AA/A	9 (4.47)	**9 (100)**	0

* $p < 0.05$ at 95% CI was considered as statistically significant.

Table 4
Association of TLR polymorphisms with development of different clinical symptoms among Dengue patients.

Gene	SNP Ref. No.	Genotype (female)/ Haplotype (male)	Statistical parameter	Symptoms (%)									
				Nausea	Rash	Myalgia	Arthralgia	Joint Swelling	Retro orbital pain	Headache	WHO-defined dengue warning signs		
											Persistent Vomiting	Abdominal pain	Haemorrhage
TLR3	**rs3775290**	CC	n/N (%)	28.57	45.71	68.57	45.71	14.3	14.3	40	40	31.42	14.3
			p value	0.65	**0.007**	0.52	0.67	0.31	0.08	0.34	**0.01**	0.06	**0.04**
			O.R.	0.82	3.007	1.30	1.17	1.83	3.03	1.46	2.70	2.29	3.88
		CT	n/N (%)	30.20	26.04	62.5	41.66	12.5	5.20	31.25	13.54	16.66	4.16
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		TT	n/N (%)	13.11	16.39	59.01	39.34	6.55	4.91	29.50	21.31	19.67	8.19
			p value	**0.01**	0.15	0.66	0.77	0.68	0.93	0.81	0.90	0.63	0.28
			O.R.	0.34	0.55	0.86	0.90	0.77	0.94	0.92	0.95	1.22	2.05
TLR7	**rs5741880**	TT/T	n/N (%)	12.5	75	87.5	75	0	25	62.5	50	37.5	12.5
			p value	0.34	**0.001**	0.24	0.06	–	**0.03**	**0.03**	**0.04**	0.19	0.55
			O.R.	0.37	9.23	3.30	4.33	–	5.55	4.20	3.96	2.58	1.92
		GG/G	n/N (%)	27.67	24.52	56.60	40.88	12.57	5.66	27.04	20.75	18.86	6.91
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		GT	n/N (%)	13.79	24.13	79.31	34.48	10.34	6.89	48.27	31.03	20.68	6.89
			p value	0.11	0.96	0.21	0.51	0.73	0.79	**0.02**	0.22	0.81	0.99
			O.R.	0.41	0.97	1.81	0.76	0.80	1.23	2.51	1.71	1.12	0.99
TLR7	**rs179010**	CC/C	n/N (%)	29.26	41.46	65.85	58.53	9.75	7.31	53.65	34.14	21.95	9.75
			p value	0.33	0.12	0.29	**0.01**	0.61	0.66	**0.003**	**0.03**	0.60	0.47
			O.R.	1.48	1.78	1.49	2.57	0.74	1.38	2.95	2.33	1.26	1.59
		TT/T	n/N (%)	21.81	25.45	56.36	35.45	12.72	5.45	28.18	18.18	18.18	6.36
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		CT	n/N (%)	35.55	17.77	84.44	28.88	8.88	8.88	28.88	26.66	24.44	6.66
			p value	0.07	0.30	< **0.01**	0.66	0.49	0.42	0.92	0.23	0.23	0.94
			O.R.	1.97	0.63	4.208	0.84	0.66	1.69	1.05	1.63	1.63	1.05
TLR7	**rs3853839**	CC/C	n/N (%)	27.41	27.41	53.22	43.54	8.06	4.83	30.64	25.80	16.12	4.83
			p value	0.43	0.90	0.11	0.80	0.71	0.71	0.52	0.67	0.58	0.71
			O.R.	1.32	1.04	0.59	1.08	0.81	0.77	0.80	1.16	0.79	0.81
		GG/G	n/N (%)	22.12	26.54	65.48	41.59	9.73	6.19	35.39	23.01	19.46	6.19
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
TLR7	**rs179008**	CC/C	n/N (%)	28.57	23.80	71.42	47.61	14.28	19.04	61.90	76.19	71.42	42.85
			p value	0.73	0.40	0.26	0.50	0.72	**0.01**	**0.003**	< **0.01**	< **0.01**	< **0.01**
			O.R.	0.83	1.51	1.81	1.35	1.26	4.26	4.33	14.55	14.70	25.05
		AA/A	n/N (%)	25.00	27.32	62.20	40.11	11.62	5.23	30	18.02	14.53	2.90
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
TLR8	**rs3764880**	AA/A	n/N (%)	38.88	33.33	86.11	38.88	8.33	19.44	55.55	66.66	61.11	19.44
			p value	0.051	0.23	**0.001**	0.65	0.68	**0.0003**	**0.001**	< **0.01**	< **0.01**	**0.007**
			O.R.	2.12	1.61	4.63	1.19	0.77	6.81	3.27	10.66	11.69	5.00
		GG/G	n/N (%)	23.02	23.68	57.23	39.47	12.50	3.94	27.63	15.78	11.84	4.60
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		GA	n/N (%)	50	0	50	100	0	0	0	0	0	0
			p value	0.37	NA	0.83	0.08	NA	NA	NA	NA	NA	NA
			O.R.	3.34	NA	0.74	7.64	NA	NA	NA	NA	NA	NA
TLR8	**rs5744080**	CC/C	n/N (%)	30.43	43.47	65.21	60.86	17.39	13.04	43.47	52.17	34.78	8.69
			p value	0.47	0.06	0.81	0.09	0.41	0.24	0.47	**0.01**	0.29	0.89
			O.R.	1.42	2.39	1.11	2.17	1.65	2.31	1.38	3.15	1.65	0.90
		TT/T	n/N (%)	23.47	24.34	62.60	41.73	11.30	6.08	35.65	25.21	24.34	9.56
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		TC	n/N (%)	26.41	22.640.80	58.49	28.30	11.32	5.66	22.64	9.43	5.66	1.88
			p value	0.68	0.90	0.61	0.09	0.99	0.91	0.09	**0.01**	0.03	0.07
			O.R.	1.17		0.84	0.55	1.001	0.92	0.52	0.301	0.18	0.18
TLR8	**rs3764879**	CC/C	n/N (%)	36.36	27.27	57.57	48.48	12.12	6.06	33.33	30.30	18.18	9.09
			p value	0.14	0.97	0.33	0.45	0.92	0.82	0.72	0.33	0.83	0.58
			O.R	1.82	1.01	0.67	1.33	0.94	0.83	1.15	1.52	0.89	1.47
		GG/G	n/N (%)	23.80	26.98	66.66	41.26	12.69	7.14	30.15	22.22	19.84	6.34
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		GC	n/N (%)	21.62	27.02	48.64	35.13	8.10	5.40	40.54	27.02	24.32	8.10
			p value	0.78	0.99	**0.04**	0.50	0.44	0.71	0.23	0.54	0.55	0.70
			O.R	0.88	1.002	0.47	0.77	0.60	0.74	1.57	1.29	1.29	1.30

*$p < 0.05$ at 95% CI was considered as statistically significant.
NA = Not applicable, "ref" = reference genotype.

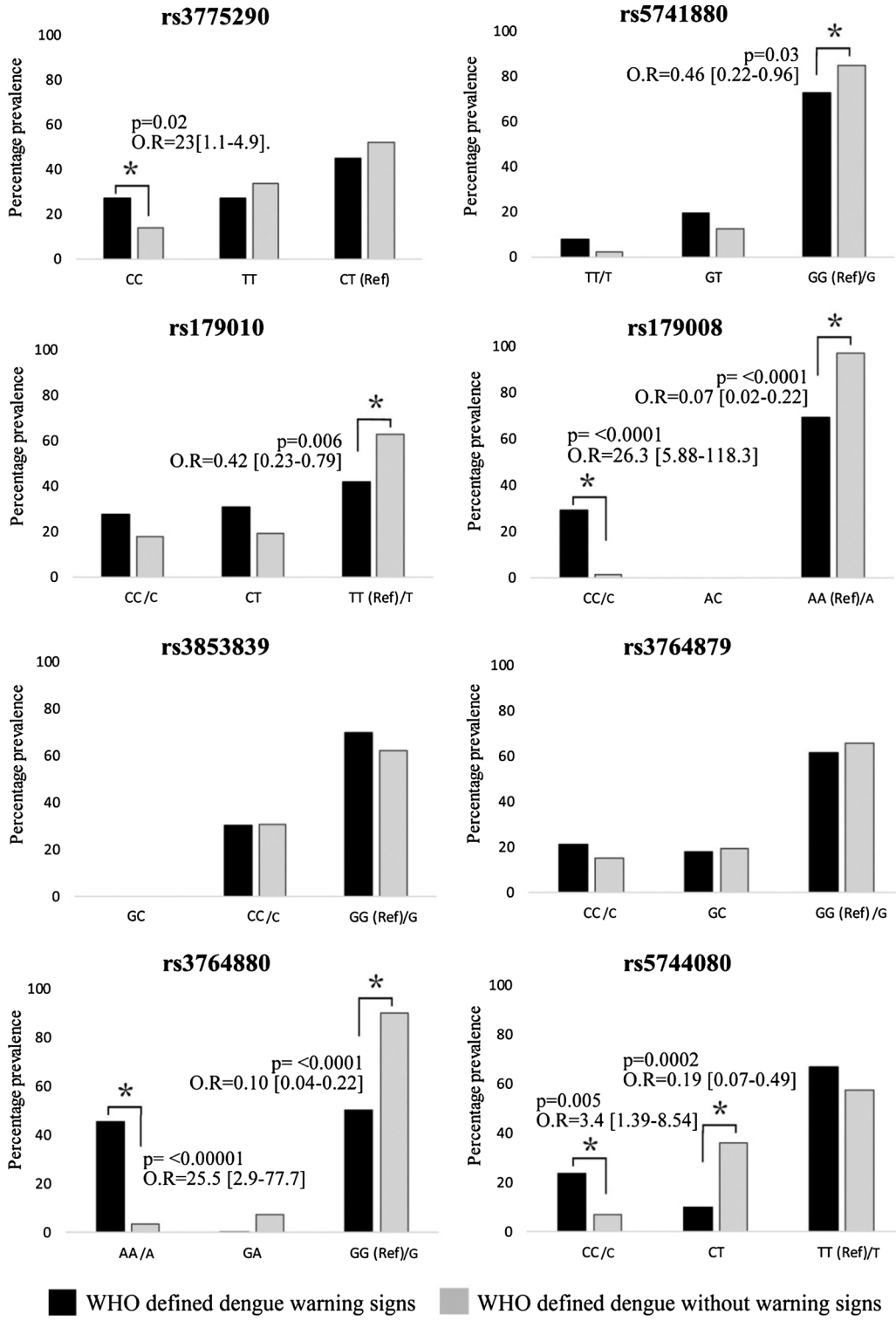


Fig. 2. Genotypic distribution pattern of the polymorphisms with/without warning signs among dengue patients.

haemorrhagic manifestation.

Association of particular genotypes with presence or absence of any WHO classified warning signs among dengue infected patients were analysed (Fig. 2). The TLR3-rs3775290-CC genotype, TLR7-rs179008-CC/C, TLR8-rs3764880-AA/A and TLR8-rs5744080-CC/C were found to be present in significantly higher frequencies among dengue patients exhibiting WHO-defined warning signs; whereas, TLR7-rs5741880-GG/G, TLR7-rs179010-TT/T, TLR7-rs179008-AA/A, TLR8-rs3764880-GG/G and TLR8-rs5744080-CT genotype, were considerably more prevalent among patients exhibiting no warning signs. Further, prevalence of specific genotypic combinations of significantly associated polymorphisms were analysed among dengue patients with/without warning signs (Table 3b). The CC/C-AA/A combination of TLR7-rs179008-TLR8-rs3764880 was predominantly present among patients with warning signs.

4. Discussion

Though TLR3, 7, 8 has long been known to be the first line of defence in innate immune recognition of dengue viral RNA genome, there has been very limited study regarding the role of TLR polymorphisms in dengue disease susceptibility. The present study clearly depicts the importance of various polymorphisms of TLR3, 7, 8 genes in determining dengue disease pathogenicity among Eastern Indian patient population.

Both TLR3 and TLR7 are important for controlling dengue virus replication and eliciting cellular antiviral response within the host (Urcuqui-Inchima et al., 2017). The CC genotype of TLR3-rs3775290 was significantly associated with dengue disease susceptibility among Eastern Indian patients, with three fold higher prevalence of this genotype among diseased population. This genotype of rs3775290 has also been previously detected to be higher among active chronic HBV patients of Turkish origin (Goktas et al., 2016). Involvement of this polymorphism has also been implicated among Tunisian chronic HCV patients and intrauterine transmissibility of HBV among Chinese patients (Huang et al., 2015; Sghaier et al., 2017).

TLR7 plays an important role in sensing viral genomic RNA which leads to the production of antiviral response (Urcuqui-Inchima et al., 2017). The CC genotypes of TLR7-rs179008 and TLR7-rs3853839 respectively of TLR7 gene demonstrated significant association with dengue infectivity among the study population. The TLR7-rs179008 polymorphism has been previously correlated with increased risk of disease progression among HCV mediated liver disease among Moroccan population and with post-bronchiolitis lung function deficiency of Finnish patients (Fakhir et al., 2017; Lauhkonen et al., 2016). Similar to the current study, the GC genotype of TLR7-rs3853839 was significantly higher among chikungunya virus infected Indian patients (Dutta and Tripathi, 2017). This TLR7 polymorphism has also been previously associated with protection against HCV persistence and susceptibility to enterovirus-71 mediated Hand foot and mouth disease among Chinese population (Yue et al., 2014).

The AA and CC genotypes of TLR8-rs3764880 and TLR8-5744080 respectively exhibited significant association among dengue infected patients. This A-allele of TLR8-rs3764880 was previously strongly associated with advanced liver disease among HCV infected Moroccan patients (Fakhir et al., 2017). Similar to this study, frequency of G-allele of rs3764880 was found to be significantly higher among pulmonary tuberculosis patients of Pakistani origin (Bukhari et al., 2015). On the contrary, this allele conferred significantly protective effect regarding progression of disease among HIV positive patients of German origin (Oh et al., 2008). The TLR8-rs5744080 polymorphism has been significantly different between osteoarthritis patients and healthy individuals of Japan (Yang et al., 2013).

Further, the present study demonstrated significantly increased risk of dengue disease susceptibility with two genotypic combinations of these polymorphisms. Contribution of such genotypic combinations of polymorphisms of JAK1 and several cytokines towards increased risk of Dengue haemorrhagic fever (DHF) development has been previously reported (Silva et al., 2010; Alagarasu et al., 2015a). Involvement of various genotypic combinations of TLR polymorphisms towards disease phenotype has also been previously demonstrated in ulcerative colitis, Crohn's disease and severe malarial anaemia (Meena et al., 2015; Munde et al., 2012).

The TLR7-rs179008-CC/C, TLR8-rs5744080-CC/C and TLR8-rs3764880-AA/A could be associated with the risk of developing WHO warning signs among dengue infected patients. Interestingly, reference genotypes of TLR7-rs5741880, TLR7-rs179010, TLR7-rs179008, TLR8-rs3764880 polymorphisms were significantly more prevalent among patients without any warning signs - thus these genotypes seemed to impart protection against development of warning signs among infected individuals. Presence of CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 among dengue patients significantly correlated with development of WHO-classified warning signs viz. persistent vomiting, abdominal pain and haemorrhage, observed in this study. The genotypic combination CC/C of rs179008 and AA/A of rs3764880 was significantly prevalent among patients with warning signs. Contribution of such genotypic combination of various SNPs towards increased risk of DHF development has been previously demonstrated among several polymorphisms of JAK1, interleukin-10 and interferon gamma (Silva et al., 2010; Alagarasu et al., 2015b).

In addition to the role of TLR polymorphisms in imparting dengue disease severity, prevalence of any specific DENV serotype or presence of secondary dengue infection among these patients was also investigated (Table S2, Fig. S1). No significant difference in DENV serotypic distribution and prevalence of secondary dengue infection was observed between patients with and without WHO-defined warning signs.

5. Conclusion

In conclusion, the present study indicated increased risk of dengue disease susceptibility among individuals with specific genetic variants of majority of the analysed TLR3, TLR7 and TLR8 polymorphisms – indicating important role of TLR polymorphisms in dengue disease pathogenesis. Increased risk of dengue infectivity was also predicted among certain genotypic combinations of these polymorphisms. Moreover, specific genotype(s) of certain polymorphism(s), alone or in combination, were associated with development of WHO-classified warning signs, which might act as potential prognostic biomarkers for predicting disease severity among dengue infected patients.

Authors' contribution

Tripathi A: Research idea formation, manuscript writing and correction and overall supervision; Mukherjee S: Performed the experiment, data analysis and manuscript writing. All authors read and approved the final manuscript.

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Ethical approval and consent to participate

All procedures performed in this study involving collection of blood from human participants were in accordance with ethical standards at the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments. The study was approved by the Research Ethics Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013). Prior to participation in the study, written consents were received from patients and healthy control individuals regarding their willingness to participate in the study, patients' rights and responsibilities, treatment information, confidentiality of the patient identity, permission to investigators to release information to the sponsors, regulatory authorities, Government agencies and ethics committee.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.08.009.

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ORIGINAL INVESTIGATION

Association of serum C-reactive protein level and polymorphisms with susceptibility to dengue infection and severe clinical outcome among eastern Indian patients

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Abstract
Dengue virus (DENV) infection is a major public health concern in India ranging from simple febrile illness to severe outcome. This study aimed to investigate association of serum CRP level and CRP gene polymorphisms towards development of dengue disease susceptibility and severity among eastern Indian patients. Blood was collected from 348 symptomatic patients. Sera was subjected to serological diagnosis for the presence of anti-dengue IgM, anti-dengue IgG antibodies and dengue NS1 antigen by ELISA. Viral RNA was extracted and the presence of DENV genome, viral load, serotypes was determined by qRT-PCR. CRP level and polymorphisms were determined by immunoturbidimetry and polymerase chain reaction–restriction fragment length polymorphism, respectively. Statistical analysis was performed by GraphPad-Prism. Among 206 dengue patients, CRP level increased significantly among patients within acute phase, and patients with qRT-PCR/NS1 antigen positivity, high viral load (HVL), secondary infection, and DENV4 and DENV2 infections. rs3091244, TT genotype positively associated with dengue susceptibility ($p = 0.03$). CT genotype of rs3093059 and TT genotype of rs3091244 were found to correlate with elevated CRP level and development of WHO-defined warning signs. TT genotype of rs3091244 was more prevalent among HVL patients. Thus, these CRP polymorphic variants and CRP concentration might act as potential prognostic biomarkers for predicting disease severity among acute-stage dengue patients.

Keywords Dengue virus · WHO-warning signs · CRP · Polymorphism · Secondary dengue infection · Serotype

Introduction

There has been rapid increase of dengue virus (DENV) infection in India during past decades [1]. This infection presents a wide spectrum of symptoms: simple dengue fever (DF), occasionally representing feverish illness, headache, joints and muscle pain, which matches with symptoms of other febrile illness, severe dengue involves haemorrhage, persistent vomiting, abdominal pain and severe plasma leakage [1–3]. Severe dengue has been associated with life-threatening disease complications, patient mortality rate lying between 10 and 20% in case of patients with dengue haemorrhagic fever (DHF) and 40% among dengue shock syndrome (DSS) patients [4]. According to World Health Organisation (WHO), simple dengue patients with warning signs are at higher risk of developing severe dengue disease (DHF and DSS) compared to patients without warning signs [5–7]. Dengue viral load and infecting viral serotypes in blood have been previously associated with the development of warning signs and disease severity of the patients [8, 9].

The acute phase response (APR) is an organism's early defence system against infections, using acute phase proteins [10]. C-reactive protein (CRP), an acute phase protein, genetically located in chromosome 1q23.2 region has been previously used as inflammatory and infectious biomarker in clinical set-up [11, 12]. CRP level might also be utilised to distinguish between viral and bacterial infections to assess severity of illness as well as to

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differentiate between dengue and other febrile illness [13, 14]**.** It is a general scavenger protein that binds toxic molecules released from damaged tissues and removes them from circulation and activates complement system to opsonise microorganisms [15]. Over time, dengue infections have left imprints in the human genome that evolved into single nucleotide polymorphisms (SNPs) of genes involved in protection/ susceptibility to dengue and SNPs in immune response genes have also been previously implicated in severe dengue development [16–18]. Severe dengue has been associated with haemorrhagic manifestation, high viral load and hepatic dysfunction, CC genotype of rs3091244 and TT genotype of rs3093059 of CRP have been previously implicated in haemorrhagic manifestation of stroke [19, 20]. Moreover, genetic variants of these two CRP polymorphisms were previously associated with altered viral load, increased CRP levels and hepatic inflammation among HBV and HCV carriers of Brazil and China [21–23]. Similarly, these genotypes of CRP might also affect development of increased serum CRP levels among dengue patients. Differential CRP level has been previously implicated between simple DF and severe dengue patients [12, 24]. As CRP plays an important role in innate immunity as an early defence system against infections, similar to other innate immunity genes, polymorphisms of CRP might also affect susceptibility towards dengue infection. Specific genotypes of rs3775290 (TLR3), rs179008, rs38353839, rs5741880 (TLR7), rs3764880, rs5744080 (TLR8) has been previously found to be associated with dengue virus susceptibility and development of WHO-defined dengue warning signs [18, 25–27]. But no detailed investigation has been done to understand the effect of genotypic distribution of CRP polymorphisms on CRP level and dengue viral replication (load) within infected patients, which in turn might affect dengue disease severity.

Being a dengue endemic county, most Indian states have been classified as having constant risk of dengue transmission [28]. Dengue disease burden in India is being poorly analysed and a study reported that actual number of dengue cases in the country were 282 times the number reported by national vector-borne disease control programme [29]. Meta-analysis has shown that case-fatality ratio (CFR) of dengue in India is 2.6% with 95%CI 2·0–3·4, which was higher than CFR (1.14%) of dengue patients worldwide [30]. Hence, identification of an early acute phase predictive marker for development of dengue disease severity could help clinicians for better clinical management.

In this current study, contribution of CRP level and CRP gene polymorphisms towards the development of dengue viral susceptibility and disease severity among infected eastern Indian patients has been investigated.

Materials and methods

Ethics statement

All experiments performed in this study, including collection of blood from patients as well as healthy controls were done according to ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013) and followed 1964 Helsinki Declaration and its later amendments. Prior to participating in this study, written consents were received from patients and healthy control individuals.

Study population and collection of patients' blood

Blood samples (5 ml) were collected from 348 symptomatic febrile patients (within 0–10 days of symptomatic onset) during post monsoon season of July 2015–October 2016, visiting Virology unit of Calcutta School of Tropical Medicine, Institute of Post Graduate Medical Education and Research and Medical College Hospital, Kolkata, West Bengal, India after obtaining institutional ethical committee approval. To carry out age-matched case–control study, bloods from 102 healthy unrelated individuals of same ethnicity, who neither had any signs and history of other infections nor were detected with dengue infection by anti-dengue IgM ELISA/dengue NS1 ELISA/quantitative RT-PCR, were collected from the same community. Symptomatic patients and age-matched healthy controls were Bengali population from same geographical location of eastern India, whose ethnicity is similar to that of Bengali Bangladeshis (BEB) of south Asian population (SAS), present within 1000 genome project of Genome Wide Association Studies (GWAS), the largest public catalogue of human variation and genotype data.

Under inclusion criteria, patients exhibited fever and at least any two of the following symptoms: aches and pain, nausea, rash, vomiting, abdominal pain, joint swelling, bleeding, retro-orbital pain were included in this study. Blood was collected from all age groups and both sexes. Sera were separated from blood by density gradient centrifugation and aliquots were stored at – 20 °C and 80 °C freezer.

Categorization of patients according to WHO (2009) classification

Clinical symptoms of dengue patients were also monitored after next 10 days (symptoms monitored during first and subsequent visit to hospital) and patients were categorised

into two groups: with and without warning signs, according to WHO classification [1].

Extraction of viral RNA and determination of DENV load

Viral RNA was extracted from 140 µL of patients' sera, using QIAamp Viral RNA Mini Kit according to the manufacturer's protocol (Qiagen, Courtaboeuf, France). Presence of DENV genome and viral load among patients' sera was determined by quantitative real-time RT-PCR (Taqman assay) in total volume of 20 µl using 8-µl viral RNA, OneStepqRT-PCR MasterMix and other components of DENV Genesig kit (Primer Design Ltd., UK) according to the manufacturer's protocol. Real-time PCR was performed on ABI Prism 7500 fast instrument [9]. A standard curve of cycle threshold (Ct) values was constructed using six tenfold dilution series of a positive control template (provided within kit). DENV RNA copy number in patients' sera was calculated from corresponding Ct values. Each sample was loaded in triplicate. Viral RNA titer ≥ 10,000 copies/ml and < 10,000 copies/ml was considered as high viral load (HVL) and low viral load (LVL), respectively [8, 9].

Detection of anti-dengue IgM and IgG antibodies and dengue NS1 antigen

Suspected patients' sera were subjected to serological diagnosis for the presence of anti-dengue IgM (96% sensitivity, 87% specificity), dengue NS1 antigen using dengue IgM MAC ELISA Kit and dengue NS1 ELISA Kit, respectively (National Institute of Virology, Pune, India) according to the manufacturer's instructions. Dengue patients sera were subjected to serological diagnosis for the presence of anti-dengue IgG antibody using Panbio dengue IgG capture ELISA kit (80.9% sensitivity, 87.1% specificity).

Determination of DENV serotypes

Dengue serotypes of PCR-positive samples was determined by real-time RT-PCR in 20-µl reaction mix, containing 8-µl viral RNA, One-Step qRT-PCR Master Mix and other components of DENV serotyping Genesig kit (PrimerDesign Ltd., UK), according to the manufacturer's protocol. Reactions were carried out in ABI 7500 fast instrument using serotype-specific DENV controls present within the kit [9].

Quantification of CRP

CRP level in patient sera was estimated once for each of the symptomatic febrile patients belonging to acute (within days 1–3 of symptomatic onset), critical (4–6 days) and late (≥ 7 days) phase of infection. Quantification of CRP in sera was done by immunoturbidimetry method using AUTOSPAN turbi gold kit (SPAN diagnostics, India) according to the manufacturer's instructions. Briefly, 3 µl of patient serum/calibrator was mixed with 500-µl ten times diluted latex reagent (containing latex particle coated with anti-human CRP antibodies) and its absorbance was measured at 550 nm wavelength after 10 s (A1) and 120 s (A2). Serum concentration (mg/l) of CRP was measure using the following formula:

Serum concentration of CRP (mg/l) = [(A2-A1) Serum sample/(A2-A1) Calibrator] x Concentration of calibrator

SNP genotyping

SNP genotyping was carried out using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP)-based procedure. Briefly, genomic DNA was extracted from blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Based on the sequences available in GenBank database, three primer pairs were designed using Primer3 software, to amplify rs3093059 and rs3091244 polymorphic regions of CRP. PCR reaction was carried out in 20-µl volume, using 1 × PCR buffer (Fermentas, USA), 1 mM of each dNTP, 1unit of Taq DNA polymerase (Phusion™ High-Fidelity DNA Polymerase, Fermentas, USA), 1.5 mM $MgCl_2$ and 20p.moles of previously mentioned primers. PCR reactions were carried out in VERITI 96-well thermal cycler (Applied Biosystems, USA) at desired annealing temperature for 30 cycles and PCR products were electrophoresed on 2% agarose gel at 5 V/cm for an appropriate time period. Respective PCR products were digested with Tas I, Bfa I (Fermentas, USA) and Taq I (Himedia, India) separately as mentioned in Table S1 and digested products were visualised on 2.5% agarose gel. Different RFLP patterns were validated by sequencing of the respective PCR products using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in an ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA).

Statistical analysis

Association of any particular genotype of respective SNPs with dengue disease susceptibility, CRP level, viral load and specific dengue symptoms (WHO-defined with/without warning signs) was calculated using SPSS version 22 and GraphPad Prism[8] software (Los Angeles, CA, USA). Allele and genotype frequencies were compared between different study groups using Pearson's Chi square test and multivariant analysis using one way ANOVA. Mean and median values of viral load and CRP level in each cohort were compared using t-test. For genotypic associations, *p*

values, odds ratio (OR) and minor allele frequency (MAF) were calculated. A p value of < 0.05 was considered statistically significant. For controls, Hardy–Weinberg equilibrium was analysed for two polymorphisms with Haploview programme.

Results

Among 348 symptomatic febrile patients, 206 (59.19%) patients were found to be dengue infected as evidenced by qRT-PCR (27.66%: 57/206), anti-dengue IgM (70.38%: 145/206) and dengue NS1 ELISA (35.43%: 73/206). Remaining dengue negative symptomatic patients ($n = 142$) might be infected by other febrile infections like chikungunya, influenza, etc. which are also common in Indian sub-continent during this season. Blood was obtained from age–sex-matched 102 healthy control volunteers with same ethnicity from same region and without any past dengue encounter, symptoms and history of dengue infection as tested by anti-dengue IgM/IgG ELISA/dengue NS1 ELISA/quantitative RT-PCR, to carry out case–control study. Detailed clinicopathological history of patients with dengue and other febrile illness has been depicted in (Table 1). Male–female ratio of dengue-infected patients was 1.14:1 (110:96), whereas that of control group was 1.04: 1 (52:50) (Table 1). Mean age of dengue patients was 32.01 ± 14.7 years (range: 7–72 years), whereas that of control was 36.2 ± 11.8 years (range: 20–68 years). Among dengue-infected patients, 44.66% (92/206) exhibited WHO-defined warning signs, whereas 55.33% (1114/206) were without any warning signs. Prevalence of more than one WHO-defined warning signs was noticeably higher among dengue patients compared to those with other febrile illness. Dengue serotyping by qRT-PCR identified DENV2 (50.87%) to be the most prevalent viral serotype followed by DENV1 (21.05%), DENV4 (19.29%) and DENV3 (8.77%) among analyzed patients. Among PCR-positive dengue patients, 56.14% (32/57) have viral load of $\geq 10{,}000$ copies/ml

Table 1 Demographic scenario and clinicopathological history of the study population

		Symptomatic patients ($n = 348$)		Healthy controls ($n = 102$)
		Dengue ($n = 206$)	[#]Other febrile illness ($n = 142$)	
Mean age (in years)		32.01 ± 14.7	34.07 ± 15.3	36.2 ± 11.8
Sex	Male	53.39% (110)	52.02% (77)	50.98% (52)
	Female	46.60% (96)	47.98% (71)	49.02% (50)
Course of illness	Acute phase	33.49% (69)	25.35% (36)	NA
	Critical phase	53.88% (111)	57.04% (81)	
	Late phase	12.62% (26)	17.6% (25)	
Sample classification according to WHO	With warning signs	44.66% (92)	21.13% (30)	
	Without warning signs	55.33% (114)	78.87% (112)	
[†]Frequency of WHO-defined warning signs	Any one warning sign	48.91% (45)	76.66% (23)	
	Any two warning signs	39.13% (36)	23.33% (7)	
	Any three warning signs	11.95% (11)	0[‡]	
Diagnostic parameters	Anti-Dengue IgM	70.38% (145)	0	
	Dengue NS1	35.43% (73)	0	
	qRT-PCR	27.66% (57)	0	
Type of infection	Primary Dengue	94.17% (194)		
	Secondary Dengue	5.83% (12)		
		qRT-PCR confirmed dengue ($n = 57$)		
Vital titter	High viral load (HVL: $\geq 10{,}000$ copies/ml)	56.14% (32)	NA	
	Low viral load (LVL: $< 10{,}000$ copies/ml)	43.85% (25)		
Circulating serotypes	Dengue 1	21.05% (12)		
	Dengue 2	50.87% (29)		
	Dengue 3	8.77% (5)		
	Dengue 4	19.29% (11)		

[#]Other febrile illness mostly comprised of viral infections viz. chikungunya, influenza and other arboviruses

[†]Warning Signs comprised of abdominal pain, persisting vomiting and bleeding/haemorrhagic manifestation

[‡]No bleeding/haemorrhagic manifestation was observed among other febrile illness group patients

of sera (HVL: ≥ 10,000 copies/ml), whereas remaining 43.85% (25/57) harboured low viral load (LVL: < 10,000 copies/ml).

During acute phase of infection (within days 1–3 of symptomatic onset), mean CRP level was significantly higher among dengue patients compared to those with other febrile illness (49.84 mg/l vs. 22.86 mg/l; $p = 0.001$). However, CRP level decreased among critical phase dengue patients and it was comparable between late phase (≥ 7 days of symptomatic onset) patients with dengue and other febrile illness (Fig. 1a). CRP concentration was also significantly higher among dengue patients with WHO-defined warning signs compared to that of patients without warning signs (median: 25.6 mg/L, range: 1.89–177.14 mg/l vs. 11.07 mg/l, range: 1.09–63.67 mg/l; $p = 0.0001$) (Fig. 1b). Markedly higher CRP concentration was observed among anti-DENV IgG-positive patients (31.20 ± 7.34 mg/l, $p = 0.0029$) compared to the negative ones (15.72 ± 1.61 mg/l) (Fig. 2a). CRP level was also significantly higher among DENV qRT-PCR (37.68 ± 3.44 mg/l, $p = 0.019$) and DENV NS1-positive patients (29.48 ± 4.5.6 mg/l, $p = 0.0007$) compared to DENV qRT-PCR (26.11 ± 3.21 mg/l) and DENV NS1-negative patients (15.67 ± 1.51 mg/l), respectively (Fig. 2c, d).

Dengue patients with HVL demonstrated significantly higher CRP concentration compared to LVL patients (median: HVL: 37.82 mg/l, range: 4.08–91.94 mg/l, LVL: 15.76 mg/l, range: 2.41–77.98 mg/l; $p = 0.03$) (Fig. 3a). CRP level of DENV4 (39.14 ± 6.37 mg/l)- and DENV2 (36.25 ± 5.11 mg/l)-infected patients was markedly higher than that of DENV1 (14.49 ± 3.03 mg/l) and DENV3 (5.87 ± 1.98 mg/l) infection.

Genotypic distribution of rs3091244 indicated significantly higher prevalence of TT genotype among dengue patients compared to that of age-matched healthy controls (OR = 3.54; 95% CI 1.02–12.23; $p = 0.03$) (Table 2). Similarly, CT genotype (OR = 1.92; 95% CI 1.03–3.55; $p = 0.04$) and C allele (OR = 1.84; 95% CI 1.05–3.21; $p = 0.03$) of rs3093059 was markedly predominant among CRP-positive dengue patients.

Dengue patients having CT genotype ($p = 0.0001$) and C allele ($p = 0.01$) of rs3093059 and TT genotype ($p = 0.0001$) and T allele ($p = 0.01$) of rs3091244 exhibited higher CRP level in serum compared to patients harbouring other genotypes of the polymorphisms (Fig. 4a). CT genotype of rs3093059 ($p = < 0.0001$) and TT genotype of rs3091244 ($p = 0.001$) were significantly more prevalent among dengue patients with WHO-defined warning signs compared to those without any such signs (Fig. 4b). Prevalence of TT genotype of rs3091244 was markedly higher among dengue HVL patients also ($p = 0.01$) (Fig. 4c).

Discussion

CRP, an acute phase protein, is generally considered as a non-specific inflammatory biomarker, which is elevated during bacterial infections. But, very limited study has been performed regarding the role of CRP in dengue disease pathogenesis.

This study depicts the importance of specific genotypes of CRP polymorphisms in determining CRP concentration in patients' blood and contribution of CRP in the development

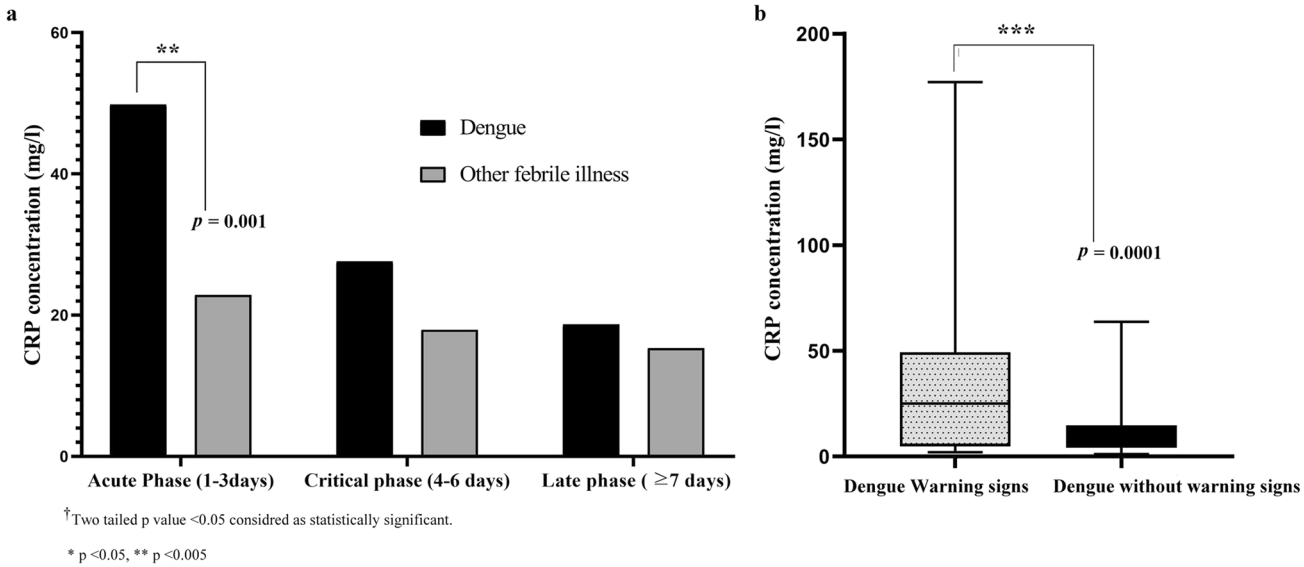


Fig. 1 Differential serum CRP concentration (**a**) among patients with dengue infection and with other febrile illness in different phases of infection (**b**) among dengue patients with and without WHO-defined warning symptoms

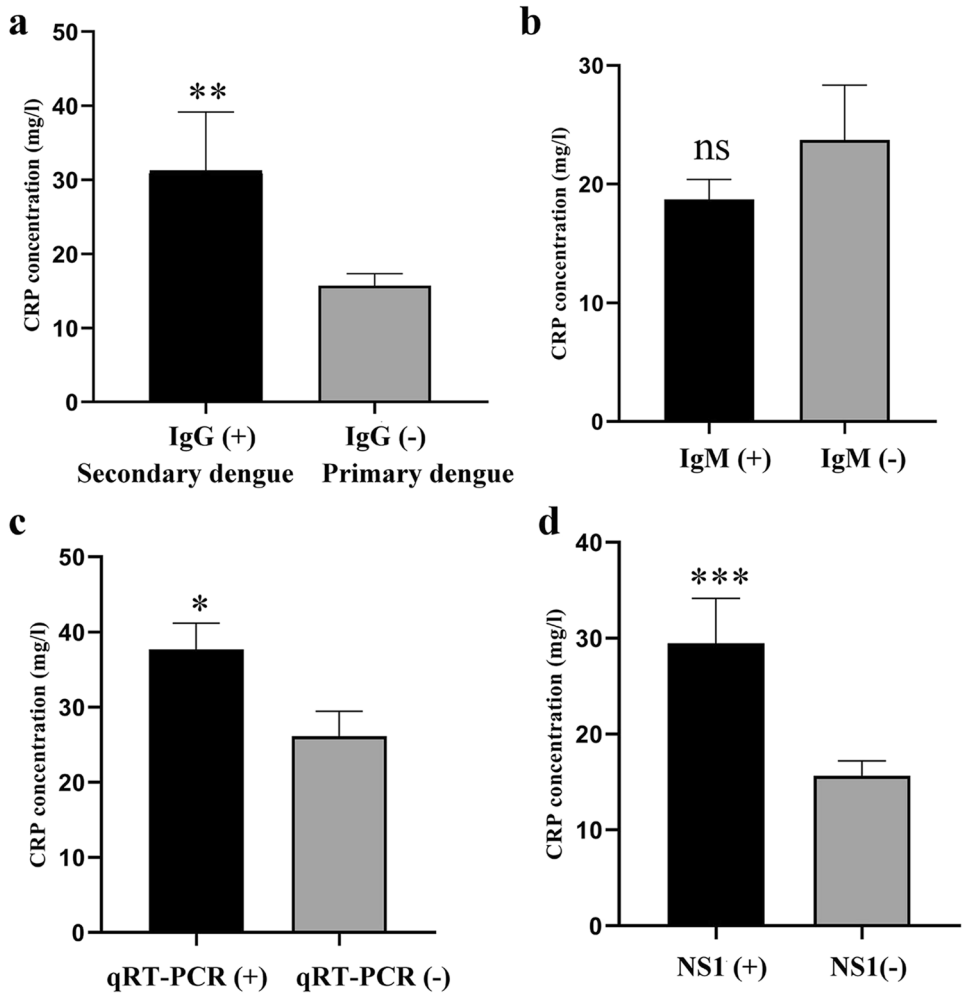


Fig. 2 Serum CRP levels among (**a**) secondary and primary dengue-infected patients, (**b**) dengue IgM-positive and IgM-negative (**c**) dengue qRT-PCR-positive and -negative (**d**) dengue NS1-positive and -negative patients

†Two tailed p value <0.05 considred as statistically significant.

‡ns = non significant.

* p <0.05, ** p <0.005, *** p <0.0005

of WHO-defined warning signs among eastern Indian patient population. CRP level increased significantly among dengue patients within acute phase of infection and among patients with qRT-PCR/NS1 antigen positivity, high viral load, secondary infection and infections with DENV2 and DENV4 serotypes. Similar to this study, higher concentration of CRP was previously reported among Taiwanese and Indonesian dengue patients within 1–3 days of symptomatic onset; previous reports from eight countries across Asia and Latin America suggested that CRP levels in first 3 days of illness could be a useful biomarker for early dengue risk prediction and may assist in differentiating dengue from other febrile illnesses [14]. Significantly higher CRP level was also detected among severe dengue patients (DHF/DSS) of Indian, Taiwanese and Venezuelan origin and among Venezuelan patients with secondary dengue infection [12, 24, 31, 32]. Due to higher prevalence of viral particles viz. NS1 antigen, viral RNA (high viral titter) during acute phase of infection, CRP level might have increased significantly to activate complement system and to elicit cellular antiviral response for opsonisation of the pathogen [15]. Similar to our findings, previous records also confirmed elevated CRP level to be associated with disease severity, secondary infection and DENV2 and DENV4 serotypes and among Venezuelan dengue patients [24]. Higher CRP levels indicated more serious tissue damage resulting from greater inflammation, which is often associated with poor clinical outcome [33, 34]. Also, in this study, mortality was reported in one dengue NS1-positive acute phase (3 day) patient with abnormally high CRP level of 177.14 mg/ml.

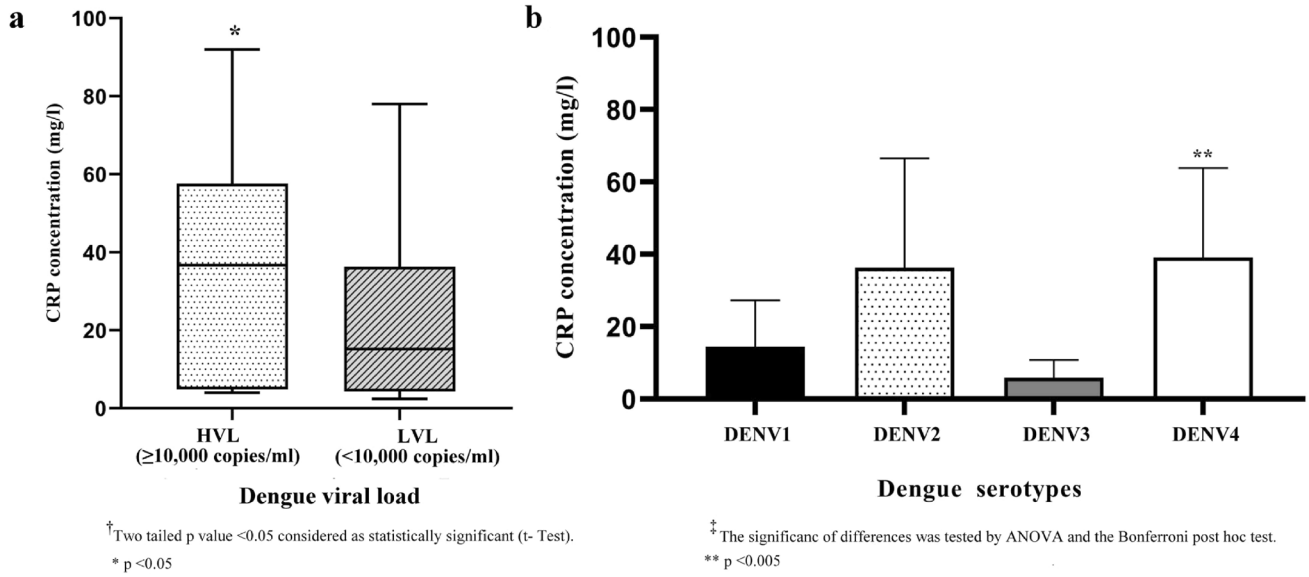


Fig. 3 Serum CRP level among dengue patients (**a**) with High Viral Load and Low Viral Load (**b**) with different infected serotypes

In this study, higher concentration of CRP was also found among dengue patients with CT genotype and C allele of rs3093059 and TT genotype and T allele of rs3091244. Comparable to this result, higher concentration of CRP was previously detected among Taiwanese patients with C allele of rs3093059 and T allele of rs3091244 [35, 36]. Though specific genotypes of rs3093059 and rs3091244 were previously associated with haemorrhagic stroke, ischemic stroke, colorectal cancer, HBV-related hepato-cellular carcinoma among Han-Chinese, American-Indian, Taiwanese and Chinese population, this is the first study to investigate the role of these polymorphisms for dengue disease susceptibility and the development of WHO-defined warning signs among dengue patients [19, 23, 37–39]. In this study, TT genotype of rs3091244, CT genotype of rs3093059 and TT genotype of rs3091244 were significantly more prevalent among dengue patients with WHO-defined warning signs and disease susceptibility. Furthermore, TT genotype of rs3091244 was more prevalent among dengue patients with high viral load. Contribution of such genotypic distribution of various SNPs towards increased risk of dengue disease severity and development of warning signs has been previously demonstrated among several polymorphisms of immune system gene viz. JAK1, interleukin-10, interferon gamma and TLRs [16–18, 40]. However, results of this study have to be validated among larger number of dengue patients with severity symptoms (DHF/DSS), which is the limitation of this study. Further, in addition to CRP, levels of several other acute phase proteins, pro-inflammatory cytokine and endothelial markers are also altered during disease pathogenesis which might act as better marker for severe dengue development.

Conclusion

This is the first study to highlight the implication of specific genotypes of CRP polymorphisms viz. rs3093059-CT and rs3091244-TT in determining CRP concentration in patients' blood and contribution of CRP in development of WHO-defined warning signs among eastern Indian dengue patients. Moreover, significantly increased CRP level among acute phase dengue patients might act as potential biomarkers for predicting disease severity.

Table 2 Genotypic and allelic distribution of CRP polymorphisms among Dengue patients and healthy controls

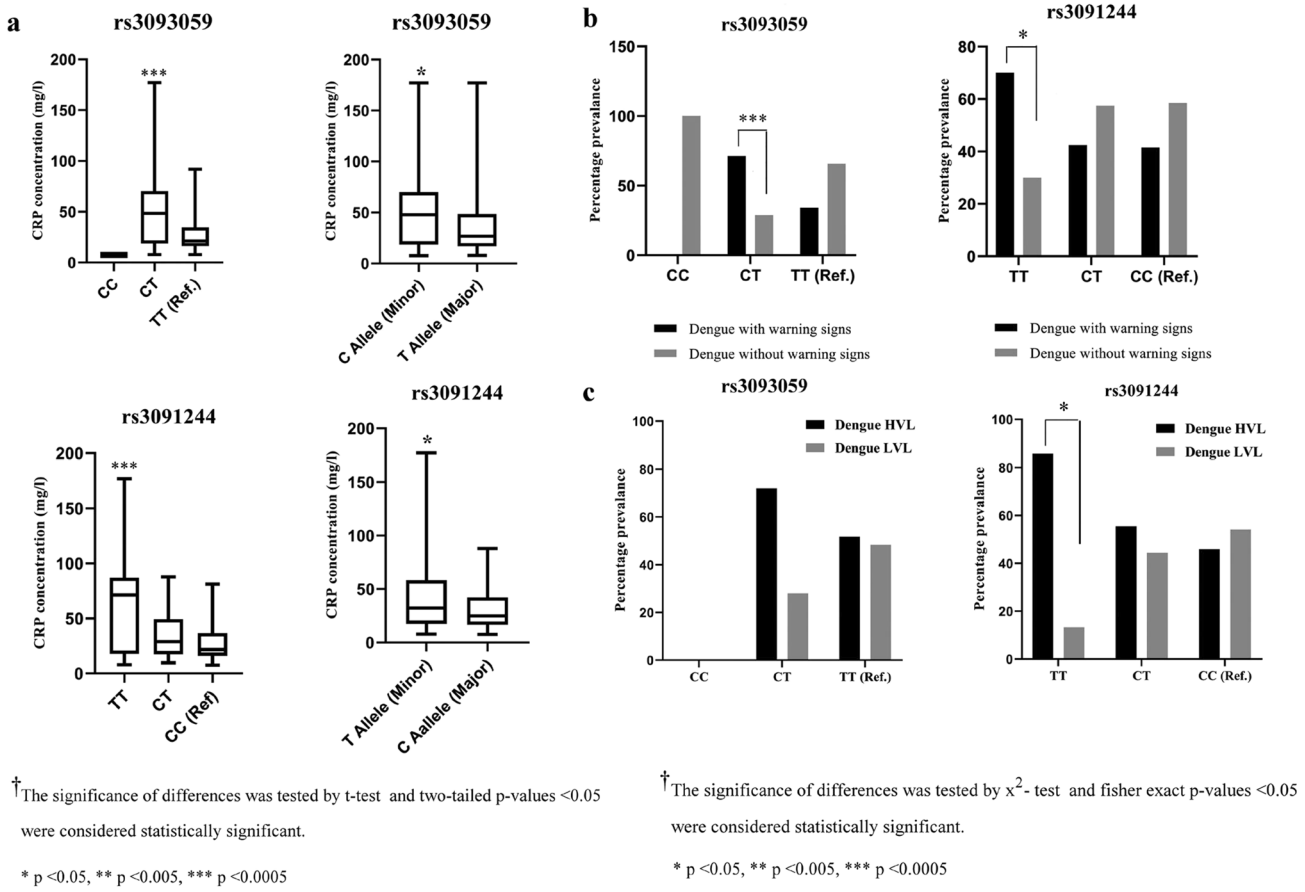
Sr. no	SNP Ref. no	Chromosome	Genotype and allele distribution	Healthy Controls (%) $N=102$	Dengue-infected patients $N=206$	Dengue infected patients CRP(+) $N=102$	Dengue-infected patients CRP(-) $N=104$	*p* value1	Odds Ratio at 95% CI	*p* value2	Odds Ratio at 95% CI
1	rs3093059	1	CC	2	1	1	0	0.25	0.24 (0.02–2.72)	0.49	3.08 (0.12–76.70)
			CT	21	59	36	23	0.16	1.54 (0.87–2.79)	0.04*	1.92 (1.03–3.55)
			TT	79	146	65	81	Ref			
			C allele	25	61	38	23	0.45	1.24 (0.75–2.04]	0.03*	1.84 (1.05–3.21)
			T allele	179	351	166	185				
2	rs3091244	1	TT	3	20	12	8	0.03*	3.54 (1.02–12.23)	0.35	1.60 (0.62–4.09)
			TC	39	80	40	40	0.45	1.21 (0.74–1.99)	0.99	1.03 (0.58–1.80)
			CC	60	106	50	56	Ref			
			T allele	45	120	64	56	0.06	1.45 (0.97–2.15)	0.33	1.24 (0.81–1.89)
			C allele	159	292	140	152				
			AC	0	0	0	0	NA	NA	NA	NA
			AA	0	0	0	0	NA	NA	NA	NA

ref reference genotype, *NA* Not applicable

*$p < 0.05$ at 95% CI was considered as statistically significant

†*p* value[1] = Healthy control vs. Dengue patients

‡*p* value[2] = Dengue-infected patients CRP (+) vs. Dengue-infected patients CRP (−)



†The significance of differences was tested by t-test and two-tailed p-values <0.05 were considered statistically significant.

* p <0.05, ** p <0.005, *** p <0.0005

†The significance of differences was tested by x^2- test and fisher exact p-values <0.05 were considered statistically significant.

* p <0.05, ** p <0.005, *** p <0.0005

Fig. 4 Genotypic distribution pattern of polymorphic variants of rs303059 and rs3091244 among dengue patients (**a**) with serum CRP levels (**b**) with and without WHO-defined warning signs (**c**) High Viral Load and Low Viral Load

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Author contributions MS: Performed the experiments, data analysis and manuscript writing. TA: Research idea formation, manuscript preparation, correction and overall supervision; All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures performed in this study involving human participants were in accordance with ethical standards at the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments.

Informed consent The study was approved by the Research Ethics Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013).

Availability of data and material All data generated and analysed during this study are included in this article.

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Research Article

Research Article

Clinical significance of differential serum-signatures for early prediction of severe dengue among Eastern Indian patients

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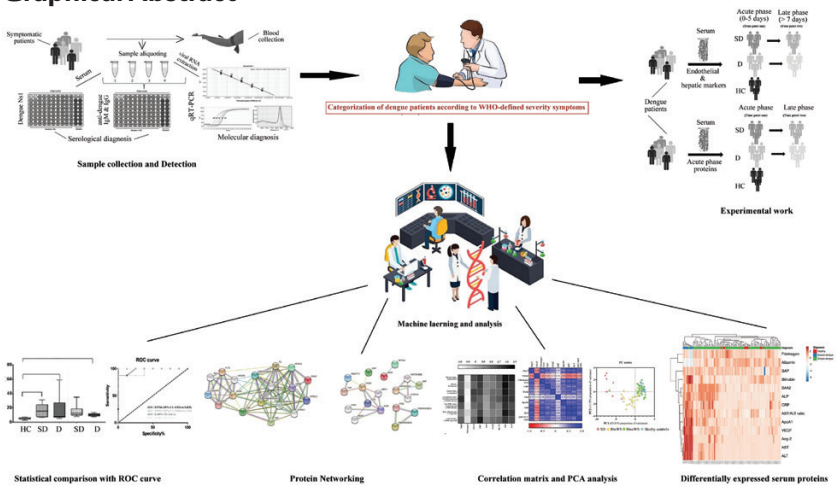
Abstract

Dengue infection can result in simple dengue fever or life-threatening severe dengue. Early identification of severe patients is needed for proper disease management. Dengue infection was screened among 168 symptomatic patients by qRT-PCR, anti-dengue IgM, and IgG ELISA. Dengue patients were categorized according to WHO classification. Viral load and dengue serotypes were determined by qRT-PCR. Levels of acute-phase-proteins (SAP, SAA2; CRP and ApoA1), endothelial (Ang2, VEGF), coagulation (fibrinogen) markers were determined by sandwich ELISA/immunoturbidimetry/western-blotting. Hepatic (ALT, AST, ALP) and other blood biochemical parameters were studied by autoanalyzer and haematology cell counter. Statistical analysis and protein–protein-interaction network were performed by GraphPad-Prism and STRINGS database, respectively. Among 87 dengue patients, significantly higher levels of Ang2, VEGF, CRP, SAA2, ApoA1, AST, ALT, and AST/ALT ratio and low level of fibrinogen were detected in severe-dengue cases compared to dengue without warning-signs, with seven of them severely altered during febrile-phase. Higher fold-change of Ang2 and VEGF as well as decreased fibrinogen were observed among patients with haemorrhagic-manifestation, clinical-fluid accumulation and thrombocytopenia. Functional network analysis predicted Ang2, VEGF, and CRP to be functionally and physically connected and SAA2 and ApoA1 to be functioning together. Correlation analyses also validated this connectivity by a strong positive correlation between Ang2, VEGF, and CRP. PCA analysis followed by hierarchical clustering heatmap analysis segregated severe-dengue patients from the rest, with VEGF, Ang2, ApoA1, AST, and ALT clearly distinguishing the severe-dengue group. Thus, serum levels of VEGF, Ang2, ApoA1, AST, and ALT might act as potential biomarkers for predicting dengue severity during the early stage.

Graphical Abstract



Keywords: severe-dengue infection, WHO classification, biomarker detection, endothelial marker, hepatic marker, acute-phase proteins

Abbreviations: ALP: alkaline phosphatase; ALT: alanine transaminase; Ang2: angiopoietin2; ApoA1: apolipoprotein A1; AST: aspartate aminotransferase; CRP: c-reactive protein; DwoWS: dengue patients without warning signs; DwWS: dengue patients with warning signs; HDL: high-density lipoprotein; HVL: high viral load; LDL: low-density lipoprotein; LVL: low viral load; SAA2: serum amyloid A2; SAP: serum amyloid P; SD: severe-dengue; VEGF: vascular endothelial growth factor; VL: viral-load; WHO: World health organization; DENV: dengue virus

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Introduction

Currently, dengue-virus (DENV) infection is an emerging arboviral threat that comprises a wide clinical spectrum, from simple-dengue fever to severe-dengue (dengue haemorrhagic fever: DHF; dengue shock syndrome: DSS) which can be life-threatening [1]. Dengue patients may progress through three clinical phases viz. febrile, critical, and recovery phases. During the early febrile phase, it is often not possible to predict clinically whether a patient with dengue will progress to severe disease. Various forms of severe -manifestations may unfold only as the disease progresses through the critical phase.

Pathophysiology of severe-dengue development is poorly understood. Several factors viz. secondary-dengue infection, circulating serotypes, viral-titter, and host immune response have played a crucial role in disease progressions [1, 2]. Three organ systems of infected patients are primarily affected in DHF/DSS: immune-system, liver, and vascular-endothelium [2]. Hepatic injury and disruption of endothelial permeability are major differential symptoms for DHF and DSS [3, 4]. Acute-phase-response (APR) is the human's first line of defence against any infection, with altered production of acute-phase proteins (APPs) mainly by hepatic-cells, comprising of a large heterogeneous group of proteins [5]. During infection, levels of certain APPs viz. CRP, SAP, SAA2, and ApoA1 are elevated; these proteins are referred to as "positive" acute-phase reactants [5]. These act as pattern recognition receptors to trigger host antimicrobial defence mechanisms upon infection by activating the complement system and opsonizing the pathogen [5, 6]. Hence, these APPs can be used as a diagnostic tool in many infectious diseases including viral infections. The significance of CRP, SAP, SAA2, and ApoA1 in host defence mechanism against viral infections like HBV, HCV, and Influenza virus has been reported [7–11]. But their beneficial role as a biomarker to dengue severity has not been well studied. The hallmark of severe dengue is increased vascular permeability, coagulation abnormalities, and fibrolysis, which has been an active area of research [1, 12]. Angiogenic-proteins viz. Angiopoietin1 (Ang1) and Angiopoietin2 (Ang2) function in maintaining vascular function and integrity; Ang2 is synthesized in endothelial cells and is a potent inducer of vascular permeability [12]. Another angiogenic-protein is vascular-endothelial-growth-factor (VEGF) that promotes the growth, proliferation, and migration of endothelial-cell [12, 13]. A regulated activation of coagulation is part of host-defence against infectious agents; but viral activation of endothelium may result in alterations in coagulation and fibrinolytic systems by degrading fibrinogen directly and prompting secondary activation of pro-coagulant homeostatic mechanisms [14, 15]. Previous studies have shown the importance of these endothelial and coagulation factors viz. Ang-2, VEGF, and fibrinogen in other viral haemorrhagic fever viz. Crimean-Congo virus and Hanta virus as potent clinical-markers [16–18]. Hence, investigation of clinical alteration of these factors among acute phase dengue patients and association of platelet degradation might be crucial for early prediction of disease severity. Hepatic-dysfunction due to reduced perfusion associated hypoxic injury, as a consequence of a severe vascular-leak, is another crucial feature of severe-dengue and according to WHO, aspartate aminotransferase (AST) or alanine aminotransferase (ALT) concentration ≥1000 units/l can be considered as a criterion for severe-dengue [1, 19, 20]. Studies have suggested that serum aminotransferases, including ALT and AST are crucial and promising in the diagnosis and assessment of viral liver diseases [21]. Identification of the kinetics in liver function tests associated with severe dengue infection is very important.

Being a dengue-endemic country, most Indian states are classified as having a constant risk of dengue transmission and more precisely, the situation has worsened in eastern India in the past few years [22]. Hence, the availability of dependable markers that predict severe dengue during the early-stages of infection might be helpful in triaging patients for better clinical management.

This study deals with the evaluation of serum levels of acute-phase serum proteins (SAP, SAA2, CRP, and Apo A1), endothelial-markers (Ang2 and VEGF), coagulation-factor (fibrinogen), and hepatic-markers (AST and ALT) among WHO classified dengue patients, which can be used as disease severity marker(s) in dengue-endemic areas during the early stage of infection.

Materials and methods

All experiments performed in this study, including the collection of patient's blood as well as healthy individuals were done accordingly to ethical standards of Clinical Research Ethical Committee, Calcutta School of Tropical Medicine, Kolkata (CREC-STM/53 dated 26.09.2013), following the 1964 Helsinki Declaration and its later amendments. Written consents were received from patients and healthy individuals prior to participating in this study.

Collection of patients' blood

Blood samples (5 ml) were collected from 168 symptomatic patients (within 0–10 days of symptomatic onset) from July 2016 to June 2017, visiting Calcutta School of Tropical Medicine, Institute of Post Graduate Medical Education and Research, West Bengal, India. To carry out an age-matched case-control study, blood was collected from 25 healthy unrelated individuals within the same community and of the same ethnicities, who neither had any signs and history of other infections nor were detected with dengue infection by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR. Sera were separated by density gradient centrifugation and aliquots were stored at –20°C and –80°C freezer.

Case definition

Clinical symptoms were monitored by clinicians and patients were categorized into three groups: dengue patients without warning signs (DwoWS), with warning signs (DwWS), and severe dengue (SD), according to WHO 2009 classification.

Extraction of viral RNA and determination of DENV load

Viral-RNA was extracted from 140 µl of patients' sera, using QIAamp Viral RNA Mini Kit according to the manufacturer's protocol (Qiagen, Courtaboeuf, France). The presence of the DENV genome and viral load among infected patients' sera was determined by qRT-PCR (Taqman assay) in 20 µl, using 8 µl viral-RNA, OneStepqRT-PCR MasterMix, and other components of DENV Genesig kit according to manufacturer's protocol (Primer Design Ltd., UK). Real-time PCR was performed on ABI Prism 7500 fast instrument. A standard curve

of cycle threshold (Ct) values was constructed using six 10-fold dilution series of a positive control template (provided with the kit). DENV RNA copy number in patients' sera was calculated from corresponding Ct values. Viral-RNA titre ≥10 000 copies/ml and <10 000 copies/ml was considered as high-viral-load (HVL) and low-viral-load (LVL), respectively.

Detection of anti-dengue-IgM and IgG antibodies and dengue-NS1 Antigen

Suspected patients' sera were subjected to serological diagnosis for the presence of anti-dengue-IgM, using dengue IgM MAC ELISA kit (National Institute of Virology, India), according to manufacturer's instructions. Patients' sera were also analysed for the presence of dengue-NS1 antigen and anti-dengue-IgG antibody using dengue-NS1 ELISA kit and dengue IgG capture ELISA kit (Panbio, Australia).

Determination of DENV serotypes

Dengue serotypes of PCR-positive samples were determined by real-time RT-PCR in a 20 µl reaction mixture, containing 8 µl viral RNA, One Step qRT-PCR Master-Mix and other components of DENV serotyping Genesig kit, according to manufacturer's protocol (Primer Design Ltd., UK). Reactions were carried out in ABI 7500 fast instrument using serotype-specific DENV controls present within the kit.

Quantification of serum protein, endothelial factors, and coagulation factor

Serum levels of SAA, SAP, Ang2, VEGF, and fibrinogen among different groups of dengue patients were determined by sandwich ELISA kits (USCN LifeScience, USA), according to the manufacturers' instructions. Absorbance was measured at 450 nm wavelength against chromogen blank. Standard-curve was drawn using GraphPad-Prism software and levels of each protein were analysed.

Quantification of CRP

Quantification of CRP was done by the immunoturbidimetry method using AUTOSPAN turbi-gold kit (ARKRAY, India), according to the manufacturer's instructions [23].

Quantification of biochemical and hematological parameters

Biochemical analysis was done by using a standard auto-analyser (ERBA EM360) which includes AST, ALT, ALP, bilirubin (Blr), albumin, HDL, LDL, total cholesterol (TC), and triglyceride. In addition, white blood cell counts, haemoglobin (Hb), and platelet count (Pt) were measured using an automatic cell counter (SYSMEX No -KX100). All tests were performed according to the manufacturer's instructions.

Determination of serum levels of Apo A1

Quantification of Apo A1 was carried out using sodium-dodecyl–sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) followed by western-blotting. Each sample was diluted and 50 µg/well sample-solution was loaded to 14% SDS–PAGE (90V/24 mA, 4 h). A transfer tank was used for transferring the proteins to polyvinylidene-fluoride (PVDF) membrane (30V/90 mA, 18 h). To block non-specific sites, the PVDF membrane was blocked with 5% BSA and then incubated with diluted primary-antibody (apoA-I:sc-376818, Santa Cruz Biotechnology, USA) (1:2000) overnight. After successive washing, PVDF membrane was incubated with HRP conjugated secondary-antibody (m-IgGκ BP-HRP:sc-516102, Santa Cruz Biotechnology, USA) (1:20 000) for an hour. After proper washing, immune-reactive bands were visualized after the addition of Electro-Chemi-Luminescence reagent (Thermo Fisher Scientific, USA), which was auto-radiographed. Quantification of Apo A1 in auto-radiographs was carried out through densitometric scanning by ImageJ software (National Institutes of Health, USA) (Supplementary Fig. S1).

Protein network analysis

Human gene symbols for studied serum-proteins uploaded to web-based STRING[10.5] tool to construct the protein–protein-interaction network and only experimentally validated interactions with a combined score >0.4 were selected as significant.

Statistical analysis

Statistical analyses were done using GraphPad Prism[9] software. A statistical correlation of serum-markers with the presence of WHO-defined dengue severity symptoms during different phases of illness was analysed using Pearson's correlation matrix and multivariant analysis was performed by using ANOVA (with Bonferroni correction). Mean values with ± SEM of serum-markers in each cohort were compared using *t*-test and $P < 0.05$ were considered to be statistically significant. Cut-off values, sensitivity, and specificity of serum-markers associated with severity were analysed by the ROC curve. Principal-component-analysis (PCA) followed by the hierarchical correlation-cluster analysis was used to reduce the number of predictive markers. We selected PCs with eigenvalues higher than or equal to 1.0. Both patients along with healthy controls and serum-markers were clustered to obtain a heatmap.

Results

In this study, the importance of serum-level of APPs, endothelial, coagulation, and hepatic factors during dengue disease pathogenesis of eastern-Indian patients was evaluated. Out of 168 symptomatic patients, 87 (51.78%) were dengue infected as confirmed by qRT-PCR, anti-dengue IgM, and dengue NS1 ELISA among which 10.34% (9/87) were a severe-dengue, 48.27% (42/87) exhibited warning-signs, whereas 41.37% (36/87) were without any warning-signs and 13.79% (12/87) were secondary-dengue infection. Detailed clinicopathological history of patients is depicted in Table 1. Percent prevalence of secondary-dengue infection was significantly higher among the SD group, whereas primary dengue cases were considerably more evident among the DwoWS group ($P = 0.02$). Co-circulation of both single and co-infection by multiple dengue serotypes were observed in this study, among which D2 (61.53%) was most prevalent among mono-infected cases, whereas D2–D4 (63.63%) combination was most predominant among co-infected patients. D2 was significantly associated with SD and DwWS development among mono-infected patients ($P = 0.01$). Since only 12 patients were detected with multiple dengue serotypes, no statistical association was found with disease severity. Among PCR positive dengue patients ($n = 50$), ~72% (36/50) had high viral-load (HVL), whereas the remaining 28% (14/50)

Table 1. Demographic setting and clinicopathological parameters of the study population

			Healthy controls (n = 25)	Dengue (n = 87)	*P*-value
Age			29.2 ± 6.90	25.08 ± 12.70	0.318
Sex	Male		60% (15)	52.87% (46)	0.74
	Female		40% (10)	47.12% (41)	
Diagnostic parameters		Dengue NS1antigen		50.57% (44)	
		qRT-PCR		57.47% (50)	
		Anti-dengue IgM antibody		60.91% (53)	
		Anti-dengue IgG antibody		13.79% (12)	

		Healthy controls (n = 25)	Patients with severe dengue (SD) (n = 9)	Patients with warning signs (DwWS) (n = 42)	Patients without warning signs (DwoWS) (n = 36)	Patients with warning signs (DwWS) (n = 42)	wPrimary dengue infection (n = 75)	Secondary dengue infection (n = 12)	*P*-value[‡]
Infection type	Primary dengue (n = 75)	NA	8% (6)	45.33% (34)	**46.66% (35)**	**0.02**[a]	NA		NA
	Secondary dengue (n = 12)	NA	**25% (3)**	66.66% (8)	8.33% (1)		NA		
Sample classification	Febrile phase (1–3 days) (n = 33)	NA	4	19	10	NA	24	9	NA
	Critical phase (4–6 days) (n = 21)	NA	5	12	4		20	1	
	Recovery phase (>7 days) (n = 33)	NA	0	11	22		31	2	
Circulating serotypes (n = 50)	Single serotype (n = 38)	D1 (n = 5)	0	20% (1)	**80% (4)**	**0.01**[a]	100% (5)	0	ns
		D2 (n = 24)	25% (6)	**66.66% (16)**	8.33% (2)		70.83% (17)	29.16% (7)	
		D3 (n = 4)	0% (0)	**75% (3)**	25% (1)		100% (4)	0	
		D4 (n = 5)	20% (1)	**80% (4)**	0% (0)		100% (5)	0	
	Multiple serotype (n = 12)	D1–D3 (n = 1)	0% (0)	0% (0)	100% (1)	0.054	100% (1)	0	ns
		D2–D3 (n = 3)	0% (0)	0% (0)	100% (3)		100% (3)	0	
		D2–D4 (n = 7)	28.57% (2)	71.42% (5)	0% (0)		71.42% (5)	28.57% (2)	
		D1–D2 (n = 1)	0% (0)	100% (1)	0% (0)		100% (1)	0	
Viral titter (n = 50)	High viral load (HVL: ≥10 000 copies/ml) (n = 36)	NA	**88.88% (8)**	**76.66% (23)**	45.45% (5)	0.07	75% (27)	**25% (9)**	0.24
	Low viral load (LVL: <10 000 copies/ml) (n = 14)	NA	11.11% (1)	30.43% (7)	54.54% (6)		92.8% (13)	7.14% (1)	
	Mean viral load (copies/ml)	NA	**260 678 ± 62273**	**58448 ± 19009**	10426 ± 1149	**<0.0001**[b]	36 785 ± 11804	**240 595 ± 56058**	**0.005**[b]

Table 1. Continued

		Healthy controls ($n = 25$)	Patients with severe dengue (SD) ($n = 9$)	Patients with warning signs (DwWS) ($n = 42$)	Patients without warning signs (DwoWS) ($n = 36$)	Patients with warning signs (DwWS) ($n = 42$)	wPrimary dengue infection ($n = 75$)	Secondary dengue infection ($n = 12$)	*P*-value‡
Clinical symptoms	Fever	NA	100% (9)	100% (42)	100% (36)	ns	100% (75)	100% (12)	ns
	Nausea	NA	33.3% (3)	40.47% (17)	22.22% (8)	ns	29.33% (22)	50% (6)	ns
	Rash	NA	**77.77% (7)**	38.09% (16)	25% (9)	**0.005**[a]	37.33% (28)	33.33%(4)	ns
	Myalgia	NA	66.6% (6)	78.57% (33)	91.66% (33)	ns	80%(60)	83.33% (10)	ns
	Arthralgia	NA	44.44% (4)	47.61% (20)	33.33% (12)	ns	41.33% (31)	41.66% (5)	ns
	Joint Swelling	NA	22.22%(2)	11.90% (5)	8.33% (3)	ns	12% (9)	25% (3)	ns
	Headache	NA	44.44% (4)	61.90% (26)	40.62% (13)	ns	49.33 (37)	50% (6)	ns
	Retro-orbital pain	NA	**55.55% (5)**	16.66% (7)	2.77% (1)	**0.024**[a]	12% (9)	**33.33% (4)**	**0.02**[a]
	Abdominal pain	NA	**77.77% (7)**	**66.66% (28)**	0%	**<0.0001**[a]	33.33% (25)	**83.33% (10)**	**0.002**[a]
	Vomiting	NA	**66.66% (6)**	**64.28% (27)**	0%	**<0.0001**[a]	32% (24)	**75% (9)**	**0.008**[a]
	Bleeding	NA	**100% (9)**	0%	0%	**<0.0001**[a]	6.66% (5)	**33.33% (4)**	**0.01**[a]
	Hepatic dysfunctions	NA	**88.8% (8)**	23.80% (10)	2.77% (1)	**<0.0001**[a]	14.66% (11)	**58.33% (7)**	**0.002**[a]
	Fluid accumulation	NA	**44.44% (4)**	11.11% (4)	0%	**<0.0001**[a]	5.33% (4)	**33.33% (4)**	**0.01**[a]
Haematological	Haemoglobin (gm/dl)	14.42 ± 0.52	13.95 ± 0.46	14.09 ± 0.36	14.183 ± 0.26	ns	14.32 ± 0.48	14.08 ± 0.31	ns
	Platelets (Pt) count ($\times 10^3 mm^3$)	166.02 ± 6.06	**112.02 ± 12.86**	160.73 ± 5.09	167.7 ± 2.68	**<0.0001**[b]	153.93 ± 8.71	138.01 ± 9.35	0.24
parameters	WBC count ($\times 10^3 mm^3$)	6.03 ± 0.143	**4.25 ± 0.198**	5.94 ± 0.098	6.06 ± 0.379	**0.02**[b]	5.97 ± 0.329	**4.41 ± 0.12**	**0.001**[b]
Biochemical parameters	ALP (IU/l)	50.52 ± 1.85	**111.7 ± 8.21**	**100.3 ± 4.45**	53.33 ± 1.05	**<0.0001**[b]	75.39 ± 3.28	**117.41 ± 8.74**	**0.0001**[b]
						<0.0001[b]			
	Bilirubin (BR) (mg/dl)	0.478 ± 0.066	**0.83 ± 0.099**	0.44 ± 0.034	0.412 ± 0.02	**0.0001**[b]	0.48 ± 0.069	0.81 ± 0.12	**ns**
	Albumin (Alb) (gm/dl)	4.45 ± 0.17	**2.89 ± 0.18**	4.24 ± 0.089	4.49 ± 0.057	**<0.0001**[b]	4.39 ± 0.078	**3.86 ± 0.21**	**0.01**[b]
Lipid profile	HDL (mg/dl)	68.412 ± 2.51	**86.85 ± 3.351**	70.19 ± 1.305	68.74 ± 1.322	**<0.0001**[b]	71.16 ± 1.89	**87.96 ± 4.12**	**0.001**[b]
	LDL (mg/dl)	114.74 ± 3.08	**88.98 ± 4.610**	110.6 ± 2.555	113.2 ± 2.91	**<0.0001**[b]	**114.35** ± 1.92	**86.29** ± 4.39	**0.0001**[b]
	Triglyceride (TG) (mg/dl)	143.91 ± 2.58	**180.8 ± 5.23**	**168.8 ± 3.16**	153.8 ± 2.09	**<0.0001**[b]	167.24 ± 1.88	176.65 ± 5.97	**ns**
						0.003			
	Total cholesterol (TC) (mg/dl)	184.84 ± 2.21	182.83 ± 2.14	189.6 ± 1.407	191.5 ± 1.766	Ns	188.32 ± 1.09	184.29 ± 2.41	ns

*$P < 0.05$ at 95% CI was considered statistically significant.
[a]Contingency chi-square test (fisher's exact).
[b]Paired *t*-test.
NA: not applicable.
ns: non-significant.
†*P*-value was calculated with respect to patients without warning signs.
‡ *P*-value was calculated in between primary and secondary dengue cases.

harboured low viral-load (LVL). The majority of SD patients (8/9) had HVL with an average viral-titter of 260 678 ± 62 273 copies/ml, which is significantly higher ($P < 0.0001$) than that of both DwWS (58 448 ± 19 009 copies/ml) and DwoWS (10 426 ± 1725 copies/ml) patients. Whereas, 70.58% (24/34) of patients with DwWS and 57.14% (4/7) DwoWS were in the HVL group (Table 1). Several clinical, haematological, and biochemical parameters had shown significant alteration among the SD patients. Clinical symptoms viz. rash, retro-orbital-pain, bleeding, hepatic dysfunction, and fluid accumulation were significantly higher among the SD patients. Leukopenia ($P = 0.02$) and thrombocytopenia ($P < 0.0001$) were significantly more prevalent among the SD patients than of DwWS and DwoWS patients. Similarly, ALP level was elevated significantly among the SD patients ($P < 0.0001$) than that of DwoWS patients. Significantly increased bilirubin ($P = 0.0001$) and decreased albumin ($P < 0.0001$) levels were exclusively observed among the SD patients. Whereas, HDL ($P < 0.0001$) and triglyceride ($P < 0.0001$) levels were markedly increased and LDL ($P < 0.0001$) level was significantly decreased, respectively among the SD patients compared to DwoWS patients.

Statistically significant difference in occurrence of clinical symptoms viz. retro-orbital pain ($P = 0.02$), abdominal pain ($P = 0.002$), vomiting ($P = 0.008$), bleeding ($P = 0.01$), hepatic-dysfunctions ($P = 0.002$) and fluid accumulation ($P = 0.01$) were observed between primary and secondary dengue cases by chi-square test (Table 1). Similarly, *t*-test (paired) identified significant difference in mean viral-load ($P = 0.005$), WBC count ($P = 0.001$), ALP ($P = 0.0001$), albumin ($P = 0.01$), HDL ($P = 0.001$) and LDL ($P = 0.0001$) levels between primary and secondary dengue cases.

Concentrations of both vascular-endothelial-markers viz. Ang2 and VEGF were significantly higher among the SD (Ang2: 1728 ± 140.9 pg/ml, *P*-value < 0.0001; VEGF: 644.1 ± 22.81 pg/ml, $P < 0.0001$) and DwWS patients (Ang2: 849.9 ± 36.12 pg/ml, $P = 0.003$; VEGF: 304 ± 17.58 pg/ml, $P = 0.0005$) than that of DwoWS (Fig. 1a). But, significantly lower concentration of coagulation factor, fibrinogen was detected only among the SD patients (43612 ± 7093 ng/ml, $P < 0.0001$) compared to DwoWS (63 381 ± 1758 ng/ml) patients (62 256 ± 1486 ng/ml). Among APPs, levels of CRP ($P < 0.0001$), SAA2 ($P < 0.0001$) and Apo-A1 ($P < 0.0001$) increased extensively among the SD patients (CRP: 66.14 ± 5.71 mg/ml, SAA2: 10 063 ± 824.6 pg/ml, ApoA1: 43.9 ± 4.8) and considerably among DwWS patients (CRP: 32.1 ± 4.7 mg/ml, SAA2: 8949 ± 618.4 pg/ml, ApoA1: 22.8 ± 1.8) compared to DwoWS patients (CRP: 10.71 ± 2.02 mg/ml, SAA2: 5211 ± 277.5 pg/ml, ApoA1: 16.76 ± 0.89). However, SAP level did not show any significant alternation. Both hepatic-markers viz. AST and ALT elevated markedly among the SD (AST: 390.6 ± 19.25, $P < 0.0001$; ALT: 236.5 ± 8.39 IU/l, $P < 0.0001$) and the DwWS patients (AST: 113.3 ± 13.61 IU/l, $P < 0.0001$, ALT: 84.06 ± 8.45 IU/l, $P < 0.0001$) compared to DwoWS patients (AST: 46.79 ± 2.24 IU/l, ALT: 41.9 ± 1.45IU/l). AST/ALT ratio indicating hepatic-dysfunction had been significantly elevated among the SD (1.64 ± 0.08, $P < 0.0001$) and the DwWS (1.3 ± 0.03, $P = 0.001$) patients compared to DwoWS patients (1.1 ± 0.028). A cut-off value of dengue disease severity was analysed by ROC curve to differentiate SD fever from less severe forms of this disease for markers significantly altered among the SD patients. Severity cut-off values were Ang2: >1129 pg/ml, fibrinogen: <53 092 ng/ml, VEGF: >526 pg/ml, CRP: >46.76 mg/ml, SAA2: >720.3p g/ml, ApoA1: >23.18%, AST: >227.3 IU/l, ALT: >205.2 IU/l, and AST/ALT ratio: >1.45 to predict SD (Fig. 1b).

During febrile-phase of infection, endothelial-markers viz. Ang2 ($P < 0.0001$), VEGF ($P < 0.0001$), coagulation-marker fibrinogen ($P = 0.01$), acute-phase glycoprotein ApoA1 ($P < 0.0001$) and all hepatic-markers viz. AST ($P < 0.0001$), ALT ($P < 0.0001$) and AST/ALT ratio ($P < 0.0001$) had shown significant alteration among the SD patients compared to DwWS and DwoWS (Fig. 2). But, CRP ($P = 0.0008$) and SAA2 ($P = 0.0017$) levels elevated significantly during critical-phase of illness among the SD group.

Significantly higher levels of Ang2 ($P = 0.005$), VEGF ($P = 0.005$), CRP ($P = 0.003$), SAA2 ($P = 0.04$), ApoA1 ($P = 0.01$), and AST ($P = 0.03$) were observed among HVL-group of patients (Fig. 3a). Levels of studied markers were also analysed among primary and secondary-infections (Fig. 3b). Significantly increased serum-concentration of Ang2 ($P = 0.04$), VEGF ($P = 0.005$), CRP ($P = 0.02$), ApoA1 ($P = 0.01$), AST ($P = 0.001$), and ALT ($P = 0.009$) were observed among secondary-dengue patients compared to primary-infections (Fig. 3b).

Heatmap analysis of studied markers with various clinicopathological manifestations contributing to dengue disease-severity revealed that Ang2, VEGF increased more than 2-fold and fibrinogen decreased 1.8-fold among patients with haemorrhagic-manifestation, clinical-fluid accumulation and low platelets-count (<100 000 cells/ml) (Fig 4a). Whereas, CRP level was remarkably elevated among patients with haemorrhage (3.13-fold), acute viremia (3.72-fold) (>50 000copies/ml), hepatic-dysfunction (2.9-fold) and clinical-fluid accumulation (2.76-fold). AST and ALT concentrations were significantly increased among patients with haemorrhage (AST: 3.9-fold, ALT: 3.7-fold), hepatic-dysfunction (AST: 4.08-fold, ALT: 2.86-fold), thrombocytopenia (AST: 3.4-fold, ALT: 3.08-fold), and hypo-albuminemia (AST: 3.75-fold, ALT: 2.90-fold).

Figure 4b depicted protein-networking generated by studied proteins using STRING database. Functional network analysis revealed Ang2, VEGF, and CRP to be functionally and physically connected and SAA2 and ApoA1 to be functioning together (PPI enrichment $P = 1.1e{-}11$). Signalling pathway analysis revealed complement and coagulation cascades, HIF-1 signalling and platelet-activation to be primarily affected during severe dengue pathogenesis – thereby affecting plasminogen-activation, blood-vessel endothelial cell migration, and fibrinolysis. Correlation analyses of all studied markers also validated this connectivity by a strong positive correlation among Ang2, VEGF, and CRP (Fig. 4c). Fibrinogen was negatively correlated with Ang2 and VEGF. Both hepatic-markers, AST, and ALT had shown a positive correlation with CRP, SAA2, and ApoA1.

PCA identified PC1 (63.14%) and PC2 (10.53%) to have the highest proportion of variance. Hence, considering components 1 and 2, PCA could distinguish between SD and DwWS/DwoWS groups, with the DwoWS group overlapping with healthy individuals (Fig. 4d). Hierarchical clustering and heatmap analysis of all serum-markers among the SD, DwWS, DwoWS and healthy groups, screened based on of fold change, almost segregated SD group from the rest, with VEGF, Ang2, ApoA1, AST, and ALT clearly distinguishing SD group [Fig. 4e]. However, further studies are required to validate correlated markers and to explore underlying functional mechanisms.

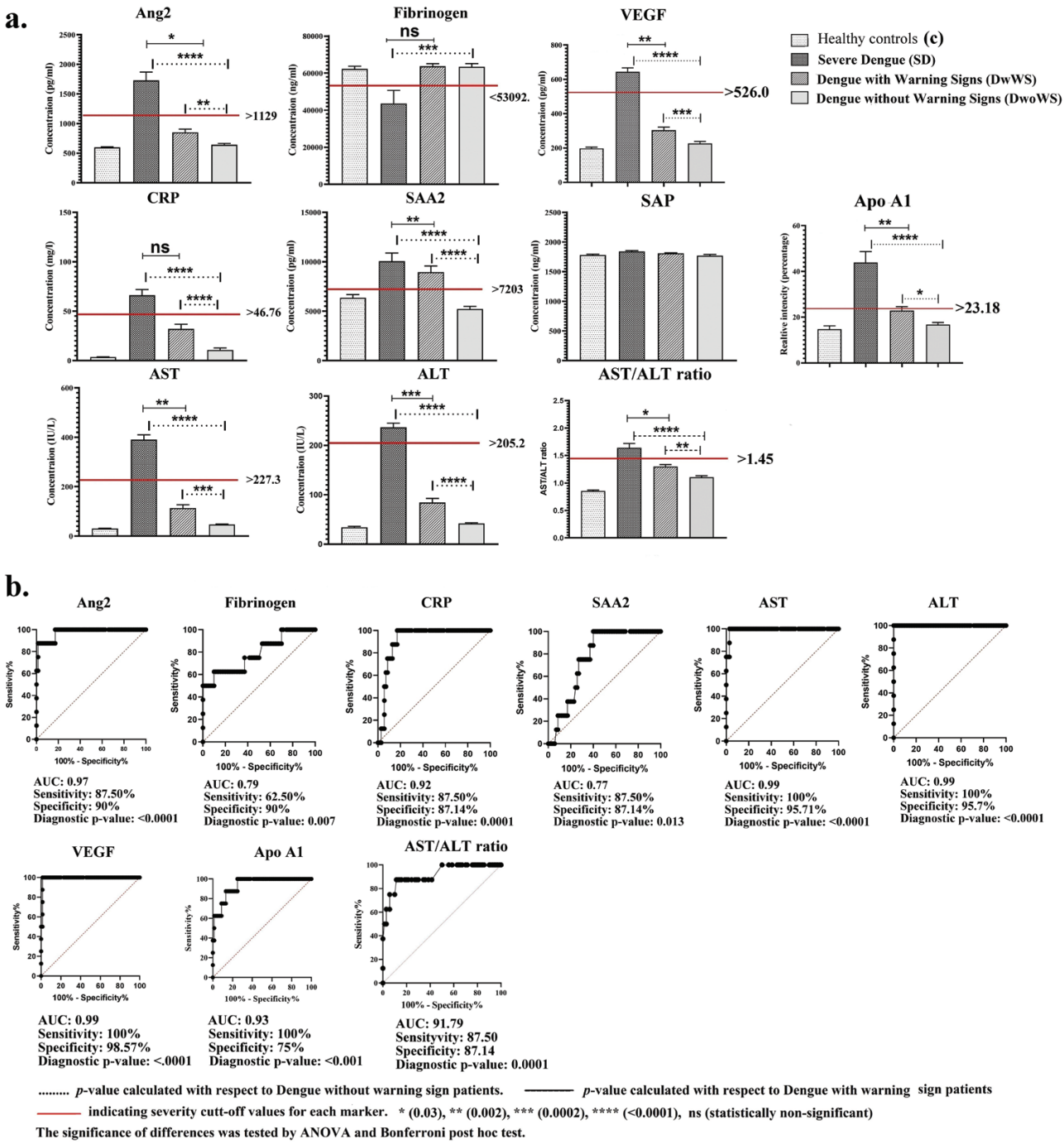


Figure 1: (a) Differential concentration of serum-markers among WHO-classified dengue samples and healthy controls. (b) ROC curve analysis of significantly altered markers.

Discussion

Among various forms of dengue disease, severe dengue is considered life-threatening with a case fatality rate of 2.6% of infected Indian patients and 10–20% patient mortality rate among DHF and 40% among DSS patients [1, 22]. Hence, early clinical prediction of severe dengue will be helpful in better patient management. To date, there are no reliable and specific biomarkers for early diagnosis and prognosis of dengue disease severity. This is the first detailed comprehensive molecular analysis of serum-markers from eastern-Indian dengue patients with molecules related to the acute phase of infection (CRP, SAA2, SAP, and ApoA1), vascular-endothelium (Ang2 and VEGF), coagulation (fibrinogen), and hepatic injury (AST, ALT, and AST:ALT ratio) for early identification of severe cases.

In this study, Ang2 and VEGF concentrations significantly increased among the SD patients compared to both the DwWS and DwoWS. Whereas, fibrinogen levels decreased among the SD patients compared to the DwoWS. These were more clearly evident during febrile-phase (0–3 days) of infection, implicating their importance to differentiate severe cases at an early stage of the disease. Higher Ang2 and VEGF levels were previously reported among Sri Lankan and Indian

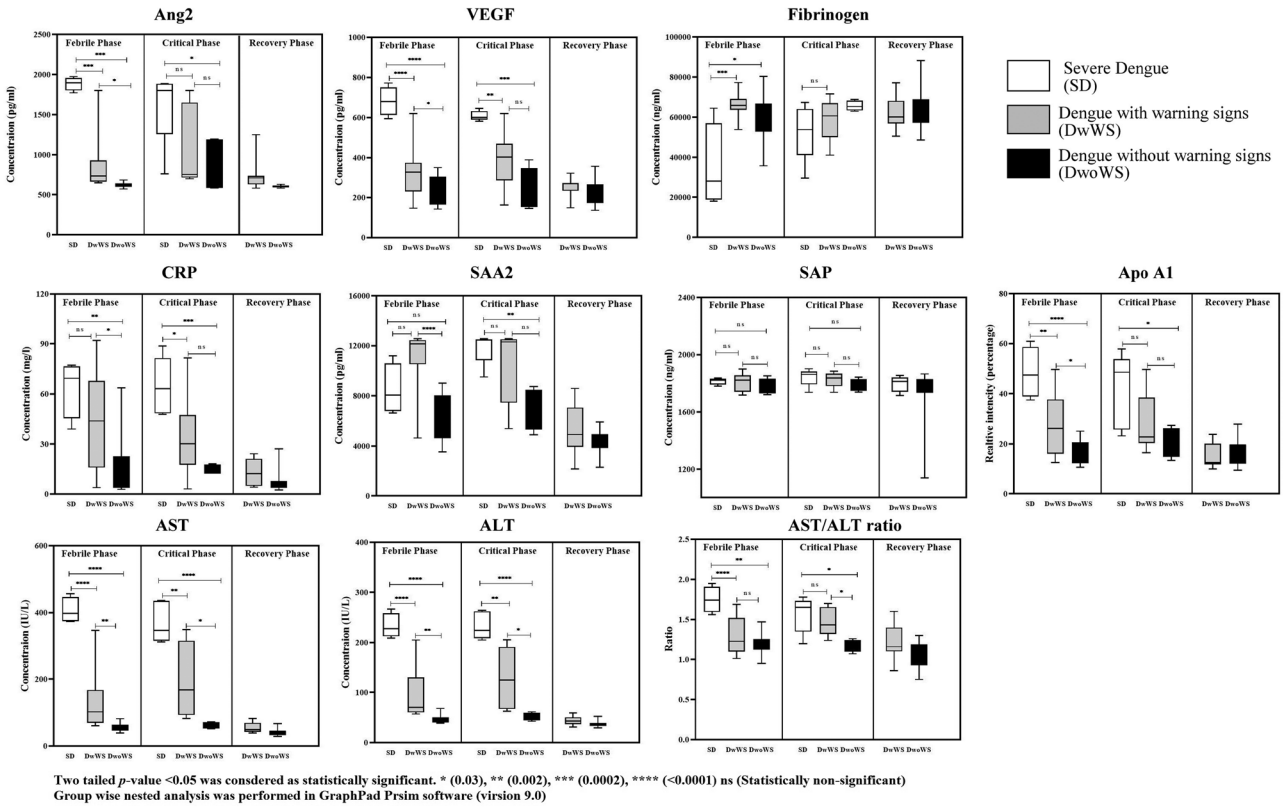


Figure 2: Comprehensive analysis of serum-markers at different stages of dengue infection.

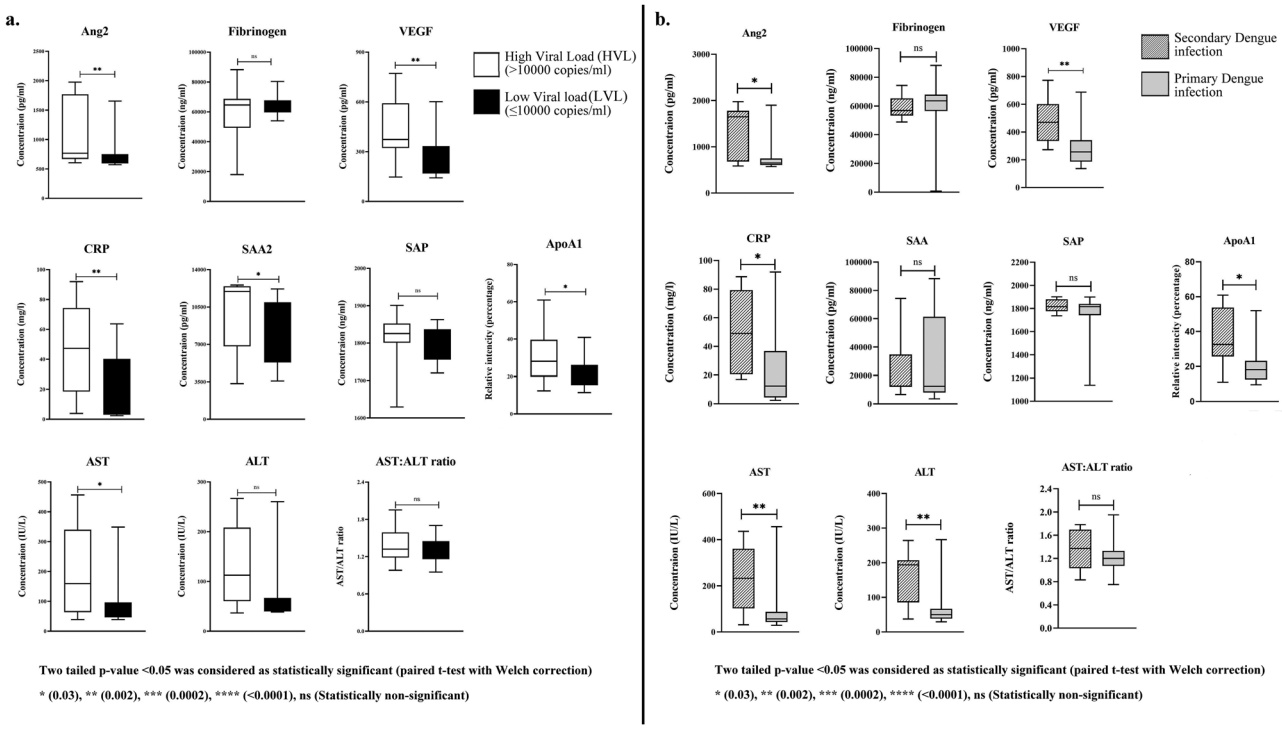
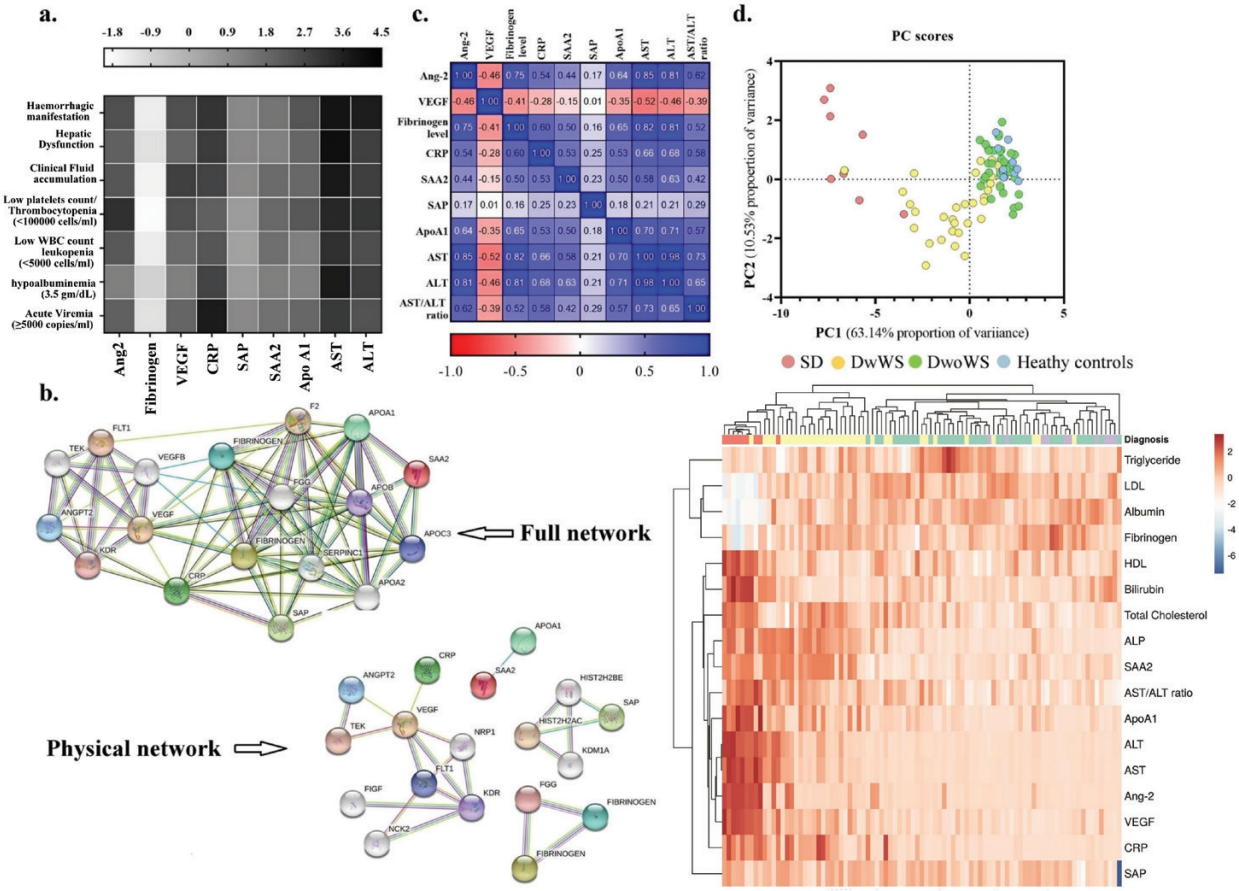


Figure 3: Serum-markers level among dengue patients (a) with high and low viral-load (b) with secondary and primary infection.

severe-dengue patients, respectively during the 9th day and significantly lower fibrinogen among Vietnamese severe-dengue patients during the 2nd day of symptomatic onset [15, 24, 25].

As hepatic cells were mainly affected in severe dengue, APPs, produced from hepatic cells provided the first-line of defence against such viral infection [5]. Significant elevation of CRP, SAA2, and ApoA1 levels were previously reported



[a] The color scale beside the heatmap represent fold change, from darker (higher fold change) to light (lower fold change)

[b] Colored lines between the proteins indicate the various types of interaction evidence. Color nodes: query proteins and first shell of interactors and white nodes: second shell of interactors.

[c] Bluer the color, higher the correlation coefficient (r-value) (strong positive correlation), while redder the color, the lower the correlation coefficient (strong negative correlation).

[d] Scatter plot of principal components show separation of data based on severe-dengue sample (indicating in red dots).

[e] In hierarchical clustering heatmap each row represents dengue samples and each column represents the serum concentration of studied markers.

Figure 4: (a) Heatmap analysis of differential fold change of serum-markers with clinicopathological parameters (b) protein–protein network analysis of studied serum proteins (c) Pearson's correlation analysis among studied serum-markers (d) variance among different group of dengue patients according to PCA analysis (e) hierarchical clustering heatmap of studied markers among different group of dengue patients.

among severe-dengue patients of Singaporean and Taiwanese origin [26, 27]. Hepatic involvement in dengue infections ranges from mild subclinical biochemical changes to severe liver disease. AST:ALT ratio was considered as a superior marker for various types of liver injuries than an alteration in AST or ALT alone. This is the first study to find a significantly higher AST:ALT ratio of ≥1.7 among patients with SD during the first 3 days of symptomatic onset. Similar to this study, significant elevation of liver enzymes was reported among severe-dengue patients from Sri Lanka [19].

Higher levels of APPs (CRP, SAA2, and ApoA1), endothelial markers (Ang2 and VEGF) and hepatic-markers, AST was reported among HVL patients compared to LVL. HVL has been previously associated with SD infection; the presence of a higher number of viral particles might have significantly triggered APR thereby activating the complement system to elicit cellular antiviral response [1, 5]. Such immune activation might have contributed to endothelial dysfunction and vascular leakage, which could induce hypoxic injury with hepatic dysfunction [19, 20]. CRP, ApoA1, AST, ALT, Ang2, and VEGF were significantly elevated among secondary dengue compared to primary infections. Previous studies have also indicated immune-complexes are implicated in mounting complement activation, vascular permeability, and coagulopathy to be significantly associated with secondary infection [28].

Decreasing fibrinogen concentration of coagulation cascade among severe-dengue patients might have activated complement system and platelet-activation, thereby liberating vasoactive peptides (anaphylatoxins) and increasing permeability of blood capillaries [29, 30]. On the other hand, an increase in Ang2 and VEGF levels might have altered HIF-1 signalling, which could have increased dengue replication in this group of patients (VL: 98 769–488 956 copies/ml) [31]. This relation was further evident in the severe-dengue patient overlapping with DwWS group in PCA [Fig. 4d], demonstrating the lowest viral load (VL: 8867 copies/ml) and Ang2, VEGF levels among the SD group. Similarly,

the DwWS patient overlapping with the SD group in PCA exhibited relatively higher VL and highest Ang2 and VEGF levels of this group. Thus, this study had important clinical significance for a better understanding of severe-dengue development among infected patients. Until now, clinically applicable biomarker(s) for severe dengue are lacking. Therefore, exploring the serum-proteomic analysis of dengue patients during the first 3 days of symptomatic onset might be a powerful platform for the discovery of novel, specific, and more reliable biomarkers for severe dengue diagnosis and prognosis.

Conclusion

The serum levels of VEGF, Ang2, ApoA1, AST, and ALT may be useful as potential and reliable biomarkers for predicting disease severity at the early stage.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Tripathi A: Research idea formation, consumable procurement, manuscript preparation, correction, and overall supervision; Mukherjee S: Performed the experiments, data analysis, and manuscript writing; Saha B: Clinical symptoms monitoring, categorization of dengue patients as per WHO-classification (2009) and clinical management. All authors read and approved the final manuscript.

Ethics approval

All experiments performed in this study, including the collection of patient's blood as well as healthy individuals were done accordingly to the ethical standards of Clinical Research Ethical Committee, Calcutta School of Tropical Medicine, Kolkata (CREC-STM/53 dated 26.09.2013), following the 1964 Helsinki Declaration and its later amendments. Written consents were received from patients and healthy individuals prior to participating in this study.

Data availability

All data generated and analysed during this study are included in this article.

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