GENETIC POLYMORPHISMS OF GENES INVOLVED IN HOST IMMUNE RESPONSE TO DENGUE SEVERITY IN NEPALESE POPULATION

M.Sc. Thesis

(2021)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement of the M Sc. degree in Biotechnology

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Date: Oct 21, 2021

RECOMMENDATION

This is to certify that the research work entiled "**GENETIC POLYMORPHISMS OF GENES INVOLVED IN HOST IMMUNE RESPONSE TO DENGUE SEVERITY IN NEPALESE POPULATION"** has been carried out by **Ms. Chetana Khanal** under our supervision.

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

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Certificate of Evaluation

This is to certify that this entitled "**GENETIC POLYMORPHISMS OF GENES INVOLVED IN HOST IMMUNE RESPONSE TO DENGUE SEVERITY IN NEPALESE POPULATION"** presented to evaluation committed by **Ms. Chetana Khanal** is found satisfactory for the partial fulfillment of Master of Science and Technology.

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Glossary Acronyms

ALT:	Alanine transaminase
APC:	Antigen Presenting Cell
ARMS:	Amplification-Refractory Mutation System
AST:	Aspartate transaminase
CCL4:	CC-chemokine ligand 4
CD:	Cluster of Differentiation
CDBT:	Central Department of Biotechnology
CDC:	Central for disease control
CMC:	Chitwan Medical College and Teaching Hospital, Chitwan
CRF:	Case report form
DALYs:	Disability Adjusted Life Years
DENRA:	Dengue Recombinant Antigen
DENV:	Dengue virus
DF:	Dengue fever
DHF:	Dengue haemorrhagic fever
DSS:	Dengue shock syndrome
ELISA:	Enzyme-linked immunosorbent assay
FDA:	Food and Drug Administration
FITC:	Fluorescein isothiocyanate
HCV:	Hepatitis C Virus
HLA-DR:	Human leucocyte antigen- DR isotype
HSC:	Human Specimen Control
IFNγ:	Interferon-γ
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IL-2:	Interleukin-2
IL-10:	Interleukin-10
ISR:	Immune Status Ratio
JEV:	Japanese encephalitis virus
MCMC:	Markov Chain Monte Carlo
MHC:	Major Histocompatibility Complex
NCA:	Normal Cell Antigen
NDEN:	Non-dengue
NHRC:	Nepal Health Research Council

NK:	Natural killer
NKT:	Natural Killer T cells
NS1:	Non-structural proteins
OD:	Optical Density
ORF:	Open reading frame
PBMCs:	Peripheral blood mononuclear cells
PCR:	Polymerase Chain Reaction
PE:	Phycoerythrin
PerCP:	Peridinin Chlorophyll Protein
RNA:	Ribose nucleic acid
RT-PCR:	Reverse transcription-polymerase chain reaction
STIDH:	Sukraraj Tropical and Infectious Disease Hospital
SNP:	Single Nucleotide Polymorphism
TBEV:	Tick-borne encephalitis virus
TMB:	Trimethyl Benzidine
TNF:	Tumour necrosis factor
UCMS:	Universal College of Medical Science
WHO:	World Health Organization
WNV:	West Nile virus
YFV:	Yellow Fever Virus
VCAM-1:	Vascular Cell Adhesion Molecule-1
VDR:	Vitamin D Receptor
VEGFR	Vascular Endothelial Growth Factor

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Abstract

In dengue, endemic country like Nepal all four serotypes (DENV1-4) of dengue virus (DENV) is found to be prevalent causing dengue fever and its severe form dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The severity of the disease is determined by virus serotype, host immune and genetic status which if known, can help in early disease management. In South Asian countries DHF and DSS are noted as leading cause of child death. Study on the severity determining marker is being carried out in endemic areas in human sample, as there is no appropriate animal model mimicking the DHF/DSS as in human and it is one of the limitations. Genetic makeup of the individual differs from place to place around the world which might be the reason for one population being more susceptible and the other being resistant to the virus. If polymorphism behind the positive or negative association between the gene and disease pathogenesis is well defined, then it could help in development of new preventive and therapeutic agents against disease. The study is focused on the severity marker prediction which might help in early diagnosis of the severe dengue cases. Genetic Marker was detected by Single nucleotide polymorphism detection by amplification refractory mutation system PCR (ARMS) of DNA extracted from selected samples.

Statistical analysis of the clinical parameters of all the case samples was performed to classify the samples in dengue hemorrhagic(DHF) 53%, severe dengue (DS) 14% and Dengue fever(DF) 33%. Serotyping by Real-time PCR and Nested PCR showed the co-circulation of DENV 2 serotype of dengue in the year 2019. Single nucleotide polymorphism(SNP) of three different genes were identified using amplification refractory mutation system PCR using the allele specific primers for TNF α (+308 A/G - rs1800629), IFNG (+874A/T − rs2430561), Interleukin 10 (IL-10) (819 C/T and 1082 A/G). CT genotype of TNF-α was observed in 60% of case population, in case of Interleukin 10 (IL-10) (819 C/T and 1082 A/G) CT and AG genotype was seen in most of the study population 71% and 62% respectively. For IFNG (+874A/T − rs2430561), AT (45%) genotype followed by AA (40%) whereas only 15% with TT genotype was reported. In case of F$c\gamma$RIIa, heterozygote genotype AG variant was detected in all study population. As the population size was small in this study, the role of SNPs in severity could not be exactly predicted but this study provides future insight in predicting the role of these biomarkers in susceptibility, progression or protection a disease

Keywords: DENV, DHF, DS, Severity markers, Single Nucleotide Polymorphism, TNFα, IFNG, Interleukin 10

Chapter I
Introduction

1.1 Background of Dengue Virus and Dengue Fever

Dengue virus (DENV) is a small, enveloped virus that contains a single-stranded, positive-sense RNA genome and is a member of the family *Flaviviridae* causing dengue fever. The term 'dengue virus' refers to a group of four genetically and antigenically related viruses that are known as serotypes (DENV-1 to DENV-4)(Kyle & Harris, 2008). These viruses are transmitted between humans predominantly by *Aedes aegypti* and *Aedes albopictus* mosquitoes and are endemic in most areas in which the vectors are present (Gubler, 2002). All four dengue virus serotypes cause the same clinical manifestations and show similar patterns of systemic dissemination, with tropism principally for monocytes, macrophages and dendritic cells (WHO,2009).

With nearly 40% of the world's population at risk for transmission of one or more of the four dengue virus (DENV) serotypes, and the continuous spread of mosquito vectors that transmit these viruses to different parts of the world, dengue fever is now becoming a global epidemic (Yung et al., 2015). Approximately 390 million human dengue infections are likely to occur annually, with 100 million clinical disease cases. Dengue virus infection causes a spectrum of clinical manifestations ranging from normal dengue fever to fatal hemorrhagic diseases. Dengue fever is clinically characterized by acute febrile illness with chills, headache, body pain retro-ocular pain and arthralgia followed by nausea and vomiting and a maculo-papular rash (S. Halstead, 2008). A severe form of dengue fever, also known as dengue hemorrhagic fever (DHF) can cause severe bleeding, a sudden drop in the blood pressure (shock) and death (Jain, 2005).

1.2. History of Dengue Infection

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

Dengue virus was first isolated by Ren Kimura and Susumu Hotta in Japan in 1943. These two scientists were studying blood samples of patients taken during the 1943 dengue epidemic in Nagasaki, Japan. An epidemic of DF involving at least 200,000 cases had occurred between 1942 and 1944 during World War II in Japanese port cities such as Nagasaki, Kobe, and Osaka (Hotta, 1952). The infections originated from persons returning from the tropics, in particular Southeast Asia and the Pacific islands. A year later, Albert B. Sabin and Walter Schlesinger independently isolated DENV-1 from Hawaiian and shortly thereafter, DENV-2 from Papua New Guinean samples (Moore et al., 2017).

The viral etiology and the transmission by mosquitoes were only deciphered in the 20th century. The socioeconomic impact of World War II resulted in increased spread globally. Nowadays, about 2.5 billion people, or 40% of the world's population, live in areas where there is a risk of dengue transmission. Dengue spread to more than 100 countries in Asia, the Pacific, the Americas, Africa, and the Caribbean (S. B. Halstead, 2007).

The origins of DENV have been the subject of speculation for decades. Phylogenetic relationships to other flaviviruses provide little insight because the closest relatives to DENV occur in several continents. It was hypothesized that endemic DENV evolved from sylvatic strains in Africa or Asia that utilize nonhuman primate hosts and gallery forest-dwelling Aedes vectors (not the endemic/epidemic vectors Ae. aegypti or Ae. albopictus) (Gubler, 1997a). The sylvatic cycle is presumed to be ancestral because efficient interhuman transmission is thought to require a minimum human population size of 10,000–1 million, which did not exist until about 4000 years ago when urban civilizations arose (Weaver & Vasilakis, 2009).

1.3 Dengue in Nepal

In Nepal, the first case of dengue virus was observed in 2004 (Takasaki et al., 2008b) and since then the dengue fever has been found rapidly spreading across the country within the short period of time. More than 1500 cases of dengue infection were reported from different districts within the last five months of the year 2016 (EDCD, 2016). Mainly the serotype 2 was seen in the year 2004, serotype 1 in 2010 and serotype 1 in the year 2013(Arya & Agarwal, 2014). The shift of the viral disease from the subtropical Terai belt to the temperate hill region of Nepal is relevant to medical science because the disease could easily spread all over the world in the future, irrespective of climate (Gupta et al., 2016).

There is an increasing trend of dengue outbreak in every three years in Nepal. Although the first dengue case was reported in 2004 from Japanese traveller, the outbreak of dengue occurred in the country in 2006 with a remarkable number of 32 confirmed cases. The virus remained almost latent for the three consecutive years from 2007 to 2009 and

reoccurred again during a massive outbreak in 2010 (Basu D. Pandey et al., 2013a). Cases of dengue continued to be reported in the subsequent year 2011 and 2012 and two major outbreaks were witnessed in 2013 and 2016, clear cyclic 3-year-amplitude demonstrated by major peaks in 2010, 2013 and 2016 (Gupta et al., 2018). Incidence of dengue cases in 2018 was found to be five times more and over 150 times in 2019 than 2016.(Rijal et al., 2021)

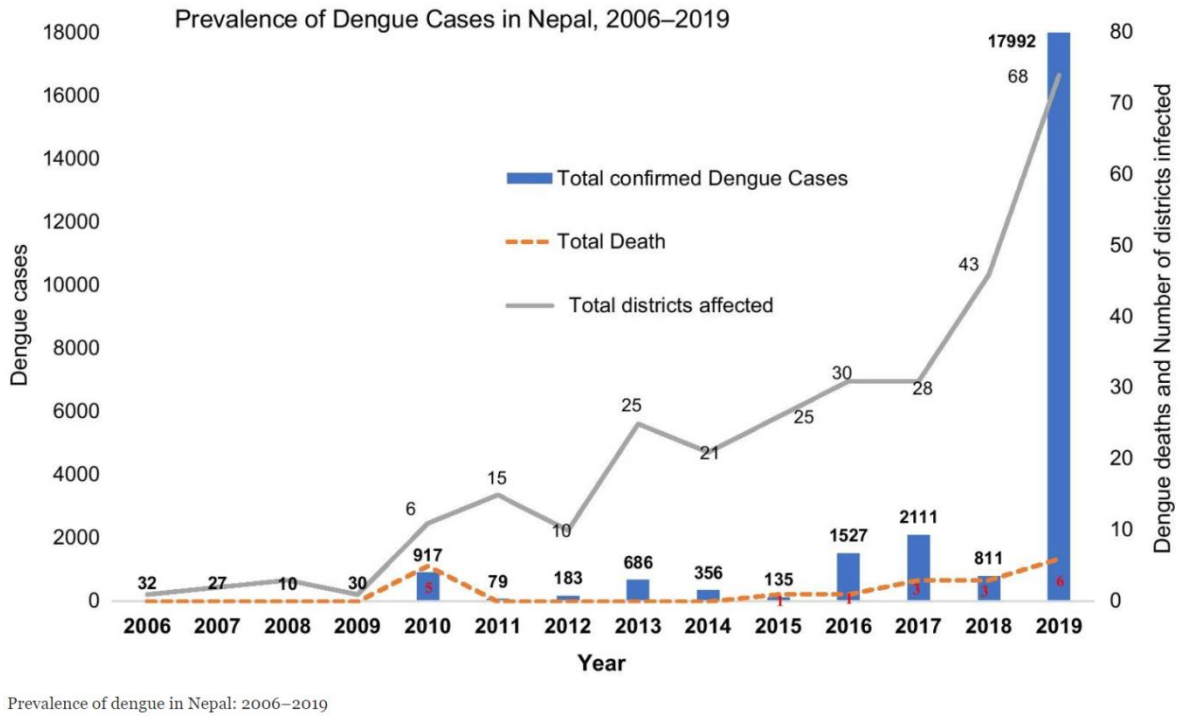


Fig 1.1. : Prevalence of Dengue Cases in Nepal 2006 to 2019 (Rijal et al., 2021)

1.4. Dengue in Global Scenario

Although DENVs achieved distribution throughout the tropics in the eighteenth and nineteenth centuries, during the twentieth and twenty-first centuries, globalization enabled their more-rapid spread and the introduction of multiple viral serotypes into permissive areas, resulting in most tropical regions becoming hyperendemic. This rapid spread began with a pandemic of dengue in South-East Asia in the 1950s that was associated with regional economic and urban growth after World War II. Epidemic activity dramatically accelerated in the 1970s and 1980s, leading to a global geographical expansion of viruses and mosquito vectors, and the consequent widespread DENV transmission across the tropics and subtropical areas. This geographical expansion resulted in increased frequency and magnitude of epidemics and increased frequency of severe disease. The principal drivers of this twentieth century pandemic were global trends, such as human population growth, urbanization, modern transportation, global trade and the absence of effective mosquito control in endemic countries. This

development led to more-frequent epidemics followed by clinically silent or undetected transmission during inter-epidemic periods. Large cities tend to be hyperendemic, with co-circulation of all four serotypes. Epidemics might occur when herd immunity to one of the four serotypes wanes and/or when a new epidemic strain of virus emerges or is introduced. Although not documented, an increase or change in vector competence of the mosquito population might also influence epidemic transmission.

The WHO estimates that more than 50 million dengue virus infections and 20,000 dengue disease-related deaths occur annually worldwide (Organji et al., 2017). A recent disease distribution model using a boosted regression tree framework estimated there to be 390 million dengue disease infections in 2010, of which 96 million are clinically apparent (Mota et al., 2017). The frequency of reported cases of dengue to WHO has increased 8 fold in the time diference of 10 years i.e.; 505,430 cases in 2000, to over 2.4 million in 2010, and 5.2 million in 2019. (*Dengue and Severe Dengue*, n.d.)

1.5 Virus taxonomy

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

There are three genera in the *Flaviviridae* family (formerly known as group B *arboviruses*) namely *Flavivirus*, *Pestivirus* and *Hepacivirus*. The DENV belongs to the group B Arbovirus, genus *Flavirus,* family Flaviviridae. The genus *Flavivirus* consists of 55 identified virus species. The word *Flavi* is a derivation from the Latin word “flavus” which means “yellow” and the type species of the genus is the yellow fever virus. The flaviviruses are thus named due to the jaundice observed in yellow fever patients. Many flaviviruses are important human pathogens, most notably the dengue viruses, yellow fever virus, Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV). The flaviviruses are predominantly transmitted by mosquitoes and ticks, whereas some have no known vector. Dengue was one of the groups classified when early researchers divided the flaviviruses serologically into eight antigenic complexes using cross-neutralization tests. However, many viruses, for example the prototype of the genus YFV, could not be affiliated with any complexes (Katzelnick, Coloma, & Harris, 2017). When sequence data became available, phylogenetic inference from molecular data showed agreement with the antigenic complex classification. In addition, it revealed the clear clustering of the *Flavivirus* genus into non vector and vector-borne virus clusters, with the latter splitting into mosquito borne and tick-borne virus clusters(Ramos-Castañeda et al.,

2017). The dengue virus was divided into four groups called *serotypes* based on antigenic properties. Subsequent evidence from molecular data reaffirmed this classification and also provided a clearer understanding of the phylogeny of the four serotypes: among the dengue viruses, DENV-4 diverged first from the common ancestor, followed by DENV-2, and finally DENV-1 and DENV-3 (Huntington et al., 2016).

There are four distinct serotypes and all of them can cause Dengue fever/Dengue hemorrhagic fever. All four serotypes have a similar natural history, and share humans as a primary host and *Aedes* mosquitoes as a primary mosquito vector. The genomic material of the DENV is contained in a positive-sense, single-stranded RNA molecule approximately 11kb in length. It consists of a single open reading frame that encodes a poly protein of between 3387 and 3392 amino acids (Gubler, 1997a).

1.6. Virus Morphology

A mature dengue virion consists of an isometric nucleocapsid 20-30nm in diameter, covered by a lipid envelope 10nm in depth. The density of the virion is 1.23g/cm^2.The envelope consists of a lipid bilayer in which is embedded as E and M part. The envelope protein has the size between 51,000 and 59,000 daltons that mediates attachment, fusion and penetration and a small non-glycosylated internal matrix protein of approximately 8,500 daltons. The envelope protein is glycosylated in most flaviviruses and is exposed on the virion surface. The lipid composition of the envelope is dictated by the composition of the host cell membrane from which the viruses bud.

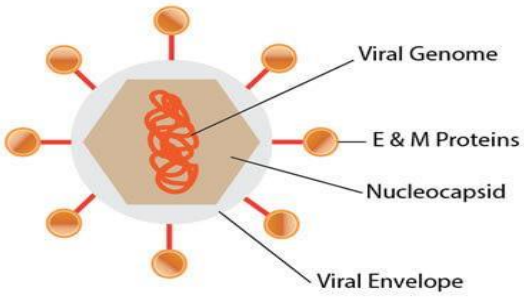


Figure 1.2. Structure of the Dengue virion ((E: enveloped protein and M: Membrane protein, the virus contains RNA as genome and different proteins as structural and non-structural proteins. There are 180 identical copies of the envelope (E) protein attached to the surface of the viral membrane by a short trans-membrane segment. (Guzman et al; 2010)

1.7. Genomic organization

The genomic organization of the dengue virus, and by extension all flaviviruses, is relatively simple compared to other arboviral families such as the *Togaviridae* (formerly known as group A *arboviruses*), *Bunyaviridae* or *Rhabdoviridae*. The DENV genome consists of a single-stranded, positive sense RNA molecule roughly 10.7 kb in size. It

contains a single translated open reading frame (ORF) that encodes a precursor polypeptide of around 3390 amino acids which is processed catalytically into ten viral proteins. There is no evidence of alternative or overlapping reading frames that are translated and there is also no hyper-variable region in the DENV genome like those reported in the Hepatitis C Virus (HCV) genome.

The virus has a genome of about 11000 bases that encodes an Open Reading Frame (ORF) flanked by 5 to 3' non-coding regions. The dengue virus ORFs are 10,888; 10,173; 10,170 and 10,158 nucleotides in length that encode a poly-protein precursor of 3396,3391,3390 and 3386 amino acid in DENV-1,-2,-3 and-4 viruses, respectively. The poly-protein is divided into 3 structural proteins, C, prM, E; 7 nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5; and short non-coding regions on both the 5' and 3'end. The structural proteins are the capsid (C) protein, the envelope (E) glycoprotein and the membrane (M) protein. The E glycoprotein is responsible for virion attachment to receptor and fusion of the virus envelope with the target cell membrane and bears the virus neutralization epitopes. In addition to the E glycoprotein, only one other viral protein, NS1 has been associated with a role in protective immunity. NS3 is a protease and a helicase, whereas NS5 is the RNA polymerase in charge of viral RNA replication. The genomic RNA of DENV contains a 5' type1 cap structure and is generally not 3' polyadenylated. Lack of poly-adenylation distinguishes the flavivirus from other positive strand RNA viruses (Rice et al., 2005).

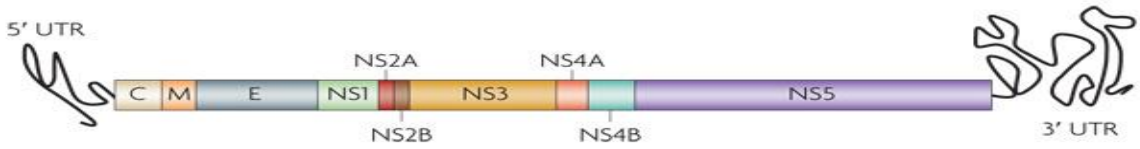


Fig 1.3: Dengue virus genome. The dengue virus genome encodes three structural (capsid [C], membrane [M], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins.(Guzman et al., 2010)

1.8. Vectors and Mode of Transmission

Dengue is transmitted from person to person through the bites of infected female mosquitoes. The etiological agent, the DENV, is believed to have been maintained in sylvatic/enzootic transmission cycles involving nonhuman primate hosts and vector species living in forests. The virus was transmitted to humans when the two came into contact and thereafter was maintained in continuous human-mosquito cycles in and/or around human population centers. Many species from the genus *Aedes* of the family *Culicidae* are known to transmit DENV, but the principal vector is *Aedes aegypti* which is also the vector of the yellow fever virus (YFV). The Australian naturalist Thomas Lane

Bancroft first suggested *Ae. aegypti* as a carrier of dengue fever in 1906 based on epidemiological grounds, and this was confirmed in 1916 by John Burton Cleland (Piovezan et al., 2016). *Ae.aegypti* is known to be a day-biting mosquito that prefers to breed in domestic and peridomestic water containers. Its adaptation to human habitats and its desiccation-resistant eggs have allowed it to flourish in urban centers. The secondary vector for dengue is *Aedes albopictus* which is commonly known as the Asian tiger mosquito. Its role as dengue vector in semi-tropical regions was first identified in Taiwan in 1917 (Seltenrich, 2016). *Ae. albopictus* serves as the primary vector for dengue in countries where *Ae. aegypti* is absent and as a maintenance vector in rural areas where both species coexist (Saito et al., 2016). In the Pacific islands *Ae. polynesiensis* has been suggested as the primary dengue vector whereas *Ae. scutellaris* was identified as the 'jungle' vector for dengue (Bharati & Saha, 2017).

Ae. aegypti and *Ae. albopictus* have both been shown to be anthropophilic, i.e. prefer to feed on humans and are widely distributed in both urban and semi-urban areas in the tropics and subtropics. Both species have also been demonstrated to pose high vector competence for the dengue virus (Akter et al., 2017). In the continued absence of vaccines and specific treatment, effective vector control (either though fogging that kills adult mosquitoes, application of larvicides that target the aquatic stage of mosquitoes, or source reduction that reduces their breeding habitat) is currently the only practical method available for reducing the incidence of dengue disease.

The larvae of the principal vector *Ae. agypti* under naturally changing temperature are capable of developing into adults in conditions lower than 10°C, whereas those of *Ae. albopictus* can survive even lower temperatures (Akter et al., 2017). Consequently the two species can be found between latitudes 35°N and 35°S, approximately corresponding to a winter isotherm of 10°C (Kumar, 2016). The southern parts of the United States and Europe, and major parts of Australia and Africa are among areas at risk of future dengue transmissions. A dengue outbreak reported in Buenos Aires, Argentina (34°36'S) in early 2009 is very close to this isotherm and is the furthest south dengue has spread. Dengue is the most common arboviral (arthropod-transmitted) disease and it also ranks as the most important mosquito-borne viral disease in the world. Some 2.5 billion people living in tropical and sub-tropical regions are at risk of dengue infection, which equates to about two-fifths of humanity (Kumar, 2016). There is an estimated 50-100 million infections occurring globally every year, with 500,000 cases requiring hospitalization and causing 24,000 deaths (Hernandez-Suarez & Mendoza-Cano, 2016). Furthermore, the number of people living in tropical and sub-tropical regions is set to double by the end of the century, thus making dengue an unqualified global threat to public health.

Female *Aedes aegypti* commonly lays eggs on the inner walls of artificial containers. When the containers filled with water, mosquito larvae hatch from the eggs. After developing through four larval stages, the larvae metamorphose into pupas. Like the larval stage, the pupal stage is also aquatic. After two days, a fully developed adult mosquito forms and breaks through the skin of the pupa. The adult mosquito can fly and has a terrestrial habitat. The dengue virus is spread through a human-to-mosquito-to-human cycle of transmission (Yung et al., 2015).

1.8.1 Transmission cycle

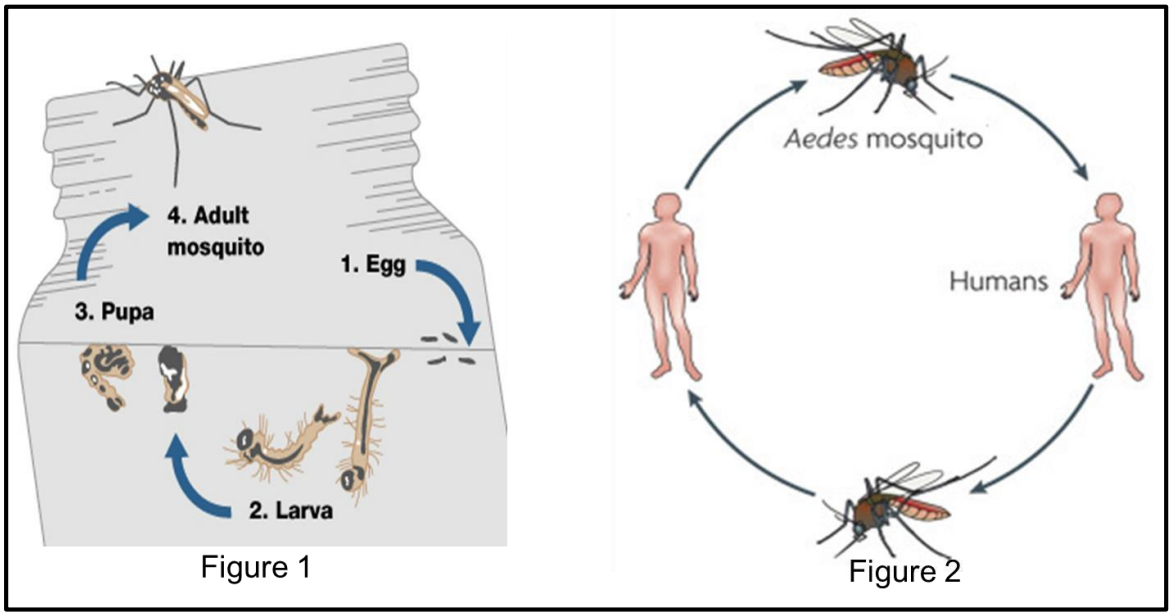


Fig 1.4. (1) *Aedes aegypti* life cycle(Guzman et al., 2010b) (2) Dengue transmission cycle

Humans are the main amplifying host of the virus. Dengue virus circulating in the blood of viraemic humans is ingested by female mosquitoes during feeding. The virus then infects the mosquito mid-gut and subsequently spreads systemically over a period of 8-12 days. After this extrinsic incubation period, the virus can be transmitted to other humans during subsequent probing or feeding. The extrinsic incubation period is influenced in part by environmental conditions, especially ambient temperature. Thereafter the mosquito remains infective for the rest of its life. Ae. aegypti is one of the most efficient vectors for arboviruses because it is highly anthropophilic, frequently bites several times before completing oogenesis, and thrives near humans. Vertical transmission (transovarial transmission) of dengue virus has been demonstrated in the laboratory but rarely in the field. The significance of vertical transmission for maintenance of the virus is not well understood. Sylvatic dengue strains in some parts of Africa and Asia may also lead to human infection, causing mild illness. Several factors can influence the dynamics of virus transmission - including environmental and climate factors, host-pathogen interactions

and population immunological factors. Climate directly influences the biology of the vectors and thereby their abundance and distribution; it is consequently an important determinant of vector-borne disease epidemics (Amin et al., 2018).

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

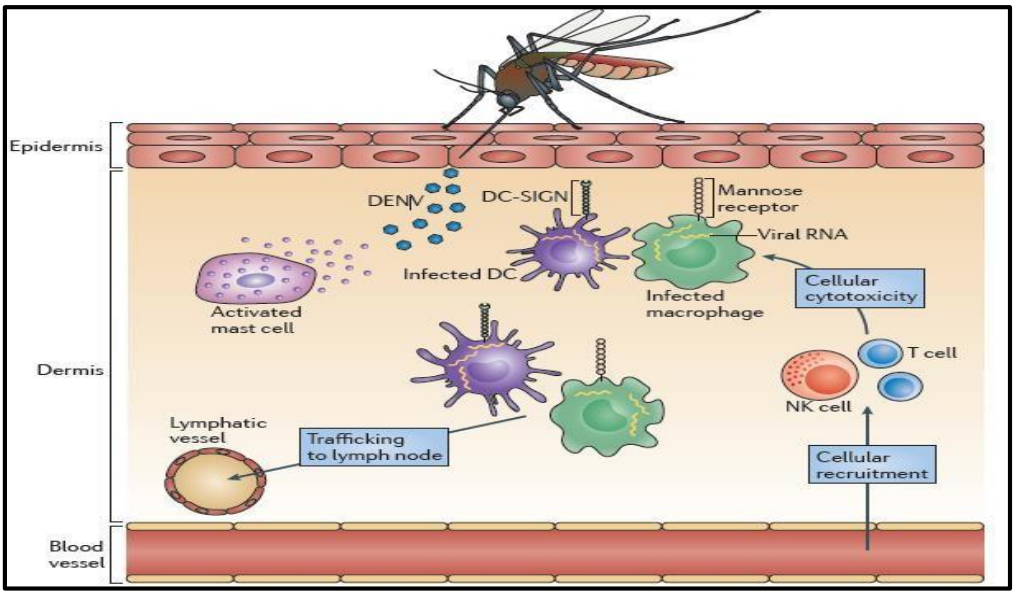


Fig 1.5. Pathway for the immuno- pathogenesis of DENV(St. John et al., 2013)

1.9 Virus Replication

The dengue virus attaches to the surface of a host cell and enters the cell by a process called endocytosis. Once deep inside the cell, the virus fuses with the endosomal membrane and is released into the cytoplasm. The virus particle comes apart, releasing the viral genome. The viral RNA (vRNA) is translated into a single polypeptide that is cut into ten proteins, and the viral genome is replicated. Virus assembly occurs on the surface

of the endoplasmic reticulum (ER) when the structural proteins and newly synthesized RNA bud out from the ER. The immature viral particles are transported through the trans-Golgi network (TGN), where they mature and convert to their infectious form. The mature viruses are then released from the cell and can go on to infect other cells (Mukhopadhyay et al., 2005).

The newly synthesized viral RNA is enclosed in the C proteins, forming a nucleocapid. The nucleocapsid enters the rough ER and is enveloped in the ER membrane and surrounded by the M and E proteins. This step adds the viral envelope and protective outer layer. The immature viruses travel through the Golgi apparatus complex, where the viruses mature and convert into their infectious form. The mature dengue viruses are then released from the cell and can go on to infect other cells.

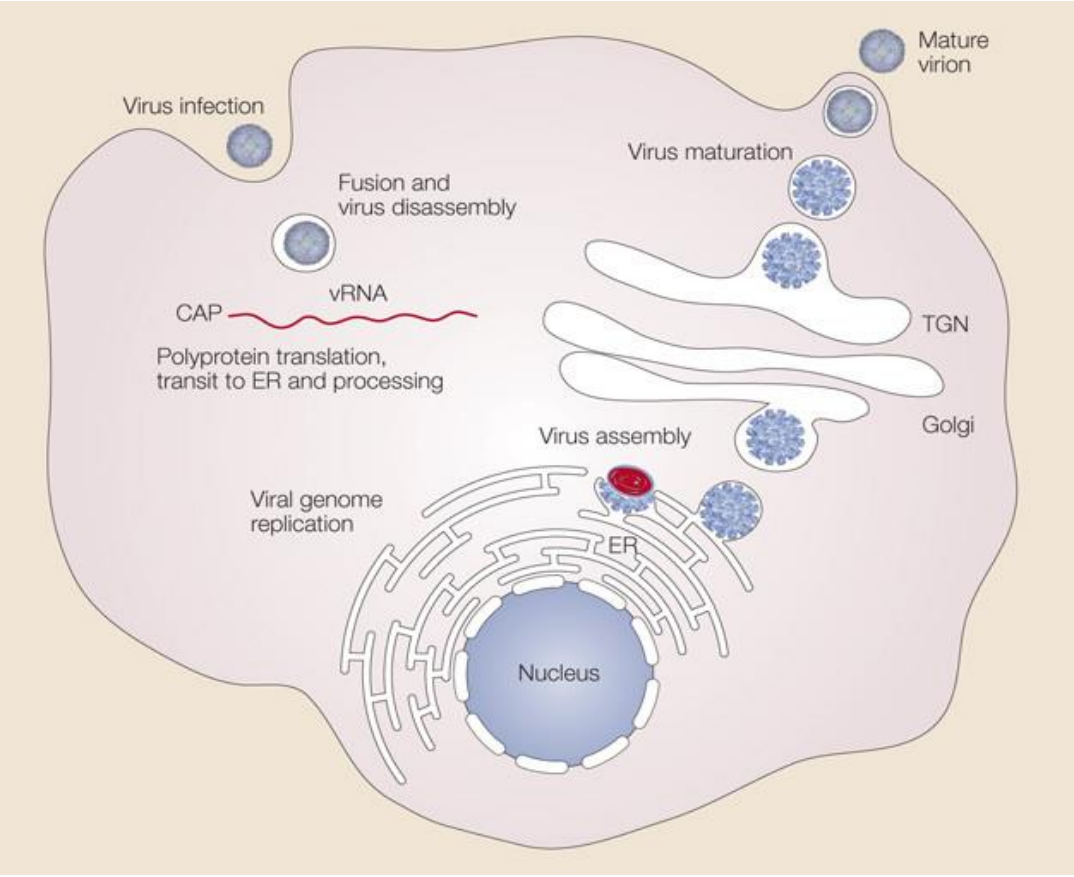


Fig 1.6. Dengue virus replication (Mukhopadhyay et al., 2005)

1.10 Dengue symptoms and clinical manifestations

Infection with any of the four serotypes (DENV1–4) of dengue virus (DV) can produce a broad spectrum of symptoms, ranging from asymptomatic infection to a severe life-threatening illness. Dengue is a dynamic illness, despite its short duration (no more than 1 week in nearly 90% of cases). Its clinical expression can change as the days go by and can also worsen suddenly. Dengue illness can evolve into three phases: the acute febrile

phase was observed in most of the patients and the critical and the recovery (convalescent) phases (WHO, 2009).

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

The critical phase coincides with the leakage of plasma that can lead to shock, which is characterized by coldness in the teguments, weak pulse, delayed capillary filling, tachycardia, oliguria and hypotension. Shock is caused by low blood volume (hypovolaemia). At the beginning, not all clinical signs of shock are observed, and, in this setting, shock can be detected by a narrowing of the differential arterial tension or pulse pressure (a difference of ≤20 mmHg between the maximum or systolic arterial tension and the minimum or diastolic arterial tension). At this stage, patients usually have a flushed face, a warm trunk, cold and clammy extremities, diaphoresis (sweating), slow venous filling, restlessness, irritability, pain in the upper and middle abdomen and decreased urinary output. In addition, patients might also exhibit signs of impaired haemostasis, including scattered petechiae on the forehead and extremities, spontaneous ecchymoses, easy bruising and bleeding at venipuncture sites, and circumoral and peripheral cyanosis (blue skin discolouration). Gastrointestinal bleeding occurs in <10% of patients and usually follows a period of uncorrected hypotensive shock. Patients with shock also experience rapid and potentially laboured breathing, a weak pulse and have a rapid heartbeat that sounds 'thready'. Finally, their livers are usually firm, tender and can become enlarged to 4–6 cm below the costal margin, the haematocrit level is increased and the platelets which were decreasing progressively reach their lowest count. In those who recover, this critical phase lasts for 24–36 hours and is followed by a rapid

convalescence. Convalescence can involve complications, such as encephalopathy, bradycardia, ventricular extarsystoles and, rarely, myocarditis and encephalitis.

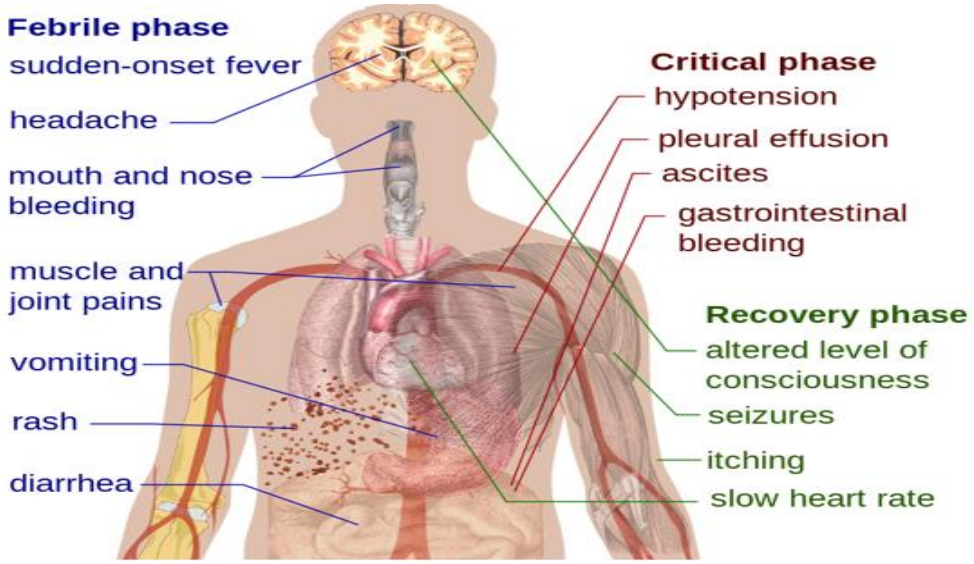


Fig 1.7. Schematic representation of dengue infection (*Schematic Description of the Symptoms of Dengue Fever (Www.Cdc.Com). | Download Scientific Diagram*, n.d.)

1.11 Classification of Dengue ailment

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

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

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

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

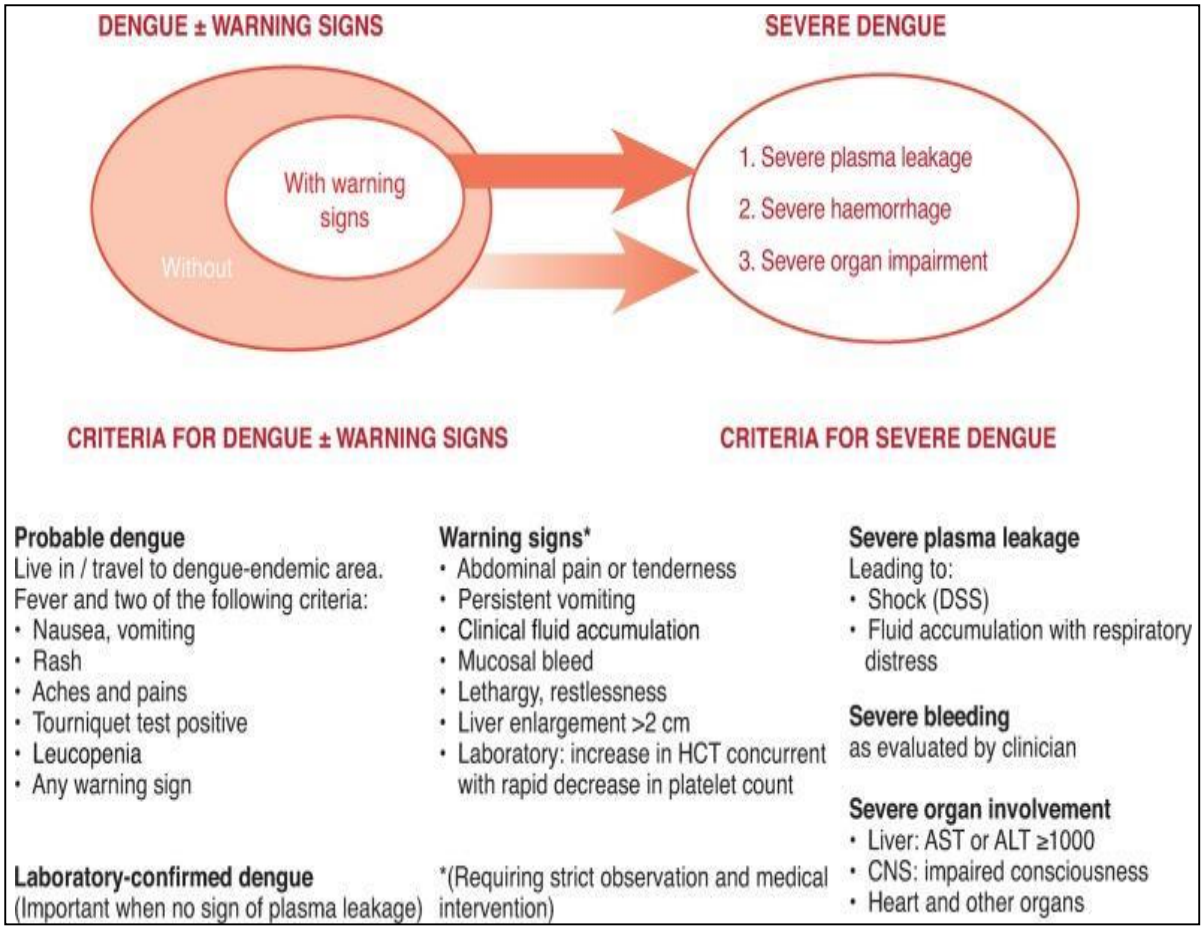


Fig 1.8. Classification of dengue according to severity(Hadinegoro, 2012)

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

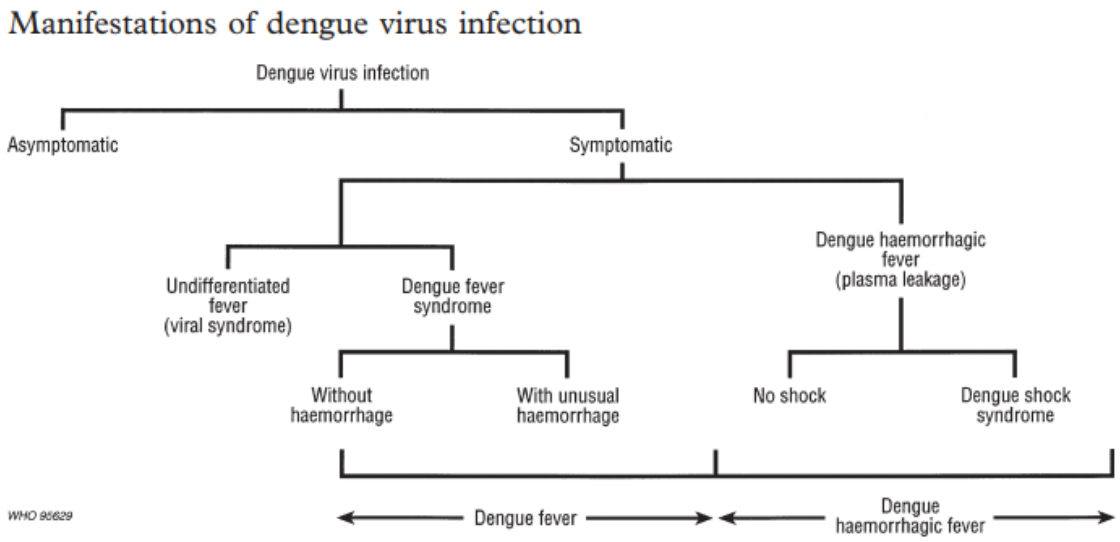


Fig 1.9: Manifestation of dengue virus infection(*Dengue Haemorrhagic Fever*, n.d.)

Disease severity and the manifestation of clinical symptoms are known to be influenced by the environmental factors, the serotype/genotype of dengue virus, the immune response and genetic background of host (Santos et al., 2017).

1.12 Diagnosis of Dengue virus

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

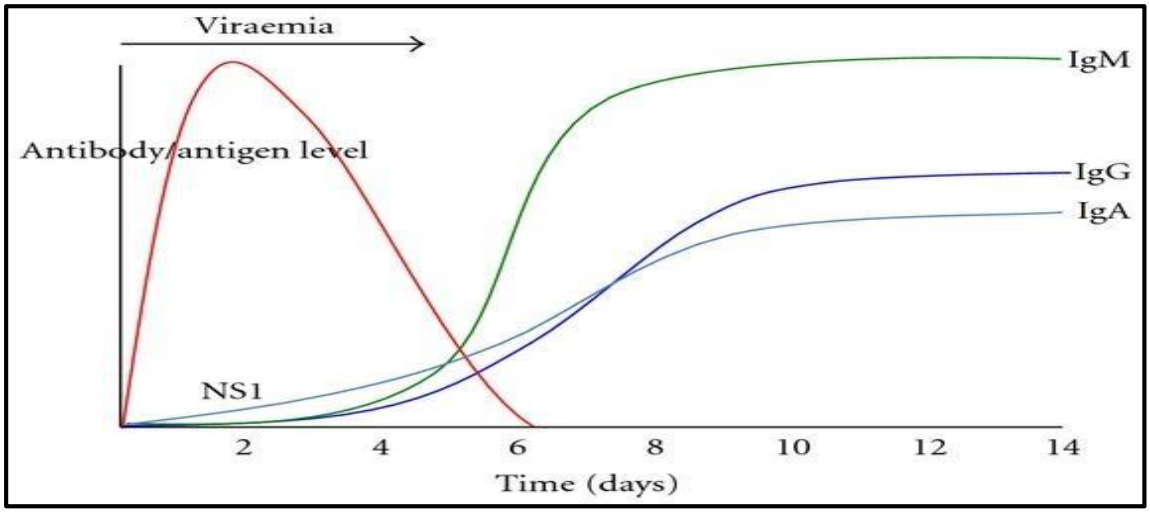


Figure 1.10.: Immune response to dengue infection (Guzman et al., 2010).

Abbreviations: DENV, dengue virus; NS1, non-structural protein.

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

Dengue can be diagnosed by isolation of the virus, by serological tests, or by molecular methods. Diagnosis of acute (on-going) or recent dengue infection can be established by testing serum samples during the first 5 days of symptoms and/or early convalescent phase (more than 5 days of symptoms). Acute infection with dengue virus is confirmed when the virus is isolated from serum or autopsy tissue specimens, or the specific dengue virus genome is identified by reverse transcription-polymerase chain reaction (RT–PCR) from serum or plasma, cerebrospinal fluid, or autopsy tissue specimens during an acute febrile illness. Methods such as one-step, real-time RT–PCR or nested RT–PCR are now widely used to detect dengue viral genes in acute-phase serum samples(CDC). Several formats of ELISA are designed for detecting DENV antibodies. Classical indirect ELISA and immunoglobulin antibody capture ELISA are the 2 most common formats. Indirect ELISA uses the viral antigens to coat the microtiter plates. Following serial incubation with patient serum and enzyme-conjugated anti-human immunoglobulin, the chromogen substrate is added during the final step for colour development. The colour is then read using a spectrophotometer. Recently, NS1 serotype-specific IgG ELISA was also found to be a reliable method for differentiating primary and secondary virus infections (Chuan-Liang Kao 1, 2005).

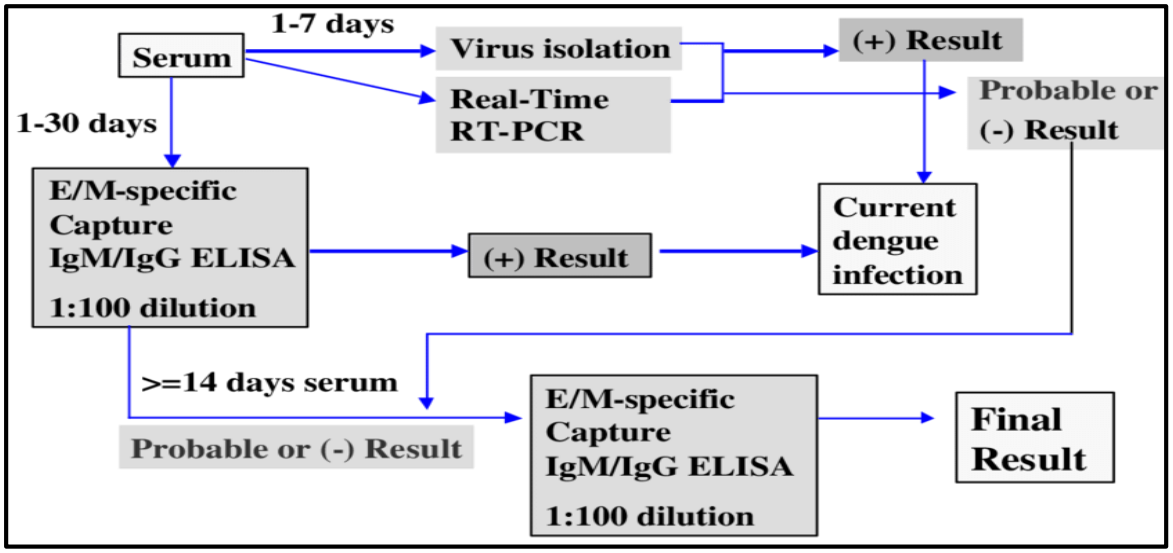


Fig 1.11.: Process for the diagnosis of Dengue (CDC).

Different types of capture ELISA like IgM, IgG and NS1 are performed for the early diagnosis and confirmation of primary and secondary infection. Besides this mid-point, ELISA is performed for the calculation of titre value. PCR is performed for the confirmation of the viral infection and serotyping is done for the confirmation of the prevalent serotype in a year and in a certain locality.

1.13 Host Immune Response

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

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

called antigens, on their surface. The Langerhans cells then travel to the lymph nodes and alert the immune system to trigger the immune response because a pathogen is in the body. Lymph nodes are small organs located throughout the body connected by vessels that form a network called the lymphatic system. The lymph nodes are stations in the body for immune cells that help fight against infections.

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

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

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

1.14 Secondary Dengue Infections

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

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

Normally after an infection with a pathogen, the body "remembers" the infection for a long time because cells called memory B cells and memory T cells remain in the body. Because they remember the first infection, these memory cells can react rapidly to provide an adaptive response when an infection strikes a second time. Memory cells can remain in a person's body for many years, even an entire lifetime. Halstead proposed a phenomenon called "antibody-dependent enhancement of infection" to explain these observations. There are four different types of dengue viruses (serotypes), but the memory cells only provide immunity from reinfection with the dengue serotype that caused the first infection. When a person is infected with a second dengue serotype, Halstead proposed that antibodies from the first infection actually help spread the dengue viral infection and increase viremia, the amount of virus in the bloodstream. This phenomenon can also happen in children who received antibodies against dengue from their mothers while in the womb. Surprisingly, instead of destroying the virus, the existing antibodies and the antibodies newly produced by the memory B cells can actually help the virus infect host cells more efficiently. Ironically, the consequence of antibody-dependent enhancement is that the body's immune system response actually makes the clinical symptoms of dengue worse and raises the risk of severe dengue illnesses. Antibody (Ab)-dependent enhancement of infection occurs when preexisting antibodies present in the body from a primary (first) dengue virus (DENV) infection bind to an infecting DENV particle during a subsequent infection with a different dengue serotype. The antibodies from the primary infection cannot neutralize the virus. Instead, the Ab–virus complex attaches to receptors called Fcγ receptors (FcγR) on circulating monocytes. The antibodies help the virus infect monocytes more efficiently. The outcome is an increase in the overall replication of the virus and a higher risk of severe dengue.

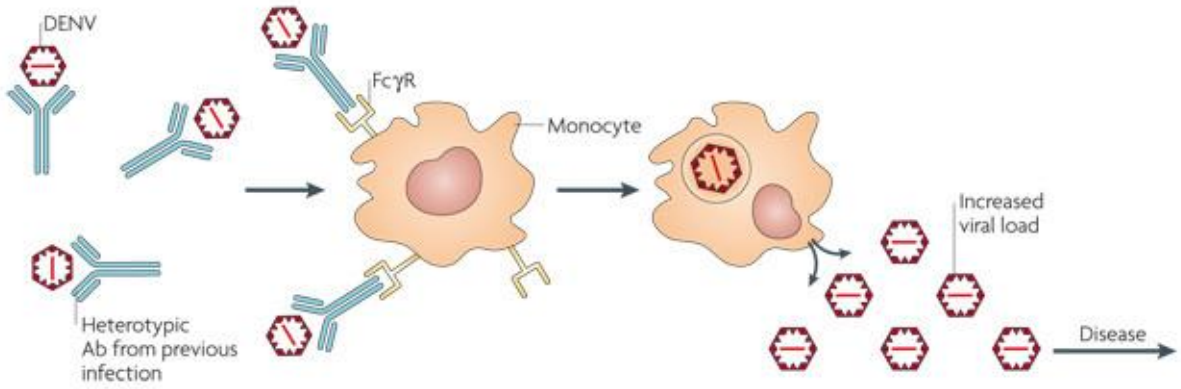


Fig 1.12.: Model of antibody-dependent enhancement of dengue infection

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

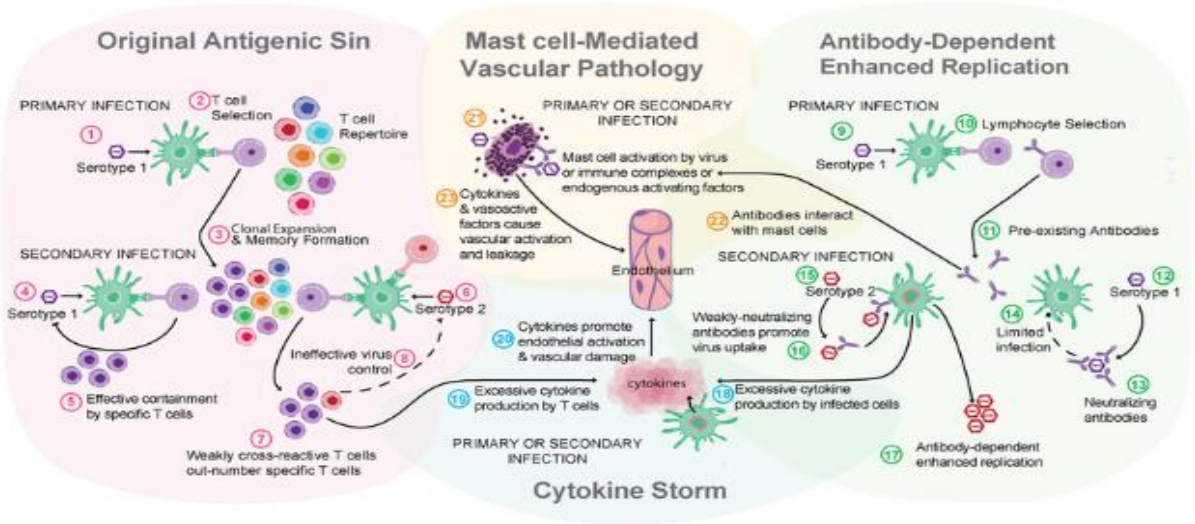


Fig 1.13 Multiple theories of dengue immune pathogenesis. (St. John, 2013)

1.15 Biomarkers

Development of the disease being associated with different factors, host immune response among them is being taken as genetic biomarker for the disease with the production of several cytokines. Single nucleotide polymorphisms (SNPs) in the genes associated with the host immune response have influence in the production and functioning of the immune molecules and their association with protection and susceptibility and the disease progression in an individual (Fang et al., 2012).

Some of the key molecules of immune system associated with the disease development of dengue fever are Human leukocyte antigen (HLA), Dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN), FCγRIIa, transporter associated with antigen processing (TAP), vitamin D receptor (VDR), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), acute plasma glycoprotein mannose binding lectin (MBL) and human platelet-specific antigens (HPA) and cytokines (Fang et al., 2012).

Cytokines are small protein molecules secreted by numerous cells and are important in cell-signaling. The biological functions of cytokines are complicated, extensive, and overlapping (synergistic or antagonistic). During dengue infection these molecules increase vascular permeability leading to hemorrhage causing DHF. These proteinaceous molecules are secreted during innate and adaptive immunological responses, acting as inflammatory mediators or modulatory molecules during dengue infection. Viral infection activates the innate immune response to express pro-inflammatory cytokines that recruit and activate cells involved in inflammation and the induction of adaptive immunity (Imad et al., 2020). There is significant difference in cytokine levels in DF and DHF patients from that of healthy individuals. The role of cytokines in DENV pathogenesis has been identified by many researchers and, based on their functions the effects of cytokines on DENV infection can be split into protection, susceptible , and mixed (Fang et al., 2012).

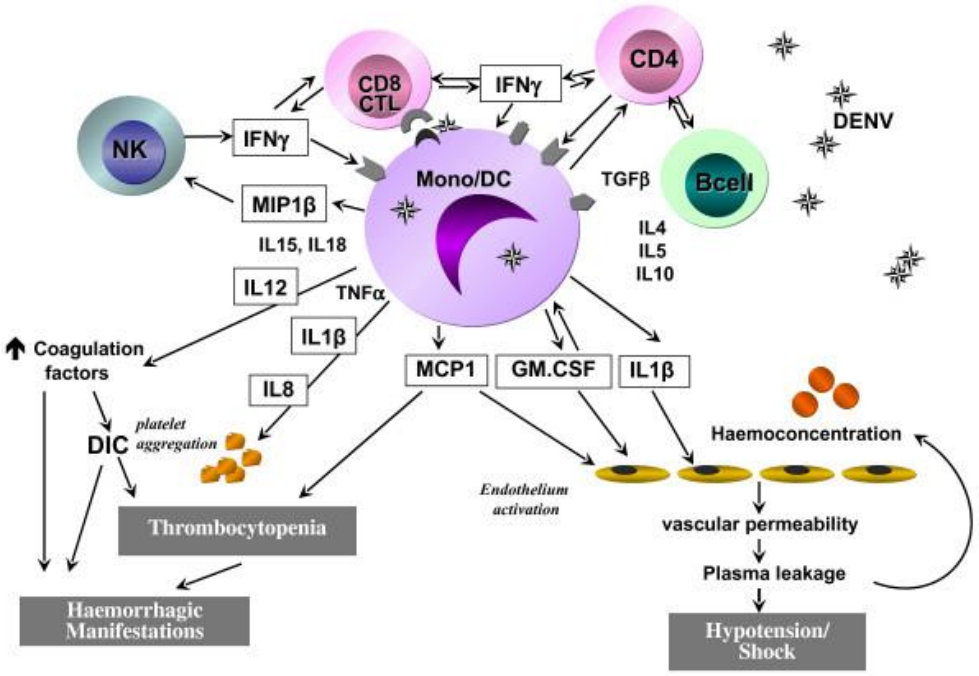


Fig 1.14. Hypothetic mechanism to explain cytokine models during dengue fever. MIP-1β is associated with a good prognostic and IFN-γ has a predictive value for severity. GM-CSF, MCP-1, IL-1β, IL-6, IL-8, IL-12, IL-13 are also playing important roles during dengue pathogenesis (Bozza et al., 2008)

Interleukin 10 (IL-10)

Interleukin 10 (IL-10) is an anti-inflammatory cytokine encoded by the *IL10* gene, chromosome 1q32.1. It is also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine primarily produced by monocytes. During infection it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for optimal pathogen clearance but also contribute to tissue damage. In consequence, IL-10 can both impede pathogen clearance and ameliorate immunopathology. Many different types of cells can produce IL-10, with the major source of IL-10 varying in different tissues or during acute or chronic stages of the same infection. First described as a product of Th2 cells that inhibited cytokine synthesis in Th1 cells, IL-10 is now known to be produced by macrophages, dendritic cells (DC), B cells, and various subsets of $CD4^+$ and $CD8^+$ T cells. Initially shown to regulate T cell responses, many of the effects of IL-10 on T cell and NK cell function are now known to be indirect, being mediated via a direct effect of IL-10 on monocyte-macrophages. Thus, IL-10 inhibits MHC class II and costimulatory molecule B7-1/B7-2 expression on monocytes and macrophages and limits the production of proinflammatory cytokines (including IL-1α and β, IL-6, IL-12, IL-18, and TNF-α) and chemokines (MCP1, MCP5, RANTES, IL-8, IP-10, and MIP-2). Importantly, autocrine IL-10 signaling in DC can inhibit chemokine production and prevent their

trafficking to lymph nodes as shown in mycobacterial infection, leading to the failure to recruit and induce Th1 differentiation of naive T cells. Nevertheless, IL-10 can act directly on $CD4^+$ T cells, inhibiting proliferation and production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α. Thus, IL-10 can directly regulate innate and adaptive Th1 and Th2 responses by limiting T cell activation and differentiation in the lymph nodes as well as suppressing proinflammatory responses in tissues, leading to impaired pathogen control and/or reduced immunopathology. IL-10 has emerged as a key immunoregulator during infection with viruses, bacteria, fungi, protozoa, and helminths, ameliorating the excessive Th1 and $CD8^+$ T cell responses (typified by overproduction of IFN-γ and TNF-α) that are responsible for much of the immunopathology associated with infections

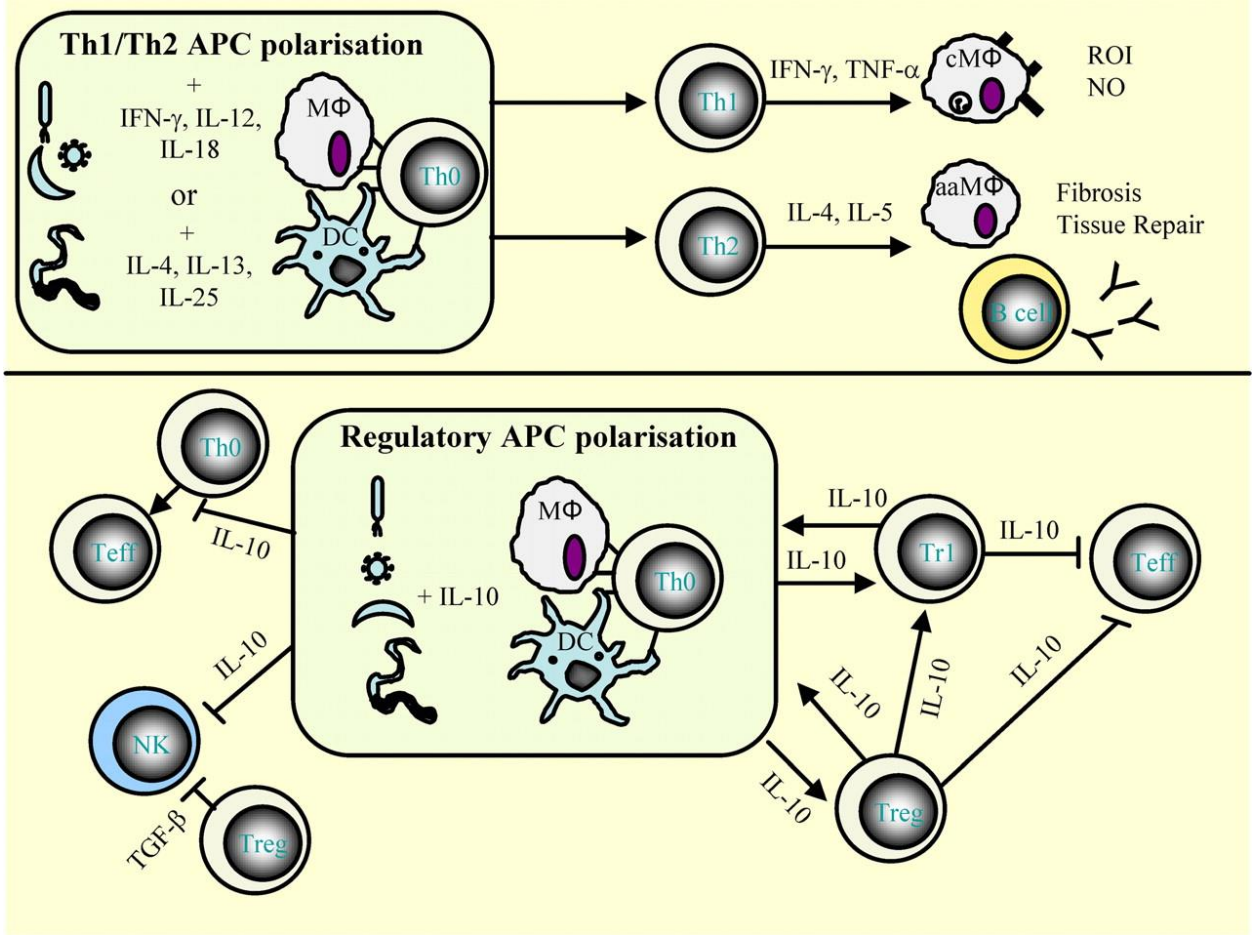


Fig 1.15. The sources and targets of IL-10 during infection.(Couper et al., 2008a)

Interactions between pathogens and APC direct the development of Th1, Th2, or Treg populations depending on the cytokine environment and the level of costimulation. APC promoting regulatory responses are induced by priming under regulatory conditions (often involving IL-10 signaling) or as a result of direct modulation of their functions by the pathogen. IL-10 may act in an autocrine manner to suppress APC proinflammatory responses, may act directly on effector T cells to limit their proliferation and function, or

may promote the differentiation of naive T cells into regulatory populations. Adaptive and natural regulatory T cells may then directly influence macrophage (MÔ), DC, and effector T cell differentiation and effector functions via IL-10-dependent mechanisms, alternatively activated macrophage; classical macrophage; ROI, reactive oxygen intermediate.

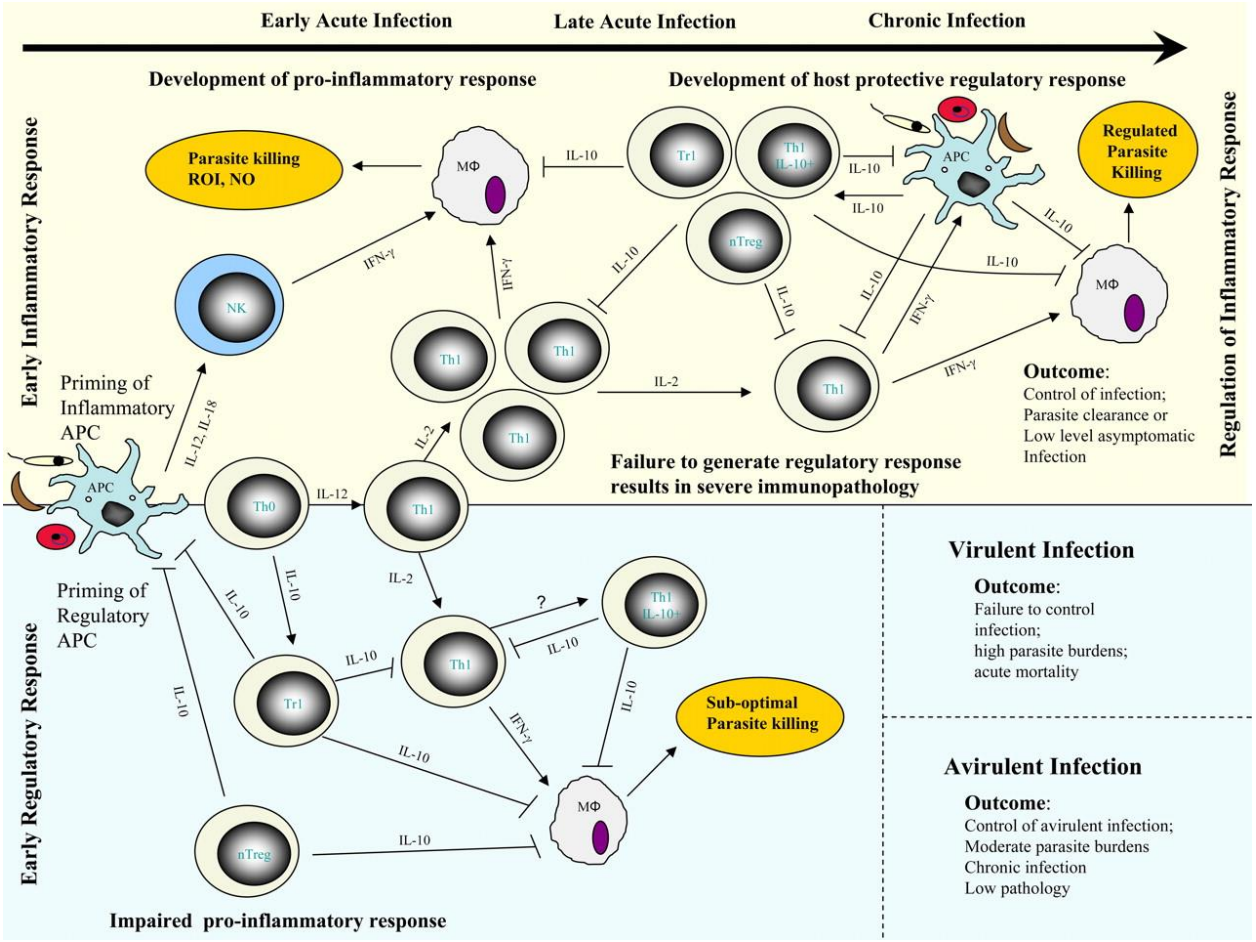


Fig 1.16 The source, timing, and magnitude of IL-10 production determines the outcome of protozoan infections.(Couper et al., 2008a)

Early priming and polarization of proinflammatory immune responses are crucial for the effective control of infection. Proinflammatory cytokine production by APC after interaction with pathogens and the subsequent activation of NK cells and Th1 cells are required to activate macrophages to eliminate microbes. Regulatory DC and macrophages and/or regulatory T cells (Tr1, IL-10-Th1, and nTreg) must then control inflammation to prevent immune-mediated pathology. By contrast, early polarization toward regulatory, IL-10-producing APC populations inhibits inflammation. In the case of avirulent infections, this will allow the pathogen to persist in the absence of tissue damage. In the case of virulent infections, this leads to uncontrolled pathogen dissemination and death. ROI, Reactive oxygen intermediate.(Couper et al., 2008b)

Interferon Gamma (IFN-γ)

Interferon gamma (IFN-γ) is a dimerized soluble cytokine that is the only member of the type II class of interferons usually secreted by activated T lymphocytes and NK. Interferon-γ is a type 1 cytokine that exhibits a wide array of proinflammatory activities. It is produced during most types of infections and plays a key role in amplifying innate and adaptive immune responses. In innate immunity, IFN-γ synergistically augments LPS-stimulated proinflammatory cytokine production, induces respiratory burst, and suppresses release of anti-inflammatory cytokines such as IL-10. IFN-γ also aids in development and shaping of adaptive immune responses. Most importantly, it enhances the function of the APC through stimulation of phagocytosis, up-regulation of MHC class I and class II molecules, and induction of the immunoproteasome. Consequently, T cells can respond more efficiently to IFN-γ-activated APCs that express MHC molecules bound with pathogen-derived peptides. Apart from Ag presentation, IFN-γ also affects adaptive immunity by promoting Th1 (cell-mediated) immune responses. Namely, IFN-γ promotes differentiation of $CD4^+$ T cells into Th1 phenotype IFN-γ-producing cells, induces B cell IgG2a Ab class switching, and stimulates $CD8^+$ T cell cytotoxicity.

IFN-γ has antiviral activity through the induction of effectors molecules (e.g., nitric oxide), and through enhanced antigen presentation and induction of apoptosis. Induction and activation of specific host molecules by IFN block virus infection at several levels, including transcription, translation, and RNA degradation.(Diamond et al., 2000)

Tumor Necrosis Factors

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine secreted by inflammatory cells. Being multifactorial cytokine TNF plays important roles in various cellular events such as cell survival, proliferation, differentiation, death and may be involved in inflammation-associated carcinogenesis (Wang & Lin, 2008) In viral disease like dengue TNF-α plays vital role in causing severe dengue disease by enhancement of vascular permeability.

$F_C\gamma$ receptor II

$F_C\gamma$ receptor is a widely distributed receptor on the surface of certain cells such as B lymphocytes, follicular dendritic cells, natural killer cells, macrophages, neutrophils, eosinophils, basophils, human platelets, and mast cells. They add to the protective functions of the immune system by binding to antibodies that are attached to infected cells or invading pathogens. It is widely distributed receptor for all subclasses of IgG, hence mediate antibody dependent enhancement binding to virus-IgG complexes (Loke et al., 2002).

1.16 Single Nucleotide Polymorphism

A Single-Nucleotide Polymorphism (SNP) sequence variation occurring in a DNA where a single nucleotide Adenine (A), Thymine (T), Cytosine (C), or Guanine (G) differs between members of a species or paired chromosomes in an individual's genome. SNPs in an individual can be vital factor on disease development and one's response to pathogens, chemicals, drug, vaccines, and other agents. One of its greatest assistance in biomedical research is for comparing regions of the genome between different ethnic groups (with and without a disease). SNPs in cytokine and other immune genes can be used as disease susceptibility and severity marker as well as tool for etiopathogenesis study and understanding. SNPs describe and demonstrate the influence of genetic polymorphism in gene transcription and its impact on inter individual variations in immune response (*Single-Nucleotide Polymorphism - ISOGG Wiki*, n.d.)

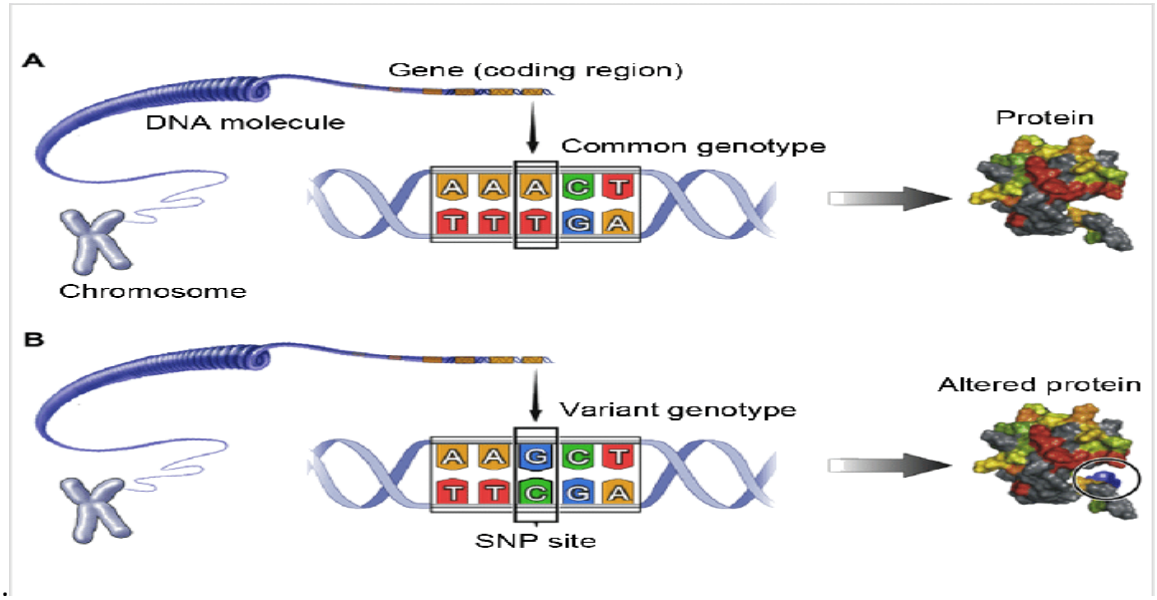


Fig 1.17 Single-nucleotide polymorphisms (SNPs) are genetic mutations that alter single base in DNA, causing sequence modification in amino acids and malfunction of a corresponding protein. (*Single-Nucleotide Polymorphisms (SNPs) Are Genetic Mutations That Alter... | Download Scientific Diagram*, n.d.)

Detection of SNPs is done by using various methods

1 **Hybridization-based methods:**

Several applications have been developed that interrogate SNPs by hybridizing complementary DNA probes to the SNP site. The challenge of this approach is reducing cross-hybridization between the allele-specific probes. This challenge is generally overcome by manipulating the hybridization stringency conditions.(*SNP Genotyping - Wikipedia*, n.d.)

a. Dynamic allele-specific hybridization
b. Molecular beacons
c. SNP microarrays

2 Enzyme-based methods

a. Restriction fragment length polymorphism
b. PCR-based methods
c. Flap endonuclease
d. Primer extension
e. 5'- nuclease
f. Oligonucleotide Ligation Assay

3 Other post-amplification methods based on physical properties of DNA

a. Single strand conformation polymorphism
b. Temperature gradient gel electrophoresis
c. Denaturing high performance liquid chromatography
d. High-resolution melting of the entire amplicon
e. Use of DNA mismatch-binding proteins
f. SNPlex
g. Surveyor nuclease assay

4 Sequencing

1.17 Amplification-Refractory Mutation System (ARMS)

The amplification-refractory mutation system (ARMS) is a tool for detection of SNPs in a gene and is also known as allele-specific polymerase chain reaction (ASPCR) or PCR amplification of specific alleles. It is a simple, rapid, and reliable method for detecting SNPs and other mutations.

ARMS uses sequence-specific PCR primers allowing the amplification of test DNA only when the target allele if present in the sample and not when the target allele is absent (Little, 1995). ARMS employs two pairs of primers to amplify two alleles in one PCR reaction. The primers are designed such that the two primer pairs overlap at a SNP location but each match perfectly to only one of the possible SNPs. The basis of the invention is that unexpectedly, oligonucleotides with a mismatched 3'-residue will not function as primers in the PCR under appropriate conditions. As a result, if a given allele is present in the PCR reaction, the primer pair specific to that allele will produce product but not to the alternative allele with a different SNP. The two primer pairs are also designed

such that their PCR products are of a significantly different length allowing for easily distinguishable bands by gel electrophoresis or melt temperature analysis. In examining the results, if a genomic sample is homozygous, then the PCR products that result will be from the primer that matches the SNP location and the outer opposite-strand primer, as well from the two outer primers. If the genomic sample is heterozygous, then products will result from the primer of each allele and their respective outer primer counterparts as well as the outer primers. (Kitching & Seth, 2005)

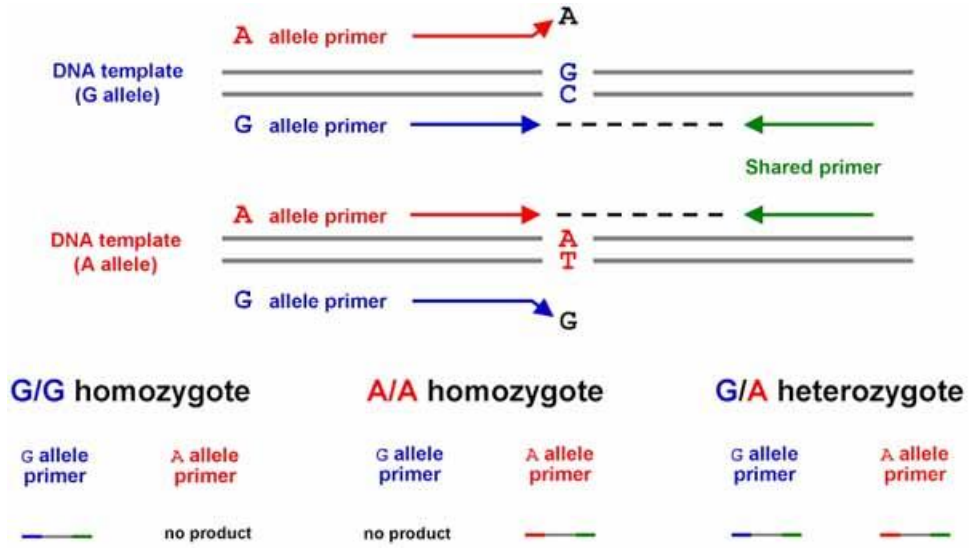
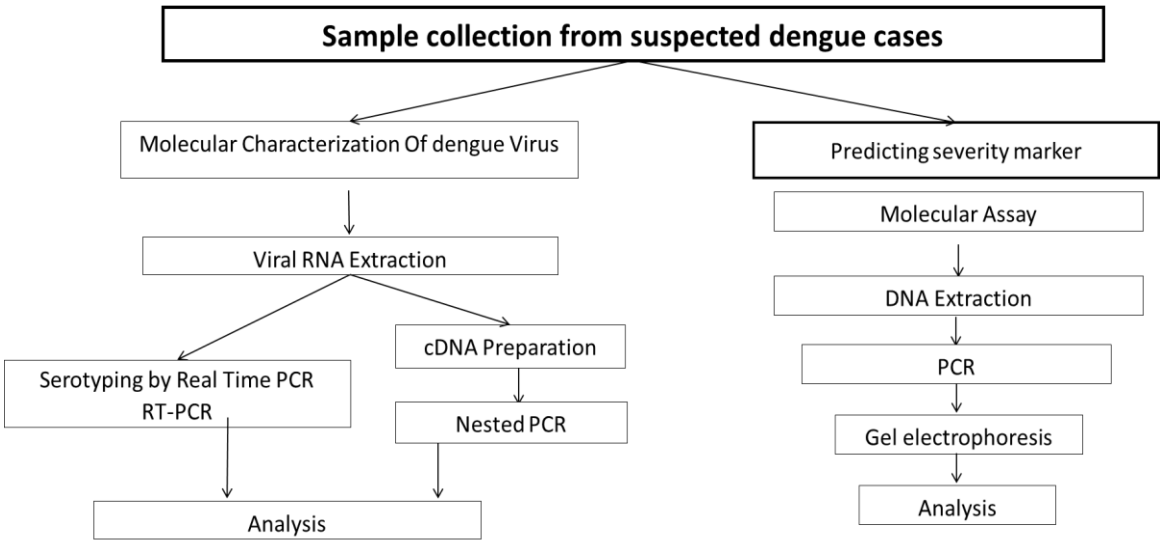


Fig 1.18. Generalized diagram of ARMS PCR design and results. Upper: Separate PCR reactions are set up to allow extension from the mutant (red) or wild-type (blue) primer only when the 3' end of the primer is complementary to the 5' nucleotide of the template DNA. In addition to the allele-specific primer, each ARMS reaction requires a second primer common to both reactions (green). Lower: the presence of amplified DNA is detected by electrophoresis of the products from the separate wild-type and mutant reaction products; wild-type (blue) amplification without mutant (red) amplification indicates wild-type homozygosity; mutant (red) amplification without wild-type (blue) amplification indicates mutant homozygosity; and amplification products in both reactions indicates heterozygosity (Kitching & Seth, 2005)

1.18 Theoretical/Conceptual Framework



1.19 Hypothesis/ Research Questions

Is host genetics associated with Dengue severity in Nepalese population?

What are the immunological and genetic differences in patients with DF, DHF and DSS?

Is there any correlation between clinical manifestations, immunological marker, gene polymorphism and virus serotype with severity?

Null Hypothesis:

Host genetics is not associated with Dengue severity in Nepalese population.

Alternative hypothesis

Host genetics is associated with Dengue severity in Nepalese population.

1.20 Research objectives

General objectives

Study of host genetics associated with dengue severity and effect of polymorphism of markers in the prognosis of severity of dengue among Nepalese population

Specific Objective

1. Identification of dengue severity based on clinical manifestation, serological and immunological analysis.
2. Molecular diagnosis and serotyping of serological positive subjects.
3. Host genome analysis on the blood samples of suspected dengue patients.
4. Study of polymorphism on the host gene, IL-10, TNF-α, INF-γ, FCγRII
5. Correlation of clinical, hematological, biochemical, immunological and polymorphism of the study subjects.

1.21 Problem statement / Rationale

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

Dengue is endemic in Terai of Nepal and recently suffered a huge outbreak affecting almost 25000 Nepalese (*EDCD|Vector Borne Disease Updates*, n.d.) Also the outbreaks have been showing some familiar pattern and there is a major outbreak in every 3 years which might lead to development of severe case in Nepal. In today's context, major focus of research on disease management is based on pathogen rather than host. Host genetics behind the disease development among Nepalese population is not yet studied which is important in understanding the disease. Prediction of severity markers help in the prognosis of dengue, dengue hemorrhagic fever or dengue shock syndrome. Synergistic effect of different markers is often misleading the clinical findings among diseased patients. This research work will resolve the discrepancy and strengthen the findings with different researchers. The cytokines and biomarkers are the new arena for the researchers for the kit and vaccine development. This research will also play pivotal role during epidemics to segregate and identify the possible patients who are at high risk of progressing to severe form or death with proper medical attention in advance.

CHAPTER II
Literature Review and Research Gaps

2.1 Dengue Virus

Dengue virus (DENV) is a mosquito-borne single stranded, positive sense encapsulated RNA virus, approximately 11 kb long. The genome of DENV codes for three structural proteins- a nucleocapsid or core (C), a membrane-associated (M), an enveloped (E) glycoprotein and seven non-structural (NS) proteins. Dengue fever caused by DENV is one of the most important arthropod-borne tropical viral infections with an estimation of 390 million infections annually (Bhatt et al., 2013). The disease is transmitted by mosquitoes, primarily *Aedes aegypti* and *Aedes albopictus*. Dengue is the most common and widespread arboviral infection in the tropical and subtropical regions with significant morbidity and mortality. The clinical picture ranges from a mild dengue fever (DF) to severe cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which might be fatal. The primary infection with any single serotype usually causes fever but the severe cases are usually an outcome of a secondary infection with a heterologous serotype along with the presence of non-neutralizing antibodies. The later phenomenon is known as Antibody Dependent Enhancement. The presence of four classical serotypes among the Dengue viruses that have no cross-protective immunity in infected patients and hence dramatically increases the complexity of this disease.

2.2 Dengue in Nepal

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

found to be co-circulating in Nepal. From the study in 2017 sample DENV-1 isolated from Nepalese population were found to be closely related to Indian isolates belonging to genotype V and DENV-2 detected was reported to be related to genotype cosmopolitan Iva which is closely related to Indonesian isolates.(Prajapati et al., 2020) With minimal cases in 2018, 2019 was reported with highest cases declaring dengue epidemic in Nepal.

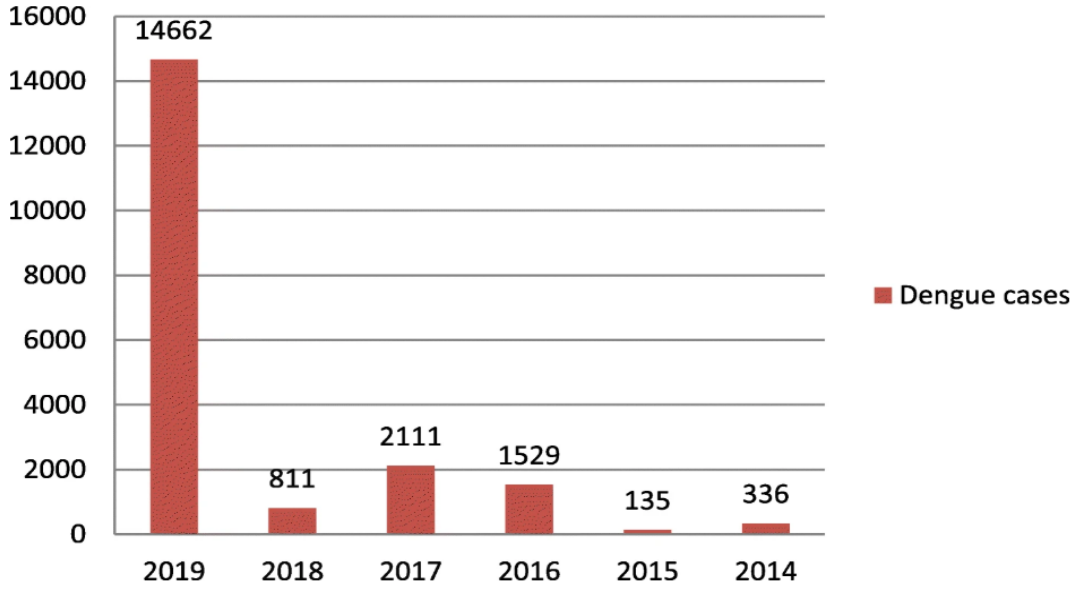


Fig 2.1. Year-wise cases of dengue cases in Nepal from 2013/2014–2018/2019 (Adhikari & Subedi, 2020)

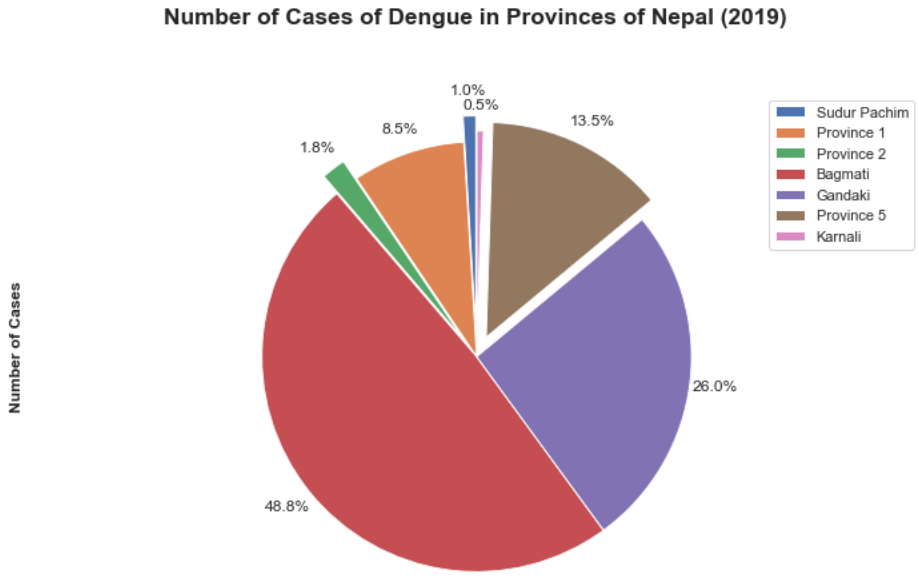


Fig2.2. Dengue confirmed Provinces in the year 2019 (Adhikari & Subedi, 2020)

2.3 Clinical manifestations of dengue

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

2.4 Laboratory diagnosis of dengue

Laboratory diagnosis methods for confirming dengue virus infection may involve detection of the virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques. After the onset of illness, the virus can be detected in serum, plasma, circulating blood cells and other tissues for 4–5 days. During the early stages of the disease, virus isolation, nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis (World Health Organization, 2009). DENV viremia is detectable 24–48 hours before fever onset and continues for 5–6 days.

During this period, infective virus, its specific RNA and the NS1 protein can be detected in patient blood, serum and plasma, and also in tissues from fatal cases (Gubler et al., 2014). Virological, molecular and serological methods are used to confirm DENV infection for epidemiological surveillance and clinical diagnosis. Anti-DENV IgM antibody detection is the most widely used test in routine practice (Tang & Ooi, 2012). Anti-DENV IgM titres in sera from patients in the acute phase of disease are measured to serologically confirmed infection, whereas patients in convalescence are identified through IgM and IgG seroconversion by comparing antibody titres in paired acute and convalescent sera (Gubler et al., 2014). For patients who are suspected of having dengue, a presumptive diagnosis can be made by the detection of anti-IgM antibodies in samples collected at day 6 of acute symptoms. Commercial kits for IgM or IgG detection in Enzyme-Linked Immunosorbent Assay (ELISA) and less-sensitive rapid test formats are available (Hunsperger et al., 2009). Reverse transcription PCR (RT-PCR), real-time RT-PCR, DENV isolation in mosquito cell lines and by mosquito inoculation facilitate confirmation and

identification of the agent virologically. Although virus isolation and identification is highly specific, it has a relatively low sensitivity and is resource-consuming and time-consuming. By contrast, DENV RNA detection provides a rapid, sensitive and specific method for virological diagnostic confirmation. NS1 protein detection provides a window of opportunity for early aetiological diagnosis. The sensitivity and specificity of DENV NS1 detection depend on the infecting serotype, the timing of sample collection and the parity of DENV infection (primary versus secondary), as well as the format of the test (Hunsperger et al.,2014).

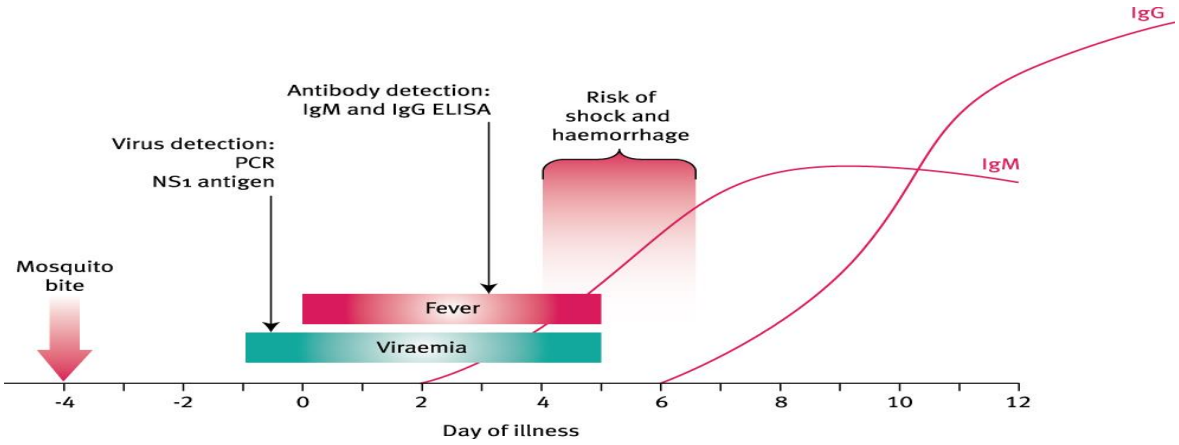


Fig 2.3. Dengue diagnosis Viremia, Non-Structural 1 (NS1) antigen and antibodies change over time; thus, different diagnostic tests will be appropriate depending on the stage of infection. ELISA, Enzyme-Linked Immunosorbent Assay; RT, reverse transcription Adapted from REF. 153, Nature Publishing Group

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

2.5. Molecular identification/ Nucleic acid detection

Reverse Transcriptase PCR and Real time PCR have been the method of choice for genome detection. Viral RNA can be extracted from serum, blood, plasma, tissues (including

formalin-fixed specimens), blood collected on filter paper, and (more recently) saliva. Since the 1990s, several reverse transcriptase-polymerase chain reaction(RT-PCR) assays have been developed. They offer better sensitivity compared to virus isolation with a much more rapid turnaround time.

Nucleic acid detection assays involve three basic steps: nucleic acid extraction and purification, amplification of the nucleic acid, and detection and characterization of the amplified product. Extraction and purification of viral RNA from the specimen can be done by traditional liquid phase separation methods (e.g. phenol, chloroform) but has been gradually replaced by silica based commercial kits (beads or columns) that are more reproducible and faster, especially since they can be automated using robotics systems. Many laboratories utilize a nested RT-PCR assay, using universal dengue primers targeting the C/prM region of the genome for an initial reverse transcription and amplification step, followed by a nested PCR amplification that is serotype-specific (Chang & Vorndamt, 1992). A combination of the four serotype-specific oligonucleotide primers in a single reaction tube (one-step multiplex RT-PCR) is an interesting alternative to the nested RT-PCR (Waggoner et al., 2013). There are other studies using various RT-PCR protocols which have reported a wide range of DENV detection rates (5% to 84%) among serologically and/or clinical confirmed dengue patients (Ahamed et al., 2017).

2.6. Severity Marker

According to WHO 2009 classification of dengue, dengue fever can be classified as severe dengue, dengue fever with or without warning symptoms. Severe dengue is defined having at least one of the following criteria: shock, fluid accumulation with respiratory distress and severe organ impairment (WHO, 2009). Severe case of dengue (DHF/DSS) with thrombocytopenia, massive multi-organ hemorrhage, circulatory failure, and neurological symptoms which usually lead to death of the patient if not treated is becoming a leading cause of child death in South East Asian countries as mentioned by Guzman et al, 2010(Guzman et al., 2010).

Factors associated with developing DHF and DSS include infection with specific viral strains, host genetics, and nutritional status but prior immunity to DENV is considered a major cause of severity. There are no defined clinical or laboratory parameters that can predict the severity of dengue cases which is most important for patient triage and give proper therapy to the patient with plasma leakage and hemorrhage (Srikiatkhachorn & Green, 2009).

Since severity is related to the immune system and the gene of the system protein, most of the severity markers are immunological cells and the gene related to them and its

polymorphism. Some of the markers which could be used in prediction of severe dengue cases are:

Clinical markers:

They are currently used marker in the health center for diagnosis of the DENV infected patient which includes marked thrombocytopenia (platelet count<100,000 cells per cu mm), Hepatomegaly and liver tenderness (Elevated liver transaminases, AST and ALT) (Srikiatkhachorn & Green, 2009).

- Platelets: One common signs for identification of DHF is the platelet count less than 100000 cells per cu mm. Lower number of platelet count is termed as thrombocytopenia and this cannot be considered as preliminary indicator of DHF as the platelet counts lower may also occur during DF. Moreover, thrombocytopenia serves as pointer referring progression towards severe forms of disease. Lower platelet counts lead to spontaneous hemorrhage in DHF patients that may further be complicated by accompanying plasma leakage and shock. Immune complexes comprising dengue antigen formed over the platelet surface tend to destruct the platelet. Platelet related antibodies also lead to decrease the platelet counts and cause clinical severity during acute secondary infections.

- Liver Enzymes: DF and DHF may cause symptoms such as hepatomegaly and liver tenderness. Elevated liver transaminases (AST and ALT) act as early indicators for severe dengue disease. Autopsy cases related to liver pathology depicted mild infiltration of monocytes and lymphocytes along with variation in necrosis patterns. In vitro, DENV-specific CD4+ T cells are observed to facilitate hepatocytes induced by bystander cytotoxicity resulting through the Fas pathway. Likewise, CD8+ T cells, in vivo involved in experimental models relating liver pathology of DENV infection in mice. Thus, this illustrates that liver pathology can be aided by both virus and immune responses mediated by host against the virus.

Biological markers:

Biological markers are the cells of the immune system that produce the signals and actively participate in the disease diagnosis and severity analysis and alteration in soluble receptors.

- Dengue Viral Products: Viral loads demonstrate whether the patients have DF or DHF. Likewise, the plasma levels of viral product, NS1 antigen also are found to be higher in DHF as compared to its DF counterparts. Timing of peak NS1 levels occurs at febrile stage while plasma leakage occurs at severe dengue

cases. Thus, higher viral load and level of NS1 antigen are considered a risk factor for the severe forms of disease and can be used as preliminary indicators.

- Cytokine signals produced by immune system: Various cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-10, IL-2, IFN-γ etc. are produced by the infection of cellular targets with DENV. Cytokines with proinflammatary and vascular permeability are found to rise in DHF however their part in plasma leakage is indistinguishable. Regardless of the fact, these are considered early signs for severe dengue disease.
- During Late Febrile stage and Defervescence phase of disease: During the secondary infection with a DENV serotype, cross reactive T cells are activated that upraise the levels of IL-2, IFN-γ, TNF- α in DHF compared to DF. IL-10 levels have been observed to increase referring to enhanced regulatory response to more immense immune activation in DHF. Cellular receptors for cytokines and other ligand in soluble forms are generated in elevated levels in the plasma of DHF patients. Increased level of TNF-α, CD4, CD8 have been reported in studies to signify the severity and form of dengue infection. Likewise, increased levels of a potent permeability enhancing cytokine, vascular endothelial growth factor (VEGF), in DHF patients during plasma leakage have also been reported. This increase was associated with a progressive decline of the circulating soluble form of VEGFR-2 receptor in the plasma of DHF patients. Thus, these allow them to be a candidate to determine DHF and DF patients (Srikiatkhachorn & Green, 2010).

Molecular/genetic markers:

They include Single Nucleotide Polymorphism (SNP) in the gene related to the key molecules associated with the host DENV immunologic defense system. They are usually seen in population of DENV endemic region which either makes an individual susceptible or protective to the severe form (DHF/DSS). Some of the genes involved are Human leukocyte antigen (HLA), Dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)- grabbing non-integrin (DC-SIGN), $F_C\gamma$RIIa, transporter associated with antigen processing (TAP), vitamin D receptor (VDR), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), acute plasma glycoprotein, mannose binding lectin (MBL) and human platelet-specific antigens (HPA) and cytokines (Fang et al., 2012).

Molecules	Function	Location and SNPs	Association with DENV	Source
FcγRIIa	IgG receptor that acts as linker between humoral and cellular immune systems	1q23 131 A/G (His/Arg)	Arg homozygote protective His homozygote increases the risk	Garcı'a et al. (2010) and Loke et al. (2002)
IL-10	Immune inhibitor	1q31-32 1082 A/G 819 C/T 592 A/C	Carriage of 1082/ 819/ 592 haplotype ACC/ATA may be a DHF risk factor	Fernandez-Mestre et al. (2004) and (Perez et al., 2010)
TNFα	pro-inflammatory cytokine	12q15 308G/A	A allele is protective against DF	(Santos et al., 2017)
IFNγ	pro-inflammatory cytokine	12q15 874 A/T	T allele is Risk for DHF A/T protective effect against DF and DHF	(Santos et al., 2017)

Re-emergence of DF and DHF is a recurring challenge globally. Various studies on dengue infection have revealed that the genetic constituents of dengue elements: virus, vector and hosts play a primeval part in pathogenesis of the disease. Evolution of these constituents has been phenomenal and extensive studies have further contributed to understanding DHF progression. Hence, analysis of genetic variability can support to identify possible factors and their mechanism of disease development. There are studies that depicts insightful influence of host's genetic sequence on resistance to infections. Susceptibility to diseases has a genetic basis and molecular epidemiology and virulence of pathogens, resistance to drugs, vaccines and antibiotics in certain conditions. From the study of Sierra et al., (2007), it was found that there is a lower probability of Africans developing DHF/DSS in comparison to Caucasian counterpart, referring that host's genetic changes have key impression on clinical features of dengue infection. Considering the studies that relates genetic polymorphism and dengue infection, association between human major histocompatibility complex (HLA) class I/II genes and dengue infection were observed (Lan & Hirayama, 2011). Likewise, tumor necrosis factor (TNF) was found to stimulate DHF infections. TNF-α- 308A allele was noted as susceptible factor for DHF development in South American while no risk to Southeast Asian patient regardless of the latter consisting larger sample size (Loke et al., 2001). Another research carried out in Brazilian population identified association of IFNG +874 A/T, TNFA-308G/A, IL-10-819 C/T genotypes as an aspect for affecting susceptibility, guard clinical phenotypes of dengue and regarded as good predictive indicators (Santos et al., 2017). Epidemiological data from

the studies carried out in Thailand illustrated that 99% of DHF cases had heterotypic antibody to the serotype of dengue virus that caused DHF. Additionally, 90% of the children infected were in a secondary infection with a serotype of DENV different from the one that caused the primary infection. Cross-reactive antibodies lack neutralizing activity are induced in primary infection. So in secondary infection, dengue virus and non-neutralizing antibodies form virus-antibody complexes which binds to Fcγ receptors on target cells and result to enhance the infection. This is one of the studies that states that improving antibodies intensify number of dengue infected cells and levels of viremia leading to DHF (Kurane, 2007). Another study established that initial activities of natural killer cells, B cells and IgM and later actions of IFN-γ and IgG play a key role in shield against DENV infection (Shresta et al., 2004). However, host's resistant responses to eternal defensive immunity by natural DENV infection is not understand well.

Despite all the knowledge about the severity markers, the proper establishment is yet to be done. As no animal models that can imitate human DHF are available, studies in dengue patients is must. The data from the human studies is difficult to interpret and assimilate due to the heterogeneity in the sample. So accuracy in the timing and the methods of sample collection and processing determine the reliability of data. The development of sequence based typing technologies in recent years has also provided new and powerful tools to study human heterogeneity in association with DENV disease by human genomic SNP analysis, such as single-stranded conformational polymorphism and sequencing-based typing (Stephens, 2009).

Interferon Gamma (INFγ):

IFN-γ is also known to supplement antibody-dependent enhancement in the course of dengue disease development. Increased serum levels of IFN-γ in naturally infected patients, and show a correlation between serum IFN-γ levels and disease severity in some study indicating the negative role of IFN-γ in dengue immunity. In the comparative study of healthy control and dengue, protective association of genotype heterozygosity of +874 A/T in INF-γ was observed. The T allele has been associated with increased indoleamine-pyrrole 2, 3-dioxygenase activity, and the growth of this enzyme activity promotes a high conversion of tryptophan to kynurenine, a molecule involved causing the vascular permeability leading to hemorrhage in DHF. From the study Brazilian population, genotypes TT,T/A and AA were associated with high, intermediate and low production of IFNG, respectively, wherein the T allele was associated high levels as it have influence in gene transcription level (Santos et al., 2017b).

Interleukin 10 (IL10):

From a study in Cuban population haplotypes ACC and ATA for the -1082 (G>A), -819 (C>T), and -592 (C>A) SNPs are found to be associated with low IL-10 expression leading to Dengue Hemorrhagic fever (DHF) due to inefficient immune regulation. A study in Indian population showed positive association of -819 SNP GA genotype against DHF than DF while in Brazilian population there was no association of IL10 -819 C>T SNP with severe dengue(Xavier-Carvalho et al., 2017).

$F_C\gamma$RIIa :

$F_C\gamma$RIIa (CD32), member of Fcγ receptor family is a low-affinity immunoglobulin G (IgG) receptor. $F_C\gamma$RIIa interacts with IgG 1–4 subtypes by binding to their Fc fragments and is expressed on the surface of most of the hematopoietic cells. A single-point mutation A > G (rs1801274) in $F_C\gamma$RIIa, replacing histidine (H) at position 131 with arginine (R), results in altered affinity of the receptor for different subclasses of IgG such that the form having histidine (genotype 131HH) interacts more efficiently with IgG2 while the one with arginine (genotype 131RR) shows more affinity for binding IgG1 and IgG3 subclasses. It was assumed that polymorphism of $F_C\gamma$RIIa (494 A/G) gene may be the reason for variability in immune response and may shed some light on the pathogenesis of severe dengue infection. Studies in Vietnamese, Cuban, Pakistani and Mexican Population supports the involvement of the gene polymorphism in dengue pathogenesis.(Mohsin et al., 2015)

A study in Vietnamese population showed the positive association of homozygosity of the amino acid Arg $F_C\gamma$RIIa-R/R131 with DHF/DSS. The significantly different frequencies of the Arg variant at position 131 around the world may be moderately endorse the distribution of DENV infection in different regions and races (García et al., 2010). A study in Cuban population also provide the evidence that the FcγRIIa-H/H131 genotype was negatively associated to dengue leading to severe cases as HH genotype was detected at an increased frequency in individuals who suffered from clinical dengue (both DF and DHF/DSS), whereas RR genotype was found predominated in those individuals with an asymptomatic dengue infection.(García et al., 2010)

TNF α:

Pro-inflammatory cytokine TNF-α, if found secreted in high level in the system induces apoptosis of endothelial cells leading to hemorrhage in a mouse model of DHF. Single nucleotide polymorphism in promoter region of TNF α gene plays role in regulation of its level in the system. Study in various populations shows the association of variant allele (A)

of TNF-α 308 with DHF. In the study performed in Sri Lankan and Malaysian Population - 308 homozygosity (GG) was found to be associated with progression of dengue fever to severe dengue disease (Alagarasu et al., 2013).

Therefore, understanding the background, procedure and analysis of dengue infection and considering the present scenario of health facilities provided to dengue patients in Nepal, the research has been carried out to understand and identify potential ambiguities that have resulted regular occurrence of dengue epidemics. Dengue epidemic has been re-emerging in Nepal every three years in past two decades. As the fundamental basis for research works in Nepal relating the causes, diagnosis, genetics and host severity has not been well determined, this research is considered to study the host genetics associated with dengue severity and effect of polymorphism of markers in the prognosis of severity of dengue among Nepalese population. This research work also resolves the inconsistency and strengthen the findings with different researchers. This research works as a basis during epidemics to segregate and identify the possible patients who are at high risk of progressing to severe form or death and provide them proper medical attention in time.

CHAPTER III
Materials and methods

3.1. Ethical approval

The ethical clearance for the molecular and immunological study of dengue has already been taken from NHRC (Reg No: 121/2019). The samples have been collected after taking written consent from the patients. The laboratory work has been done in Central Department of Biotechnology, TU which is well equipped and in accordance with the ethical guidelines of NHRC. The universal safety guidelines were followed for the transportation of samples and laboratory works.

3.2. Site selection

The study was hospital based and four major hospitals from four districts, where most of referral cases from eastern to central regions of Nepal were found were included. The samples were collected from Sukraraj Tropical and Infectious Disease Hospital (STIDH) - Kathmandu, Chitwan Medical College and Teaching Hospital (CMC)-Chitwan, Bijayapur Hospital (BH)-Dharan and Hetauda Hospital (HH)-Hetauda**.** The samples were collected in the four-month time duration from June to October, which were the peak months for dengue epidemics of the year 2019. The study was designed as a cross-sectional descriptive study.

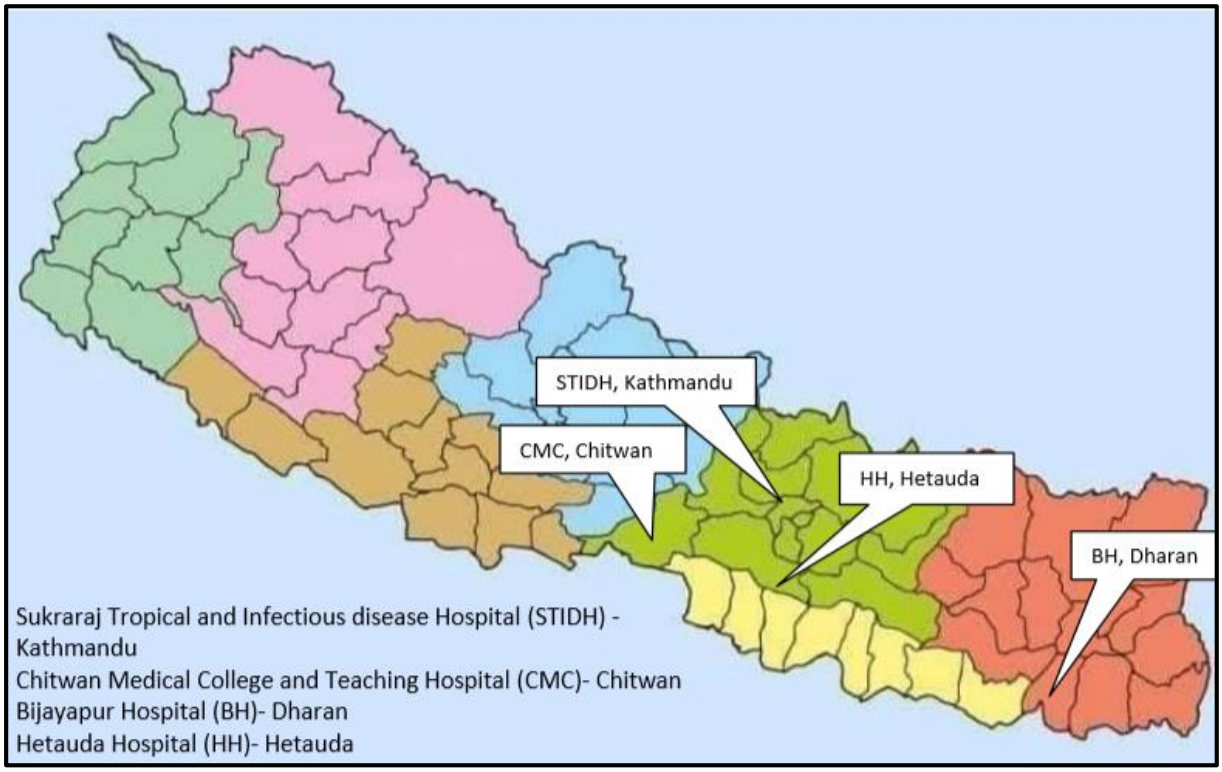


Fig 3.1. Hospitals where samples were collected: STIDH, CMC, BH and HH

Patients with an acute febrile illness of 2-7 days' duration and showing the symptoms of dengue fever were enrolled in the study. WHO, 2009 guidelines were strictly followed for determination of dengue-case identification. Only those subjects were enrolled who had fever with two of the following symptoms: nausea, vomiting, rashes and aches and pains. The other recorded symptoms were abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, liver enlargement >2cm, severe bleeding, shock, organ failure or fluid accumulation with respiratory distress. Demographic information like age, sex and address, symptoms and diagnosis were recorded by attending physicians in the Study CRF (Case report form).

3.3 Sample collection and storage

The whole blood samples from NS1 Positive patients by RDT test were collected and stored at -80°C until further processing. In case of the samples from STIDH, blood was collected and immediately transferred to CDBT for further processing. In other collection sites, whole blood was transported to CDBT and further processing was done there. The samples were classified according to WHO classification their severity as Dengue without Warning signs (Fever with two of the following symptoms: nausea, vomiting, rashes, muscles and joints pain), Dengue with Warning signs (abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy or liver enlargement >2cm) and Severe Dengue (severe bleeding, shock, organ failure or fluid accumulation with respiratory distress).

A total of fifteen healthy control samples were included in this study. The healthy controls were healthy people who did not show any symptoms of illness and they were negative to the rapid diagnostic tests for NS1, IgM and IgG tests of dengue. Among them, six samples were collected from Bijayapur hospital, Dharan which is an endemic area for dengue in the year 2019 and nine samples were from Central Department of Biotechnology, Kathmandu which is non-endemic for dengue. The healthy controls were used for the study of change in clinical parameters with respect to dengue and non-dengue cases.

3.4 Molecular Assay

Viral RNA Isolation by QIAamp® viral RNA Mini kit

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

Preparation of reagents

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

RNA isolation by using Spin protocol

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

Serotyping by Reverse Transcriptase Real-Time Polymerase Chain Reaction

Dengue diagnosis kit provided by Center for Disease Control and Prevention (CDC), USA were used for identification of DENV serotypes keeping separate dengue controls (DENV 1 to 4) catered in the kit. A master mix at the rate of 20 µL per well were prepared [RNase-free water-3.7µL, 2Xs Buffer-12.5, Primer Forward and Reverse for DENV 1-4 (D1 F/R–D4F/R)-100 µM, Probe for DENV 1-4 (P1-P4) -10 µM, Superscript III Platinum one-step qRT-PCR system enzyme (Invitrogen Cat No. 11732-020)-0.5 µL] in two separate labeled Eppendorf tubes for DENV reaction and Human Specimen Control (HSC) reaction. The PCR

plate was loaded to Real-Time PCR machine (BIORAD-CFX96 Touch™ Real-Time PCR Detection System) having program set of reverse transcription at 50°C for 15 min, inactivation at 95°C for 2 min followed by 45 PCR amplification cycles (melting temp. 95°C for 15 sec with ramp rate 4.4°C/s, and annealing and extension temp. 60°C for 1 min. with ramp rate 2.2°C/s), melting curve set for 1 cycle (melting temp. 95°C for 10 sec, and annealing and extension temp. 65°C for 1 min.; continuous acquisition mode at 97°C); cooling program set at 50°C for 20 min with ramp rate 2.2°C/s) and finally the reaction was left at 4°C. The results were interpreted as positive to DENV 1, 2, 3 and 4 if the amplification of probes; FAM (Blue), VIC (Green), Texas Red (Red) and Cy5 (Purple) curve were amplified respectively within CT value 37.

Table 3.1: Master-mix preparation for multiplex PCR (DENV1-4)

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

Table 3.2: Master-mix preparation for multiplex PCR (DENV1-4)

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

Viral RNA Isolation by TRIzol

This protocol uses TRIzol (also known as TRI REAGENT) for the isolation of total RNA. Trizol is a mixture of guanidine thiocyanate and phenol, which effectively dissolves DNA, RNA and protein on homogenization or lysis of sample. After adding chloroform, centrifugation was performed and the mixture separates into 3 phases with the upper clear aqueous phase containing the RNA. The next steps in the extraction are washes and precipitation of the RNA. The first part of the protocol from the homogenized tissue in Trizol to the point of an RNA pellet in 75% ethanol, the whole process takes less than 1 hour. The RNA can then be stored for longer periods of time at -80°C.

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

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

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

3. RNA Precipitation: The RNA was precipitated from the aqueous phase by mixing with 300µL of isopropyl alcohol. The mixture was centrifuged for 10 minutes at 10,000 rpm (The RNA precipitate forms a gel-like pellet on the side of the tube at bottom).

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

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

cDNA preparation

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

Table 3.3: Components used for cDNA Synthesis

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

The complete reaction mix was incubated in a thermal cycler using the following protocol:

Table 3.4: Reaction Protocol

Priming	5min at 25°C
Reverse transcription	20min at 46°C
RT- inactivation	1min at 96°C
Optional step	Hold at 4°C

Nested RT-PCR:

Nested PCR of the cDNA was performed for envelope protein primers (Table 3.3) designed by targeting partial region of envelope and Solis Biodyne -5x FIREPol® Master Mix (Cat. No. 04-11-00125). Primers AA6EP_F (10 pm/µl) and AA7EP_R (10 pm/µl) were used for the first round PCR. The PCR conditions were set to initial denaturation at 98°C for 5 min followed by 35 cycles of the PCR with cycling conditions of denaturation 95°C, annealing 59°C and extension 72°C. The final extension was done at 72°C for 5 min and the reaction was kept at hold at 4°C. The PCR product was diluted in the ratio 1:10 and used as template for second round PCR using serotype-specific primers (AA8EP_F & AA9EP_R for DENV1, AA10EP_F & AA11EP_R for DENV2, AA12EP_F & AA13EP_R for DENV3 and AA14EP_F & AA15EP_R for DENV4). The PCR conditions were same as of the first round and only annealing temperature was different for different serotypes (54.5°C, 53.5°C, 56°C and 53.5°C for DENV1, DENV2, DENV3 and DENV4 respectively). Agarose gel electrophoresis was run in 1.5% agarose and the bands were visualized under UV transilluminator.

Table 3.5. Primers used for dengue virus confirmation and serotype specific PCR

Primer	Sequence	Location (w.r.t. ref. seq.)	Ref. seq. (GenBank accession no.)	Expected size (bp)
AA6EP_F	TGGCTGGTGCACAGACAATGGTT	616-638	MF381049.1	600
AA7EP_R	GCTGTGTCACCCAGAGTGGCCAT	2146-2168	KY849753.1	
AA8EP_F	GGGGCTTCAACATCCCAAGAG	667-687	MH680237.1	500
AA9EP_R	GCTTAGTTTCAAAGCTTTTTCAC	1170-1148	MG933845.1	
AA10EP_F	ATCCAGATGTCATCAGGAAAC	808-828	MH110734.1	337
AA11EP_R	CCGGCTCTACTCCTATGATG	1153-1134	MG895167.1	
AA12EP_F	CAATGTGCTTGAATACCTTTGT	893-914	MG895205.1	189
AA13EP_R	GGACAGGCTCCTCCTTCTTG	1089-1071	MH173166.1	
AA14EP_F	GGACAACAGTGGTGAAAGTCA	953-973	MH178419.1	138
AA15EP_R	GGTTACACTGTTGGTATTCTCA	1095-1074	MG895393.1	

Table 3.6: Master-mix preparation for PCR

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

Table 3.7.: PCR Program for Envelope Protein of Dengue

PCR Steps	**Temperature of all 4 Dengue serotype**				**Time**
	DENV1	**DENV2**	**DENV3**	**DENV4**	
PCR1					
Initial Denaturation	95°C	95°C	95°C	95°C	5 min
Denaturation	95°C	95°C	95°C	95°C	1 min
Annealing	59°C	59°C	59°C	59°C	45 sec
Extension	72°C	72°C	72°C	72°C	45 sec
Final extension	72°C	72°C	72°C	72°C	5 min
Hold	4°C	4°C	4°C	4°C	
PCR2					
Initial Denaturation	95°C	95°C	95°C	95°C	5 min
Denaturation	95°C	95°C	95°C	95°C	1 min
Annealing	**54.5°C**	**53.5°C**	**56°C**	53.5°C	45 sec
Extension	72°C	72°C	72°C	72°C	45 sec
Final extension	72°C	72°C	72°C	72°C	5 min
Hold	4°C	4°C	4°C	4°C	

Human Genomic DNA Extraction

Genomic DNA was extracted from the whole blood by non-enzymatic salting out method.

Preparation of reagents

The reagents were prepared as described below:

a. TKM 1 Buffer / Low salt buffer (500 ml): 0.605 g of TrisHCl (10mM) pH 7.6, 0.372 g of KCl (10 mM), 1.016 g of $MgCl_2$ b (10 mM), 0.372g of EDTA (2mM) was dissolved in 500ml of distilled water
b. TKM 2 Buffer / High salt buffer(100 ml): 0.121 g of TrisHCl (10mM) pH 7.6, 0.074 g of KCl (10 mM), 1.203 g of $MgCl_2$

c. SDS: One gram of sodium dodecyl sulphate was dissolved in 10ml distilled water. (10 mM), 0.074 g EDTA (2mM), 0.467 g of NaCl (0.4 M) was dissolved in 100ml of distilled water
d. 6M NaCl: 8.765 g of NaCl was dissolved in 25 ml of distilled water.
e. TE Buffer: 0.030 g of TrisHCl (10mM) pH 8.0, 0.009 g of EDTA (1mM) was dissolved in 100ml of distilled water

DNA extraction Protocol

RBC Lysis

500 µl of TKM 1 and 15 µl of 1x Triton-X were added to 500 µl of heparinized blood in a clean 1.5 ml Eppendorf and was mixed by inversion. Cells were centrifuged at 2200 rpm for 10 minutes and the supernatant was discarded. Pellet was washed by adding 500 µl TKM 1 and centrifuged at 2200 rpm for 10 minutes. This step was repeated until RBC lysis was complete and a white pellet of WBCs was obtained.

Cell Lysis

To the cell pellet, 80µl of TKM 2 and 5 µl of 10% SDS were added and mixed thoroughly followed by incubation at 37° C for 5 minutes. At the end of incubation, 100 µl of 6M NaCl was added and vortexed to precipitate the proteins. Cells were centrifuged at 12000 rpm for 5 minutes.

Precipitation of DNA

The supernatant was transferred into a new Eppendorf tube containing equal volume of isopropanol. DNA was precipitated by inverting the Eppendorf slowly and Eppendorfs were centrifuged at 12000 rpm for 5 minutes to pellet down the DNA. Supernatant was discarded, 70% ethanol was added and mixed slowly to remove any excess salts. Finally, the tubes were centrifuged at 12000 rpm for 5 minutes to pellet down the DNA. Supernatant was discarded and DNA air-dried. After thorough drying, 50 µl of TE buffer was added to dissolve the DNA.

Agarose gel electrophoresis was done to check presence of DNA and remaining DNA was stored at -20°C for further experiments.

3.5 Single Nucleotide Polymorphism Detection

Polymorphism of three different genes were identified using amplification refractory mutation system PCR (ARMS PCR) using the allele specific primers for each gene.

Interleukin 10 (IL-10): SNP at two different locations 819 C/T and 1082 A/G were determined in all the individual included in the study using primer sequences as follows: IL-10(1082) primer G allele '5 -CTACTAAGGCTTCTTTGGGAG-3'; IL-10 Primer A allele, '5ACTACTAAGGCTTCTTTGGGAA-3'; IL-10(1082) Generic primer (antisense), '5 -CAGTGCCAACTGAGAATTTGG-3'; IL-10 (819) Primer C allele : 5' -CCCTTGTACAGGTGATGTAAC-3'; IL-10 (819) Primer T allele, '5-ACCCTTGTACAGGTGATGTAAT-3'; IL-10(819) Generic primer (antisense): 5'-AGGATGTGTTCCAGGCTCCT-3' with common Internal control primer; sense: 5' -GCCTTCCCAACCATTCECTTA-3' and antisense: 5' -TCACGGATTTCTGTTGTGTTTC-3'. Amplification of target DNA was performed under following conditions: The PCR conditions were set to initial denaturation at 98°C for 2 min followed by 35 cycles of the PCR with cycling conditions of denaturation 95°C, annealing 61°C and extension 72°C. The final extension was done at 72°C for 10 min and the reaction was kept at hold at 4°C. PCR amplicons were then submitted to a 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized under ultraviolet light.

Table 3.8.: Primers used for polymorphism study in IL-10

	Primer	Sequence	Expected size (bp)
IL-10 (1082)	G allele	5'-CTACTAAGGCTTCTTTGGGAG-3'	258
	A allele,	5'-ACTACTAAGGCTTCTTTGGGAA-3'	
	Generic primer	5 -CAGTGCCAACTGAGAATTTGG-3'	
IL-10 (819)	C allele	5'-CCCTTGTACAGGTGATGTAAC-3'	233
	T allele	5'-ACCCTTGTACAGGTGATGTAAT-3'	
	Generic primer	5'-AGGATGTGTTCCAGGCTCCT-3'	
Internal Control	Forward	5'-GCCTTCCCAACCATTCECTTA-3'	429
	Reverse	5'-TCACGGATTTCTGTTGTGTTTC-3'	

Table 3.9: Master-mix preparation for PCR

Reagents	**Vol. per reaction**
Master mix (5x)	5µl
G/A allele/ Sense Primer (10pM/ µl)	1µl
Generic/ antisense primer /(10pM/ µl)	1µl
Template cDNA	2.5µl
DNA grade water	15.5µl
Total	**25µl**

Table 3.10.: PCR Program for Interleukin 10 (IL-10) gene:

PCR Steps	Temperature	Time
Initial Denaturation	95°C	2 min
Denaturation	95°C	30sec
Annealing	61°C	1min
Extension	72°C	1min
Final extension	72°C	10 min
Hold	4°C	∞

Interferon γ (INF γ):

The polymorphism of IFN-γ (+874 AT – rs2430561) gene was identified by using the primer sequences were as follows: IFN-γ primer A allele, 5'-TTCTTACAACACAAAATCAAATCA-3'; IFN-γ primer T allele, 5'-TTCTTACAACACAAAATCAAATCT-3'; GH (growth hormone) internal control forward, 5'-GCCTTCCCAACCATTCCCTTA-3'; GH (growth hormone) internal control reverse, 5'-TCACGGATTTCTGTTGTGTTTC-3'; and IFN-γ generic primer, 5'-TCAACAAAGCTGATACTCCA-3'. Amplification of target DNA was performed under following condition: heating at 95°C for 3 min, 10 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 50 s, elongation at 72°C for 40 s, followed by 20 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 50 s, elongation at 72°C for 50 s, final elongation at 72°C for 7 min, and final hold at 4°C for 5 min. Agarose gel electrophoresis was run in 1.5% agarose and the bands were visualized under UV transilluminator.

Table 3.11.: Primers used for polymorphism study in IFN-γ

Primer	Sequence	Expected size (bp)
A allele	5'-TTCTTACAACACAAAATCAAATCA-3'	261
T allele,	5'-TTCTTACAACACAAAATCAAATCT-3'	
Generic primer,	5'-TCAACAAAGCTGATACTCCA-3'	
GH internal control Forward	5'-GCCTTCCCAACCATTCCCTTA-3'	429
GH internal control Reverse	5'-TCACGGATTTCTGTTGTGTTTC-3'	

Table 3.12.: Master-mix preparation for PCR

Reagents	Vol. per reaction
Master mix (5x)	5µl
A/T allele/ Sense Primer (10pM/ µl)	1µl
Generic/ antisense primer /(10pM/ µl)	1µl
Template DNA	2.5µl
DNA grade water	15.5µl
Total	**25µl**

Table 3.13.: PCR Program for interferon gene:

PCR Steps	Temperature	Time	Cycle
Initial Denaturation	95°C	3 min	-
Denaturation	95°C	15 sec	10
Annealing	65°C	50 sec	
Extension	72°C	40 sec	
Denaturation	95°C	20 sec	20
Annealing	55°C	50 sec	
Extension	72°C	50 sec	
Final extension	72°C	10 min	-
Hold	4°C	∞	-

Tumor Necrosis Factor Alpha (TNF α): The polymorphism of TNF α (+308 A/G - rs1800629) gene was identified by using the primer sequences were as follows: F1 5'-TCTCGGTTTCTTCTCCATCG-3', R1 5'-ATAGGTTTTGAGGGGCATGG-3', R2 5'-ATAGGTTTTGAGGGGCATGA-3', R3 5'-GAGTCTCCGGGTCAGAATGA-3'. Amplification of target DNA was performed under following conditions: The PCR conditions were set to initial denaturation at 98°C for 2 min followed by 30 cycles of the PCR with cycling conditions of denaturation 95°C, annealing 62°C and extension 72°C. The final extension was done at 72°C for 10 min and the reaction was kept at hold at 4°C. PCR amplicons were then submitted to a 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized under UV transilluminator.

Table 3.14.: Primers used for polymorphism study in Tumor Necrosis Factor Alpha (TNF α)

Primer	Sequence	Expected size (bp)
Generic primer (F)	5'-TCTCGGTTTCTTCTCCATCG-3'	184
G allele (R1)	5'-ATAGGTTTTGAGGGGCATGG-3'	
A allele(R2)	5'-ATAGGTTTTGAGGGGCATGA-3'	
Internal Control (F)	5'-TCTCGGTTTCTTCTCCATCG-3'	531
Internal Control (R3)	5'-GAGTCTCCGGGTCAGAATGA-3'	

Table 3.15.: Master-mix preparation for PCR

Reagents	Vol. per reaction
Master mix (5x)	5µl
F1 Primer (10pM/ µl)	1µl
R1/R2/R3 antisense primer /(10pM/ µl)	1µl
Template DNA	2.5µl
DNA grade water	15.5µl
Total	**25µl**

Table 3.16. PCR Program for Tumor Necrosis Factor Alpha (TNF α) gene:

PCR Steps	**Temperature**	**Time**
Initial Denaturation	94°C	2 min
Denaturation	95°C	30sec
Annealing	62°C	1min
Extension	72°C	1min
Final extension	72°C	10 min
Hold	4°C	∞

F_C-gamma receptor IIa ($F_C\gamma$RIIa) :

The polymorphism of $F_C\gamma$RIIa (494 A/G) gene was identified by PCR using sense primer P63 (5'-CAAGCCTCTGGTCAAGGTC-3') and antisense primer $F_C\gamma$RII-30 (5'-CAATGACCACAGCCACAATC-3'). Nested PCR was performed using the specific sense primers 494A and 494G (5'-ATTCTCCC[A/G]TTTGGATC-3'), respectively and P52 as an antisense primer (5'-GAAGA GCTGCCCATGCTG-3'). PCR products were run on agarose gel in a DNA electrophoresis, and the allelic forms of the $F_C\gamma$RIIa gene of each individual were determined.

Table 3.17.: Primers used for polymorphism study in F_C-gamma receptor IIa ($F_C\gamma$RIIa)

Primer	**Sequence**	**Expected size (bp)**
FCyRIIP63F	CAAGCCTCTGGTCAAGGTC	230
FCyRIIP63R	CAATGACCACAGCCACAATC	
FCyRII494A	ATTCTCCCATTTGGATC	
FCyRII494G	ATTCTCCCGTTTGGATC	150
FCyRIIP52	GAAGAGCTGCCCATGCTG	

Table 3.18.: Master-mix preparation for PCR

PCR1		**PCR2**	
Reagents	**Vol. per reaction**	**Reagents**	**Vol. per reaction**
Master mix (5x)	5µl	Master mix (5x)	5µl
Sense primer P63 (10pM/ µl)	1µl	Specific sense primers 494A/G (10pM/ µl)	1µl
Antisense primer $F_C\gamma$RII-30 (10pM/ µl)	1µl	P52 (antisense primer) (10pM/ µl)	1µl
Template DNA	2.5µl	Diluted PCR 1 (1:10)	1µl
DNA grade water	15.5µl	DNA grade water	17µl
Total	**25µl**	**Total**	**25µl**

Table 3.19 PCR Program for F_C-gamma receptor IIa ($F_C\gamma$RIIa) gene:

PCR Steps for PCR1	**Temperature**	**Time**
Initial Denaturation	94°C	2 min
Denaturation	95°C	30sec
Annealing	62°C	1min
Extension	72°C	1min
Final extension	72°C	10 min
Hold	4°C	∞

PCR Steps for PCR2	**Temperature**	**Time**
Initial Denaturation	94°C	2 min
Denaturation	95°C	30sec
Annealing	62°C	1min
Extension	72°C	1min
Final extension	72°C	10 min
Hold	4°C	∞

3.6 Statistical Analysis:

Data obtained in this study were recorded in MS-Excel 2010 and statistical analyses were performed using Graph-pad Prism V 7.0 software. The descriptive statistics were reported using the Mean ± Standard deviation for descriptive parameters. Genotype frequencies were calculated by direct counting and compared between study groups.

CHAPTER IV
Results

4.1 Study Population

Whole blood samples were collected from afore mentioned hospitals that were reported with positive rapid diagnostic test for NS1 antigen of dengue. Out of total samples (n=129), twenty-one samples are included in the study. Among twenty-one samples, ten samples were from Sukraraj Tropical and Infectious Disease Hospital, five from Chitwan Medical College (CMC), four from Hetauda Hospital(HH), Makawanpur and 2 from Bijayapur Hospital(BH), Dharan. The distribution of the study samples included twenty districts all of the provinces except Province-6 and samples included in the experiment till now are from eight different districts.

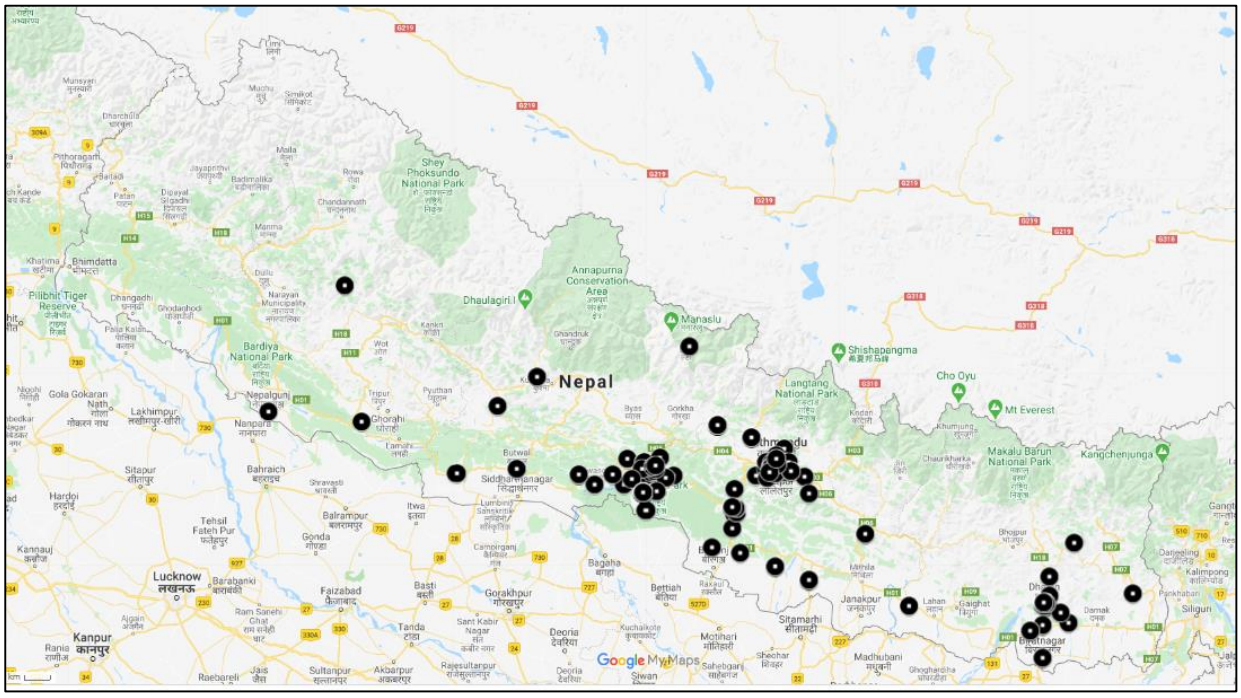


Fig 4.1. Sample Distribution Sites. The samples were from 20 districts of Nepal including all the provinces except province-6 and 7.

The study consisted of 60% males (n=77) and 40% (n=52) females with the ratio of male: female as 3:2. The age group ranged from 3 years to 80 years with mean age 31.905.

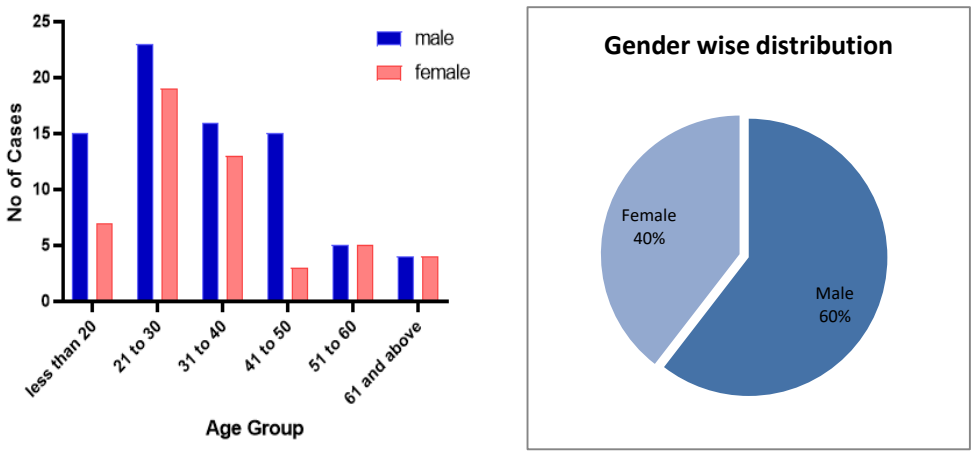


Fig 4.2 Population distribution among different age group for male and female of suspected cases (n=129)

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

Real Time RT-PCR

PCR of nine NS1 positive samples were performed. All nine samples were positive to Real-Time PCR performed by using Center for Disease Control (CDC), USA serotyping kit (Table 2) and were found to be DENV2.

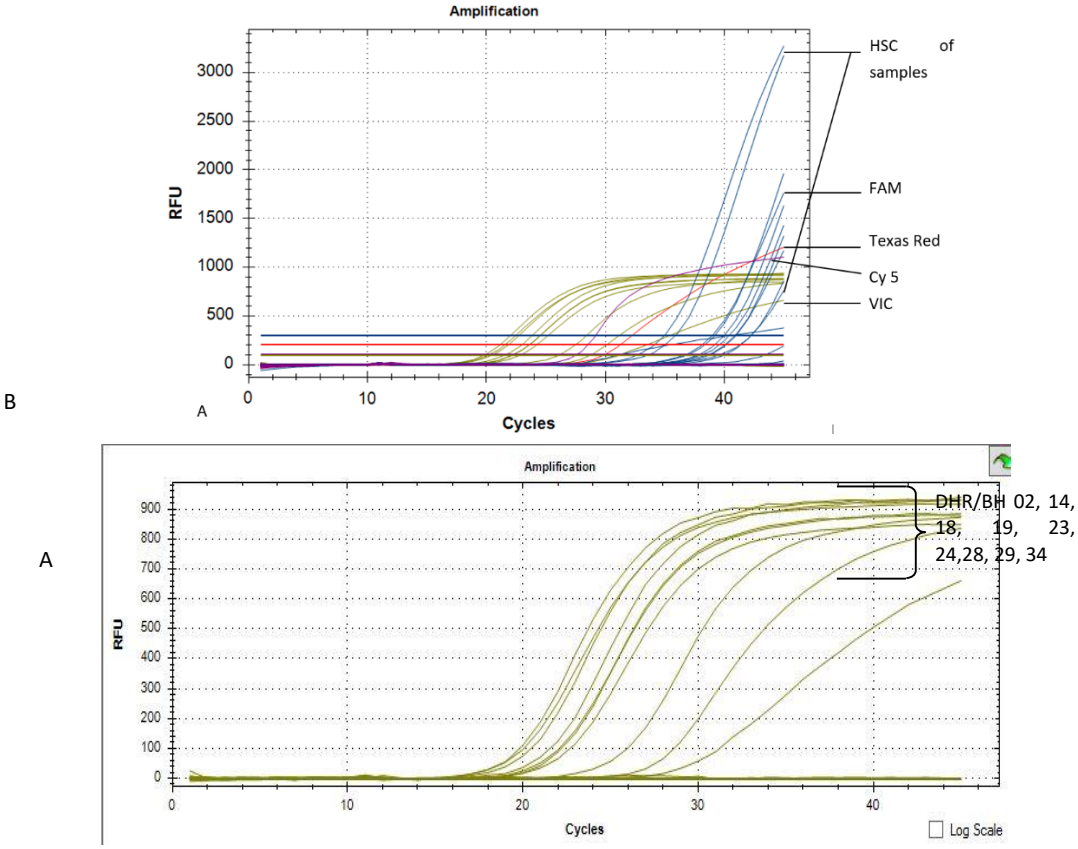


Fig 4.3 Serotyping by Real time PCR. FAM fluorophore blue in colour is specific for DENV1. VIC fluorophore green in colour is specific for DENV2, Texas Red fluorophore is specific for DENV3. (A) Graph showing the amplification of positive control and (B) Graph showing the amplification of samples

Table 4.1: Cycle threshold (CT) values for Real time PCR positive samples

Subject ID	Fluorophore	Serotype	CT value
DHR/BH 02	VIC	DENV2	22.71
DHR/BH 14	VIC	DENV2	20.32
DHR/BH 18	VIC	DENV2	21.39
DHR/BH 19	VIC	DENV2	19.65
DHR/BH 23	VIC	DENV2	28.63
DHR/BH 24	VIC	DENV2	22.07
DHR/BH 28	VIC	DENV2	26.01
DHR/BH 29	VIC	DENV2	22.06
DHR/BH 34	VIC	DENV2	20.04

Nested PCR

Serotype-specific nested PCR performed in the Real-Time PCR positive samples and other remaining samples showed amplicons of sizes of 337 for DENV2. The conventional PCR verified the CDC's serotype specific Real Time RT PCR serotypes circulating in Nepal. No case of concomitant infection with more than one serotype was observed.

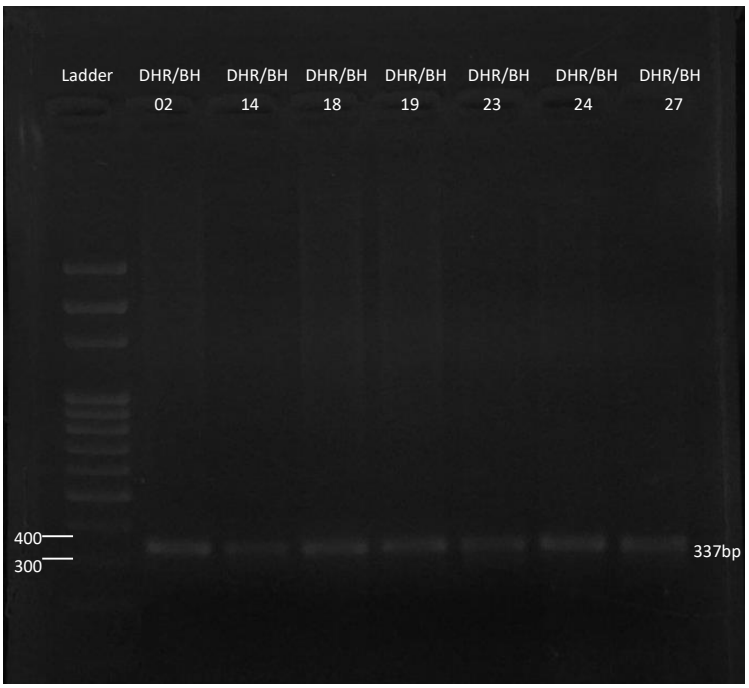


Fig 4.4 Gel images under UV Transilluminator of the second round nested PCR of envelope gene that 337 bp amplicon corresponding to the expected size of DENV2. 100bp ladder [Thermo Scientific Generuler 100 bp DNA ladder, ready to use (Cat. No. #SM0243) was run along with the PCR product.

4.3 Severity study based on clinical pecularity

Only twenty-one samples from the total collected samples were selected for the SNP analysis based on their clinical peculiarity as dengue fever, dengue hemorrhagic fever and severe dengue cases. Most of the samples selected were DHF (11) cases followed by DF (7) cases and severe dengue (3) cases.

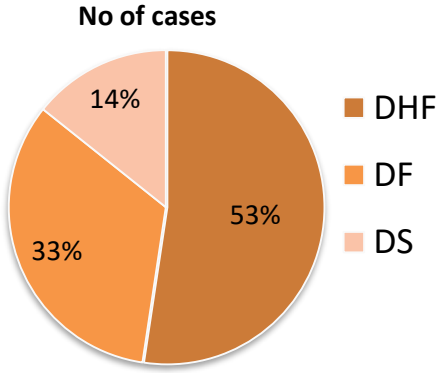


Fig 4.5. Sample distribution based on Clinical Pecularity. Based on the clinical peculiarity most of the cases selected were of DHF (Dengue Hemorrhagic Fever) 53%, followed by DF(Dengue Fever) 33% and DS(Severe Dengue) 14%.

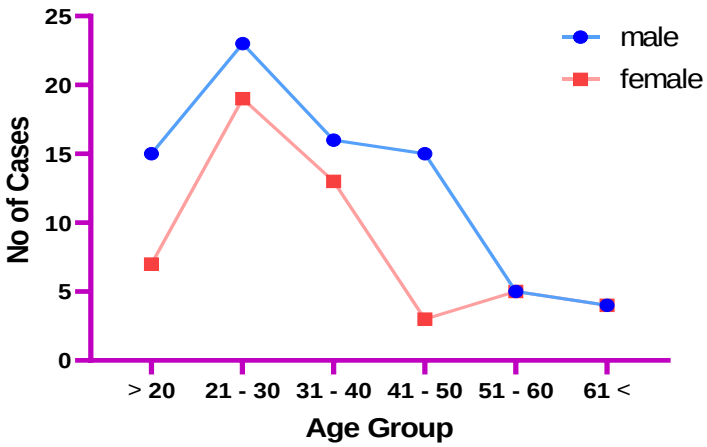


Fig 4.6: Sex and age wise distribution of selected dengue cases. The age varied from 18 years to 61 years.

Selection of the sample was done on the basis of their clinical manifestation and laboratory findings. All of the patients visiting hospital had fever (100%). Most commonly observed symptoms were muscles and joint aches (80.96%) lethargy (71.32%), nausea (47.7%) vomiting (47.7%) and rashes (48.93%). According to the symptoms observed and clinical findings 33%, 53% and 14% cases were respectively classified as dengue fever, dengue hemorrhagic fever and severe dengue respectively.

Symptoms	% of patients included in study (n=21)
Fever	100% (21)
Rash	9.52% (2)
Nausea	47.7% (10)
Vomiting	47.7% (10)
Muscles and joints aches	80.96% (17)
Abdominal pain or tenderness	42.85% (9)
Hemorrhage	28.57% (6)
Gum bleeding	9.52% (2)
Liver enlargement (>2 cm)	9.52% (2)
Lethargy	71.32% (15)
Fluid accumulation with respiratory failure	9.52% (2)
Organ failure	0
Shock	4.76%(1)
Bleeding	0
Classification of the cases	
Dengue Fever(DF)	33%(7)
Dengue Hemorrhagic Fever (DHF)	53% (11)
Severe Dengue(DS)	14% (3)

Hematological test and biochemical tests result of the total NS1 positive and selected cases were compared.

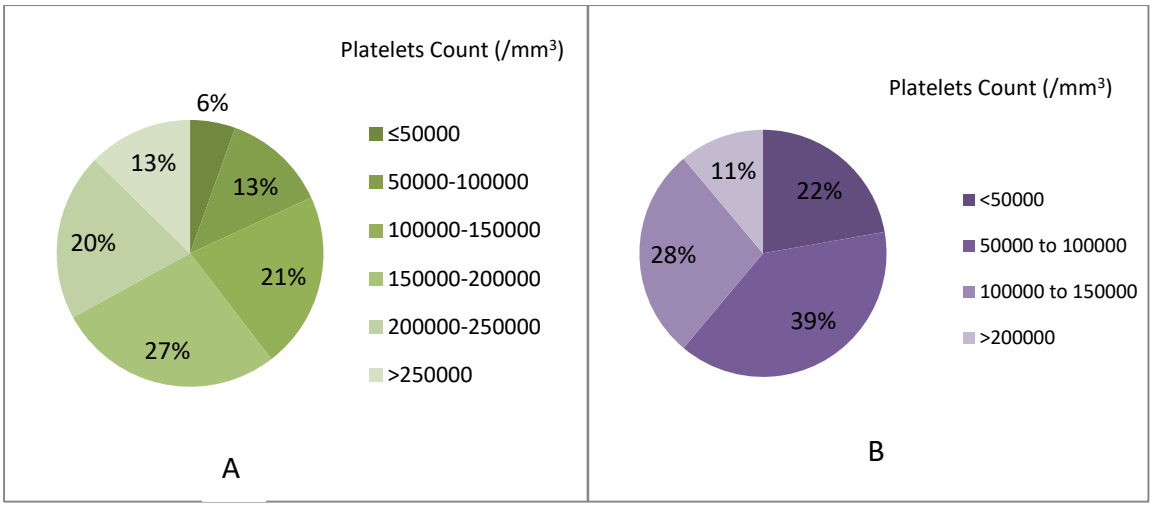


Fig 4.7 Distribution of the platelets count in collected NS1 positive samples and the selected twentyone samples. A: NS1 Positive Samples, B: Selected Cases.

Most of the selected samples had platelets count between 50000 and 100000 (39%) followerd by 100000 to 150000(28%), less than 50000 (22%) and 11% had greater than 200000/mm^3. Patients having less than 100000 /mm^3 platelets count were having dengue hemorrhagic fever and severe dengue.

4.4 Single Nucleotide Polymorphism Analysis

Polymerase chain reaction(PCR) using the afore mentioned primers were performed on selected samples and PCR product was observed under UV transilluminator as below

4.4.1 Tumor Necrosis Factor Alpha (TNF-α):

ARMS PCR using fore mentioned primer was performed for Tumor Necrosis Factor Alpha (TNF-α) and based on the gel image observed of the PCR amplicon the allelic frequencies were noted and analyzed.

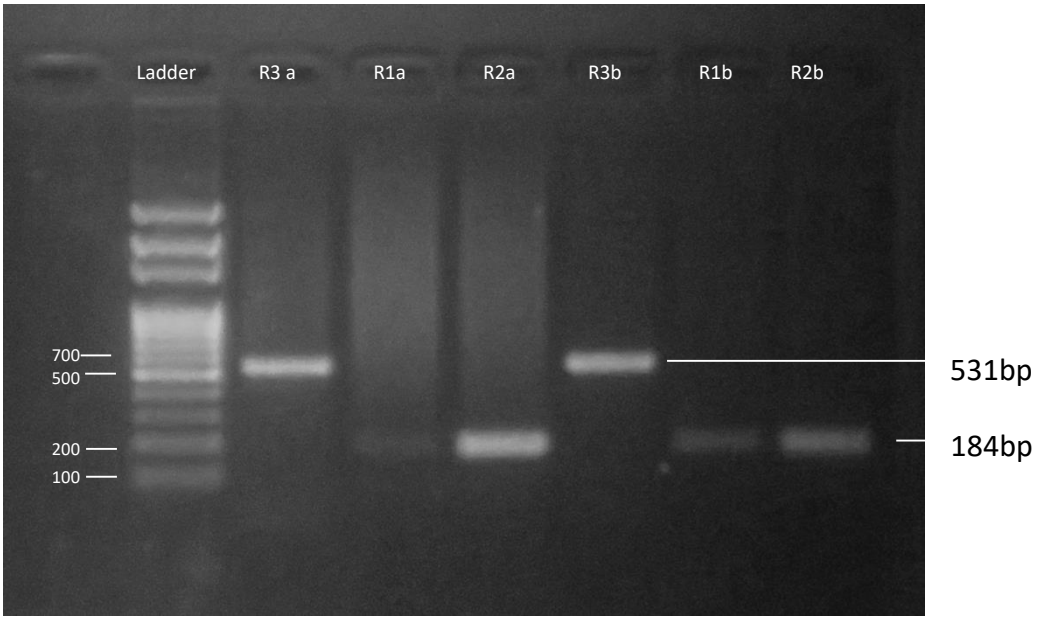


Fig 4.7. Gel image under UV Transilluminator of the PCR product of TNFα -308 (G/A) that gave 531 bp and 184 bp amplicon corresponding to the expected size of Internal control and genotype GG or GA respectively. 100bp ladder [Thermo Scientific Generuler 100 bp DNA ladder, ready to use (Cat. No. #SM0243)] was run along with the PCR product. PCR was similarly done for all of the genes and the allelic frequency were noted and analyzed.

Table 4.2: Genotypic frequency of TNFA (308**)** dengue patient groups [n(21)].

Clinical Peculiarity	**TNFA -308(G/A)**		
	GG	GA	AA
DHF	3(27.0)	8(73.0)	-
DS	2(100.0)	-	-
DF	3(43.0)	4(57.0)	-

From the result of ARMS PCR, 73% of DHF carry GA genotype whereas only 27% carry GG genotype, among Dengue fever patient almost equal 43% and 57% for GG and GA genotype.

4.4.2 Interferon α (INF-α):

ARMS PCR was similarly done for Interferon α (INF-α) gene and the allelic frequency were noted and analyzed.

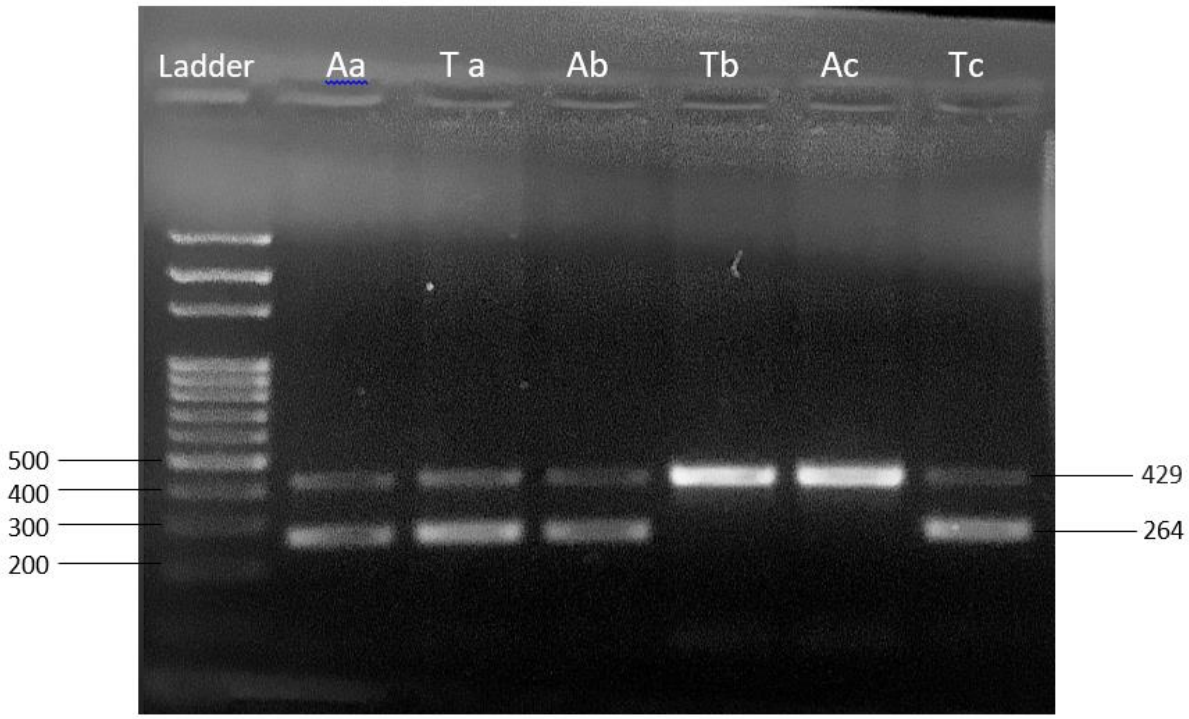


Fig 4.8. Gel image under UV Transilluminator of the PCR product of INF-γ (+874A/T) that gave 429 bp and 264 bp amplicon corresponding to the expected size of Internal control and genotype AA, TT or AT respectively. 100bp ladder [Thermo Scientific Generuler 100 bp DNA ladder, ready to use (Cat. No. #SM0243)] was run along with the PCR product.

Table 4.4: Genotypic frequency of IFNG in dengue patient groups [n (21)].

Clinical	**IFNG +874(A/T)**		
Peculiarity	AA	AT	TT
DHF	4(40.0)	6(60.0)	-
DS	1(33.0)	2(67.0	-
DF	3(43.0)	1(14.0)	3(43.0)

In case of IFNG +874(A/T) case frequency of DHF and DS is mostly distributed in AA and AT genotype. Only individuals with dengue fever (DF) have TT genotype (43%)and no cases from DHF and DS showed TT genotype.

4.4.3 Interleukin 10 (IL-10)

Genotypic frequency of IL-10(819) and IL-10(1082) were noted and analysed observing the gel image of the PCR amplicon obtained from the ARMS PCR.

Table 4.3: Genotypic frequency of IL-10(819) and IL-10(1082) dengue patient groups [n (21)].

Clinical Peculiarity	IL 10-819(C/T)			IL 10-1082 A/G		
	CC	CT	TT	AA	AG	GG
DHF	2(18.0)	9(82.0)	-	3(30.0)	7(70.0)	-
DS	1(33.0)	2(67.0)	-	-	3(100.0)	-
DF	-	5(71.0)	2(29.0)	2(28.571)	3(42.857)	2(28.571)

Here from the result of the ARMS- PCR of IL 10-819(C/T), frequency of CT genotype is greater than CC or TT genotype in all three disease conditions DHF, DF and DS. There is no CC genotype present in any of the severe cases and hemorrhagic cases. Again CC genotype is not recorded in dengue fever cases. In case of IL 10-1082 A/G, AG genotype is seen in most of the case with 100% in case of severe dengue.

4.4.4. F_C-gamma receptor IIa ($F_C\gamma$RIIa)

The ARMS-PCR of $F_C\gamma$RIIa (494 A/G) of all the cases showed AG genotype in all the three disease conditions DHF, DF and DS. No evidence of AA or GG was seen in the selected subject.

Clinical Peculiarity	$F_C\gamma$RIIa (494 A/G)		
	AA	AG	GG
DHF	-	11(100.0)	-
DS	-	3(100.0)	-
DF	-	7(100.0)	-

CHAPTER V
Discussion

Nepal is an endemic nation for many vector borne diseases, like malaria, kala-azar, Japanese encephalitis, lymphatic filariasis and dengue fever (Dhimal et al., 2015). Dengue is one of them which was reported in 2004 for the first time in Japanese traveller followed by annual reports of indigenous circulation from the year 2006 (Malla et al., 2008). Dengue has infected 32 out of 75 districts of the country spreading rapidly all over the country from the East to the West encompassing even the temperate hilly regions (Khetan et al., 2018).

From study in 2019, dengue cases were found distributed in 68 districts comprising all seven provinces showing rapid geographical expansion of dengue infection. During outbreak of dengue in 2019, eight death cases were reported from 5 districts of Nepal (2 deaths in Chitwan, and 1 death in Sunsari, Sindhupalanchock, Kathmandu and Doti).(Adhikari & Subedi, 2020) The endemicity of dengue continued to spread towards highland hilly regions in our study too and has been found in Dhankuta and Panchthar districts which is really a serious concern as the disease could be extended pandemic to Nepal. The major reasons behind may be rapid urbanization, quick transportation facility and better adaptation of *Aedes spp* mosquito to the comparatively cold environment. However, gender-wise infection verified higher male patients than female accordance with the previous report by Gupta et al., 2018. This could be attributed to the two facts; one the men are outdoor field workers to be exposed to the vector and the next, men have preferentially quick access to the healthcare system than females do. Further, the most productive age group (20 to 45 years old) population of the nation is being affected potentially crippling the economy of the country by increasing DALYs (Disability Adjusted Life Years) of the nation.

Results obtained from all of the polymorphisms investigated from the twenty-one samples from three different dengue cases i.e. DF, DHF and DS were compared. Serotyping of some samples was carried out due to time and resource limitation in which DENV2 was found in all the samples subjected to serotyping.

An allele A is known to be stronger transcriptional activator causing high TNF-α secretion resulting the activation of immune system during the infection leading to the induction of cytokine expression which finally activate the endothelial cells to produce vasodilator substances associated with the disease severity. (MT et al., 2004). Study conducted in Sri Lanka have shown that the GG genotype is associated with risk factor in progression of disease to DHF and severe dengue whereas in Cuban and Venezuelans AA genotype is associated with the disease severity, and in Thai and Mexican population no association

of this cytokine gene polymorphism with disease was found.(Santos et al., 2017a) In our study, almost 38% of cases showed GG genotype and 60% of the study population showed GA genotype. GA genotype is known to be associated with protective immunity in most of the population and in our study GA was found in higher percentage of the infected cases it could be the reason behind the severity.

In a study done on Brazilian population, CC genotype of IL-10 819 was found to be protective against severe dengue cases whereas TT is found to be susceptible for dengue infection. CT genotype is seen to be playing role in disease progression to severity(Perez et al., 2010). In our study most of the cases showed CT genotype (71%) whereas protective CC genotype was found to be present in 14% of the cases. Further the people having CC genotype could have chance of severity. As most of the infected cases showed CT it could be concluded that severity is also affected by this genotype.

In case of IL 10-1082 A/G, enough study has not been done but the combination of IL 10-1082 and 819 AC/AT may be a risk factor for dengue progression(Perez et al., 2010). In our study population 22% showed AA genotype while 62% showed AG genotype. An A allele being reason behind the disease progression which might define the clinical peculiarity of DHF, DF and DS cases in the study.

For INFγ, +874 A/T polymorphism influence the level of INFG production in the system where TT is associated with high production followed by AT and AA (Perez et al., 2010). In our study, most of the individuals showed AT (45%) genotype followed by AA (40%) whereas only 15% with TT genotype.

Heterozygote genotype AG variant was found in all the study population in case of $F_C\gamma$RIIa (494 A/G) gene. Both Histidine and arginine aminoacid is expressed in 131 position. Presence of histidine or arginine alters the affinity of $F_C\gamma$RIIa feceptor to IgG1 and IgG3 subclasses. Histidine was present in most of the severe cases while arginine was in most of the asymptomatic dengue cases in the study done in Pakistani, Cuban and Vietnamese population.

As the population size was small in this study, the role of SNPs in severity could not be exactly predicted but this study provides future insight in predicting the role of these biomarkers in susceptibility, progression or protection a disease. A subtle change in multiple genes most probably influence the degree of severity. In our study influence of TNF-α, IL-10 and INF-γ cytokines gene and $F_C\gamma$RIIa gene polymorphism in dengue disease cases were observed.

CHAPTER VI
Conclusion

Dengue disease is one of the most important infectious diseases in tropical and subtropical countries and is becoming a matter of serious health concern in Nepal also. Hospital based study across Nepal shows huge number of clinical case reports of patient showing fever and symptoms closely related to dengue many of which remain undiagnosed. Most of the cases showing the symptoms when diagnosed were dengue febrile illness even though similar symptoms are seen in other viral diseases. As foreseen in previous studies regarding cyclic outbreak of dengue, the year 2019 was predicted to be epidemic and as expected Nepal was hit by the disease with the largest no of dengue cases till date with highest morbidity. In 2017, co-circulation of 3 different serotype of DENV (DENV-1, DENV-2, DENV-3) were reported in Nepal, whereas in 2019 DENV-2 is confirmed in most of the cases whose sequencing is yet to be known.

In Nepal, diagnosis of dengue fever is done primarily by clinical parameters like hemoglobin, hematocrit, AST and ALT along with platelets count which is then confirmed by Rapid test kit for NS1 antigen if available. Reason behind the progression of dengue disease after viral infection to Dengue Fever (DF), Dengue hemorrhagic Fever (DHF) and severe dengue (DS) cases is yet to be done for getting concrete result. Population where DF, DHF and DS are expanding and the cases are increasing year after year. Different research on dengue viruses are being done but understanding the pathogenesis behind the severe dengue disease is not yet clear which might be important for the development of better treatment and the prevention strategies of viral infection like dengue. Host genetics study in the Nepalese population will help in knowing the reason behind the disease progression and might help in the clinical management of the patient. The severe form of dengue if not managed in time one can lose his/her life not getting the basic treatment. In the polymorphism study done in various genes, IFNG gene at the locus +874 showed polymorphism of AA and AT among the clinically severe dengue cases of DHF. The gene TNFA at the locus 308 with GG and GA express the severity towards the DSS and DHF while AA polymorphism was absent in the samples. Both in IL 10-819 and IL10 – 1082 gene, heterologous allele C/T expressed the higher risk for dengue severity. In Nepalese population, the heterologous AG polymorphism at 494 locus of FcG RIIa gene showed risk of severity.

LIMITATIONS OF THE STUDY

1. The study was carried out in limited number of samples.
2. Sequencing of circulating DENV 2 could not be done.
3. The study of cytokines level was not included.
4. Expression analysis of the genes was not done.

RECOMMENDATIONS/ FUTURE PERSPECTIVES

1. Dengue should be incorporated in National Disease Control Program being a major public health issue with its endemicity spreading towards hilly regions like Dhankuta and Panchthar.
2. Host genetics study in different ethnic groups should be done in Nepalese population to identify risk groups in a dengue exposed population and develop effective immunotherapy to control the viral infection.
3. The study based on clinical parameters of dengue and non-dengue other febrile illness should be carried out in large number of samples.
4. There should be at least one sophisticated and well-equipped lab in each province for the diagnosis and management of dengue.
5. Effective epidemiological surveillance for dengue fever must include the ability of routine laboratory confirmation and monitoring of circulating serotypes.
6. Expression analysis of the molecules involved in causing dengue severity should be done.
7. Further studies on the cellular immune response of the host should be done.

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Appendices

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 394

8 August 2019

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

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

Dear Mr. Rauniyar,

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

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

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

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

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

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

Thanking you,

Prof. Dr. Anjani Kumar Jha
Executive Chairperson

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

अनुसन्धान अध्ययनमा सहभागिता जनाउने मञ्जुरीनामा फाराम

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

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

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

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

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

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

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

हुन्छ ☐ हुदैन ☐

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

हुन्छ ☐ हुदैन ☐

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

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

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

....

मिति बिरामीको नाम

....

ठेगाना : सम्पर्क नं

....

मिति साक्षीको नाम

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

....

मञ्जुरी लिनेको नाम सम्पर्क नं

....

मिति हस्ताक्षर

Form No:-.....

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

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

PATIENT INFORMATION

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

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

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

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

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

Fever:- Intermittent Continuous Remittent .....

PRESENTING SYMPTOMS

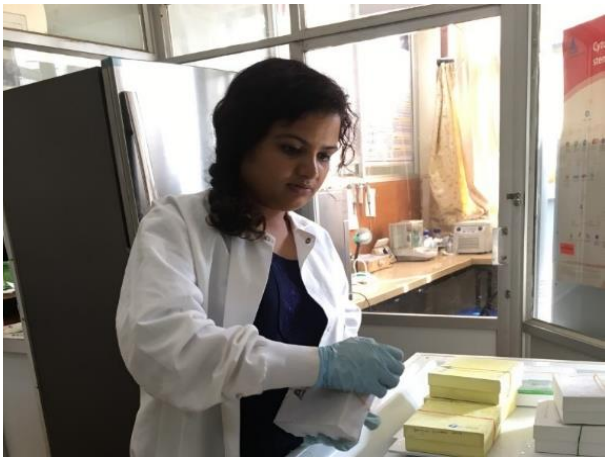
Hemorrhagic manifestations:- Yes NO
Severe bleeding:- :- Yes NO
Typeof haemorrhage:- Petechiae Purpura Echymosis Epistaxis Gum bleeding Hematesis Melena
Rash:- :- Yes NO
Itching sensation:- :- Yes NO
Burning/Piercing sensation:- Yes NO
(Mention the whole body/body parts) .....
Shock:- :- Yes NO
Headache:-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

CLINICAL COURSE

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

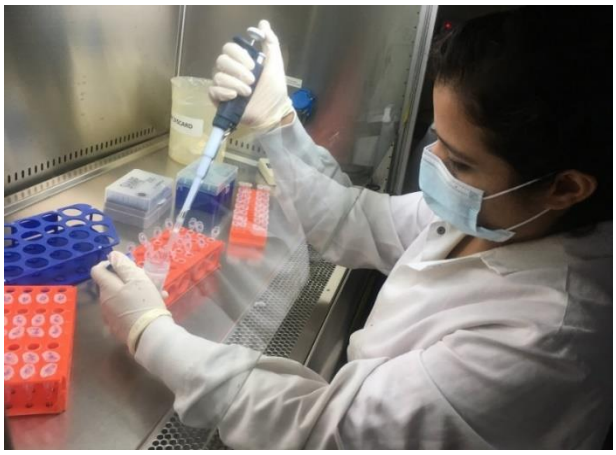
Remarks:- .....

Sample Collection



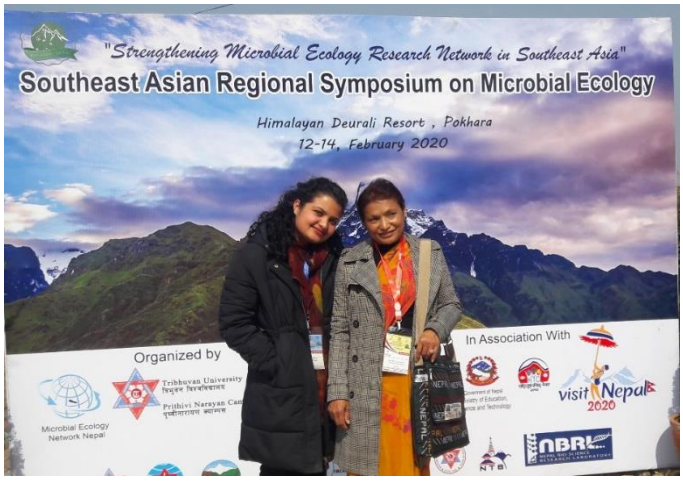
Sample Storage

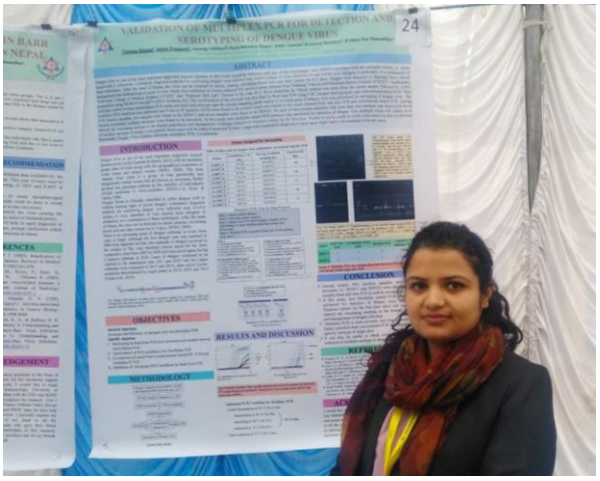
Analysis



Lab Work

Winter School





Participation

Team Virology