SEROTYPING OF DENGUE VIRUS AND ESTIMATION OF T CELLS IN CIRCULATING PERIPHERAL BLOOD OF DENGUE PATIENTS

M.Sc. Thesis

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For the partial fulfillment of the requirement for the

Master of Science in Biotechnology

Submitted to:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

Submitted by

Sudiksha Chaulagain
Exam Roll No.: BT 218/071
T.U. Registration Number.: 5-2-282-23-2010

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Supervisor

Prof. Dr. Krishna Das Manandhar
Central Department of Biotechnology
Tribhuvan University

Dedicated
To
My
Beloved
Mummy
and Daddy

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Glossary Acronyms

%	Percentage
ADE	Antibody Dependent Enhancement
APC	Antigen Presenting Cells
BSL	Biosafety level
C	Capsid
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CDBT	Central Department Of Biotechnology
CDC	Centre for Disease Control and Prevention
cDNA	complementary Deoxyribnucleic Acid
Cq	Quantification cycle
DENV	Dengue Virus
DENV1	Dengue virus serotype 1
DENV2	Dengue virus serotype 2
DENV3	Dengue virus serotype 3
DENV4	Dengue virus serotype 4
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
E	Envelope
FACS	Fluorescence Associated Cell Sorting
FAM	6-carboxyfluorescein
FDA	Food and Drug Administration
FSC	Forward Scatter
HB	Hemoglobin
HSC	Human Specimen Control
Kb	Kilo base

MHC	Major Histocompatibility Complex
NC	Negative Control
NHRC	Nepal Health Research Council
NS	Non Structural
OD	Optical Density
PAHO	Pan American Health Organization
PC	Positive Control
PrM	Premembrane
QC	Quality Control
RNA	Ribonucleic Acid
RP	RNAse P
Rpm	Revolutions per Minute
Tm	Melting temperature
UTR	Untranslated Region
WHO	World Health Organization
µl	Microllitre

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ABSTRACT

Dengue is a mosquito borne viral disease mostly transmitted through bites of infected *Aedes aegypti*. Epidemiological studies suggest that dengue has been rapidly spreading in tropical and sub-tropical countries imposing a significant health, economic and social burden. The geographical expansion of four dengue virus serotypes (DENV1-4) signifies the need for serotyping of dengue virus for proper surveillance and epidemiological studies. Dengue virus serotypes share 65% similarities in their genome but genetic variation can be observed even within single serotype. The understanding of changing pattern of dengue virus serotypes is important. Cellular immunity plays a vital role during dengue infection. CD8 T cells have been responsible for both protection and immunopathology in dengue patients. Though various epidemiological studies regarding dengue has been carried out but the study about immunological response in dengue patients is not done in Nepal.

In this study, serotyping of dengue virus was performed and the change in pattern of serotype was observed. We used selective clinical samples available from two different hospitals of Nepal in 2016 and 2017. RNA was extracted from serum samples and Real-Time PCR was performed. The serum samples were collected from confirmed dengue cases. The samples labeled with the antibodies lysed with FACS lysing solution after incubation , were stained and run in a flowcytometer. Then the data was obtained for further analysis in Flowjo. The flow cytometer analysis of dengue positive samples from 2017 was carried out for the study of immunological response in dengue patients in BD FACS Calibur. The percentage of CD4 and CD8 T cells in dengue patient was estimated.

Change in pattern of dengue serotype was observed between two different years. DENV1 was found prevalent in 2016 whereas DENV2 was found prevalent in 2017. The percentages of CD4 cell of samples were similar to that of control whereas the percentage of CD8 cells in dengue positive samples were higher than that of control.

Hence, Real-Time PCR is rapid and effective method for serotyping of dengue virus. There was a pattern change observed in dengue virus serotype in the samples of two consecutive years. The increase in percentage of CD8 cells was due to dengue infection.

Keywords: Dengue, Serotyping, Real Time PCR, FACS, T cells

CHAPTER ONE

INTRODUCTION

1.1 Background

In the last five decades, dengue has appeared as one of the most important infectious diseases. Evidence indicates the expansion of dengue virus endemic areas and consequently the exponential increase of dengue virus infections across the subtropics is in increasing trend. The clinical manifestations of dengue virus infection include sudden fever, rash, headache, myalgia and in more serious cases, spontaneous bleeding. These manifestations occur in children as well as in adults. Defining the epidemiology of dengue in a given area is critical to understanding the disease and devising effective public health strategies (Kosasih et al., 2016).

Today, 40% of world's population is at risk of dengue infection. Dengue is endemic in at least 100 countries in Asia, the Pacific, the Americas, Africa, and the Caribbean. The World Health Organization (WHO) estimates that 50 to 100 million infections occur yearly, including 500,000 DHF cases and 22,000 deaths, mostly among children. The global spreading of principal vector of dengue virus led to rapid spread of dengue virus all over the world. Various human activities like slave trade, migration, intercountry and continental commerce and two devastating world wars led to major impact of dengue virus globally in early and mid-20^{th} century. It was found that from 1960 to 2010 there has been more than 30 fold escalation in dengue incidence (Gubler, 1998).

1.2 Burden of disease:

Dengue imposes a significant health, economic and social burden on the populations of endemic areas. The estimated number of disability-adjusted life years (DALYs) lost to dengue in 2001 was 528 at global level. Annually about 0.4 to 1.3 million cases were found to be reported in WHO in the decade 1996 - 2005. The number of dengue cases varies significantly from year to year as it is an infectious disease. The full burden of dengue is yet to be understood due to the misdiagnosis and underreporting of many cases of dengue infection which is posing a problem to understand the exact burden of dengue (http://www.who.int/tdr). The overall cost of officially reported dengue is of US$ 440 million. This estimate ignores the underreporting of cases and substantial costs associated with dengue surveillance and vector control programs. The various studies are being

conducted which led to a conclusion that a treated dengue case imposes significant costs on both the health sector and the overall economy. The availability of vaccine could have prevented much of this burden of dengue infection. There is a higher risk of severe dengue to occur in children (Guzmán et al., 2002).

Severely ill patients require intensive care, including intravenous fluids, blood or plasma transfusion and medicines. Dengue troubles mostly all peoples of society but the burden may be higher among the poor people due to the improper manangement of waste and water supply in their areas which may favour a rapid multiplication of the main vector, *Ae.aegypti* (Campos et al., 2017).

1.3 The virus:

Family	:Flaviviridae
Genus	:Flavivirus
Species	:Dengue viruses (DENV)
Serotype	:(DENV-1,2,3,&4)
Nucleic Acid type	:Single stranded RNA
Sense	:Positive sense
Nucleotides	:10.7 kb

Dengue virus (DENV) is a small single-stranded RNA virus which consists of four distinct serotypes (DENV-1 to 4). The four serotypes of the dengue are closely related and belong to the genus Flavivirus, family Flaviviridae. The virion of the dengue virus is spherical with a diameter of 50nm which contains a single copy of a positive-sense, single-stranded RNA genome, multiple copies of the three structural proteins and a host-derived membrane bilayer. The genome of dengue virus is cleaved by host and viral proteases in three structural proteins (capsid, C, prM, the precursor of membrane, M, protein and envelope, E) and seven nonstructural proteins (NS). Easily distinguishable genotypes have been identified within each serotype, which highlights the extensive genetic variability of the dengue serotypes. The viruses that are “fit” for both human and vector are evolved and maintained which is based on purifying selection that appears to be a dominant theme. The “Asian” genotypes of DENV2 and DENV-3 are frequently associated with severe disease that accompanies secondary dengue infections. Intra-host viral diversity has also been described in human hosts which are termed as quasispecies.

1.3.1 Virion Morphology:

The virion of dengue virus, is spherical with 40-50 nm diameter. It is a nucleocapsid of about 30 nm diameter that is enclosed in a lipid envelope. The nucleocapsid contains the viral capsid and RNA genome. The lipid envelope consists of a lipid bilayer, an envelope protein between 51,000 and 59,000 daltons which plays significaaant role in 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 (Schmaljohn& McClain, 1996 and Suleman et al., 2016).

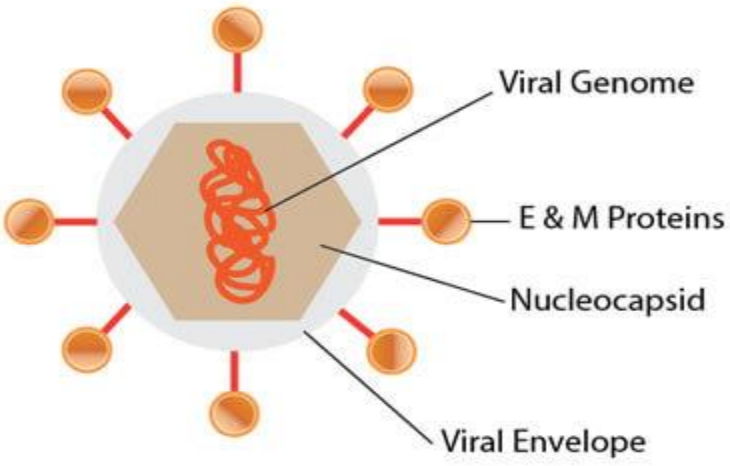


Figure 1. 1 Structure of the dengue virion (E: enveloped protein and M: Membrane protein, the virus contains RNA as genome and different proteins such as structural and non structural proteins

1.3.2 Genomic Organization of dengue virus

Genome of dengue virus is single stranded , positive sense RNA, which is 10 to 11 Kb in length. The genome is enveloped with lipid membrane and is spherical with an icosahedral symmetry (Disabelle, n.d.). A short transmembrane segment helps in attachment to the surface of viral membrane. The genome encodes a single polyprotein which is cleaved into ten mature peptides. Ten mature peptides include 3 structural and 7 non- structural proteins. Three structural proteins include capsid(C) protein, the envelope(E) glycoprotein and the membrane (M) protein. Furine mediated cleavage from prM precursor leads to the formation of membrane protein. The role of E glycoprotein bears neutralization epitopes and helps in attachment of virion to receptor and fusion of target cell membrane with virus envelope. Seven non structural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. A short non coding region is present on both 5’ and 3’ end. (“ http://www.denguevirusnet.com/,”)

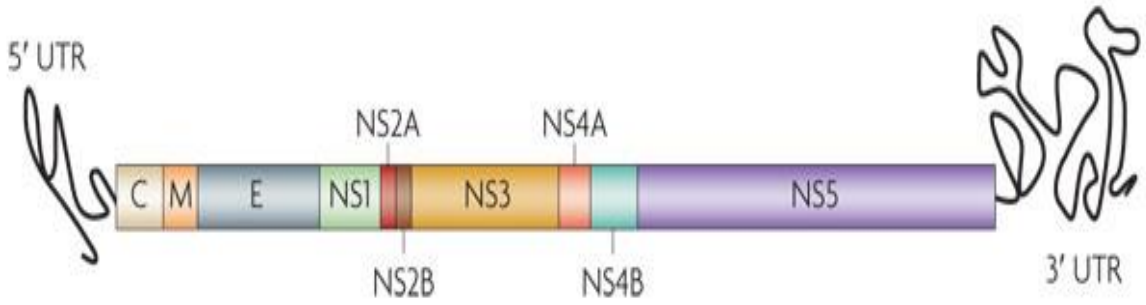


Figure 1. 2 Genomic Structure of Dengue virus

The genomic organization of the dengue virus, is relatively simple in comparasion to other arboviral families. It contains a single translated open reading frame (ORF) that encodes a precursor polypeptide of around 3390 amino acids which is organized catalytically into ten viral proteins. There is no evidence of alternative or overlapping reading frames that are translated.

The DENV ORF is flanked at its 5’ terminus by an untranslated region (UTR) of about 100 nucleotides and a longer UTR of about 500 nucleotides at its 3’ terminus. The 5’ terminus of the genome has a type I cap (m7GpppAmp) and there is no polyadenylation of the 3’terminus. The translated polyprotein is cleaved co- and post-translationally by viral and host proteases into ten viral proteins: three structural proteins including C,capsid; prM/M, precursor of membrane; E, envelope encoded at the 5’ end of the ORF, and seven non-structural proteins including NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 encoded at the 3’ end (Figure 1.3). The three structural proteins comprises of the DENV virion: the capsid protein surrounds the viral RNA genome to form the nucleocapsid, whereas the prM and E proteins are embedded in the lipid bilayer that forms the viral envelope. The cleavage of the prM into the membrane (M) 13 protein by furine which takes place during viral release has been shown to be a prerequisite for the production of mature infectious virions. E protein is the most studied among three structural proteins as it is the major constituent of the virus envelope. It is glycosylated at two sites and is responsible for attachment of virus to receptors of susceptible host cells and for fusion with cell membranes. The E glycoprotein also contains the main epitopes recognized by neutralizing antibodies (Campos et al., 2017). Such epitopes are also found to a lesser extent on the M glycoprotein (Filomatori et al., 2017).

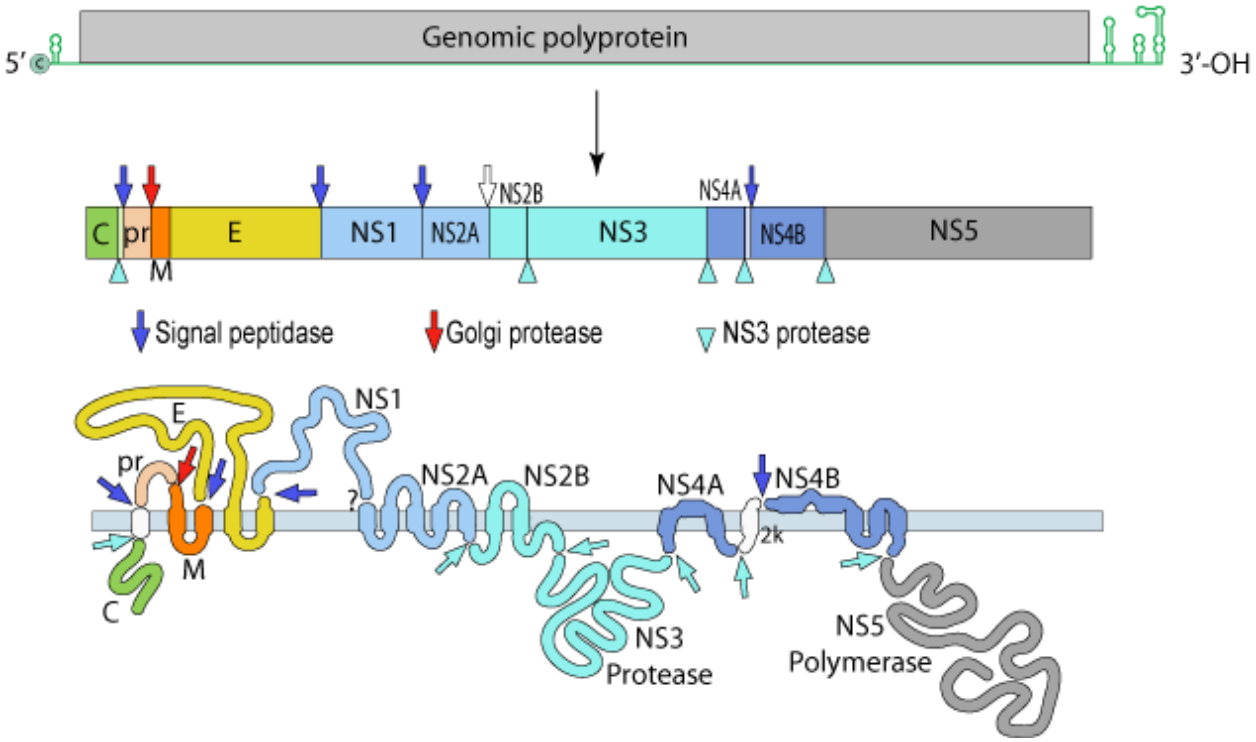


Figure 1. 3 Schematic diagram of genomic organization of Dengue virus (C: Capsid protein; PrM: Pre-Membrane protein; E: Enveloped protein; NS1-5: Non-Structural protein 1-5)

1.3.3 Serotype

In 2004, the first case of Dengue fever was reported in Nepal. The isolation of dengue virus type-2 was done from a dengue patient returning to Japan from Nepal in October 2004. The virus was found to be dengue virus serotype 2. This was the first isolate of dengue virus from Nepal whose nucleotide showed closest homology to dengue virus serotype 2 isolate from India in 2008 (Takasaki et al., 2008).

DENV-1, DENV-2, DENV-3, DENV-4 are causative of dengue virus infection which are closely related. Each virus has different interactions with antibodies in human blood serum due to which four viruses are called serotypes. The four viruses has approximately 65% similarities in their genome but genetic variations can be observed even within single serotype. Though there is genetic variation, infection with all 4 dengue serotype results in same disease and same range of clinical symptoms.

1.4 The vectors:

The different serotypes of the dengue virus are transmitted to humans through the bites of infected *Aedes aegypti*. *Aedes aegypti* is a tropical and subtropical species which has wide distribution around the world, mainly between latitudes 35 0N and 35 0S. *Ae. Aegypti is* not

found above 1000 meters due to lower temperatures. The larvae are found in water-filled habitats, mostly in artificial containers in close association with human dwellings. Various studies says that *Ae. Aegypti* may spend their lifetime around the area where they emerge as adults which suggests that not mosquitoes but people move the virus within and between communities.

Aedes albopictus, *Aedes polynesiensis* and several species of the *Aedes scutellaris* complex has a particular ecology, behaviour and geographical distribution and are associated with Dengue outbreaks. It was reported that in recent decades *Aedes albopictus* has spread from Asia to Africa, the Americas and Europe, especially aided by the international trade in used tyres which contained deposited eggs in rainwater. This spread might have happened as eggs can remain viable for many months in the absence of water.

1.5 The host

When infection by any of the four virus serotypes of Dengue virus takes place, after an incubation period of 4-10 days, a wide spectrum of illness, although most infections are asymptomatic or subclinical occurs. A lifelong protective immunity to the infecting serotype is induced after Primary infection (Halstead, 1974).

Within 2-3 months of the primary infection the infected individual is protected from clinical illness with different serotype but there is no long-term cross-protective immunity.

The various risk factors associated with different individual determine the severity of disease. The factors include secondary infection, age, ethnicity and associated chronic diseases (bronchial asthma, sickle cell anemia and diabetes mellitus) with the individual. Young children are at high risk of severe dengue as they may be less able than adults to compensate for capillary leakage.

Seroepidemiological studies suggests that secondary heterotypic infection poses a great threat being a risk factor for severe dengue, even though there are a few reports of severe cases associated with primary infection(Halstead et al, 1970).

During primary infection of infants who are born to dengue-immune mothers the infants frequently have severe dengue. Antibody-dependent enhancement (ADE) is the mechanism to explain severe dengue which usually takes place in the course of a secondary infection and in infants with primary infections.

In this model, during a primary infection, non-neutralizing, cross-reactive antibodies are raised or acquired passively at birth, which bind to epitopes on the surface of a

heterologous infecting virus and ease virus entry into Fc-receptor-bearing cells. The leads to increase in number of infected cells which result in a higher viral burden and induction of a vigorous host immune response that includes inflammatory cytokines and mediators, some of which might contribute to capillary leakage . Cross-reactive memory T-cells are also rapidly activated, proliferated, express cytokines and die by apoptosis in a manner that generally correlates with overall disease severity during secondary infection. The clinical outcome of infection might be due to influence of host genetic determinants but this issue is yet to be addressed properly (Kouri et al. , 1989).

Studies in the American region shows that the rates of severe dengue is lower in individuals of African ancestry than those in other ethnic groups (Sierra et al., 2007).

While an infected mosquito is taking a blood meal, the dengue virus enters via the skin. The virus is present in the blood during the acute phase of illness and defervescence occurs during its clearance. The association of plasma leakage is found to be with functional effects on endothelial cells. Endothelial cell dysfunction might have been caused due to activation of infected monocytes and T cells, the complement system and the production of mediators, monokines, cytokines and soluble receptors. Alterations in megakaryocytopoieses by the infection of human haematopoietic cells and impaired progenitor cell growth, might lead to Thrombocytopenia resulting in platelet dysfunction, increased destruction or consumption.

In summary during severe dengue, a temporary and reversible imbalance of inflammatory mediators, cytokines and chemokines occurs which might have been driven by a high early viral burden, and leading to dysfunction of vascular endothelial cells, derangement of the haemocoagulation system then to plasma leakage, shock and bleeding.

1.6 Transmission

The multiplication of virus takes place majorly inside human host. During feeding female mosquitoes ingests dengue virus circulating in the blood of viraemic humans, which then infects the mosquito mid-gut and subsequently spreads systemically over a period of 8-12 days. The virus is then further transmitted to other hosts during subsequent mosquito feeding. Ambient temperature influences the extrinsic incubation period. Afterwards, the mosquito remains infective for the rest of its life.

The anthropophilic nature of *Ae.Aegypti* makes it one of the most potent vector for dengue infection as it frequently bites several times before completing oogenesis, and blooms in close proximity to humans. The significance of vertical transmission of the virus is not well understood. Sylvatic dengue strains may also lead to human infection that might cause mild

illness. Hostpathogen interactions , environmental and climatic factors and population immunological factors has great influence on the dynamics of virus transmission. Vector-borne disease is directly influenced by climate as it plays vital role in biology of the vectors and also their abundance and distribution.

1.7 Dengue Infection

Dengue is a mosquito-borne viral disease that is transmitted by female mosquitoes mainly of the species *Aedes aegypti* and, to a lesser extent, *Ae. albopictus*. This disease is generally spread throughout the tropical area, with local variations in risk influenced by rainfall, temperature and unplanned urbanization("WHO | Dengue and severe dengue," 2017). Dengue is caused by one of the four serotypes of the dengue virus (DENV 1-4) which is a RNA virus that belongs to the genus Flavivirus of the family *Flaviviridae* (Lam et al., 2013).

Dengue virus being a leading cause of illness and death in the tropics and subtropics more than one third of world's population are living at area of risk of its infection.("Dengue | CDC," n.d.)

Dengue virus infections may be asymptomatic or may lead to undifferentiated fever, dengue fever (DF) or dengue haemorrhagic fever (DHF) with plasma leakage that may lead to hypovolemic shock called dengue shock syndrome (WHO) and (TDR), 2009).

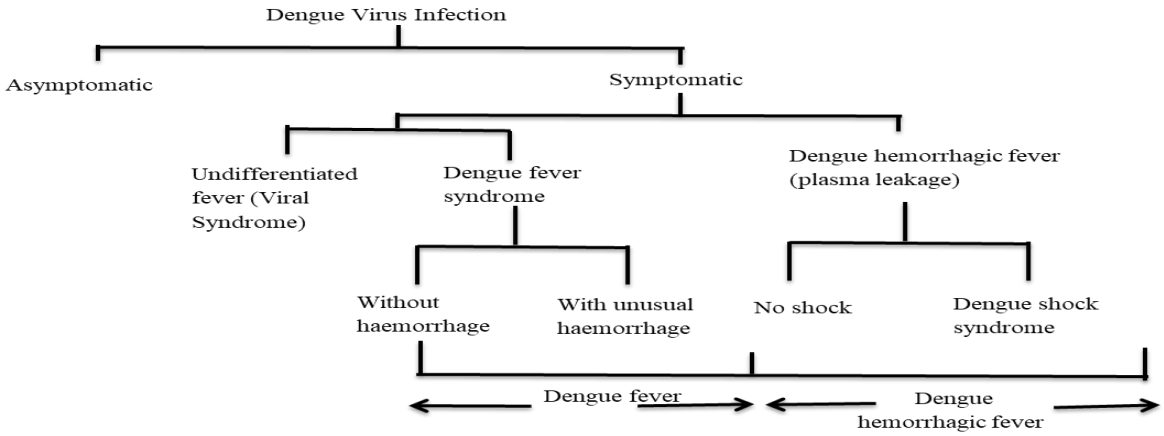


Figure 1. 4 Chart showing manifestation of Dengue infection(World Health Organization (WHO) and the Special Programme for Research and Tropical Diseases (TDR), 2009)

1.7.1 Dengue Fever (DF)

Dengue fever is mostly found to have occurred in children and adults that is characterized by sudden onset of fever and various signs and symptoms that are non-specific. This

includes nausea and vomiting, retro-orbital pain, weakness, joint pains and rash("Dengue haemorrhagic fever," 1997).The temperature during dengue fever may rise to 105 degree Fahrenheit lasting for 2 to 7 days. Despite fever, a relative bradycardia in which a person has slow heart rate may also be noticed. In addition, lymphadenopathy, a disease of lymph nodes, is commonly found. Rashes can be variable and found as either early or late eruption in about 50 % of the patients; and when temperature of body falls to or below normal, scattered or confluent petechiae may appear. Dengue fever is rarely fatal and is self-limiting. The disease could have acute phase that lasts for 3 to 7 days and the convalescent phase that lasts for several weeks. In adults, convalescent phase is reported to be associated with weakness and depression (Gubler, 1988). With dengue viral infection, no permanent sequelae are known yet.

1.7.2 Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome(DSS)

Major clinical manifestation of DHF includes high fever, hemorrhagic phenomena, and often, hepatomegaly and circulatory failure. DHF is primarily characterized by sudden onset of fever. Dengue Shock Syndrome (DSS) is a syndrome due to the dengue virus that tends to affect children under 10, causing abdominal pain, hemorrhage (bleeding) and circulatory collapse (shock). It is difficult to distinguish from dengue fever during acute phase of illness as no signs or symptoms of dengue hemorrhagic fever is observed. Although no pathognomonic signs and symptoms are observed during acute stage, when fever remits, plasma leakage leads to clinical manifestation which can lead to accurate clinical diagnosis. Defervescence time is regarded as the critical stage of DHF. After temperature falls to normal or below, signs of circulatory failure or hemorrhagic manifestation can sometimes occur from 24 hours before to 24 hours after the temperature becomes normal. During classic DHF, hemoconcentration indicating plasma leakage occurs but is more severe in patients who have shock syndrome. Hemoconcentration can be observed as an evidence of vascular leakage syndrome. Ecchymoses, petechiae, purpuric lesions are included as common hemorrhagic manifestations. Thrombocytopenia occurs between 3 to 8 days of illness during which platelet count is ≤100,000/mm. Hepatomegaly though is not constantly found but it is a said to be common in patients with DHF. Capillary fragility is indicated by tourniquet test.(Gubler, 1998)

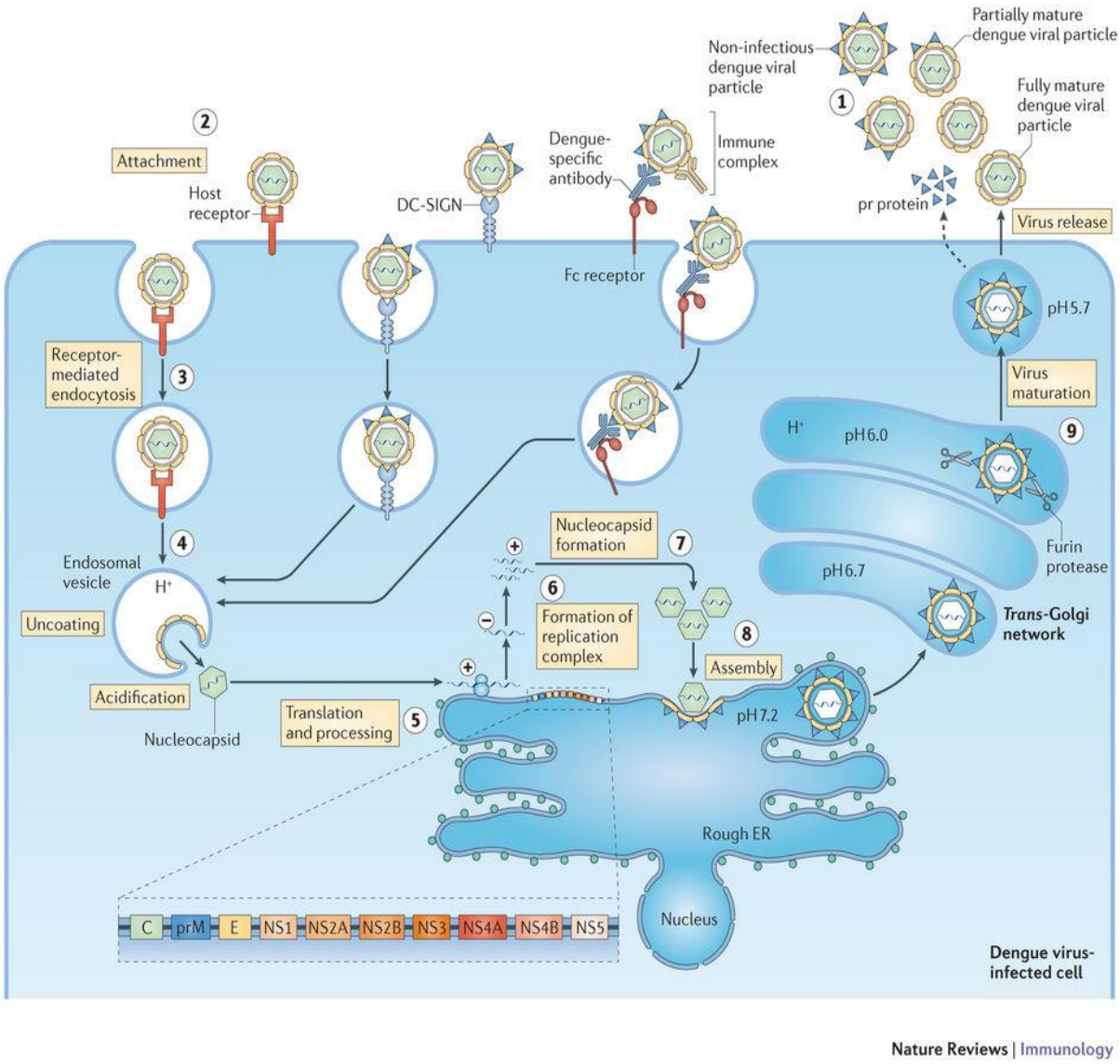


Figure 1. 5 Schematic diagram showing dengue infected cell

1.8 Real Time PCR

Real time PCR is the most reliable method of detection and measurement of products generated during PCR which is equivalent to the amount of template present at start of PCR process. TaqMan probe is a fluorogenic non extendable probe which has a fluorescent reporter dye attached to its 5' end and a quencher dye at its 3' end. The fluorogenic probe anneals downstream from primer site which is cleaved by the 5'nuclease activity of Taq polymerase enzyme during extension phase when the target sequence is present. When the target sequence is not present ,the probe is intact, fluorescence resonance energy transfer occurs which leads to the absorbance of the fluorescence emission of the reporter dye by the quenching dye. When taq polymerase cleaves probe during PCR the separation of reporter and quencher dyes takes place which increases fluorescence from reporter dye.

After the cleavage, the probe is removed from target strand, which allows primer extension to continue to end of template strand which does not interfere with exponential accumulation of PCR product. The additional reporter dye molecules are cleaved in each consecutive cycle which in turn leads to increase in fluorescence intensity that is proportional to amount of amplicon produced.

Taqman Probe: A forward primer, a reverse primer and a probe is used. The assay is dependent on 5' to 3' nuclease activity of Taq Polymerase. The probe has Fluorescent reporter and quencher dye covalently bonded at the 5' and 3' ends respectively. The intactness of probe leads to occurence of FRET which is due to proximity of the reporter and quencher dye as a result of which flouorescence emission does not occur. When target sequence is present, during PCR the annealing of probe to target takes place and taq polymerase cleaves probe allowing an increase in fluorescence emission. The intensity of fluorescence is increased in proportion to amount of amplicon produced.(Arya et al., 2005)

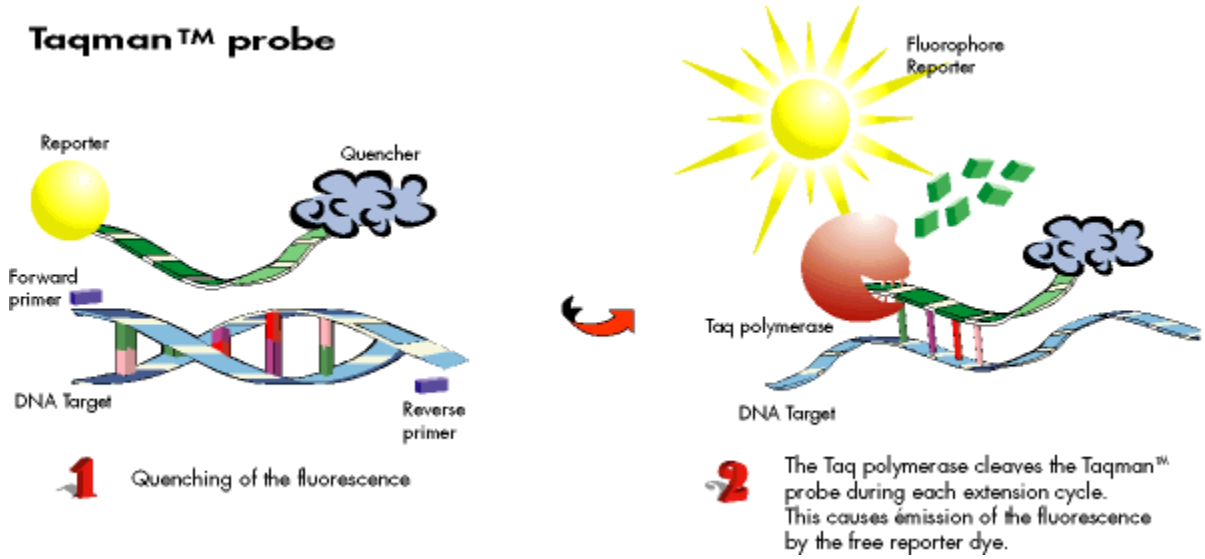


Figure 1. 6 Schematic diagram shoeing quenching of fluorescence and emission of fluorescence

On the basis of fluorescence emission data the computer software constructs amplification plots. Baseline describes about the PCR cycles in which a reporter fluorescence signal accumulates but is below the limit of detection by the machine.

Δ RN: It is the difference between fluorescence emission of product at each time point and fluorescence emission of baseline.

Threshold: It is arbitrary value choosen by computer being based on variability of baseline. The threshold cycle for a sample is a fluorescent signal which is detected above the threshold.

Ct: Ct is defined as the fractional number of PCR cycle at which reporter fluorescence is greater than the threshold (minimum detection level). The essential component in production of accurate and reproducible data is based on Ct which is a basic principle of Real time PCR (Higuchi et al., 1993). The lower Ct value indicates greater amount of target nucleic acid present in the sample.

During the early cycles of PCR exponential amplification of target sequence takes place which is the time when Ct value occurs. If more template is present at beginning of the reaction, a fewer number of cycle can lead to a point at which fluorescent signal is recorded statistically significant above the background (Higuchi et al., 1993).

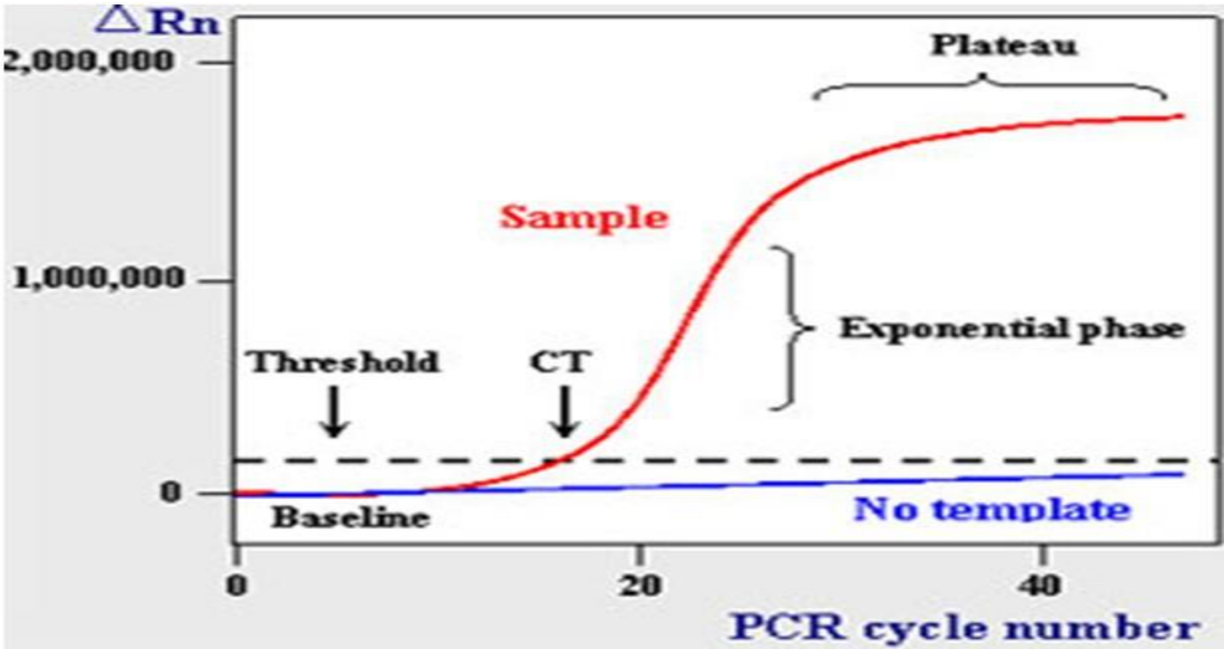


Figure 1. 7 Schematic diagram of the Real Time PCR reaction run

1.9 Immunopathology of Dengue Virus

When mosquito takes a blood meal infection initiates in host skin and immune cells and viral replication and inflammation will begin in organs such as liver and spleen. The dissemination of viraemia leads to production of CC chemokines in blood, liver and spleen which leads to local damages.

Since T cells are major sources of IL-17 in the liver which along with CC chemokines contribute to the massive inflammatory response observed during DHF/DSS, targeting the

CC chemokines, IL-17A and IL-1 family of cytokines may represent an effective adjunct therapy to attenuate the severity of disease manifestations observed in DHF/DSS (Guabiraba & Ryffel, 2014).

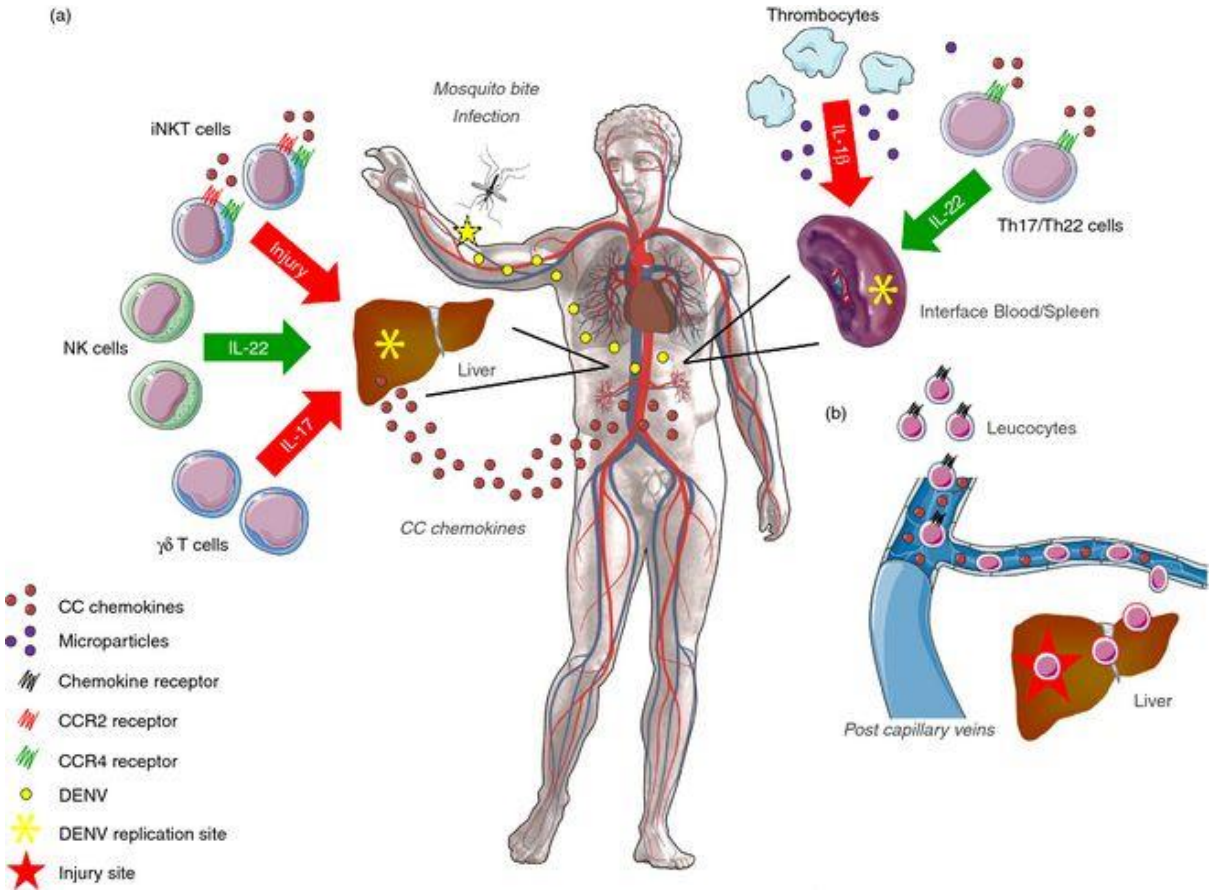


Figure 1. 8 Schematics showing the intricate role of chemokines, cytokines and inflammatory leucocytes in the pathogenesis of dengue virus (DENV) infection(Guabiraba & Ryffel, 2014)

1.10 Immunity against dengue virus

Humoral immune responses are thought to contribute to virus clearance via the generation of neutralizing antibodies and cellular immune responses are thought to contribute to virus clearance via the activation of CD4+ and CD8+ T lymphocytes. Innate host defence also plays role in limiting infection by the virus. After infection, serotype specific and cross-reactive antibodies and CD4+ and CD8+ T cells are found in body for years. The immune response, the genetic background of the individual and the virus characteristics may all

contribute to severe dengue which is characterized by plasma leakage, haemoconcentration and abnormalities in homeostasis.

1.10.1 CD4 T cells

CD4 is considered as a glycoprotein encoded by a gene called CD4 gene in humans which plays a major role in the immune system and lies on the surfaces of some immune cells T helper cells. Antigens presented by Major Histocompatibility Complex (MHC) Class II are recognized by CD4+ T cells and are activated in order to kill intracellular microorganisms by releasing cytokines (Acharya et al., 2016).

1.10.2 CD8 T cells

CD8+ (cytotoxic) T cells, are generated in the thymus and express the T-cell receptor. They express a dimeric co-receptor, usually composed of one CD8α and one CD8β chain. The peptides presented by MHC Class I molecules are recognized by CD8+ T cells. CD8 T cells are vital for defense against viruses, bacteria and other intracellular pathogens.

When a CD8+ T cell recognizes its antigen and becomes activated, it secretes cytokines, primarily TNF-α and IFN-γ, which have anti-tumour and anti-viral microbial effects. The role of CD8 T cells in immune defense against viruses is very important and it can also result in excessive immune response which further leads to immunopathology, or immune-mediated damage.(“CD8+ T Cells | British Society for Immunology,”)

1.11 Flow cytometry

Flow cytometry is very importat immunological technology used for rapid cell count analysis. It forms the pillar for a basic blood count test. Enumeration of cells is done in a liquid flow using flow cytometry. The cells to be enumerated are either tagged with fluorescent antibodies or are stained and suspended in a liquid stream within a flow cell. The sheath buffer maintains pressure in such a way that ensures the cells flow in a narrow stream toward the laser source, and the beam hits only one single cell at a time. The light which is incident on the cell is then scattered forward as well as sideways. The scattered light is then captured by the optics in the analyzer and is directed to the detector. The cells are generally analysed on the basis of their shape, size as well as internal complexity (“http://www.thyrocare.com”).

Multicolor T-lymphocyte marker panels employs Flow cytometric cell sorting of T-lymphocytes (CD3-positive) into CD4-positive and CD8-positive cell populations The addition of antibodies against additional CD molecules allows further characterization of each sub-population through quantification and evaluation of different T-cell subsets. From T-cell population count we can conclude that depletion of a specific cellular population can

indicate of progression of a disease, while increment of another cell population may be indicative of a response to treatment.

Fluorometric detection for flow cytometry: Use of directly labeled primary antibodies are more preferred because non-specific binding is avoided, multiplexing is possible with antibodies from the same species as there is no secondary antibody incubation step and therefore fewer wash steps are present. However, the main reason for this is that direct labeling of primary antibodies with detection moieties such as fluorescent dyes can be a complicated and time-consuming process, which requires specialist knowledge (“Flow cytometry for CD4+ and CD8+ T cells – Expedeon,”).

1.12 Rationale of the study:

Dengue being a major public health issue of Nepal, the first goal of this research is to determine serotype of dengue virus by using Real Time PCR. The serotype of dengue virus keeps on changing in every outbreak so this study aims at determining prevalent serotype and its comparison with serotype that occurred during previous outbreak. Mostly, the infection with one serotype does not provide immunity against other serotype. If a patient is initially infected with dengue then during secondary infection the possible chances of fatal outcomes can be avoided. Therefore, it is very vital to know about the affecting serotype for efficient treatment of the patients. Real Time PCR is one of the efficient technique which is approved by FDA and supported by CDC. The test performed by using CDC kit is most reliable.

Estimation of T cells present in circulating peripheral blood of dengue patients is second aim of our research. In Nepal till date immune studies in dengue patients are not studied so this is the first attempt to study immune cells involved in protection and immunopathology of dengue patients. This research will provide a picture of immunity in Nepalese population against dengue. The kind of research being carried out in our department has good impact in dengue research field of Nepal.

The proper understanding of the cellular immunology during dengue infection will be valuable to correlate protective T cell immunity and to find immunologic markers predictive of disease severity which can further help in vaccine development.

The proper understanding of the contribution of cell-mediated immunity, particularly the T cells response, in protection and severity of secondary dengue infection has become very necessary.

1.13 Objectives

1.13.1 General Objectives

- Serotyping of dengue virus and estimation the T cells involved during dengue infection.

1.13.2 Specific Objectives

- Serotyping of dengue virus by one step Real-Time PCR.
- Estimation of T-cells circulating in peripheral blood of dengue patients by using BD FACS Calibur.
- Analysis of sub population of T cells (CD4 and CD8) in peripheral blood of dengue patients.
- Comparison of cellular immune response in dengue patients and healthy control.

1.14 Research Hypothesis

1.14.1 Null Hypothesis

- There is not presence of different serotypes in the sample of year 2016 and 2017.
- CD8 T cells will be same in both control and samples

1.14.2 Alternate Hypothesis

- There is presence of different serotypes in the sample of year 2016 and 2017.
- CD8 T cells will be higher in sample than in controls.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of global spread of dengue virus types

The four dengue viruses were found to be originated in monkeys which later independently jumped to humans in Africa or South East Asia between 100 and 800 years ago. Until the middle of the 20th century dengue was considered as a relatively minor, geographically restricted disease. The transportation of *Aedes* mosquitoes during the Second World War are thought to have played a crucial role in the dissemination of the viruses. Dengue Hemorrhagic Fever (DHF) was first documented only in the 1950s during epidemics in the Philippines and Thailand. It was not until 1981 that large numbers of DHF cases began to appear in the Caribbean and Latin America, where highly effective *Aedes* control programs had been in place until the early 1970s ("Dengue | CDC," n.d.).

2.1.1 DENV1

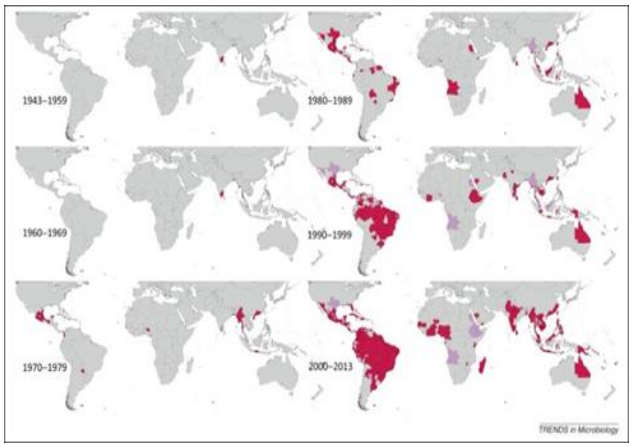


Figure 2. 1 Global spatial distribution of confirmed DENV1 occurrences by time period

The first reporting of DENV1 was in 1943 in French Polynesia and Japan. It was followed by reports in Hawaii in 1944 and 1945. The reporting of DENV1 in the Asian region started after late 1950s. It was first reported in Africa in 1984 in Sudan. DENV1 was not reported in the Americas until 1977, when it was recorded in Barbados, Cuba, French Antilles, Grenada, Paraguay, and Puerto Rico. When initial reporting of dengue in various regions occured, reporting increased continously across the region over the next few decades, mostly in Brazil, Mexico, and Puerto Rico. In the 1980s and 1990s, Colombia, Costa Rica, French Guiana, Paraguay, Peru, and Venezuela began having more sustained reports .The reporting

of DENV1 peaked in 2005–2006, which might have happened due to recorded occurrences in the Americas. It was after 1983 when the Pan American Health Organization (PAHO), in collaboration with the US Centers for Disease Control (CDC) Dengue Branch in Puerto Rico, the proper laboratory surveillance networks was initiated with technical assistance which increased the reporting of DENV cases.

2.1.2 DENV 2

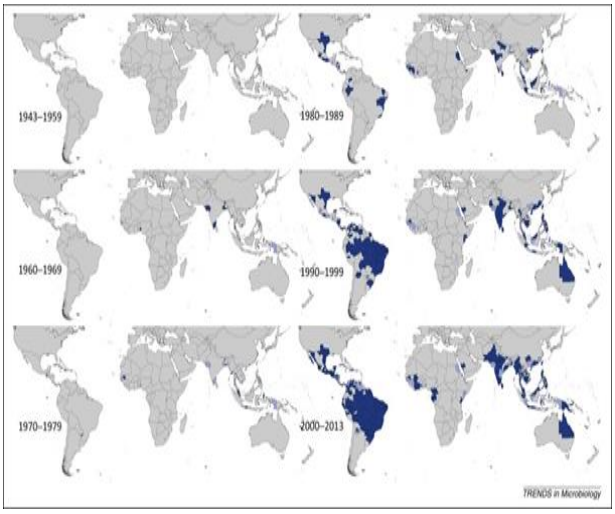


Figure 2. 2 Global spatial distribution of confirmed DENV2 occurrences by time period

In 1944 in Papua New Guinea and Indonesia, DENV2 was first reported which was followed by the Philippines in 1954 and 1956. Since the early 1960s Malaysia and Thailand have reported many consecutive years of DENV2 occurrence, and since the early 1970s Indonesia reported many consecutive years of DENV2 occurrence .In China, India, the Philippines, Sri Lanka, and Singapore, DENV2 is reported to have occured continuously since the 1980s. DENV2 was reported in Nigeria multiple times between 1964 and 1968, but has not since been reported there in Africa. Sporadic occurrences have since been reported in the African region from Goban in 2010 and Kenya in 2013. Though not continuous, DENV2 was reported in the Americas as early as 1953 in Trinidad and Tobago., but during late 1960s and early 1970s, DENV2 was notably reported in Puerto Rico. Frequent reporting of DENV2, occurred in Latin American countries since this time with continuous reporting in Brazil in particular since 1984 accounting for the majority of reporting of this type globally. An increase in the number of the more severe hemorrhagic fever (DHF) cases in the Americas occurred in the 1990s, which might have happened due to the replacement of the American DENV2 genotype with an imported and more virulent Asian one (Hesse et al., 1997). A noticeable rise in DENV2 reporting occured in this region since that time, which might be due to increase in DHF cases. DENV2 has been reported in largest number in 2005, with over 100 areas worldwide, primarily in the Americas.

2.1.3 DENV3

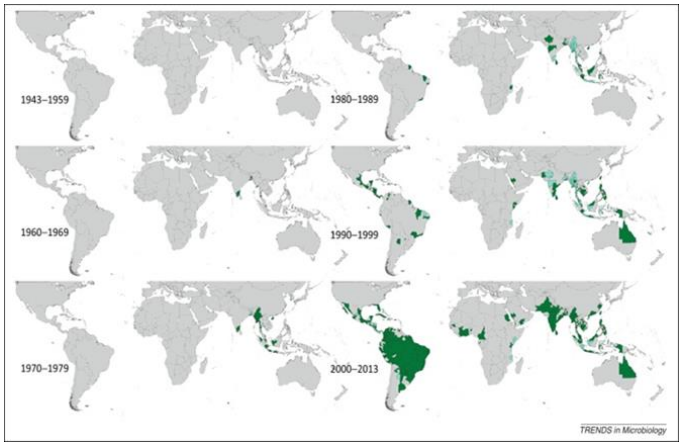


Figure 2. 3 Global spatial distribution of confirmed DENV3 occurrences by time period

DENV3 been reported in Asia every year since 1962 and was first reported in 1953 in the Philippines and Thailand. Though DENV3 has been reported in many countries in Asia throughout study period, Thailand most notably reported DENV3 every year between 1973 and 2010, with the most widespread reporting occurring between 1999 and 2002. It has been reported frequently since the 1970s in Malaysia and Indonesia and since the early 1980s in Sri Lanka. Since the mid-1990s, DENV3 is being consistently reported in China, Vietnam, Cambodia, and Singapore .It was first reported in the Americas in Puerto Rico in 1963, until 1978, and then again from 1994 to 2008 owing to the introduction of a new DENV3 genotype from Asia. Between the late 1980s and early 2000s majority of other countries in the Americas started reporting dengue infections. In the mid-2000s widespread reporting occurred in Brazil. In Africa, DENV3 has rarely been reported since the first reports in 1984–1985 in Mozambique, most occurence being sporadic, with the exception of more frequent reporting between 1994 and 2009 in Saudi Arabia (Messer, Gubler, Harris, Sivananthan, & de Silva, 2003).

2.1.4 DENV4

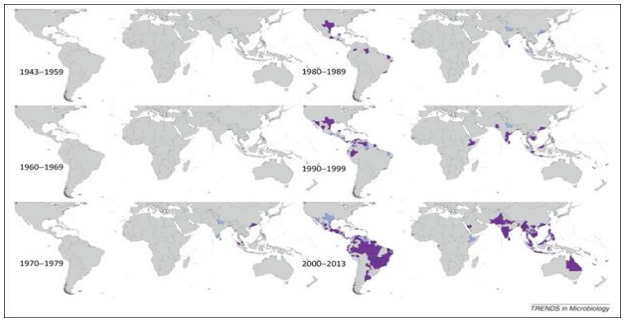


Figure 2. 4 Global spatial distribution of confirmed DENV4 occurrences by time period

The reporting of DENV4 took place first in 1953 in the Philippines and Thailand after which yearly reporting of DENV4 has occurred in this region. The most frequent reporting took place in Thailand whose most widespread reporting occurred between 1999 and 2002. DENV4 has been also reported almost yearly in Sri Lanka since 1978. Until 1981, DENV4 was not reported in the Americas. After 1981 reporting has occurred yearly in Brazil, Cuba, Dominica, Puerto Rico, and the US Virgin Islands.

Although reporting by country has not been as consistent as for other DENV types, periods of more frequent reporting have occurred in the Indochina region as well as Indonesia, India, Myanmar, and French Polynesia. DENV4 was not reported in the Americas until 1981, when it was reported in Brazil, Cuba, Dominica, Puerto Rico, and the US Virgin Islands.(Messina et al., 2014)

2.2 Dengue in Nepal

The study of Dengue virus and its infection was the immediate need from the very beginning when the infection was seen. The study was thus initiated when 876 patients of Kathmandu valley were considered but the study did not provide any indicative result. Among screened sera from 103 Patients presenting acute fever, eight individuals were tested positive for Dengue-specific IgM which led to a conclusion that DENV was transmitted in Kathmandu and surrounding hills as published in report of 2007 (Blacksell et al., 2007). About 80% of positive cases were found to be from terai people (Malla et al., 2008). In 2009 from June to September, cross sectional study was carried out and overall anti- IgM positivity was 12.17% with greatest occurrence in Kanchanpur which was followed by Chitwan (Poudel et al., 2012). Again, independent study was carried out in Nepal from 2010 January to 2011 December. This study showed 8.5% overall seropositivity among clinically suspected patients. During December 2010 when most number of cases were occurred the rate rose to 90.4%. This is supposed to be the largest outbreak of dengue in Nepal to date. Significant irregularities in case reporting have been found to have occurred at official level due to various lacking programmes and policies. For 2010 epidemic, DENV1 was found to be responsible which showed phylogenetically close relationship to predominant Indian strain (Pandey et al., 2013).

More than half of the Nepalese current population lives in areas where they are at risk of infection. Though the major factor related with emergence and rapid spread of dengue is yet to be analyzed, the most probable cause might be the increase in number and geographical range of vectors which is facilitated by their transmission due to suitable conditions. The febrile nature of Dengue and its undifferentiated and nonspecific disease symptoms overlap with other viral infections (Fernandez et al., 2013). The report are mostly

focused to the economically strong areas and does not include areas of relative social deprivation where disease is more likely to occur which can lead to a conclusion that the accurate burden of dengue in Nepal yet remains unknown (Vector Borne Diseases,).

2.3 Serotyping of Dengue virus

There are four serotypes among the Dengue viruses (DENV-1, 2, 3, & 4) that have no cross-protective immunity in infected patients which in turn increases the complexity of this disease. Recently antigenically distinct sylvatic strains contributing to human cases was observed which signifies existence of fifth serotype of dengue virus (Perdomo-Celis et al, 2017).

In 1991, a report on the development and application of a rapid assay for detecting and typing dengue viruses was published in which oligonucleotide consensus primers were designed to anneal to any of the four dengue virus types and amplify a 511-bp product in a reverse transcriptase-polymerase chain reaction (PCR). In this process a cDNA copy of a portion of the viral genome was produced in a reverse transcriptase reaction in the presence of primer D2 and a standard PCR (35 cycles of heat denaturation, annealing, and primer extension) with the addition of primer D1 was carried out. The produced DNA was then subjected to dot blot hybridization with virus type-specific probes or a second round of nested PCR was carried out with type-specific primers which amplified the particular serotype of DENV from viremic human serum samples (Lanciotti et al, 1992).

Nepal being a Himalayan country, initially dengue was recognized in tropical and subtropical areas. Later on, infection spread all over the country including the capital city Kathmandu which is a hilly area. Initially the available clinical measures were found insufficient for control of dengue outbreak. The outbreak of 2010 and 2013 led to analysis that the capacity of dengue infection could be explosive and challenging (Vector Borne Diseases, n.d.).

When a Japanese worker stayed in Nepal for few months and returned back to his homeland, he was tested dengue positive (Pandey et al., 2004). In Nepal, DF is rapidly expanding its geographical range from south to north. Extraction of RNA was performed with a High Pure viral RNA extraction Kit. The genome of dengue virus was detected by TaqMan RT-PCR. The isolated serotype was dengue virus 2 which showed 98 % similarity with DENV2 of India. It was thus assumed that the disease might have been transmitted through undiagnosed individual or might be through translocation of infectious mosquito (Takasaki et al., 2008).

The light on occurrence of dengue infection in Nepal was illuminated thereon. Till the diagnosis of DENV in Japanese worker, most of dengue cases that might have occurred remained undiagnosed. The assumption is that the clinicians could not suspect the dengue disease because of its similarity with other viral disease as well as there was limited medical

laboratory facilities. In 2006, from September to October, blood samples of suspected case from Banke and other districts were sent to National Public Health Laboratory in Kathmandu or the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand where, Reverse Transcription–PCR, (RT-PCR), ELISA or both were performed for analysis of dengue infection. Blood samples were collected from patients who had acute febrile illness for 2 to 7 days and two or more than two days of clinical manifestations such as headache, retro orbital pain, muscle or joint pain and rash. RNA from the samples were isolated using QIAGEN Viral RNA Extraction Kit per manufacturer's instructions (QIAGEN, Germantown, MD, USA) and RT PCR and nested PCR were conducted for the detection of virus serotype. During this study, all four serotype were found to be prevalent in Nepal which poses the emergence of more severe dengue disease in Nepal (Malla et al., 2008). Dengue was first observed as an outbreak in 2006 in nine lowland districts of Nepal. Later in 2010, larger epidemic occurred in Nepal with 917 serologically-confirmed cases and five deaths as reported in government hospitals of Nepal. The genetic characterization of dengue viruses isolated from patients in four major outbreak areas of Nepal was thus performed which suggested that the DENV-1 strain was responsible for the 2010 epidemics (Pandey et al., 2013).

All four serotypes circulation were found in 2006 in Nepal which showed heterogeneity of multiple dengue serotypes. Thereafter, only a single serotype was found as predominant as in the year 2010 and 2016, it was DENV-1 while in 2004, 2013 and 2014, it was DENV-2. The same type of serotype prevalence was seen in Delhi, India where all four serotypes were seen in 2003 followed by predominant serotype 3 in 2004 and 2005 (Gupta et.al, 2006). The understanding of molecular epidemiology of dengue is very important in Pakistan as dengue has been recognized as one of the major public health threats in Pakistan. A study was carried out which included 200 sera obtained from dengue-suspected patients from 2006 to 2011. DENV infection was confirmed in 94 (47%) sera by a polymerase chain reaction assay which included 36 (38.3%) DENV-2, 57 DENV-3 (60.6%) and 1 DENV-4 (1.1%) cases. DENV-2, DENV-3 and DENV-4 in Pakistan from 2006 to 2011 shared 98.5-99.6% nucleotide and 99.3-99.9% amino acid similarity with those circulated in the Indian subcontinent during the last decade (Koo et al., 2013). Though all DENV serotypes are found endemic in Jakarta on the basis of analysis of the viruses isolated from DHF patients in Indonesia from 1975 to 1978 and from 2005 to 2010 demonstrated that, but DENV-3 is the most predominant. A study was carried out in Jakarta from 2009 to 2010 which included adults with fever of less than 48 hours, 72% were confirmed dengue infection from a total of suspected 190 dengue patients. RT-PCR was performed, which showed that 16 patients were infected with DENV-4 with or without co-infection with other serotypes. It was found that (73%) were infected with DENV-4 alone, while mixed infections were present in more severe cases of dengue infection (Dewi et al., 2014).

Although Hong Kong is not endemic for dengue, it is located in Southeast Asia and is geographically close to countries endemic for dengue. Frequent travel to dengue endemic areas might be the risk factor associated with dengue infection as it was found that majorities of cases were imported from nearby Asian countries. In 2002, a DF outbreak involving 16 cases occurred in Ma Wan, an island in Hong Kong, and was caused by DENV serotype 1. Three more sporadic local cases caused by DENV serotypes 1 and 2 were reported in other districts in the same year. It was found that a fourfold increase (from 11 to 49 cases) in the incidence rate was recorded from 2000 to 2003. More than 30 cases were reported annually during 2004–2012, and 83 cases were documented in 2010 (Lo et al., 2013).

2.4 Dengue infection and the immune system associated

T cells complete their development in thymus and enter the bloodstream which are further carried through circulation. When T cells reach peripheral lymphoid organ they migrate through lymphoid tissue which return to bloodstream and keep circulating between blood and peripheral lymphoid tissue till the time they encounter their specific antigen. Naive T cells are the mature T cells that have not encountered their antigens. When a naive T cell encounters its antigen it proliferates and takes part in clearence of the antigen after which it becomes armed effector T cells which is then said to be taking part in adaptive immune response. Armed effector T cells acts upon target cells.

When any antigen is encountered by immune system, the antigen-presenting cell (APC) are activated .The antigens are carried to cell surface with the help of MHC class I molecules and are presented to CD8 T cells when Peptides from intracellular pathogens that multiply in the cytoplasm.

When Peptide antigens from pathogens that multiply in intracellular vesicles, and those derived from ingested extracellular bacteria and toxins are present as antigens , they are carried to the cell surface by MHC class II molecules and presented to CD4 T cells (Janeway, et al., 2001)**.**

The induction of a robust immune response and massive T cell activation occurs during during dengue infection. It is very important to understand why dengue can be fatal in some cases. Using flow cytometer, it was demonstrated that CD8+ T cell, expand and become highly activated, during the days following the onset of dengue fever symptoms. At the expansion important role of CD8 T cells which brings decrease in dengue virus load in patients blood is seen (Janeway et al., 2001).

Though various epidemiological studies regarding dengue has been carried out the study about immunological response in dengue patients is minimal in Nepal. Cellular immunity plays a vital role during dengue infection. CD8 T cells have been incriminated for both protection and cytokine –mediated immunopathology in dengue patients.

Though dengue virus immunity involves both innate and adaptive immune response, CD8 T cells are very important because they help in elimination of virus-infected targets through cytotoxic effector function and vital for vaccine development as well. The secretion of cytokines upon in vitro stimulation with heterologous viral antigen by the dengue-specific memory T cells, led to a guess that the “cytokine storm” induced by activated T cells may contribute to the immunopathology of dengue. The fact that CD8 T cell expansion peaks before or around the time of the peak of clinical disease and that the frequencies of activated CD8 T cells and cytokine producing cells were somewhat higher in patients with severe forms of the disease also supports the above mentioned role of CD8 T cells (Janeway et al., 2001). Though many studies are being carried out, the understanding of CD8 T cell properties during the febrile phase of dengue disease is still unclear (Chandele et al., 2016).

CHAPTER THREE

METHODS AND METHODOLOGIES

3.1 Sampling Location

Samples were collected from Chitwan Medical College Teaching Hospital, Chitwan in 2016 and Sukraraj Tropical and Infectious Disease Hospital, Kathmandu in 2017 and further research activity was carried out at Central Department of Biotechnology, Tribhuvan University, Kritipur.

3.2 Ethical Considerations

For the collection and processing of blood specimens, approval was taken from Nepal Health Research Council and our Department has coordinated with the hospitals from where samples were taken. Before collection of samples, consents were taken from the patients in written form where they need to do their signature. The consent form explains that their blood samples are being used for research purpose and they will not be paid for this. For patients below age of 18 or those who cannot read and write, their guardians or close relatives who are with them in hospital need to explain the things written in the consent form to them and if the subject agrees then the consent is finally taken.

3.3 Transportation and preservation of samples

Blood samples were collected from hospitals and transported to Central Department of Biotechnology, Tribhuvan University, Kritipur as soon as possible to prevent possible degradation of samples and immediately processed. In case of samples from Chitwan Medical College and Teaching Hospital, samples were drawn ,processed and aliquoted and stored over there and brought to Central Department of Biotechnology, Tribhuvan University, Kritipur when time is convenient. Similarly in case of Sukraraj Tropical and Infectious Disease Hospital, Kathmandu samples were drawn and immediately tansported to Central Department of Biotechnology, Tribhuvan University, Kritipur and centrifuged for further processing.

For flowcytometry analysis 200 μl of blood was aliquoted. Blood samples were centrifuged in 2500rpm for 10 minutes and then serum was aliquoted in small vials for further RNA extraction. The aliquoted RNA was further stored in -80 °C for further use. The aliquot of samples, master mix preparation and RNA extraction was performed in BSL-2 .

3.4 RNA Extraction of serum samples

RNA of dengue virus was isolated by using Qiagen viral RNA isolation kit and the extracted RNA was soon stored in the freezer to prevent further degradation of viral RNA for further molecular research.

First of all, 310 µL AVE Buffer was added to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl and allowed to dissolve thoroughly. Then, the solution was aliquoted (50µl), and stored at –20°C. AVL Buffer was observed to see whether precipitated, and if necessary was incubated at 80°C for 3-5 minutes for dissolving the precipitate if present.

Volume of Buffer-AVL (560 µl/sample) and carrier RNA mixture solution (10 µl/ml of AVL Buffer) required per batch of samples was calculated and aliquoted. Similarly, appropriate amount of Ethanol were added to the AW1 and AW2 buffers respectively to prepare the Buffers.

Buffer-AVL containing carrier RNA (560 µl) was dispensed into a 1.5 ml microcentrifuge tube and 140 µl serum was added. The solution was mixed by pulse-vortexing for 15s and incubated at room temperature (15–25°C) for 10 min. The tubes were then centrifuged briefly to remove drops from the inside of the lid and 560 µl of ethanol (96–100%) was added to the sample, and then mixed by pulse-vortexing for 15 seconds. After mixing, the tube was briefly centrifuged to remove drops from inside the lid. Then 630 µl of the solution was added to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim 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 discarded the tube containing the filtrate. The QIAamp Mini column was carefully opened, and the step was repeated again. The QIAamp Mini column was carefully opened and 500 µl of Buffer AW1 was added and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini column was placed in a clean 2 ml collection tube (provided), and discarded the tube containing the filtrate. The QIAamp Mini column was carefully opened and 500 µl of Buffer AW2 was added and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 min. The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube. The old collection tube containing the filtrate was discarded. QIAamp Mini column was opened carefully and 60 µl of Buffer AVE equilibrated to room temperature was added. The cap was then closed, and the tubes were incubated at room temperature for 1 min. Finally the tube were centrifuged at 6000 x g (8000 rpm) for 1 min and RNA collected in the microcentrifuge tube was stored at -80°C till further use.

3.5 Quantification of RNA by Nanodrop

RNA was eluted in buffer AVE, so the same buffer was used for the measurement of blank. Then after setting blank , 1 microliter of extracted RNA was used for quantification of extracted RNA.

3.6 Real Time PCR of RNA Samples

One step Real Time PCR was performed as per the instructions in CDC Kit. The prepared master mix of virus was mixed with the enzymes Superscript III and the one step Real Time PCR was performed along with positive and negative controls available in the kit.

3.6.1 RT-PCR Protocol Reagent (CDC Dengue Kit)

Reaction Mixture for	DENV-1, 2, 3 & 4
No. of Reaction Tubes	20

Reagents	**Conc.**	**Vol/Rxn**	**Total vol**
RNAse Free Water		2.2	44
2X Premix		12.5	250
Primer D1-F	100 µM	0.5	10
Primer D1-R	100 µM	0.5	10
Primer D2-F	100 µM	0.25	5
Primer D2-R	100 µM	0.25	5
Primer D3-F	100 µM	0.5	10
Primer D3-R	100 µM	0.5	10
Primer D4-F	100 µM	0.25	5
Primer D4-R	100 µM	0.25	5
Probe DENV-1	10 µM	0.45	9
Probe DENV-2	10 µM	0.45	9
Probe DENV-3	10 µM	0.45	9
Probe DENV-4	10 µM	0.45	9
Superscript III RT/ Platinum Taq Mix		0.5	10
Total		**20**	**400**

Reaction Mixture for	Human Specimen P gene		
No. of Reaction Tubes	20		
Reagents		Vol/Rxn	Total vol
RNAse Free Water		5.5	110
2X Premix		12.5	250
Primer RP-F		0.5	10
Primer RP-F		0.5	10
Probe RP		0.5	10
Superscript III RT/ Platinum Taq Mix		0.5	10
Total		**20**	**400**

Table 3. 1 Mastermix Preparation for Real Time PCR

3.6.2 RT-PCR plan for Dengue RNA

First of all, the Reaction table was prepared. Then the working table was prepared by wiping with by 70% Ethanol and all the articles to be used in master mix preparation were wiped. Separate space was prepared for keeping sterile reagents, and test samples and control positives. In sterile site Nuclease Free Water, 2X Premix, Primers and Probes were kept and at contaminated site test samples, dengue positive control and HSC positive control were kept. Two separate buckets of ice were kept for separate sterile and contanminated reagents. After that 5 μL of test samples were dispensed in each well and DENV 1-4 control, HSC positive, H_2O were dispensed in their respective wells of PCR plate. Two tubes were labeled for master mix preparation for DENV rxn and HSC rxn. Master mix were prepared separately for the DENV and Human Specimen control(HSC) in their respective Eppendorf. As last reagent, the SuperScript IIIRT enzyme was brought in cold chain box and required volume was dispensed to the master mix tubes. The tubes were vortexed once and 20 μL of mix was dispensed to each of respective wells by changing the tip every time. The plate was sealed with plalstic sticker and was rolled to press the plastic sticker. The plate was loaded to Biorad thermocycler for reaction.

3.6.3 Thermocycler Program

Table 3. 2 PCR Program for one step Real Time PCR

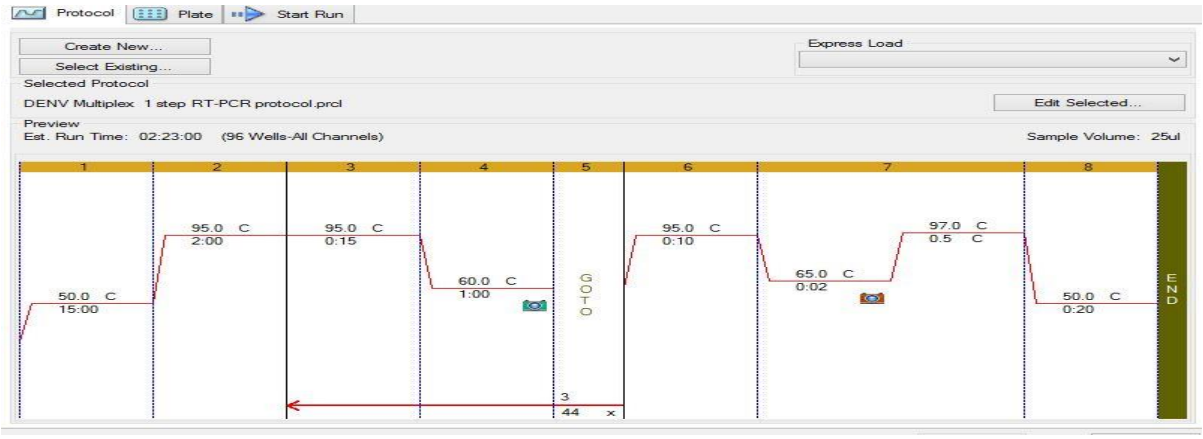
Target (^{0}C)	Acquisition mode	Hold(hh:mm:ss)	Ramp Rate(^{0}C/s)	Acquisition(per ^{0}C)	Set Target(^{0}C)	Step size(^{0}C)	Step Delay(Cycles)
95	None	00:00:15	4.4	-	0	0	0
60	Single	00:01:00	2.2	-	0	0	0

Program

Target (^{0}C)	Acquisition mode	Hold(hh:mm:ss)	Ramp Rate(^{0}C/s)	Acquisition(per ^{0}C)	Set Target(^{0}C)	Step size(^{0}C)	Step Delay(Cycles)
95	None	00:00:10	4.4	-	-	-	-
65	None	00:01:00	2.2	-	-	-	-
97	Continuous	-	0.02	5	-	-	-

Cooling

Target (^{0}C)	Acquisition mode	Hold(hh:mm:ss)	Ramp Rate(^{0}C/s)	Acquisition(per ^{0}C)	Set Target(^{0}C)	Step size(^{0}C)	Step Delay(Cycles)
50	None	00:00:20	2.2	-	0	0	0



Thermocycling parameters were as follows: reverse transcription at 50 °C for 15 min, inactivation at 95 °C for 2minutes, followed by 44 cycles of fluorescence detection at 95 °C for 15 s, and annealing at 60 °C for 1 minute. The baseline and threshold were set using the auto-baseline and threshold feature in system. Samples were considered positive if target amplification was recorded within 37 cycles. The CDC DENV-1-4 Real-Time RT-PCR Assay was performed in multiplex reactions following the manufacturer's instructions (Centers for Disease Control and Prevention) in 25 µL volumes using the SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen™). Amplification and detection were performed in a Biorad CFX96 PCR instrument .

The data analysis were performed as described in the manufacturer's guidelines. In short, the threshold was adjusted to fall within the PCR exponential phase in the linear view. The manufacturer's instructions specifies that a specimen is considered positive for either DENV1,2, 3, or 4 if the amplification curve crosses the threshold line within 37 cycles (Cq < 37).

3.7 Flow Cytometry analysis

3.7.1 Quality Control

Quality control is the process before performing an operation in FACS to check whether the machine is working properly or not. It is used to check if FL channels are working properly or not. The quality control was performed by using rainbow beads. These rainbow beads were used to check the peaks. There must be 8 peaks and the machine is thus said to be ready for further processing.

3.7.2 Compensation

There are four FL channels in our FACS machine and they are FL-1, FL-2, FL-3, FL-4 which are respectively aligned with flurochromes FITC, PE, PerCP and APC. The compensation of the machine was performed by using calibrate beads. In the graph we performed compensation by aligning the cells in their respective quadrant according to the experiment and the beads used. When compensation was performed properly the further processing of our sample was done. Adjustment of voltage was done which is vital during compensation.

3.7.3 Sample preparation for absolute cell counting by lyse No wash method

First of all 50 µl of whole blood was taken by pipetting method. Then 20 µl of antibody cocktail was added and was mixed by vortex. It was then incubated in dark for 15 minutes at room temperature. Then 450 microliter of 1X BD FACS lysing solution was added and vortex was done. It was then incubated in dark for 15 minutes. The preparation was then run on flowcytometer and counting of cell was then performed.

3.7.4 Analysis of Data

The obtained data was then analyzed in FlowJo and the estimation of percentage of CD4 and CD8 T cells were performed. Gating of targeted cells was done and then histogram plots were plotted. The histograms displayed a single measurement parameter (relative fluorescence or light scatter intensity) on the X-axis and the number of events (cell count) on the Y-axis. The data that is expressed in a histogram represents all the selected (gated) population. Histogram is simple and useful for evaluating the total number of cells in a sample that express the marker of interest. (“Single Parameter Or Univariate Histograms - Flow Cytometry Guide | Bio-Rad,”.)

CHAPTER FOUR

RESULTS

4.1 Gender Susceptibility of samples

Among all the samples used, 9 samples from Chitwan (Chitwan Medical College Teaching Hospital), 33 % of samples were female whereas 67% were males. All the patients from Sukraraj Tropical and Infectious Disease Hospital, Kathmandu were males. Thus, this report shows males are more affected by dengue in comparison to female.

4.2 Clinical symptoms of study subject

In case of samples from 2016 which were from Chitwan Medical College Teaching Hospital, all the patients i.e. 100% had symptoms of fever, headache and joint pain. Similarly, in case of samples from 2017 which were from Sukraraj Tropical and Infectious Disease Hospital, Kathmandu only six patients had symptoms of fever, headache and joint pain.

4.3 Biochemical Analysis reports

4.3.1 Hemoglobin

The normal level of hemoglobin per 100 ml of blood is 13-16gm in men and 11-13gm in women. It is said that a patient with 10 % increase in average hemoglobin level, should be rushed to the hospital though platelet count is normal (http://www.thehealthsite.com/news). Among all the patients two female patients SU8 and SU9 expressed slight increase in the hemoglobin level. Similarly, in case of male, hemoglobin seems to lie within the normal range.

Table 4. 1 Table showing hemoglobin percentage in dengue patients

Sample ID	Sex	HB(gm%)	Sample ID	Sex	HB(gm%)
SU1	Male	15.6	SU10	Male	16.2
SU2	Male	13.9	SU11	Male	14.6
SU3	Male	13.9	SU12	Male	14.7
SU4	Male	12.2	SU13	Male	14
SU5	Female	12.3	SU14	Male	15
SU6	Male	12.2	SU16	Male	11.8

SU7	Male	12.2	SU17	Male	14.5
SU8	Female	15.1	SU18	Male	11.7
SU9	Female	15.5			

4.3.2 Percentage of Neutrophils, Lymphocytes and Eosinophils in Dengue patients

Neutrophils are the most common type of white blood cell in the body with levels between 40% to 80% in the bloodstream. In case of all the patients neutrophils were within the reference range. A normal lymphocyte range is generally between 20% and 40 % in healthy individuals. In case of 2016 samples only one sample (SU9) had a slightly greater percentage of lymphocytes whereas in case of 2017 samples**,** five of the samples i.e (SU10, SU11, SU12, SU14, SU17) had shown increased lymphocyte percentage. Eosinophils are about 0.0% to 6% of blood. Among all the patients one patient sample (SU14) had high level of eosinophils.

Table 3. 3 Percentage of Neutrophils, Lymphocytes and Eosinophils in Dengue patients

Sample ID	Neutrophils	Lymphocytes	Eosinophils	Sample ID	Neutrophils	Lymphocytes	Eosinophils
SU1	70	29	1	SU10	45	45	0
SU2	67	29	1	SU11	39	50	1
SU3	67	32	1	SU12	50	45	0
SU4	79	20	1	SU13	56	34	0
SU5	79	20	7	SU14	34	46	12
SU6	79	20	1	SU16	51	29	0
SU7	79	20	1	SU17	40	46	4
SU8	54	40	6	SU18	75	19	0
SU9	52	43	4				

4.3.3 Platelets Count

Platelet in human body lasts for about 5 to 10 days and is replenished when required. Dengue virus when in its effective stage destroys the body's capacity to produce new platelets .The platelet count for a normal person varies from 150,000 - 250,000 per µl (micro litre = mm^3 or cubic millimeters) of blood. When a person is infected with dengue, their platelet count starts falling. A platelet count below 100,000 per µl (mm3) is alarming which requires immediate medical attention. A platelet count below 50,000 can be fatal. In case of 2016 samples, all the patients had somewhat low platelet count whereas two samples had platelet count below 50,000 which could be fatal. In case of 2017 patients

there was decrease in platelet count in all patients and three of them had platelet count below 50,000.

Table 4. 2 Platelet count in Dengue patients

Sample ID	Platelets/cumn	Sample ID	Platelet9/cumn
SU1	186000	SU10	27000
SU2	161000	SU11	100400
SU3	319000	SU12	87000
SU4	75000	SU13	44000
SU5	140000	SU14	44000
SU6	80000	SU16	111000
SU7	45000	SU17	80000
SU8	37000	SU18	61000
SU9	98000		

4.4 Quantification of extracted RNA

Quantification of RNA performed by using Nano drop. If RNA sample had shown the ratio of 260/280 below 2 then the protein contamination is performed. Similarly, the ratio of 260/230 if had value below 1.8 show organic contamination. The sample RNA that was extracted during my experiment thus showed protein and organic contamination. The concentration of nucleic acid measured in ng/µl showed low concentration of viral RNA in our samples. The table containing concentration of all RNA samples and the ratio of protein and organic contamination is given below. Similarly, the graph below represents the curve that is obtained during quantification of extracted RNA.

Table 4. 3 Quantification of RNA Samples

Sample ID	NA Concentration(ng/µl)	OD260/280	OD260/230
HSC RNA	57.94	0.62	0.19
Positive Control	46.37	0.56	0.21

SU1	55.23	0.65	0.11
SU2	50.72	0.69	0.11
SU3	50.71	0.69	0.11
SU4	58.66	0.65	0.13
SU5	49.72	0.7	0.49
SU6	65.14	0.63	0.15
SU7	32.78	3.5	0.41
SU8	65.14	0.63	0.15
SU9	16.67	3.1	1.92
SU10	14.55	3.11	1.02
SU11	18.66	1.23	0.33
SU12	19.22	0.7	0.49
SU13	25.5	0.9	0.47
SU14	4.56	0.65	0.25
SU15	18.99	0.67	0.44

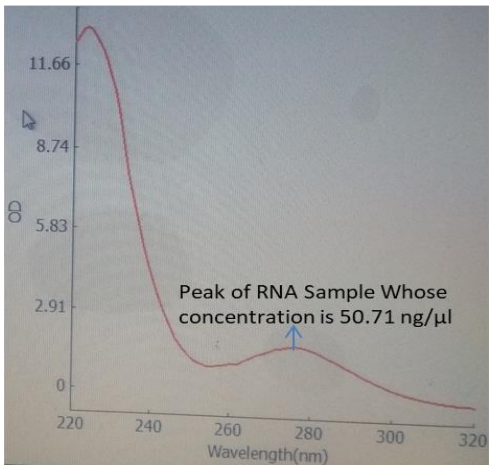


Figure 4. 1 Grraph obtained after RNA Quantification

4.5 Real Time PCR

The curves were obtained after the completion of the PCR program which were interpreted below.

4.5.1 Positive Control

Positive control virus mix included in CDC kit, which consisted of heat-inactivated DENV-1 Haw, DENV-2 NGC, DENV-3 H87, and DENV-4 H241 were amplified and expressed by all four colored curves obtained as in the picture below (Figure 4.2). Four colored graph was obtained when PCR of Positive control was performed. DENV1 positive sample is indicated by blue colored curve (FAM). DENV2 positive sample is indicated by green colored curve which represents VIC. DENV3 positive sample indicated by red colored curve which represents Texas Red. DENV4 positive sample is indicated by purple colored curve which represents Cy5. The fractional number of PCR cycle at which reporter fluorescence is less than the threshold value(Cq) (i.e. 37) are taken as positive. In our analysis the Cq values for DENV1, DENV2, DENV3, DENV4 positive control were 27.16, 30.39, 28.32 and 28.85 respectively.

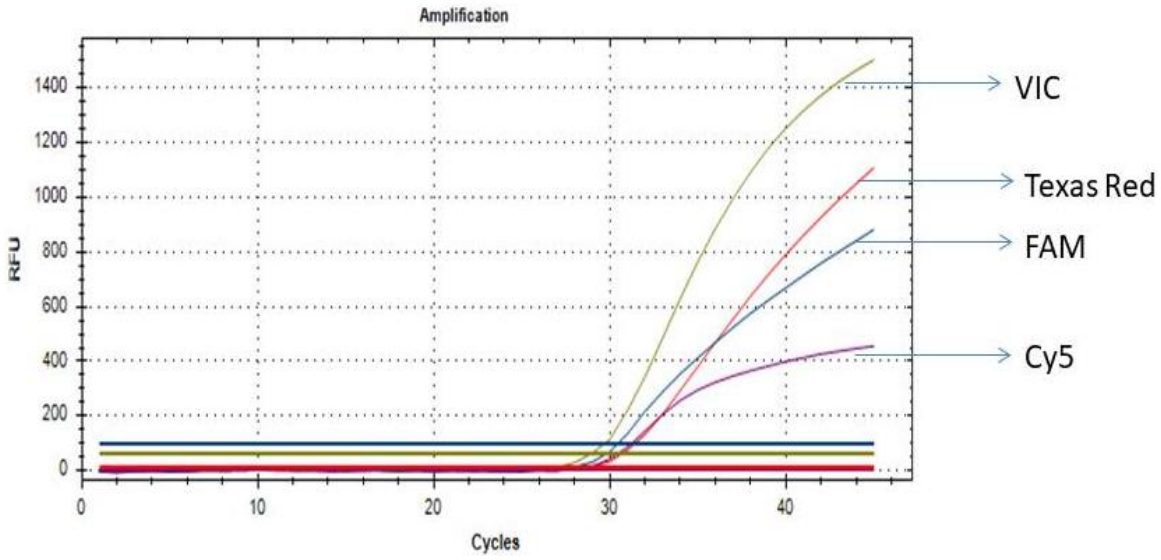


Figure 4. 2 Graph of Positive Control amplification of DENV

4.5.2 Negative Control

The negative control containing RNA of HSC was amplified when primer and probe of RP was used while it was not amplified when all DENV primers and probes were used. The Cq values were not applicable in case of Negative Control.

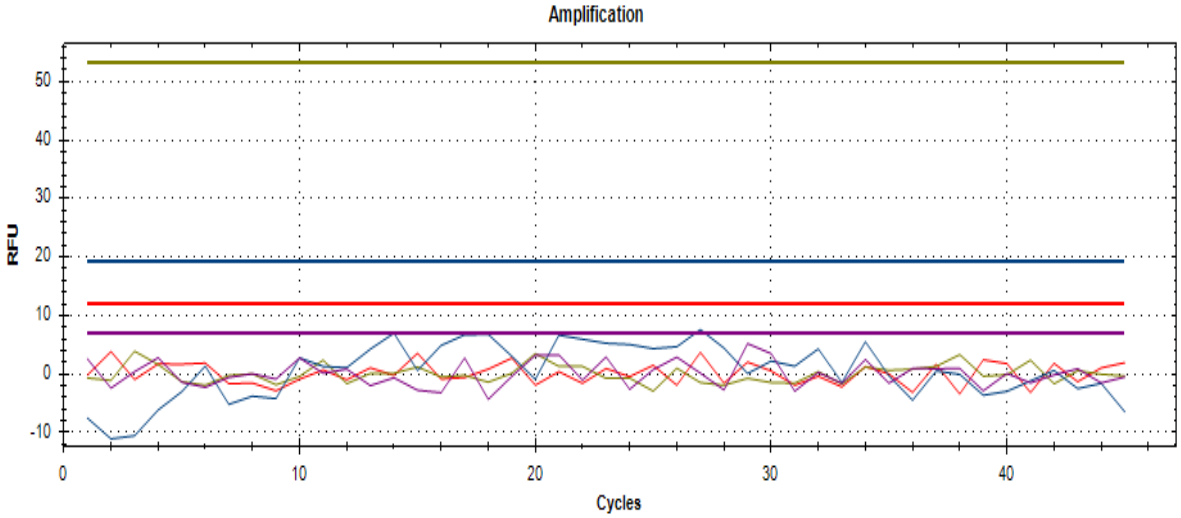


Figure 4. 3 Graph showing curves of Negative Control

4.5.3 Serotyping of DENV from 2016 samples

The samples of 2016 that were taken from Chitwan Medical College and Teaching Hospital for serotyping showed amplification by FAM. The samples were identified as DENV 1 serotype. All the nine of the samples showed the amplification curve (Figure 4.4) and their Cq value (Table 4.4)denoted that they were DENV 1 positive.

Table 4. 4 Table showing Cq values of DENV Samples of 2016

Fluor	Sample	Cq
FAM	SU1	20.67
FAM	SU2	24.24
FAM	SU3	25.21
FAM	SU4	19.01
FAM	SU5	23.89
FAM	SU6	17.35
FAM	SU7	21.77
FAM	SU8	15.71
FAM	SU9	32.98

The curve below shows the amplified DENV 1 samples of 2016.

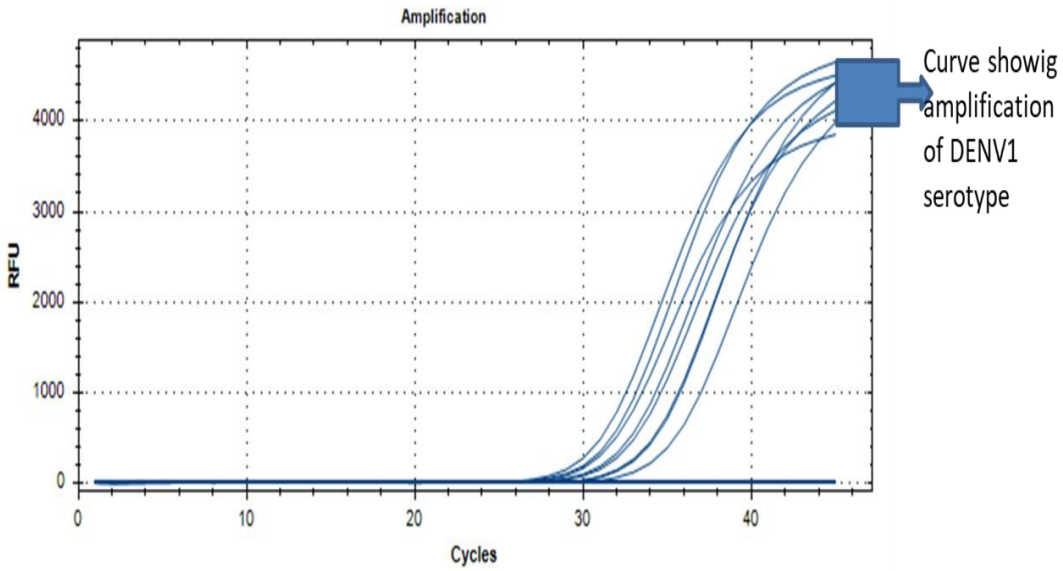


Figure 4. 4 Graph showing curve of amplified DENV1 virus

4.5.4 Serotyping of DENV from 2017 samples

The samples of 2017 that were taken from Sukraraj Tropical and Infectious Disease Hospital, Kathmandu for serotyping showed amplification by VIC. The samples were identified as DENV 2 serotype. Three of the samples were negative for serotyping as they did not show amplification by FAM in the wells containing primers and probes for HSC. This shows that RNA was not extracted properly. The remaining six samples showed the amplification curve (Fig. 4.5) and their Cq values (Table 4.5) denoted that they were DENV 2 positive. The six samples SU10, SU11, SU12 SU13 SU14 SU15 showed Cq values at 25.24, 31.36, 28.03, 23.38, 26.04, and 14.61 respectively.

Table 4. 5 Table showing Cq values of DENV Samples of 2017

Fluor	Sample	Cq
VIC	SU10	25.24
VIC	SU11	31.36
VIC	SU12	28.03
VIC	SU13	23.38
VIC	SU14	26.04
VIC	SU15	14.61

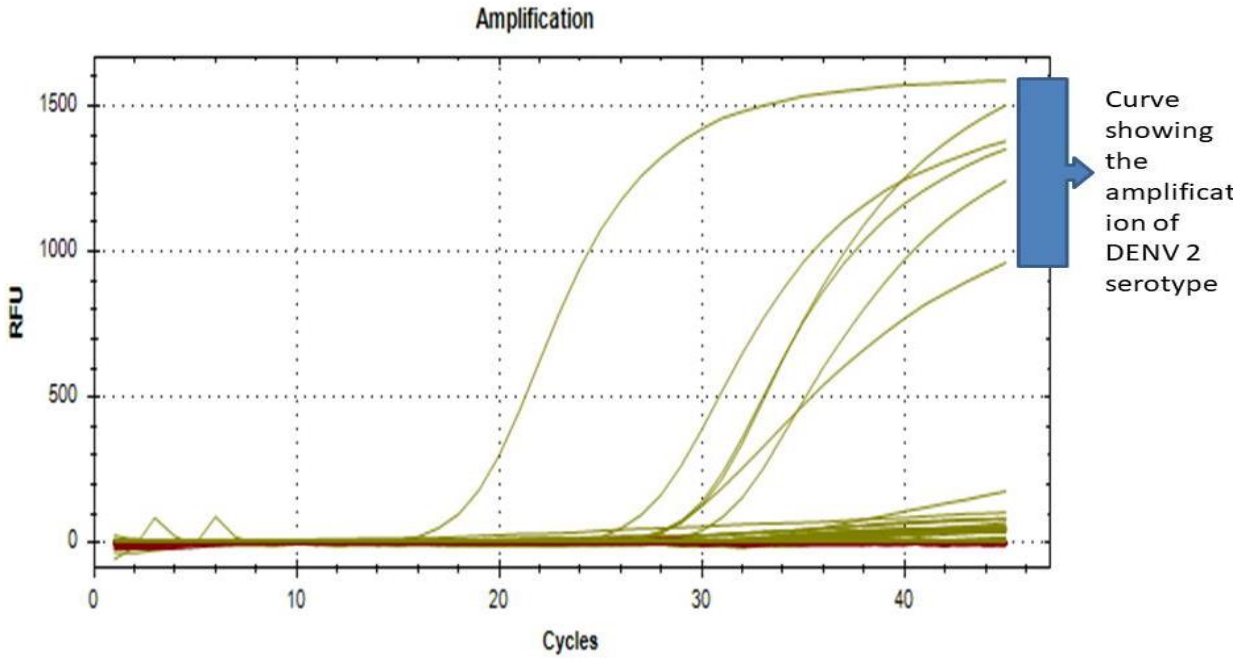


Figure 4. 5 Graph showing curve of amplified DENV2 virus

4.5.5 Result of all the amplified curves during Real Time PCR

All the samples which were used during our research were run along with the RP primers and probes to check the integrity of extracted RNA. As a result we found that three of the samples did not show any amplification curve during the Real Time PCR and this explains that the viral RNA was not extracted properly. Therefore we can conclude from this experiment that the Real time PCR is a very sensitive method for serotyping of DENV and there are many check points to correct the experiments and to eliminate the false positive results. The amplification curve of the extracted RNA is denoted by FAM and is indicated in the figure below (Figure4.6).

In the (Figure 4.7), curve of all the samples and control used in the Real time PCR and their amplification is shown. The four curves of FAM, VIC, Texas Red and Cy5 showed the positive control curves. Similarly all the lines near the base line can be considered as the background signals. The samples which did not contain DENV were not amplified thus their Cq values are given by the system as Non Applicable.

Table 4. 6 Table showing Cq values of ALL samples amplified by RP primers and probes

Sample ID	Fluor	Cq	Sample ID	Fluor	Cq
SU1	FAM	16.73	SU10	FAM	22.89
SU2	FAM	20.28	SU11	FAM	18.55

SU3	FAM	21.14	SU12	FAM	4.08
SU4	FAM	14.96	SU13	FAM	6.35
SU5	FAM	22.6	SU14	FAM	4.77
SU6	FAM	24.6	SU15	FAM	17.34
SU7	FAM	18.21	SU16	FAM	N/A
SU8	FAM	11.73	SU17	FAM	N/A
SU9	FAM	29.09	SU18	FAM	N/A

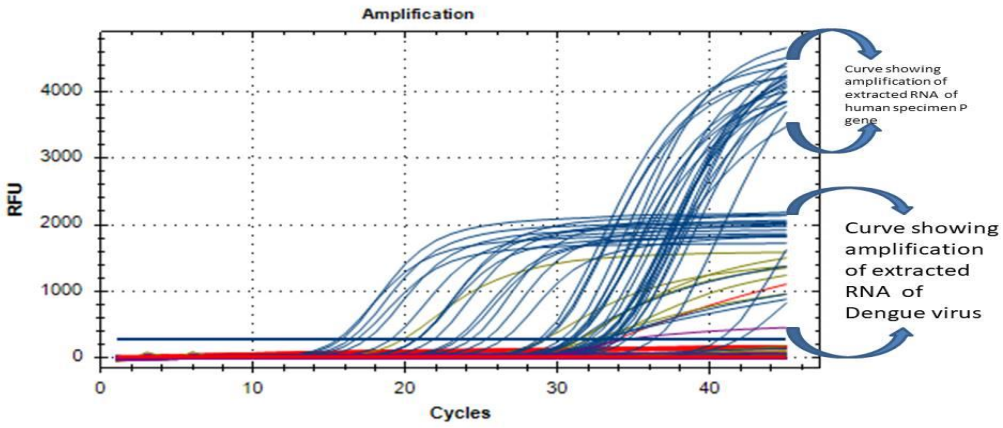


Figure 4. 6 Graph showing curve of amplified HSC RNA , positive controls and DENV1 and DENV2 virus

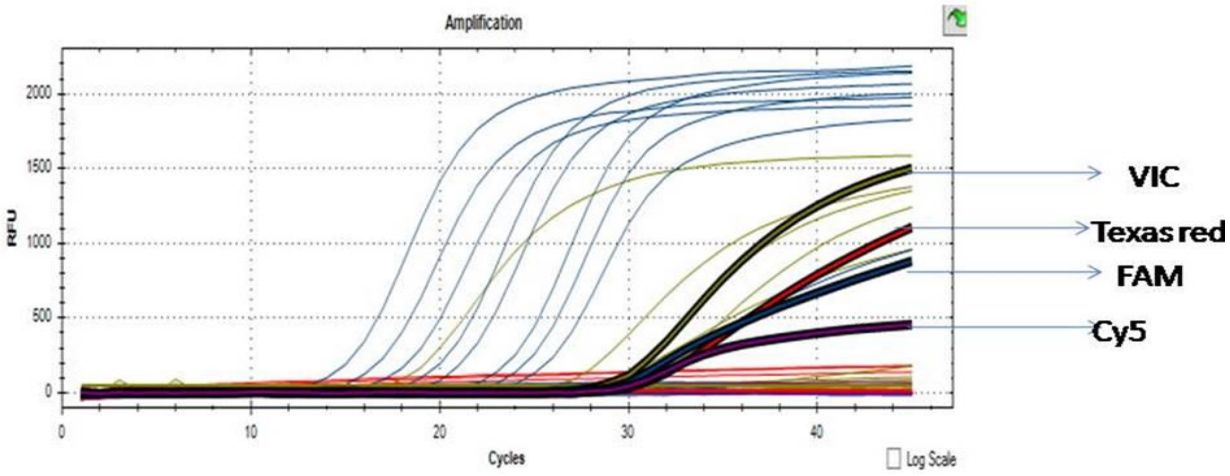


Figure 4. 7 Graph showing positive DENV samples along with positive controls

4.6 Result of Flow cytometry

4.6.1 Quality Control

For quality control the rainbow beads were used and the eight different peaks were obtained. There are four different FL channels (FL1, FL2, FL3, Fl4) so each of them showed 8 peaks and the machine is thus said to be ready for further processing. The figure (4.8) below indicates the picture taken after QC was performed by using rainbow beads.

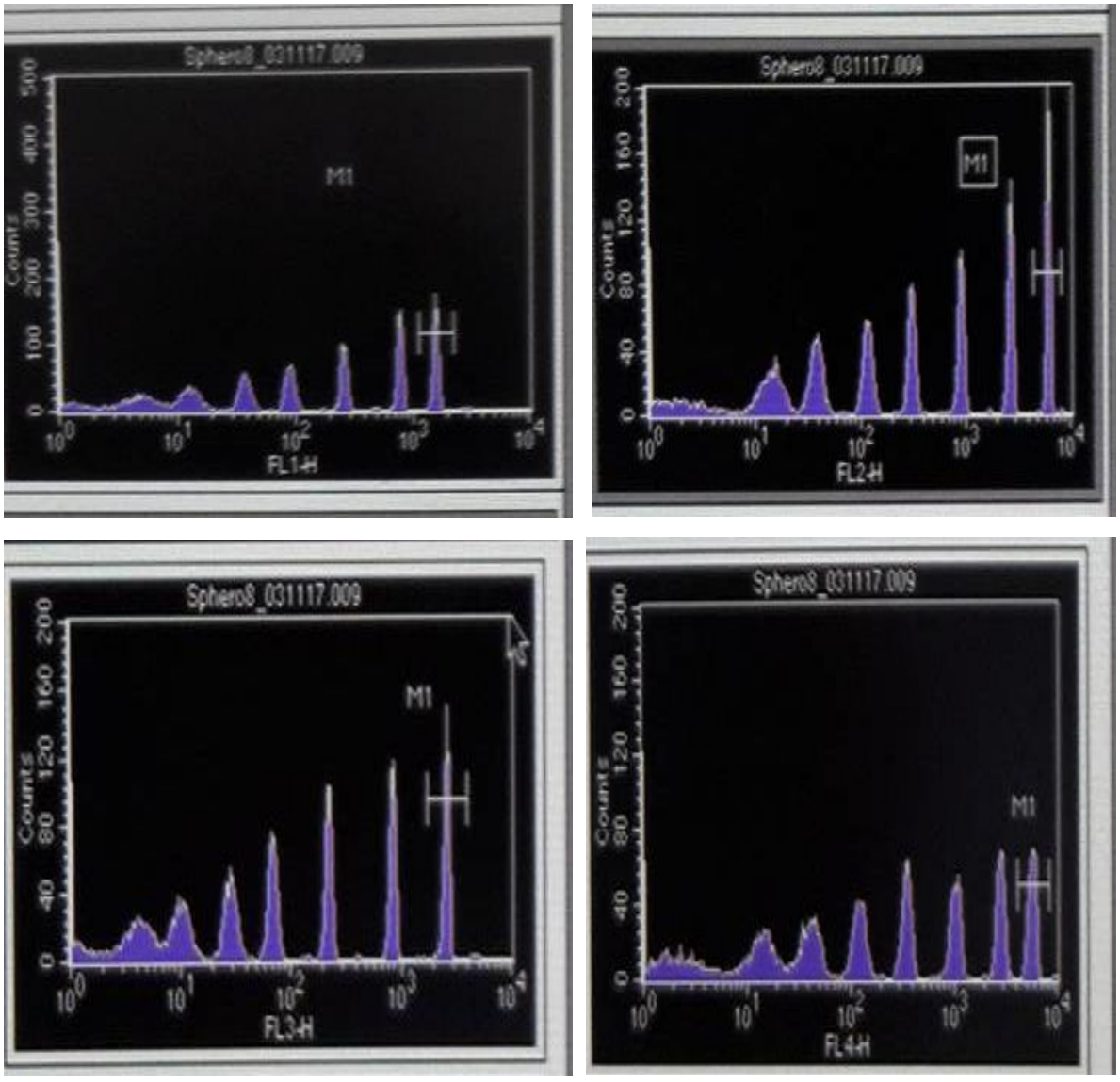


Figure 4. 8 Picture showing peaks of all FL channels after performing Quality Control

4.6.2 Estimation of T cells of control using blood of a person who is DENV negative

The control was used for the estimation of CD4 and CD8 cell percentage so that we could differentiate the cell percentage of healthy and dengue infected patients. The percentage of CD4 and CD8 cells were 24.21 % and 33.76 % respectively.

Table 4. 7 Table showing CD4 and CD8 cells in DENV negative sample

Sample	Antibody used	CD4 events	CD4 (%)
Control	CD3CD4	1617	24.21
		CD8 events	CD8(%)
Control	CD3CD8	1916	33.76

The dot plot below is plotted with side scattered in Y-axis and Forward scattered in X-axis which showed the different populations of blood cells and debris as shown in the figure (Figure 4.9). The lymphocyte population is gated as R1 is shown in the dot plot (Figure 4.10) in which the CD3 and CD4 T cell population are plotted in FL1 and FL2. The CD3 population was confined to the upper left quadrant. The upper right quadrant contained both CD3 and CD4 positive cells whereas the lower right quadrant had all the debris and unstained cells in it. The percentage of the CD4 together with CD3 was found to be 24.21% and indicates the population of CD4 T cells which is gated among the segregated blood cells. The population of CD4 cells below is that of control samples which are DENV negative.

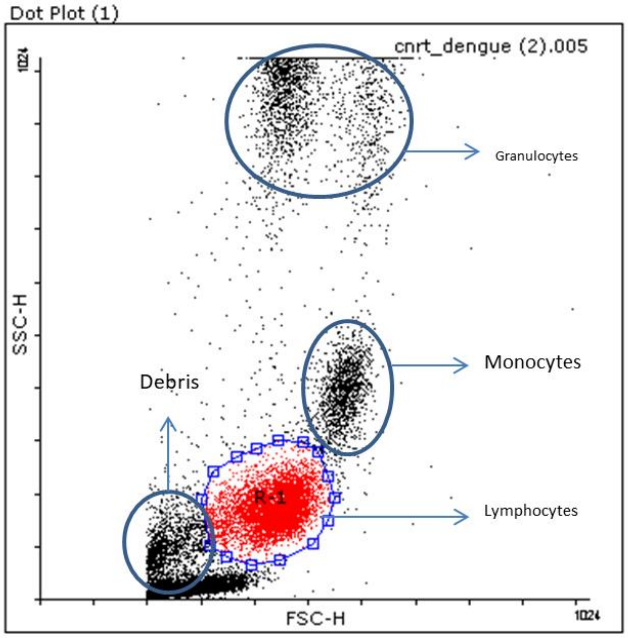


Figure 4. 9 Dot plot showing lymphocyte population and other blood cells

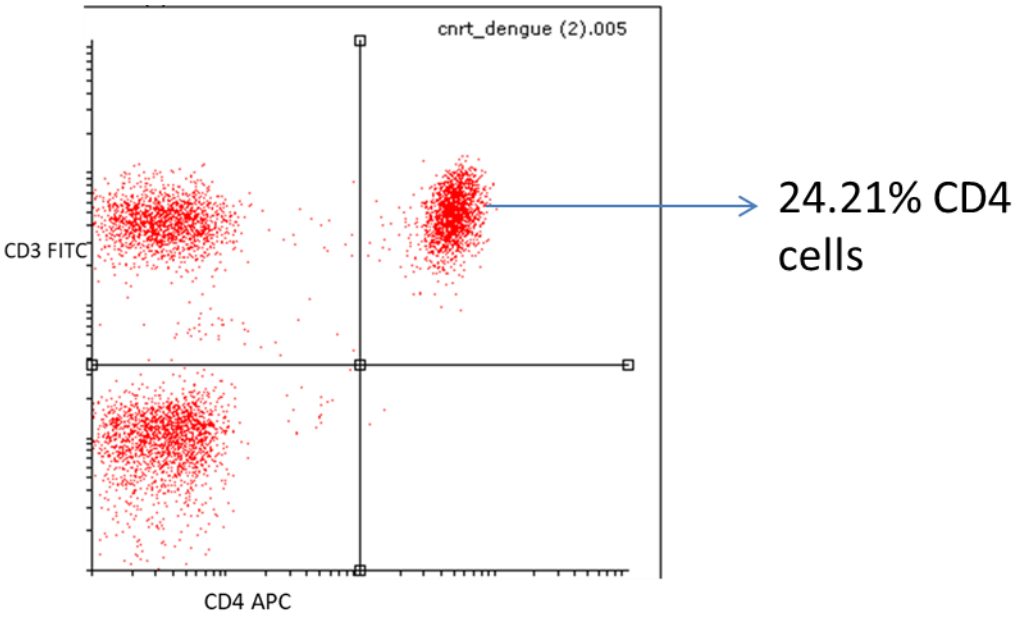


Figure 4. 10 Dot plot showing different T cell population

The lymphocyte population gated as R1 is shown in the dot plot (Figure 4.11) in which the CD3 and CD8 T cell population are plotted in FL1 and FL2. The CD3 population was confined to the upper left quadrant. The upper right quadrant contained both CD3 and CD8 positive cells whereas the Lower right Quadrant had all the debris and unstained cells in it. The percentage of the CD8 together with CD3 was found to be 33.76% and indicates the population of CD8 T cell population which is gated among the segregated blood cells. The population of CD8 cells is higher than that of control samples which are DENV negative**.**

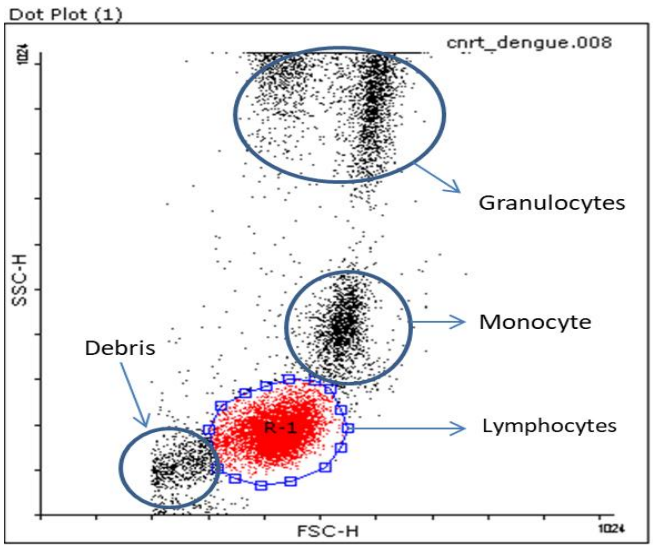


Figure 4. 11 Dot plot showing lymphocytes and other blood cells

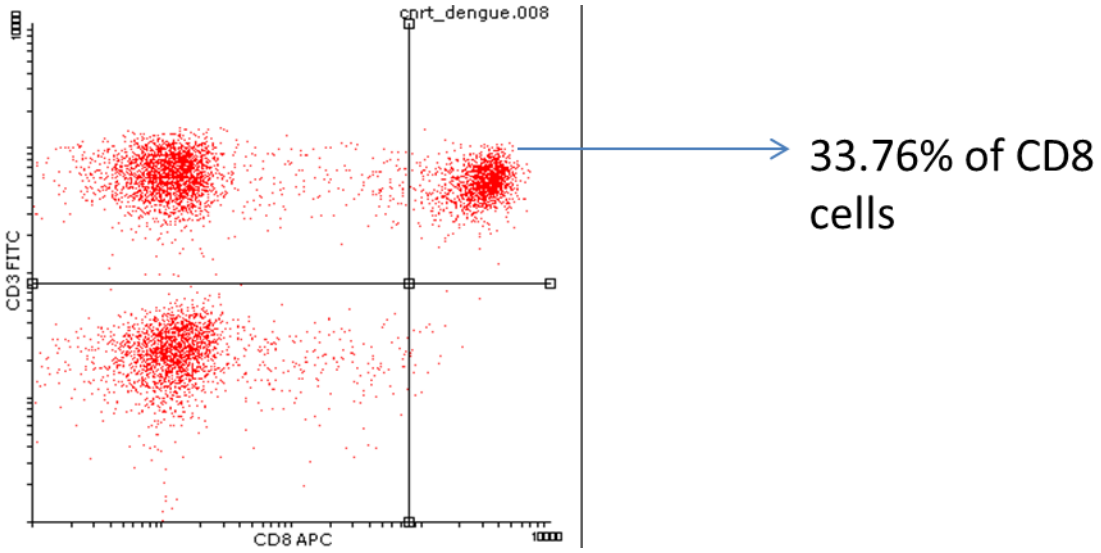


Figure 4. 12 Dot plot showing different T cell population

4.6.3 Estimation of T cells using blood of a person who is DENV positive

The samples labeled with the antibodies, lysed with FACS lysing solution after incubation, stained and run in a flow cytometer obtained the data for further analysis in FlowJo. The sample number 2 had CD4 and CD8 cell percentage 42.76% and 39.43% while the sample number 3 showed 39.61% and 47.28% respectively and sample number 6, showed 53.66% and 28.11% respectively. Similarly, sample number 4 showed 80.85% of CD8 cells and sample number 5 showed 46.27% of CD4cells. (Table 4.8)

Table 4. 8 Table showing CD4 and CD8 cell percentage in DENV positive samples

Sample number	Antibody used	CD4 events	CD4 percentage	CD8 events	CD8 percentage
2	CD3CD4	1138	42.76		
2	CD3CD8			965	39.43
3	CD3CD4	1037	39.61		
3	CD3CD8			1230	47.28
4	CD8APC			397	80.85
5	CD3CD4	1062	46.27		
6	CD3CD4	739	53.66		
6	CD3CD8			352	28.11

4.6.4 Comparison of CD4 and CD8 T cells in dengue patients and healthy control.

The graph (Figure 4.13) represents overall comparison between CD4 and CD8 cell percentage in between samples and control. It is shown that there is increase in the overall percentage of CD8 T cells than that of control whereas the percentage of CD4 cells is similar to that of the control.

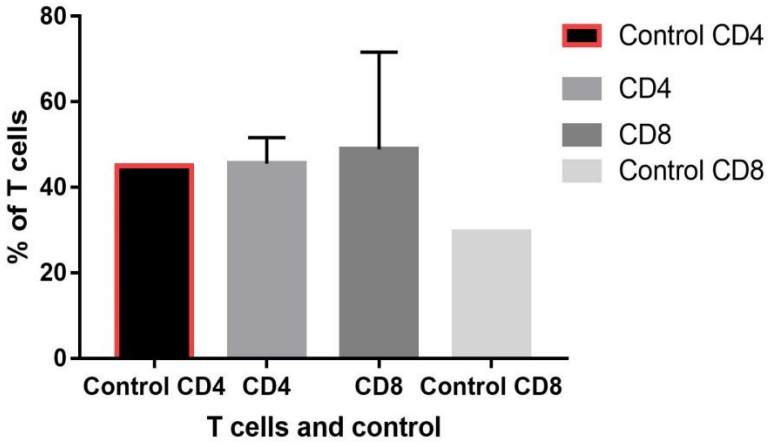


Figure 4. 13 Graph showing CD4 and CD8 T cell population along with controls

4.6.5 Histogram plots of the CD4 and CD8 cells of dengue patients

The histogram plots of all the samples whose CD4 and CD8 cell percentage are determined are given below. The two peaks are of positive and negative dataset. In these plots, the more shifting of curve towards X-axis, the more is the fluorescence and thus the fluorescence is proportional to the amount of CD4 or CD8 cells present in sample. In case of sample number 2, 3 and 4 the increase in CD8 cell percentage is observed. Relative fluorescence or light scatter intensity on the X-axis is seen higher and shifted towards right which represents a gated population.

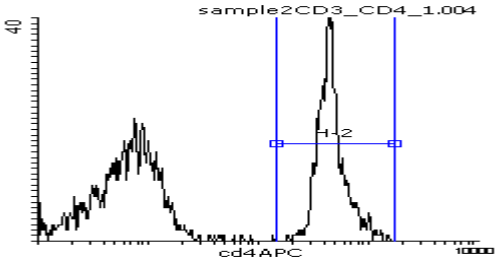


Figure 4. 14 Histogram plot of Sample number 2 showing CD4 cells among the CD3 population

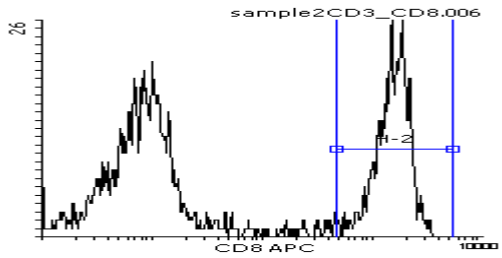


Figure 4. 15 Histogram plot of Sample number 2 showing CD8 cells among the CD3 population

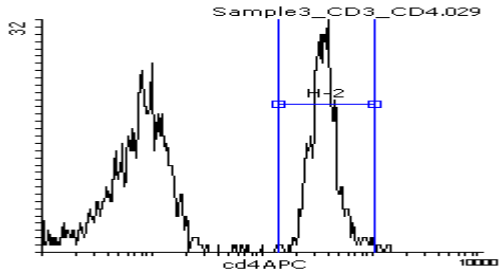


Figure 4. 16 Histogram plot of Sample number 3 showing CD4 cells among the CD3 population

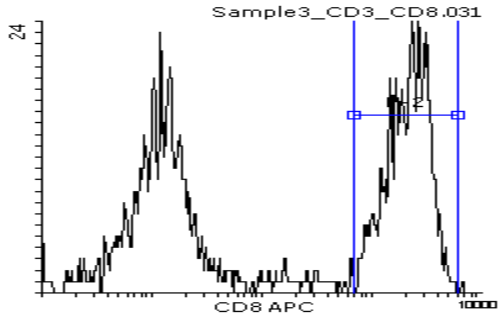


Figure 4. 17 Histogram plot of Sample number 3 showing CD8 cells among CD3 population

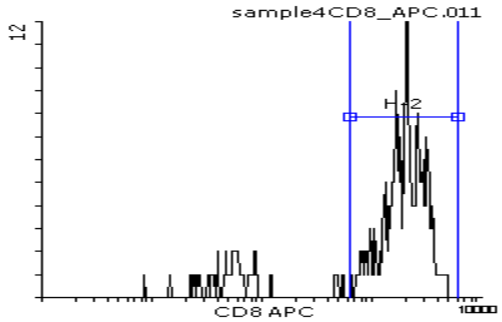


Figure 4. 18 Histogram plot of Sample number 4 showing CD8 cells

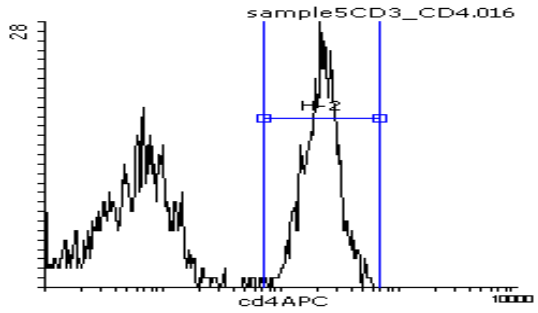


Figure 4. 19 Histogram plot of Sample number 5 showing CD4 cells among CD3 population

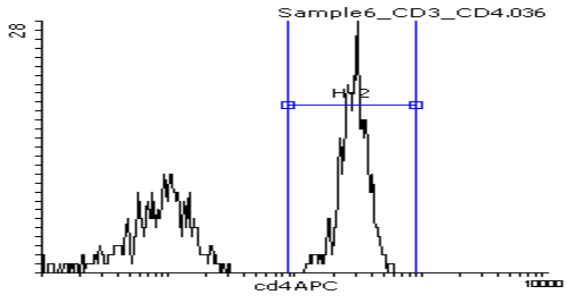


Figure 4. 20 Histogram plot of Sample number 6 showing CD4 cells among CD3 population

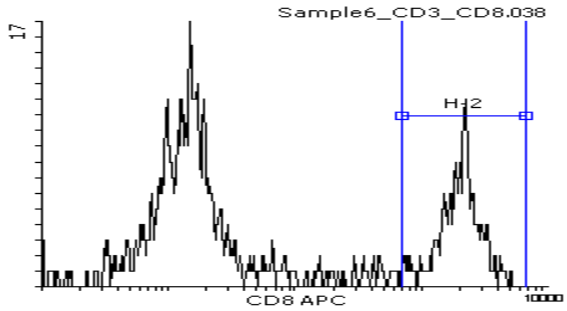


Figure 4. 21 Histogram plot of Sample number 6 showing CD8 cells among CD3 population

CHAPTER FIVE

DISCUSSION

Dengue is major public health issue in Nepal and is dangerous vector borne disease. The cross-reactive dengue infection and immunopathology associated with the infection has a higher tendency to be fatal. Currently, the emerging dengue infection cases are increasing day by day and its transmission in infected area is in increasing trend. Though, immunological detection of antibody present in patient's serum is easily available and cheaper technique for detection but the serotyping of dengue virus yields comparatively more significant result.

The fatal cases that might occur after the cross infection need to be addressed very carefully for the proper surveillance of disease and to prevent the possible risks related with infection. The Identification of dengue virus serotype will facilitate the development of novel ideas related to the severe dengue cases and may help to improve the currently available treatment for dengue patients.

The immune cells associated with virus clearance are yet to be understood in case of Nepalese dengue patients. Estimation of percentage of T cells that might help in viral clearance will facilitate the development of novel vaccination approaches that are being studied and may improve responses to currently available therapies.

In this study, we performed the serotyping of dengue virus of selected samples of two different years from different cities of Nepal. The Serotyping refers to the identification of the type of dengue virus (DENV1, DENV2, DENV3, DENV4) that causes the dengue infection. Serotyping of DENV is best performed during acute phase of illness when DENV circulates in blood. A previous study demonstrated that change in pattern of DENV occur in every outbreak and the antibody produced against first serotype won't provide long term immunity to the second infecting serotype. During my research Real Time PCR and FACS machine was first operated for a thesis experiment in Central Department of Biotechnology. In 2004, DENV2 was first serotype identified in Nepal, in 2006 all four serotype of DENV were found circulating in Nepal whereas in the year 2010 and 2016, it was DENV-1 and in 2013 and 2014, it was DENV-2. This is in accordance with our study because sample of 2016 are DENV1 positive and samples of 2017 are DENV2 positive. This is the first time DENV serotype was reported in Nepal among samples of the year 2016 and 2017 samples. The estimation of the CD4 and CD8 cell percentage is also the first immunological work performed in our lab and is first attempt of immunological analysis in dengue patients.

This report in earlier studies is consistent with our finding that the change in serotype pattern occurs. Based on our data, we can purpose that in Nepal change in dengue virus serotype in 2016 and 2017 samples might increase the cases of cross infection which leads to antibody dependent enhancement mechanism leading to DHF or DSS.

Through the method of flow cytometry analysis, we estimated the percentage of T- cells (CD4/CD8) present in circulating peripheral blood of dengue patients. Similarly, T cells (CD4 T cells, CD8 T cells) are the immune cells which play a vital role during the dengue infection. The fact that CD8 T cell expansion peaks before or around the time of the peak of clinical disease and that the frequencies of activated CD8 T cells and cytokine producing cells were somewhat higher in patients with severe forms of the disease also supports the above mentioned role of CD8 T cells (Janeway et al., 2001). The recent studies in Indian population showed increase in CD8 cell percentage during severe cases.

Based on our data we can conclude that the CD4 cell percentage is around normal range but CD8 T cells are found to have increased in dengue positive samples which is in accordance with the research previously performed.The gating of lymphocytes was performed in FlowJo and percentage of CD4 were 42.76, 39.61, 46.27, 53.66 while that of CD8 were 39.43 ,47.28, 80.85, 28.11 respectively. The T-cell count was performed among dengue patients samples and result showed the increase in percentage of CD8 T cells in few patients.

Thus we can say that, in case of Nepalese population who are infected with dengue virus the percentage of CD8 T cells are found to have increased. This might help to understand the immune status of dengue patients involved in the study.

The identification of the serotype of dengue virus is a costly affair to be performed in the laboratory. Since there was no much knowledge about the estimation of the percentage of T cells involved in immunity of dengue patients it was very difficult to operate the FACS machine and to get everything right. The reference range of CD4 cell in percentage is (31-58)% while that of CD8 is(19-43)%.

Thus, this study gave information about serotyping and immunology of dengue that will serve as vital data to understand the immune mechanism in Dengue patient of Nepal. Thus there is utmost need to seek standardization of cheaper alternatives for serotype detection and to study the different cytokines involved for the pathogenesis in details.

We had only CDC kit to be used for confirmation of serotype which is costly hence we limited the sample size. The sample screened for FACS was small due to expensive antibodies, thus the obtained data was not conclusive but a general map idea for further research. Thus my idea opens door to the importance of serotyping of DENV and poses a significant scope for analysis of immune cells involved in study of dengue patients in Nepalese population.

CHAPTER SIX

SUMMARY

Dengue is one of the most rapidly spreading arboviral disease found in tropics and subtropics. Dengue is caused by four serotypes of Dengue virus (DENV1, DENV2, DENV3, DENV4) which has 65%similarity with each other. Today, 40% of world's population is at risk of dengue infection. The virion of the dengue virus is spherical with a diameter of 50nm which contains a single copy of a positive-sense, single-stranded RNA genome. T cells response is vital in protection and severity of dengue infection.

Serotyping of Dengue Virus

First of all, this research is mainly focused on determination of prevalent DENV serotype in Nepalese population in 2016 and 2017. The study aims on the identification of different serotypes in Nepal in two different years. Since serotype of DENV keeps on changing it is very important to determine the serotypes that are circulating in Nepal which can be compared with previous serotype and thus chances of cross infection can be predicted and possible precautions can be taken.

Among total of 18 samples, 9 samples were collected from Chitwan Medical College Teaching Hospital, Chitwan in 2016 and 9 samples were collected from Sukraraj Tropical and Infectious Disease Hospital, Kathmandu in 2017 .

RNA extraction was done from the serum sample by using Qiagen viral RNA isolation kit and quantification of RNA was done by using Nano drop. The extracted RNA was then subjected to one step Real Time PCR which was performed as per the instructions in CDC Kit. The CDC DENV-1-4 Real-Time RT-PCR assay was performed in multiplex reactions following the manufacturer's instructions (Centers for Disease Control and Prevention) using the Superscript® III Platinum® One-Step qRT-PCR Kit (Invitrogen™). Amplification and detection were performed in a Bio-Rad CFX96™ Real Time System. The resulting data were analyzed as per instruction in the kit.

Among nine dengue positive samples from 2016, all of the samples were found DENV1 positive. Similarly, among nine dengue positive samples from 2017, six of the samples were found DENV2 positive whereas three of the samples did not show the positive result.

The DENV serotype when keeps on changing in every outbreak, there is a greater risk of secondary dengue infection which might lead to DHF or DSS. When a serotype is predetermined in patients serum, the possible risks can at least be avoided. The serotyping of DENV from 2017 samples thus led to a determination of DENV serotype circulating in 2017 in Nepal and we are the first to report this. The dengue vaccine Dengvaxia, provides protection against all four variants of the dengue virus which is because of the protection required against all four serotype of dengue virus. This explains the importance of serotyping of dengue virus.

Estimation of T cells in dengue patients

Secondly, the study of immunological cells involved in protection and immunopathology of dengue virus has become very vital. The estimation of T cells percentage was done in order to note the increase or decrease in the CD4 T cells and CD8 T cells percentage among sample and controls.

The sample used for flow cytometer analysis for estimation of T cells were from 2017 samples that were brought directly from Sukraraj Tropical and Infectious Disease Hospital, Kathmandu and sample preparation was done for absolute cell counting by lyse no wash method. Quality Control checking and compensation was performed before the sample was run in flow cytometer. After completion of the experiment the obtained data were further analyzed in FlowJo 10.2.

The estimation of percentage of CD4 T cells and CD8 T cells in dengue patients were performed and the percentage was compared with that of the control sample who is DENV negative. The percentage of CD4 T cells was around normal range in the samples. In few patients the increase in number of CD8 T cells was observed which is very significant because there is involvement of CD8 T cells in the protection and immunopathology during dengue infection. This was the first immunological study performed in the Dengue patients of Nepal.

Thus, circulation of two different serotypes DENV1 and DENV2 were found among Nepalese population in 2016 and 2017 respectively. The percentage of CD8 cells was found above the normal range which is evident with rise in the CD8 cell percentage in dengue patients during infection of dengue.

CHAPTER SEVEN

CONCLUSION

The data that was collected and interpreted from my research signifies that DENV1 was found infecting Nepalese population in 2016 and DENV2 infected Nepalese population in 2017. Furthermore, the CD8 T cells were found to have increased in dengue infected patients.

The result showed change in pattern of dengue serotype between two different years. The cross infection of two different dengue serotype can often lead to fatal cases so it is very vital to identify serotype of dengue virus infecting the patients. The CD4 and CD8 T-cell count was performed among dengue patients samples and result showed the increase in percentage of CD8 T cells.

Hence, Real-Time PCR is rapid and effective method for serotyping of dengue virus which is very vital in future research of disease and treatment. The pattern change observed in dengue virus serotype in the samples of two consecutive years indicates the chances of cross reactivity and ADE. The circulation of multiple serotypes of Dengue virus might worsen the outbreaks by increasing risks to recurrently infected individual which can ultimately lead to severe dengue.

The fact that T cells plays important role in virus clearance and immunopathology during dengue infection was supported by the data obtained during research. The increase in percentage of CD8 T cells in blood of dengue patients might be due to dengue infection.

Lack of proper funding for medical treatment, poor facilities for disease diagnosis are the problems faced in current scenario of Nepalese dengue patients.

The Government of Nepal should initiate more effective vector control programs and proper sanitation facilities must be maintained in the economically poor areas to prevent the spread of virus .

Recommendations:

- Additional research using different antibodies and efficient data analysis would be a good addition to the experiment.
- The significance of this experiment would be improved if the samples from overalll country were taken for serotyping.
- Cost effective methods for serotyping of dengue virus should be standardized for efficient serotyping of all dengue cases.

CHAPTER EIGHT

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APPENDICES

Below is the sample of Consent form that was filled up by the subjects before giving blood samples.

अनुसन्धान अध्ययनमा सहभागिता जनाउने मञ्जुरीनामा

परियोजना संयोजक,
प्राध्यापक कृष्णदास मानन्धर
जैविक प्रविधि केन्द्रीय विभाग
त्रिभुवन विश्वविद्यालय
किर्तिपुर, काठमाडौं, नेपाल

परियोजना शिर्षक: नेपालमा पाईने डेङ्गु तथा डेङ्गु जस्ता विमारीहरुको अध्ययन

तपाँई र/वा तपाँईको बालकलाई त्रिभुवन विश्वविद्यालय जैविक प्रविधि केन्द्रीय विभाग-नेपाल, अमेरिकाको La Jolla Institute for Allergy and Immunology र KariusDx Inc. बीचको संयुक्त प्रयासमा नेपालमा फैलिएको डेङ्गु जरोबारे बृस्तीत अध्ययन गर्न संचालन भएको "नेपालमा पाईने डेङ्गु तथा डेङ्गु जस्ता बिमारीहरुको अध्ययन" विषयको परियोजनामा सहभागि हुन आमन्त्रण गरेका छौं । यस अध्ययनबाट प्राप्त जानकारीहरुले डेङ्गु तथा डेङ्गु जस्ता बिमारीहरुबारे र तपाँई/तपाँईको बालकको राम्रो उपचार, त्यस्ता रोगहरुको खोपको विकाश र रोग पता लगाउने किट(उपकरण)बनाउनमा सहयोग पुऱ्याउनेछ । तपाँई/तपाँईको बालकलाई .....अस्पतालको जाँचमा डेङ्गु तथा डेङ्गु जस्तै रोगको लक्षण देखा परेकोले यस परियोजनामा सहभागिताको लागि छानिएको छ । यस परियोजनामा सामेल हुने वा नहुने तपाँई/तपाँईको बालकको इच्छा हो र रोजाईको कारणले यहाँले पाउने उपचारमा कुनै किसिमको असर पर्ने छैन । यस अध्ययनमा सहभागिता जनाए पनि परियोजनाको कुनै पनि समयमा तपाँई/तपाँईको बालक यस अनुसन्धानबाट हट्न सक्नु हुनेछ । यस अध्ययनमा दुई भाग छन् जुन तपाँईलाई भन्नेछौं र तपाँई/तपाँईको बालकले एक वा दुबैमा सहभागिता जनाउन सक्नुहुनेछ ।

भाग १: नमुना संकलन तथा भविष्यमा अनुसन्धान गर्न भण्डारणको लागि मञ्जुरीनामा

यदी यस अनुसन्धानमा सामेल हुन चाहनु भएमा हामी तपाँई/तपाँईको बालकको स्वास्थ्य, जस्तै पुराना स्वास्थ्य सम्बन्धि समस्याहरु र जाँचबाट पत्ता लगाएका उपायहरु र प्रयोगशालाका नियमित परिक्षणका विवरण सम्बन्धि कुराहरु बुझ्नेछौं । यदी तपाँईले सामेल हुने निर्णय गरिसक्नु भएको भए अस्पतालमा नियमित परिक्षणको समयमा तपाँई/तपाँईको बालकको २ पटकको रगतको नमूना संकलन गरिनेछ । बच्चाको भए करिब १ चिया चम्चा (५ मि.लि.) र बयस्क व्यक्ति भए ३ चिया चम्चा (१५ मि.लि.) रगत लिईनेछ । पहिलो रगतको नमूना तपाँई पहिलो पटक अस्पतालमा जाँच गराउन आउँदा र दोस्रो पटक ४ हप्ता पछी डेङ्गु तथा डेङ्गु जस्तै रोग बाँकी छ कि छैन भनी जाँच गराउन आउँदा लिईनेछ । यदी अध्ययनमा समावेस हुनु भएकोछ भने तपाँई/तपाँईको बालकलाई दोस्रो पटक रगतको नमूना लिन बोलाईनेछ । यसमा सहभागि भए बापट कुनै पैसा लाग्ने छैन ।

संकलित रगतको नमूनाको जाँचबाट डेङ्गु तथा डेङ्गु जस्तै रोगसंग तपाँई/तपाँईको बालकको शरिरले गरेको प्रतिक्रिया र भाईरस बारे थप जानकारी प्राप्त गर्न सजिलो हुनेछ । साथ साथै रगत परिक्षणबाट अझ राम्रो डेङ्गु रोग पत्ता लगाउन सहयोग मिल्नेछ । अनुसन्धानका क्रममा केही रगत परिक्षण त्रिभुवन विश्व विद्यालय जैविक प्रविधि केन्द्रीय विभाग, नेपालमा हुन्छ भने केही नमूना La Jolla Institute for

Allergy and Immunology तथा KariusDx Inc. मा गर्न अमेरिकामा पठाईनेछ । KariusDx Inc.मा १८ वर्ष र माथिका व्यक्तिहरुको रगतको नमूना मात्र परिक्षणको लागि पठाईनेछ ।

तपाँई/तपाँईको बालकको अस्पतालमा जाँच भएको रगतको विवरण डाक्टरले उपलब्ध गराउनेछ तर अनुसन्धानको विवरण भने तपाँईलाई दिईने छैन तथापि यसबाट तपाँईको उपचारमा कुनै पनि बाधा पर्ने छैन ।

तपाँई/तपाँईको बालकलाई दुई वटा कुरामा एक वटा छान्न अनुरोध गरिनेछ, क) तपाँई/तपाँईको बालकको रगतको नमुना भविष्यमा अध्ययनको लागि पनि राख्ने वा ख) तपाँई/तपाँईको बालकको रगतको नमुना यस परियोजना पछी नष्ट गर्ने । यदी तपाँई आफ्नो नमूना भविष्यमा गरिने परिक्षणको लागि भण्डारण गर्न रोज्नु भएको छ भने यस परियोजना पछीको बाँकी नमूना र विवरणलाई KariusDx Inc ले अनुसन्धान तथा रगत परिक्षणबाट संक्रमित रोगहरु पत्ता लगाउने कार्यको लागि प्रयोग गर्न सक्नेछ ।

कृपया तलका कोठामा चिनो लगाउनु होस जसले तपाँई/तपाँईको बालकको रगतको नमूना भविष्यमा गरिने अनुसन्धानको लागि राख्ने/नराख्ने भन्ने रोजाई देखाउँछ ।

☐ म/मेरो बालकको स्वास्थ्य विवरण र बाँकी रगतको नमुना भविष्यमा गरिने अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छ ।**

☐ म/मेरो बालकको स्वास्थ्य विवरण र बाँकी रगतको नमुना भविष्यमा गरिने अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छैन ।।**

भाग २: बंशानुगत अध्ययनको लागि थप मञ्जुरी

हामी, तपाँई/तपाँईको बालकको रगत नमूना भविष्यमा गरिने बंशानुगत अनुसन्धानको लागि पनि राख्न मञ्जुरी चाहन्छौ । बंशानुगत अनुसन्धान जिन (Gene)को अध्ययन हो । Genes मा डि.एन.ए. (DNA) र आर.एन.ए. (RNA) हुन्छ । DNA ले हाम्रो शरिरले कसरी काम गर्ने र रोग तथा बाहिरी वातावरणलाई प्रतिक्रिया दिने कामको आदेश दिन्छ भने RNA ले बाहकको काम गर्छ जसले आदेश र बंशानुगत विवरणहरु शरिरको आवश्यक भागहरुमा पुऱ्याउँछ । Genes बंशानुगत भएकोले आमा बुबाबाट बच्चामा आएको हुन्छ । यस अनुसन्धानमा ती Genes हरुको अध्ययन गरिन्छ जसले डेङ्गु तथा डेङ्गु जस्ता रोगहरु अधिकतम रुपमा ग्रसित गर्न जिम्मेवार छन् साथ साथै स्वास्थ्यसंग सम्बन्धित अन्य Genes को पनि अध्ययन गर्नेछ । यो अनुसन्धान गर्न धेरै वर्ष लाग्ने अनुमान गरिएकोछ । गोप्यताको बारेमा भाग १ मा उल्लेख गरिए जस्ते व्यवस्थित गरिनेछ । यदी तपाँईलाई यस अनुसन्धानमा सहभागिता जनाउन मन नलागेमा माथि उल्लेखित भाग १ को लागि मात्र सहभागिता जनाउन सक्नु हुनेछ र यस रोजाईले तपाँई/तपाँईको बालकको स्वास्थ्य उपचारमा कुनै बाधा पर्ने छैन ।

यदी यस जाँच तथा अनुसन्धान (भाग २)मा तपाँई/तपाँईको बालक संलग्न हुने निर्णय गर्नू भएको छ भने रगतको नमूनाहरुमा तपाँई/तपाँईको बालकको नाम र ठेगाना नखुल्ने गरी सांकेतिक अंक राखिईनेछ र नेपालको त्रिभुवन विश्वविद्यालय, अमेरिकाको La Jolla Institute for Allergy and Immunology तथा KariusDx Inc. मा पठाईनेछ । तपाँई/तपाँईको बालकको पहिचान परियोजनाका अनुसन्धानकर्तालाई हुनेछैन । अर्को शब्दमा तपाँई/तपाँईको बालकको नाम अनभिज्ञ रहनेछ ।

तपाँईको नमूनाहरु र विवरणहरु अनुसन्धानको लागि प्रयोग हुनेछ र हुन सक्छ यसबाट नौलो चीज, रोग जाँच र औषधि उपचारका नयाँ प्रविधि पत्ता लाग्न सक्छ । तपाँई/तपाँईको बालकलाई यस अनुसन्धानबाट कुनै किसिमको सिधै वा नगदी फाईदा भने हुने छैन तर भविष्यका सन्ततीलाई यसबाट आर्जित ज्ञान फाइदाजनक हुनसक्छ । तपाँई/तपाँईको बालकलाई यस बंशानुगत अध्ययनको परिणाम बारे जानकारी गराइनेछैन र तपाँई/तपाँईको बालकको उपचारमा उपयोग गरिनेछैन ।

यदी पछी पनि तपाँईलाई तपाँई/तपाँईको बालकको रगत नमूना र विवरण भविष्यमा गरिने बंशानुगत अनुसन्धानको लागि राख्न चाहनु भएन भने कृपया हामीलाई खबर गर्नु होला हामी तपाँई/तपाँईको बालकको रगनबाट थप अध्ययन नगर्न भरसक प्रयास गर्नेछौं ।

कृपया तलका कोठामा चिनो लगाउनु होस जसले तपाँई/तपाँईको बालकको DNA/RNA को नमूना भविष्यमा गरिने बंशानुगत अनुसन्धानको लागि राख्ने/नराख्ने भन्ने ईच्छा देखाउँछ ।

☐ म/मेरो बालकको स्वास्थ्य विवरण र DNA/RNA भविष्यमा गरिने बंशानुगत अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छ** ।

☐ म/मेरो बालकको स्वास्थ्य विवरण र DNA/RNA भविष्यमा गरिने बंशानुगत अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छैन** ।।

यदी यस अध्ययनमा तपाँई/तपाँईको बालक संलग्न हुने निर्णय गर्नू भएको छ भने यसमा धेरै खटरा र फाईदा छन् । सम्भाव्य खटरामा रगत निकाल्दा सियो घोचेको स्थानमा क्षणिक सुनिनु र चिलाउनु र कहिले काहीं धाउ बन्नु हुन् । ती सम्भाव्य खटराहरुलाई कम गर्न निपुण नर्स/दक्ष स्वास्थ्य कर्मिद्वारा स्टेराईल सियो प्रयोग गरी रगत निकालिनेछ ।

अनपेक्षित घटना भई तपाँई/तपाँईको बालकलाई रगत दिदै वा दिएको कारणबाट चोट लाग्न गएमा अस्पतालमा उपचारको व्यवस्था गरिनेछ । उपचार वापट लाग्ने खर्च La Jolla Institute for Allergy and Immunology, अमेरिकाले व्यहोर्नेछ । तर तपाँईलाई विरामी अवधिभर कुनै किसिमको खर्च दिइनेछैन । यस सम्वन्धि अन्य कुनै प्रश्न छ भने अस्पतालको तल उल्लेखित डाक्टरलाई सम्पर्क राख्नु होस ।

यस अध्ययनबाट प्राप्त तपाँई/तपाँईको बालकको स्वास्थ्यका विवरणहरु गोप्य राखिनेछ र तपाँई/तपाँईको बालकको नाम र पहिचान अध्ययनको कुनै पनि प्रतिवेदनमा उल्लेख गरिने छैन । सबै विवरणहरु र नमूनाहरु सांकेतिक अंक राखेर प्रयोग तथा भण्डारण गरिनेछन । सो सांकेतिक अंकको पहिचान परियोजनाका संयोजक र प्रमुख व्यक्तिहरुलाई मात्र थाहा हुनेछ । तपाँई/तपाँईको बालकको पहिचान बाहिर आउने सम्भावना असम्भव जस्तै छ तथापि हामी सम्भावनाहरु न्यून गर्न लागि परेका हुनेछौं ।

यदी तपाँईसंग यस अध्ययन सम्वन्धि कुनै प्रश्न, जिज्ञासा तथा समस्या छ भने त्रि.वि. जैविक प्रविधि केन्द्रीय विभागका प्रा.डा. कृष्णदास मानन्धरलाई फोन नं. १ ४३३६२२१ मा सम्पर्क राख्न सक्नु हुनेछ । यदी तपाँईलाई यसमा संलग्न भए वापट आफ्नो अधिकार सम्वन्धि प्रश्न छ भने यस अस्पतालका डाक्टर श्री ..... लाई अस्पतालको फोन नं/ईमेल ....., ... मा सम्पर्क राखी सोध्न सक्नु हुनेछ । यदी तपाँईलाई यस अनुसन्धानमा सहभागिता जनाउन मन नलागेमा माथि उल्लेखित डाक्टर वा सेवामा रहेकी नर्सलाई सम्पर्क राखी जानकारी दिन सक्नु हुनेछ ।

यस अध्ययनबाट प्राप्त ज्ञानले डाक्टरलाई नेपाल र संसारभरका बालक तथा वयस्क व्यक्तिहरुको डेङ्गुको उपचार गर्न मद्दत पुग्नेछ, रोग परिक्षण गर्ने नयाँ विधिको परिक्षण गर्न सकिनेछ र यस रोगको भ्याक्सीन बनाउन वैज्ञानिक आधारहरु तयार गर्न सहयोगि हुनेछ । फेरि पनि एक पटक भन्न चाहन्छु कि यस अध्ययनमा संलग्न हुनु भनेको स्वयंसेवकको काम गर्नु हो र तपाँई कुनै पनि बेला यसबाट अलग हुन सक्नु हुनेछ ।

तपाँईसंग कुनै प्रश्न छ ?

यस मञ्जुरी पत्रमा लेखिएका सबै कुराहरु मलाई पढेर सुनाईयो । मेरो प्रश्नहरुको चित्त बुझ्दो जवाफ पाएँ र मैले थाहा पाएँ कि म/मेरो बालकको यस अध्ययन अनुसन्धानमा सहभागिता स्वयंसेवकको रुपमा हो र म/मेरो बालकको स्वास्थ्य उपचारमा कुनै असर नपर्ने गरी सहभागित जनाउन पनि सक्छु र कुनै पनि समयमा नाम फिर्ता पनि लिन सक्छु ।

_____ _____

सहभागिको नाम अध्ययनको साँकेटिक अंक

_____

बाबु आमा वा अभिभावकको नाम (आवश्यक भए)

_____ _____

बाबु आमा वा अभिभावकको दस्तखत मिति

सहभागि बच्चालाई पनि बुझाउने काम _____ पुरा भयो वा _____ पुरा गरिएको छैन ।

यदी पढ्न र लेख्न असक्षम छ भने:

म यस मञ्जुरीनामाको साक्षीले यसमा उल्लेखित कुराहरु ठिक संग पढेर संभाव्य सहभागि व्यक्तिलाई सुनाएँ र नीजले यसबारे प्रश्न गर्ने मौका पाए । म आश्वस्त पार्छु कि नीजले मञ्जुरी आफ्नो इच्छा बमोजिम दिएका हुन् ।

_____

साक्षीको नाम

_____ _____

यस अध्ययनबाट प्राप्त ज्ञानले डाक्टरलाई नेपाल र संसारभरका बालक तथा बयस्क व्यक्तिहरुको डेङ्गुको उपचार गर्न मद्दत पुग्नेछ, रोग परिक्षण गर्ने नयाँ विधिको परिक्षण गर्न सकिनेछ र यस रोगको भ्याक्सीन बनाउन वैज्ञानिक आधारहरु तयार गर्न सहयोगि हुनेछ । फेरि पनि एक पटक भन्न चाहन्छु कि यस अध्ययनमा संलग्न हुनु भनेको स्वयंसेवकको काम गर्नु हो र तपाँई कुनै पनि बेला यसबाट अलग हुन सक्नु हुनेछ ।

तपाँईसंग कुनै प्रश्न छ ?

यस मञ्जुरी पत्रमा लेखिएका सबै कुराहरु मलाई पढेर सुनाईयो । मेरो प्रश्नहरुको चित्त बुझ्दो जवाफ पाएँ र मैले थाहा पाएँ कि म/मेरो बालकको यस अध्ययन अनुसन्धानमा सहभागिता स्वयंसेवकको रुपमा हो र म/मेरो बालकको स्वास्थ्य उपचारमा कुनै असर नपर्ने गरी सहभागित जनाउन पनि सक्छु र कुनै पनि समयमा नाम फिर्ता पनि लिन सक्छु ।

_____ _____

सहभागिको नाम अध्ययनको साँकेटिक अंक

_____

बाबु आमा वा अभिभावकको नाम (आवश्यक भए)

_____ _____

बाबु आमा वा अभिभावकको दस्तखत मिति

सहभागि बच्चालाई पनि बुझाउने काम _____ पुरा भयो वा _____ पुरा गरिएको छैन ।

यदी पढ्न र लेख्न असक्षम छ भने:

म यस मञ्जुरीनामाको साक्षीले यसमा उल्लेखित कुराहरु ठिक संग पढेर संभाव्य सहभागि व्यक्तिलाई सुनाएँ र नीजले यसबारे प्रश्न गर्ने मौका पाए । म आश्वस्त पार्छु कि नीजले मञ्जुरी आफ्नो ईच्छा बमोजिम दिएका हुन् ।

_____

साक्षीको नाम

_____ _____

सहभागि/बाबु आमाको अंगुठाको छाप

मैले यस मञ्जुरीनामा यस परियोजनाको संभाव्य सहभागिलाई ठिकसंग पढेर सुनाएँ र नीजले यस अध्ययन सम्बन्धि प्रश्नहरु सोध्ने मौका पनि पाए । म आश्वस्त पार्छु कि सहभागिले मञ्जुरी आफ्नो ईच्छा बमोजिम दिएका हुन् ।

[_____]

मञ्जुरीनामा सहयोग गर्ने व्यक्ति

_____ _____

दस्तखत मिति

यस मञ्जुरीनामाको एक प्रति सहभागिको बुबा आमा वा अभिभावकलाइ दिईयो । (अनुसन्धानकर्ता वा सहयोगीको दस्तखत सहित)

Consent to Participate in a Research Study

Project Coordinators
Prof. Krishna Manandhar
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

Title of Study: Dengue fever and Dengue fever like illness in Nepal

You and/or your child are being invited to participate in a research study, which is a collaboration between Tribhuvan University, La Jolla Institute for Allergy and Immunology, and Karius, Inc. designed to learn more about Dengue and Dengue Fever-like Illnesses in Nepal. Information from this study will be used to help improve understanding of the disease, allow provision of better care for patients with Dengue, and contribute to the development of a vaccine for Dengue and better tests to diagnose Dengue. You/your child has been selected because you/your child is suspected of having Dengue at the [_____] Hospital. The choice to participate in this study is yours and will not affect the medical care that you/your child receives. At any point during this study, you/your child may choose to stop taking part in the study without penalty. The study has two parts, which we will explain to you. You/your child may choose to participate in only the first part of the study or in both parts of the study.

Part 1: Consent for Sample Collection and Storage of Samples for Future Research

If you agree for you/your child to take part in this research, we will collect information about you/ your child's health such as medical history and findings from exams and lab tests done as part of regular care. If you decide to take part in the study two blood samples will be collected while you/your child is being treated for Dengue or possible Dengue Fever-like illness. The blood samples will be collected at the same time as samples that are part of regular care. The amount of blood collected for the study will be about a teaspoon for children less than 15 years old and up to 3 teaspoons for anyone older than 15 years.

The first sample for the study will be collected when you first come to the hospital for care. The second blood sample will be collected at a follow-up visit four weeks after the beginning of the illness. If you join the study, you/your child will be asked to return for collection of the second blood sample. There will be no cost for participating in the study.

The tests done on blood collected for this study will help to learn how you/your child's body responds to Dengue and more about the virus itself. This study will also help develop better blood tests for Dengue. Some of the blood samples collected for the study will be tested at Tribhuvan University in Nepal and some will be sent to the United States for testing at the La Jolla Institute in La Jolla, California and Karius in Menlo Park, California, and may be sent to other research partners collaborating with us in the future. Only blood collected from adults 18 years or older during the acute phase of the illness or during the first visit will be sent to Karius for testing.

Your/your child's doctor will give you information about the results of the clinical tests, but you will not be informed of results from the research tests and these results will not affect the treatment you will receive.

We are asking you to choose one of two options: 1) that your/your child's samples may be stored to be used for future research or 2) that your/your child's samples are destroyed by adding bleach after the completion of this study. If you agree to have your samples stored for future research, leftover samples and information from this study could be used by Karius for research and development of blood tests for infectious diseases.

Please check the box below that indicates your willingness to provide your/your child's samples and clinical information for future research.

☐ I **DO AGREE** to allow my or my child's clinical information and any left-over samples to be used in future research.

☐ I **DO NOT AGREE** to allow my or my child's clinical information and left-over samples to be used in future research.

Part 2: Additional Consent for Genetic Research

We would like to have permission to store your/your child's clinical information and blood samples for future genetic research. Genetic research is the study of genes. Genes contain DNA (instructions for how the body works and responds to disease and the environment) and RNA (that acts as a messenger that carries instructions and genetic information around the body). Genes are passed on from parents to children. This research will look at genes that might make people more or less likely to develop severe Dengue or other infectious diseases and other genes related to health. This research is expected to take many years to complete. Confidentiality will be maintained in the same manner as described in the consent form for Part 1 of the study. If you decide not to give permission, you can still participate in Part 1 of the study and it will not affect the medical care that you/your child receives.

If you decide to participate in this part of the study (Part 2), the blood samples and clinical information collected for the study will be assigned a unique identification number and sent, without your/your child's name or address for storage and testing at Tribhuvan University in Nepal and to the United States to the La Jolla Institute in La Jolla, California and Karius Inc. in Menlo Park, California, and may be sent to other research partners collaborating with us in the future. Researchers will not have access to the key that links the identity of your/your child to the information and samples. *In other words, your/your child's name will be kept secret.*

Your samples and information will be used for research and could possibly result in inventions and discoveries that could become new products, diagnostic tests, or medications. You/your child will not benefit directly or financially from this research, but future generations may benefit from the knowledge that is gained. You/your child will not be informed of the results from the genetic research. The results will not be used in your/your child's medical treatment.

If you decide later that you do not want your/your child's blood samples and information to be used for future genetic research, please tell us and we will do our best to prevent their use in any additional studies.

Please check the box below that indicates your willingness to provide your/your child's DNA/RNA samples and clinical information for future genetic research.

☐ I **DO AGREE** to allow my or my child's samples, information, and the DNA/RNA in the samples to be used for future genetic research.

☐ I **DO NOT AGREE** to allow my or my child's samples, information, and the DNA/RNA in the samples to be used for future genetic research.

If you choose to participate or have your child participate in the study, there are several risks and benefits. The risk in drawing blood is temporary soreness and bruising; rarely, it may result in infection. These risks are minimized by having experienced nurses do the procedures and by using sterile technique.

In the unlikely event that you/your child is injured as a result of taking part in this study, medical care will be made available to you/him/her. The costs of this care will be covered by the La Jolla Institute for Allergy and Immunology in California, US, but you will not receive any money. You will not receive money for participating in the research. If you have any questions regarding this, you may consult the below mentioned doctor at your Hospital.

Information collected during this study will be kept confidential. Your/your child's name or information that identifies you/your child will not be used in any reports of the study. All the information will be processed using codes and stored in a database that only the project coordinator and key study personnel have access to. There is the unlikely possibility that your/your child's confidentiality will be compromised; however, measures have been taken to minimize this risk.

If you have any questions, concerns, or problems related to this study, please contact Pr. Krishna Manandhar at Tribhuvan University at the following numbers: +977 1 4336221. If you have any questions about your rights as a research subject, please contact Dr. [_____] of the Hospital at the following numbers or email [_____,_____]. If you wish to stop participating in this study, please communicate with a study doctor or nurse.

The knowledge gained from this study will help doctors give better care to children and adults with Dengue in Nepal and the rest of the world, will allow evaluation of important new tests, and will contribute to scientific knowledge needed for development of Dengue vaccines. Once again, participation in this study is voluntary and you may choose to stop taking part in this study at any time.

Do you have any questions?

By signing this consent form, I acknowledge that I have had the consent form read to me. My questions were answered to my satisfaction and I recognize that my/my child's participation in this study is voluntary and that I/my child can participate or withdraw at any moment without affecting my/my child's medical care.

Name of Subject _____ Study code _____

Name of Parent or Guardian (if applicable) _____

Signature of Subject/Parent or Guardian _____ Date _____

An assent process HAS _____ or WILL NOT BE _____ completed

If unable to read and/or write:

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of Witness _____

Signature _____ Date _____

Thumb print of subject/parent

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given assent freely.

[_____]
Person conducting the Consent

Signature _____ Date _____

A copy of this Informed Consent Form has been provided to the parent or guardian of the participant_____ (initialed by researcher/assistant)

EXTRA THESIS ACTIVITIES

PUBLICATION

VirusDis.
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CrossMark

REVIEW

Emergence of dengue in Nepal

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Abstract Dengue virus is a major health problem in Nepal. The endogenous dengue appeared in 2006 in the country with reported outbreaks in 2010, 2013 and 2016. Eleven years vertical data show there were sporadic cases in all the years and mostly adults between 25 and 40 years of age were infected with dengue virus. Compared with primary infections, secondary infections were observed in relatively larger numbers during the period of 2008–2016. Most of the cases had symptoms of dengue fever; while 7 and 19 cases demonstrated dengue hemorrhagic fever/-dengue shock syndrome in 2010 and 2013 respectively. The proportion of dengue hemorrhagic fever amongst all cases of dengue fever was 2.5:4.7% in 2010 and 2013. We found there is shift of serotype from dengue virus serotype-1 (DENV-1) in 2010, DENV-2 in 2013 and DENV-1 in 2016. We feel there is urgent need for better community, hospital and laboratory based surveillance system capable of monitoring the circulating dengue virus (DENV) ser-otypes in different districts of Nepal. With improvement in surveillance system and efficient management of cases, the case fatality rate due to severe dengue can be reduced.

Introduction

Dengue remains a major public health problem worldwide and the virus has been described as one of the most important arthropod-borne disease viruses [21]. The fever ailment, dengue fever (DF) is prevalent in sub-tropical and tropical countries with over 3.9 billion people being at risk of dengue virus infection worldwide [4]. WHO estimates that 50–100 million cases of DF and thousand cases of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) occur each year [1]. It has been particularly identified as a major problem of recent decades in the Asia Pacific and the Caribbean [12, 18]. However in Africa, the specific epidemiology of dengue is not clear despite the vivid presence and distribution of its vector (*Aedes* spp.) which has been identified as the major risk factor associated with the prevalence of the viral infection in most part of the continent. The prevalence of dengue has been promoted by uncontrolled population growth, ineffective vector control, global warming as well as inadequate public health facil-ities [16]. Subsequently, this has led to a significant

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EXTRA THESIS ACTIVITIES

Memories from my M. Sc Biotechnology, Tribhuvan University