SEROPREVALENCE AND SEROTYPING OF DENGUE INFECTION IN NEPAL

M.Sc. Thesis

(2019)

Submitted to:

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

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For the partial fulfillment of the requirement of the

Master of Science in Biotechnology

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BY

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GLOSSARY ACRONYMS

%	Percentage
ADE	Antibody Dependent Enhancement
APC	Antigen Presenting Cells
BSL	Biosafety level
C	Capsid
ANOVA	Analysis of Variance
CDBT	Central Department of Biotechnology
CMC	Chitwan Medical College and Teaching Hospital, Chitwan
DALYs	Disability Adjusted Life Years
DENRA	Dengue Recombinant Antigen
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 hemorrhagic fever
DSS	Dengue Shock Syndrome
Env	Envelope
EC	Endemic Control
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISR	Immune Status Ratio

Infxn	Infection
Kb	Kilo base
MHC	Major Histocompatibility Complex
NC	Negative Control
NEC	Non -Endemic Control
NEP	Nepal
NCA	Normal Cell Antigen
NHRC	Nepal Health Research Council
NS	Non-Structural
OD	Optical Density
PAHO	Pan American Health Organization PC Positive Control
PC	Positive Control
PCR	Polymerase Chain Reaction
PrM	Premembrane
QC	Quality Control
RNA	Ribonucleic Acid
Rpm	Revolutions per Minute
RT-PCR	Reverse transcription-polymerase chain reaction
STIDH	Sukraraj Tropical and Infectious disease Hospital
TMB	Trimethyl Benzedrine
TNF	Tumor necrosis factor
UTR	Untranslated Region
WHO	World Health Organization
µl	Microliter

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ABSTRACT

SEROPREVALENCE AND SEROTYPING OF DENGUE INFECTION IN NEPAL

Dengue fever represents a serious emerging infectious disease and has become a global epidemic with almost half of the world's population at risk of infection. Dengue is arthropod-borne viral disease caused by RNA virus of the family Flaviviridae and spread by *Aedes* mosquitoes mainly, *Aedes aegypti* and *A. albopictus* and are prevalent in tropical and sub-tropical countries. There are 4 distinct, but closely related, serotypes of the virus that cause dengue (DENV-1, DENV-2, DENV-3 and DENV-4). As It is one of the emerging viral diseases in Nepal, people living mainly in Terai region are at high risk. Furthermore, co-circulation of multiple serotypes may worsen any outbreak significantly by increasing the risk of patients which might lead to death due to immune pathology driven complications.

This study aims to determine the antibody titer against dengue serotypes circulating in Nepalese population by serotype specific In-House ELISA and helps to know whether Nepalese population have protective antibodies against dengue virus. In this study, In-house ELISA was performed by using serotype specific antigens for dengue virus. The study consisted of total sample size of 32 among them 62.5 % were males (n=20) and 37.5% were females (n=12) with the ratio of male: female as 1.67:1. The age group ranged from 14 years to 72 years. Antibody production against different serotypes of dengue were found to be 62.5%, 68.75%, 6.25% and 56.25% respectively for DENV-1, DENV-2, DENV-3 and DENV-4 respectively. In case of In-House ELISA, the OD values were found to be higher for DENV-1 antigen and DENV-2 and lower for DENV-3. The statistical analysis was also performed by unpaired t-test. The p-value was found to be <0.05 for all the controls and samples and there was significant difference between the mean of the positive and negative samples. Most of the samples which were positive in In-Bios Kit has shown to have higher antibody titer for all antigens. The samples which were negative for the kit also showed lower antibody titer. Out of 32 samples 15 samples were found IgM positive while only two samples were positive for IgG ELISA, higher IgM titer showed that patients had a recent acute dengue infection and IgG positive showed that person had an infection sometimes in past. Calculating the ratio between IgG and IgM, it was found that most of cases were of primary infection. Molecular serotyping was done by Nested Reverse Transcriptase PCR (RT-PCR), using dengue specific primers for part of envelope region, which showed the circulation of DENV-1 in the year 2016 in NEPAL.

The developed In-House ELISA was able to determine the antibody produced against the serotypes of dengue virus (DENV 1-4) in Nepalese population. Till now ELISA kits for NS1 antigen and antibody detection (IgG and IgM) kits are available, however, no any serotype specific antibody detection ELISA has been developed. Hence this study might be useful in serotype identification which is important in epidemiological and pathological analysis.

Key Words: DENV, RT-PCR, In-House ELISA, Serotyping

Chapter I

INTRODUCTION

1.1 Background of Dengue Fever

Dengue is one of the most important mosquito-borne viral disease, caused by any one of the four Dengue virus (DENV) serotypes (DENV-1, -2, -3, and -4) and is an expanding public health problem in the tropics and subtropics (WHO,2000). The infection is transmitted by bite of female mosquito of the species *Aedes aegypti* or *A. albopictus* (Gupta *et al.,* 2016). Dengue virus infection causes a spectrum of clinical manifestations ranging from normal dengue fever (DF) to fatal haemorrhagic diseases.

Dengue illness is clinically classified as either dengue with or without warning signs or severe dengue (WHO, 2009). Dengue illness can also be divided into three separate phases: the acute (febrile) phase, the critical (plasma leakage) phase and the convalescent or reabsorption phase. The 2009 classification replaced the previous 1997 WHO system that addressed and underscored the two pathological phenomena associated with the disease: plasma leakage and abnormal haemostasis. Under this classification, patients were designated as having either dengue fever — a nonspecific febrile illness and the most common manifestation of DENV infection — or dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS) — a combination of plasma leakage and coagulopathy, sometimes accompanied by bleeding that can lead to a rapid fall in blood pressure and consequently to circulatory shock and organ impairment (WHO Press, 1997).

The global incidence of dengue infection is in increasing trend, with an estimated incidence range of 50 million to 100 million cases per year (WHO, 2016). Dengue was also responsible for 1.14 million (95% CI,0.73 million – 1.98 million) disability-adjusted-life- years (DALY) in 2013 (Stanaway *et al.,* 2016) out of which Asia alone bore 70% of this burden (Bhatt *et al.,* 2013) It is estimated that up to 75% of the population living in the Asia-Pacific region are potentially exposed to the disease (WHO, 2012).

Dengue infection is one of the top public health problems among individuals travelling to endemic areas (Ferguson *et al.,* 2016) and the leading cause of febrile illness among international travelers (Ratnam *et al.,* 2013), which accounts for 2% of overall illnesses in travelers returning from dengue-endemic areas (Wilder-Smith, 2013).

1.2 Brief History of Dengue Infection

The first recorded symptoms compatible with dengue were noted in a Chinese medical encyclopedia in 992 ADS, however originally published by the Chin Dynasty centuries earlier (265–420 AD), prior to being formally edited. The disease was referred to as 'water poison' and was associated with flying insects (Gubler, 1998). Epidemics that resembled dengue, with similar disease course and spread, occurred as early as 1635 and 1699 in the West Indies and Central America, respectively. A major epidemic occurred in Philadelphia in 1780 and epidemics then became common in the USA into the early 20th century, the outbreak occurring in 1945 in New Orleans. The viral etiology and the transmission by mosquitoes were only finally determined in the 20th century (Wilder-Smith *et al.,*2008). Dengue virus was first isolated by Ren Kimura and Susumu Hotta in Japan in 1943. These two scientists were studying blood samples of patients taken during the 1943 dengue epidemic in Nagasaki, Japan. An epidemic of DF involving at least 200,000 cases had occurred between 1942 and 1944 during World War II in Japanese port cities such as Nagasaki, Kobe, and Osaka. The infections originated from persons returning from the tropics, in particular Southeast Asia and the Pacific islands (Hotta *et al.,* 2000). A few months after the first isolation of DENV-1 in Japan, the same serotype was isolated from Hawaiian and shortly thereafter, DENV- 2 from Papua New Guinean samples (Sabin *et al*., 1945).

Expansion of the disease heightened during World War II (WWII), By the end of the war, transportation and rapid urbanization led to increased transmission of dengue and hyperendemicity (multiple serotypes present) in most South East Asian countries, with subsequent emergence of the severe forms of dengue, many countries of Asia were hyperendemic with the co-circulation of all four virus serotypes. It was during this period that the first documented epidemics of dengue hemorrhagic fever (DHF) occurred, first in the Philippines (1953–1954) and Thailand (1958), followed in the 1960s by Singapore, Malaysia and Vietnam, and Indonesia and Burma (Myanmar) in the 1970s (WHO,1980). As urbanization and commerce grew, the frequency and magnitude of epidemic disease continued to increase. By the 1980s, DHF had become a leading cause of hospitalization and death among children in many countries of Southeast Asia. The origins of DENV have been the subject of speculation for decades. Phylogenetic relationships to other flaviviruses provide little insight because the closest relatives to DENV occur in several continents. It was hypothesized that endemic DENV evolved from sylvatic strains in Africa or Asia that utilize nonhuman primate hosts and gallery forest- dwelling Aedes vectors (not the endemic/epidemic vectors *Ae. aegypti* or *Ae. albopictus* (Gubler, 1997).

1.3. History of Dengue in Nepal

Nepal was dengue-free, or was considered as such, until the first recognized suspected incidence in 2004. This case was that of a Japanese visiting worker who stayed in Nepal for several months and tested seropositive for the disease on return to his home country and was identified by genomic analysis as DENV-2 (GenBank accession no. AB194882), which showed 98% genomic similarity with DENV-2 isolates from India, from which the reasonable conclusion was drawn that it originated from India and passed into Nepal(Pandey *et al.,* 2004).

Virus sequencing of patient serum samples obtained during the 2006 outbreak showed that DENV strains from all four serotypes were circulating in the nine Terai regions of southern Nepal, however, no detailed epidemiologic data was collected. A small number of dengue disease cases were reported in 2007–2009 followed by major outbreaks in 2010 and 2013 with the prevailing serotypes DENV-1 and DENV-2 respectively (Malla *et al.,* 2008). Another major dengue outbreak occurred in 2016, with clinical cases reported from several districts of Nepal. Circulation of DENV-1 was also documented during 2014–2016 in several countries neighboring Nepal including India, China, Pakistan, Bangladesh and Sri Lanka (Tazeen *et al.,* 2017).

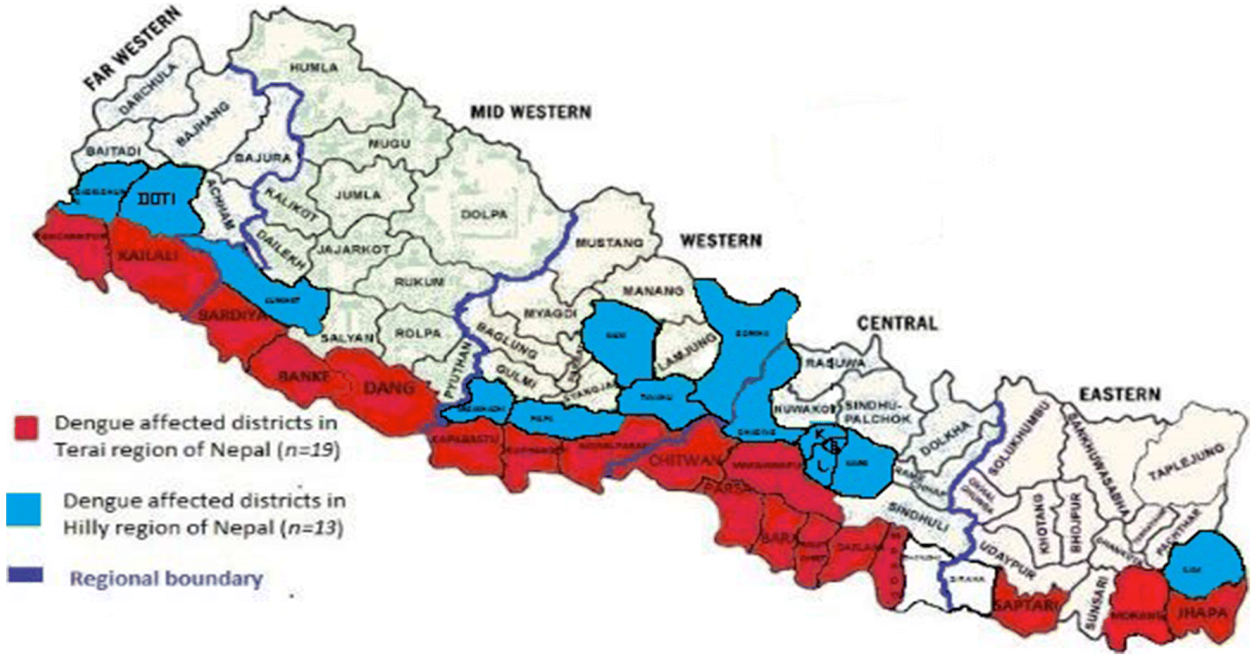


Fig. 1.1 Distribution of dengue cases from 2006 to 2016 in Nepal (Gupta *et al.,* 2018)

1.4. Systemic position of Aedes aegypti

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Diptera
Family:	Culicidae
Genus:	*Aedes*
Subgenus:	*Stegomyia*
Species:	*Ae. aegypti*
Binomial name:	*Aedes aegypti*

1.5. The Virus

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

1.6. Structure of DENV

The dengue viruses have a structure similar to other flavi-viruses; they are spherical, about 40–50nm in diameter, with a lipid envelope in which is embedded an Envelope (E) and Membrane (M) part, which is apparently derived from the host cell membrane from which the viruses bud. The envelope encloses an iso-metric nucleocapsid core of 30–35 nm in diameter, which consists of a capsid protein and single-stranded, positive- sense ribonucleic acid (RNA) genome (Perera and Kuhn, 2008). The E glycoprotein is responsible for virion attachment to receptor and fusion of the virus envelope with the target cell membrane and bears the virus neutralization epitopes (Modis *et al.,* 2003).

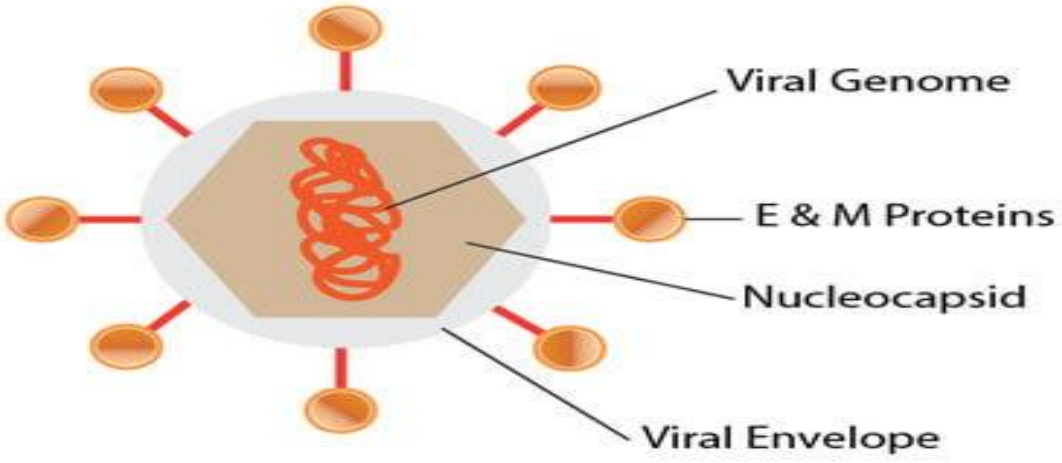


Figure 1.2. Structure of the dengue virion (Guzman *et al.,*2010)

1.7. Genome of DENV

Dengue virus has a single- stranded, Positive - sense RNA genome of 10.7 kilobases in length, surrounded by a nucleocapsid and covered by a lipid envelope that contains the viral glycoproteins. The RNA genome contains a single open reading frame that encodes a single polyprotein that is cleaved into the capsid (C), membrane (M), and envelope (E) structural proteins and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Structural glycoprotein E is responsible for cell recognition and for promoting entry, which is mediated by a fusion process between the viral envelope and the cell membrane, while the NS proteins aid viral genome replication (Alvarez *et al.,*2005).

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 (Friebe et al, 2010). Translation of the viral messenger RNA (mRNA) is initiated at the 5' end, and the resulting polyprotein goes through extensive co-translational and post-translational proteolytic processing and cleavage to form at least 10 mature viral proteins (Kuhn *et al.,* 2002; Chiu *et al.,* 2005). Both viral and host proteases appear to be involved in processing the dengue polyprotein. A C-terminal hydrophobic membrane anchor domain in the capsid, pre-membrane and envelope proteins function both as internal signal sequences involved in the transfer of the polypeptide into the lumen of the rough endoplasmic reticulum, and as membrane anchor domains (Markoff *et al.,* 1994).

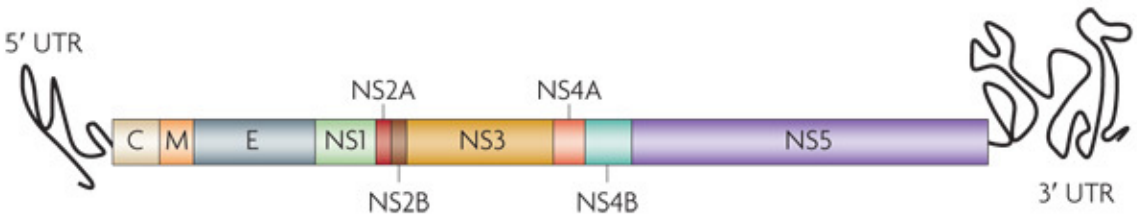


Fig 1.3. Schematic diagram of genomic organization of Dengue virus (C: Core protein; PrM & M: Membrane associated protein; E: Enveloped protein; NS1-5: Non-Structural protein 1-5) (Source: www.nature.com/ scitable content/ne000/F2_dengue_1_2. jpg)

1.8. Vectors and Transmission Cycles

The dengue virus is carried and spread by mosquitoes of the genus *Aedes*, which includes a number of mosquito species. The primary vector of the dengue virus is the species Aedes aegypti. It is the principal dengue vector responsible for dengue transmission and dengue epidemics. Other mosquito species in the genus *Aedes*; including *Aedes albopictus, Aedes polynesiensis*, and *Aedes scutellaris* — have a limited ability to serve as dengue vectors (WHO,2009).

A. aegypti, the principal vector, is a small, black-and-white, highly domesticated tropical mosquito that prefers to lay its eggs in artificial containers commonly found in and around homes, for example, flower vases, old automobile tires, buckets that collect rainwater, and trash in general. Containers used for water storage, such as 55-gallon drums, cement cisterns, and even septic tanks, are important in producing large numbers of adult mosquitoes in close proximity to human dwellings. The adult mosquitoes prefer to rest indoors, are unobtrusive, and prefer to feed on humans during daylight hours (Gubler *et al.,* 2010).

Dengue viruses exist in nature in three basic maintenance cycles (Vasilakis *et al.,* 2011). The primitive forest cycle involves canopy-dwelling mosquitoes and lower pri-mates. A rural cycle, primarily in Asia and the Pacific, involves peridomestic mosquitoes (*A. albopictus* and *A. scutellaris* spp.) and humans (Vasilakis *et al.,* 2010). The urban cycle, which is the most important epidemiologically and in terms of public health impact, involves the highly domesticated *A. aegypti* mosquito and humans (Halstead, 2008). Multiple virus serotypes are maintained in an endemic cycle in most large urban centers of the tropics, with epidemics occurring at periodic intervals.

When a mosquito bites a person who has dengue virus during a condition called viremia, that lasts for 2- to 10-days in which there is a high level of the dengue virus in the blood , it

becomes infected. Once the virus enters the mosquito's system in the blood meal, the virus spreads through the mosquito's body over a period of eight to twelve days, depending on ambient temperatures, the mosquito vector and the virus strain, the virus will disseminate and infect other tissues, including the mosquito salivary glands (Halstead, 2008). An infected mosquito can later transmit that virus to healthy people by biting them. It remains infected for life and can continue transmitting the dengue virus to healthy people (Gubler *et al.,*2010) None vector borne transmission was identified as vertical transmission that is from mother to child during pregnancy or birth, transplantation related transmission and needle stick related transmission has been reported (Wiwanitkrit, 2009). The lifecycle of a mosquito includes four separate stages; egg, larva, pupa, and adult, the first three stage require aqueous environment. The developmental stage depends on environment's temperature and availability food at the larval stage (Gubler *et al.,* 2010).

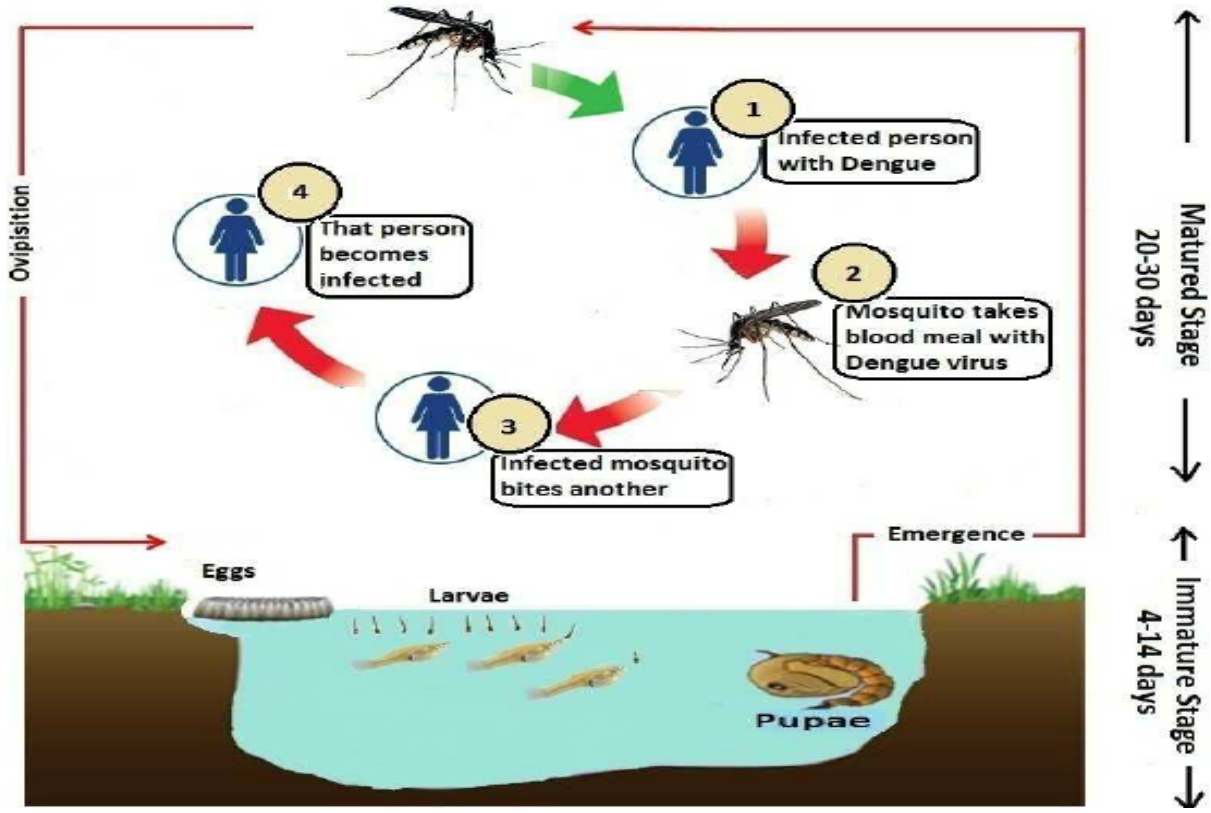


Figure 1.4. Mosquito life cycle with predator and disease transmission among human

Available from: https://www.researchgate.net/figure/Mosquito-life-cycle-with-predator-and-disease-transmission-among-human_fig1_270573038 [accessed 21 Jan 2019]

1.9. Host-Pathogen Interaction

The pathogenesis of dengue involves a complex interaction between virus and host factors, but it is still not clearly understood. The severe manifestations of DENV infection are observed at the point the virus is being cleared from the host by the immune response as opposed to when the viral load is highest, suggesting that the immune system plays a key role in disease pathogenesis (Green and Rothman, 2006). During the feeding of mosquitoes on humans, DENV is presumably injected into the bloodstream, and has been shown to infect many cell types. The dendritic cells of epidermis (epidermal dendritic cells [DC]) and keratinocytes are possible first target (Limonta *et al.,* 2007). Infected cells then migrate from site of infection to lymph nodes, where monocytes and macrophages are recruited, which become the major targets of infection. Consequently, infection is amplified, and virus is disseminated through the lymphatic system to cells of the mononuclear lineage, including blood-derived monocytes, myeloid DC and macrophages in spleen and liver (Durbin *et al.,* 2008). The humoral immune response is vital for controlling DEN virus infection and for the expression of acquired immunity. The basis for homotypic immunity is believed to be virus-neutralizing antibodies. The leading explanation for increased risk of disease in secondary infection is that non-neutralizing, cross- reactive antibodies elicited by a primary infection bind the virus which then have greater potential to infect Fc-receptor bearing cells. This phenomenon, called antibody-dependent enhancement (ADE), potentially increases the risks of developing severe disease by virtue of increasing the number of virus infected cells and there- fore the viral biomass in vivo (Hatstead *et al.,* 2003) Cellular immune responses are also suggested to play a role in clearing virus infection and potentially triggering the development of severe disease. Activated memory T cells recognizing both conserved and altered peptide ligand epitopes, are suggested to be involved in the development of plasma leakage. It is proposed that the expression of viral epitopes on the surface of infected cells trigger the proliferation of memory T cells and the production of pro-inflammatory cytokines that have an indirect effect on vascular endothelial cells resulting in plasma leak. The level of T cell response is thought to correlate to disease severity. This is suggested to delay viral clearance, and via cytokine-mediated effects, potentially increase the risk of severe manifestations of disease. (Mongkolsapaya *et al.,* 2003)

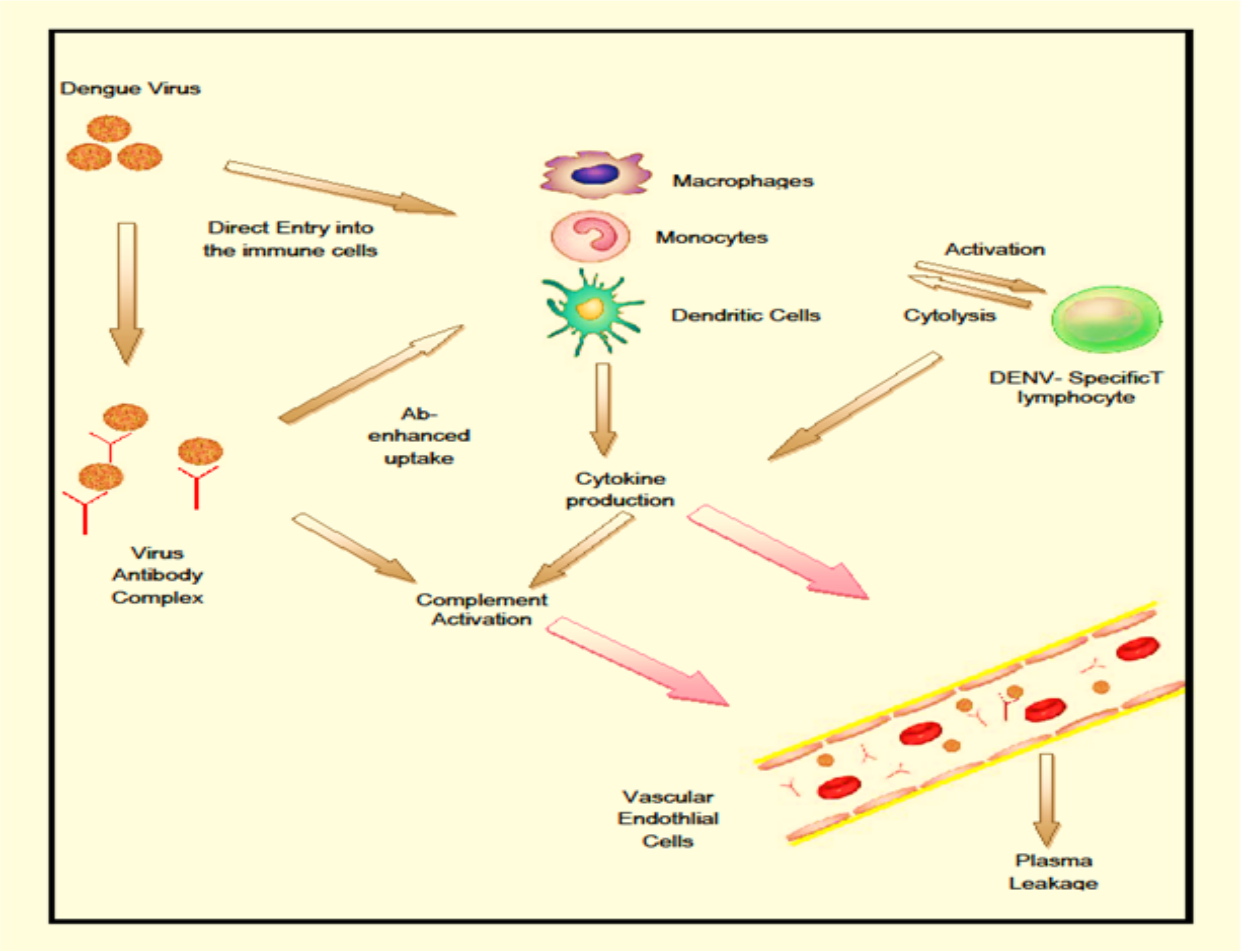


Fig 1.5. Pathway for the immuno- pathogenesis of DENV; Source: www.Google.com/ immuno- pathogenesis of Dengue virus

1.10. Dengue Sign and Symptoms

Patients infected with DENV have a wide spectrum of clinical manifestation, ranging from silent infections with no symptoms to a mild flu-like syndrome, dengue fever (DF), or severe dengue disease (SDD), including dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (WHO,2009). Infection with any of the four serotypes causes a similar clinical presentation that may vary in severity, depending on a number of risk factors. The incubation period varies from 3 to 14 days (most often 4 to 7 days) (Sabin *et al.,*1945) A range of other clinical features may be observed, and include sore throat, diarrhea, vomiting, anorexia and conjunctival injection. Rashes are commonly seen in dengue: early in the course of illness the skin often appears flushed with petechiae developing in the 'critical' phase and a macular rash occurring in the convalescent period. Severe arthralgia can be a feature of the illness and explains the use of 'break-bone fever' as a descriptive term for dengue (McBride, 2010). The clinical features differ between age groups with clinical features such as cough, vomiting and

abdominal pain being more frequently observed in children (WHO,2009). Dengue illness can evolve into three phases: the acute febrile phase — observed in most of the patients — and the critical phase and the recovery (convalescent) phases (WHO, 2009)

During the acute phase of illness fevers occurs during which body temperature may rise to 102 to 105°F, and fever may last 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. Rash is variable but occurs in up to 50% of patients as either early or late eruptions. Different types of aches and pain are also common this includes retro-orbital pain, joint pain, loss of appetite, nausea, vomiting and abdominal pain (WHO,1997) Dengue fever is rarely fatal and is self- limiting. The differential diagnoses during the acute phase of illness should include measles, rubella, influenza, typhoid, leptospirosis, malaria, other viral hemorrhagic fevers, and any other disease that may present in the acute phase as a nonspecific viral syndrome. 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).

The critical stage is characterized by signs of circulatory failure or hemorrhagic manifestations that may occur from about 24 h before to 24 h after the temperature falls to 37.5-38°C (WHO,2009). Increase in capillary permeability with an associated rise in haematocrit can be observed. The degree of plasma leakage is variable. If a critical volume of plasma is lost patients will develop clinical shock. Thrombocytopenia is almost universally seen in dengue infection and minor mucosal bleeding can be a feature of uncomplicated infection. Severe haemorrhage can occur – gastrointestinal bleeding is well described in those with a history of peptic ulcer disease (Tsai *et al.,* 1991). Intracerebral and pulmonary haemorrhage can occur (Kumar *et al.,* 2009). Blood tests usually show that the patient has thrombocytopenia (platelet count, ≤100,000/mm^3) and hemoconcentration relative to baseline as evidence of a vascular leak syndrome. Common hemorrhagic manifestations include skin hemorrhages such as petechiae, purpuric lesions, and ecchymoses. Epistaxis, bleeding gums, GI hemorrhage, and hematuria occur less frequently. The primary pathophysiologic abnormality seen in Dengue Hemorrhagic Fever and Dengue Shock Syndrome is an acute increase in vascular permeability that leads to leakage of plasma into the extravascular compartment, resulting in hemoconcentration and decreased blood pressure (Innis *et al.,* 1995). Plasma volume studies have shown a reduction of more than 20% in severe cases. Supporting evidence of plasma leakage includes serous effusion found postmortem, pleural effusion on X-ray, hemoconcentration, and hypoproteinemia.

The recovery phase occurs if the patients survives the 1-2 days critical phase with the reabsorption of the leaked fluid into the bloodstream, which usually lasts two to three days. During this stage appetites return, gastrointestinal symptoms abate, another rash may occur with either a maculopapular or a vasculitis appearance, which is followed by peeling of the skin (WHO,2009).

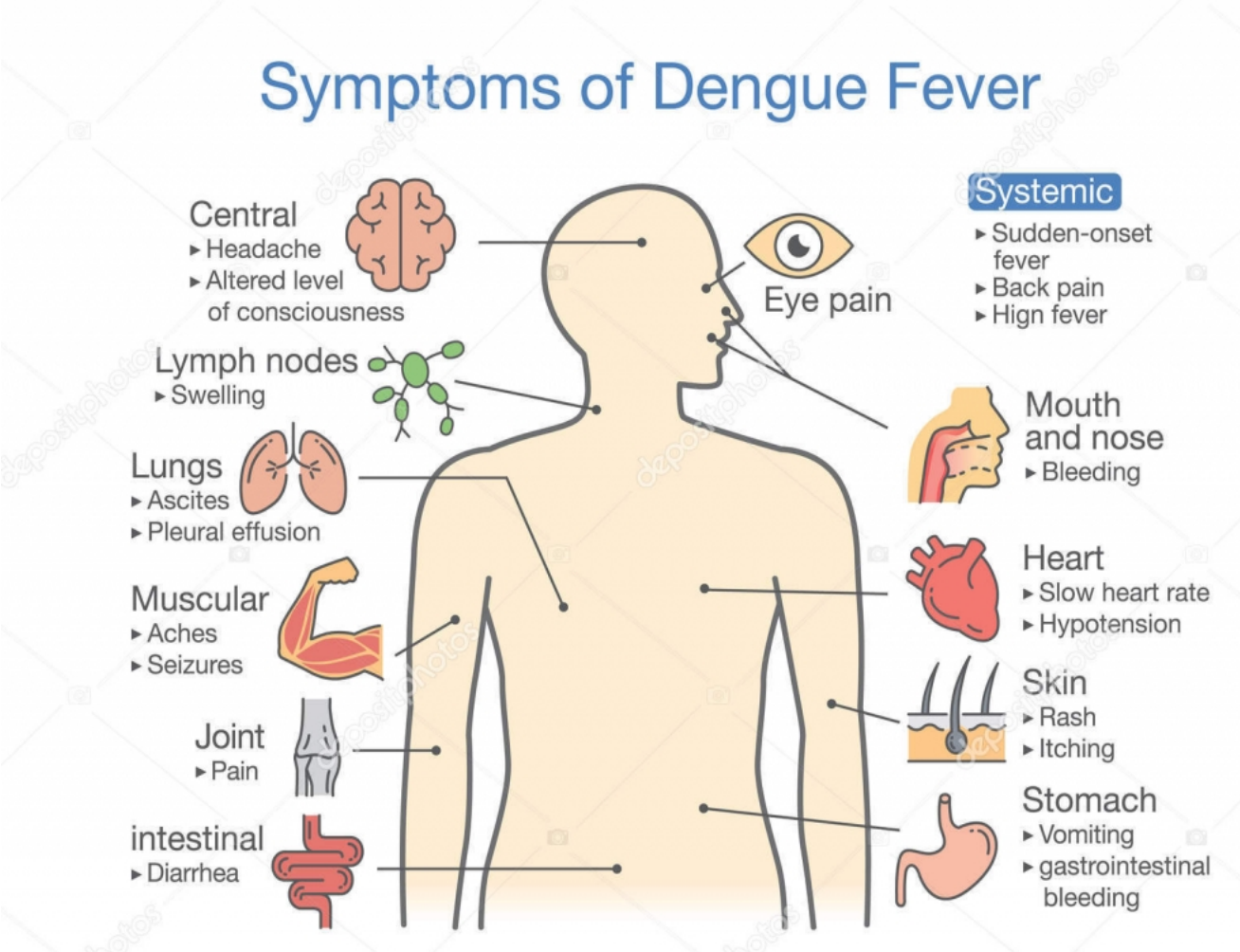
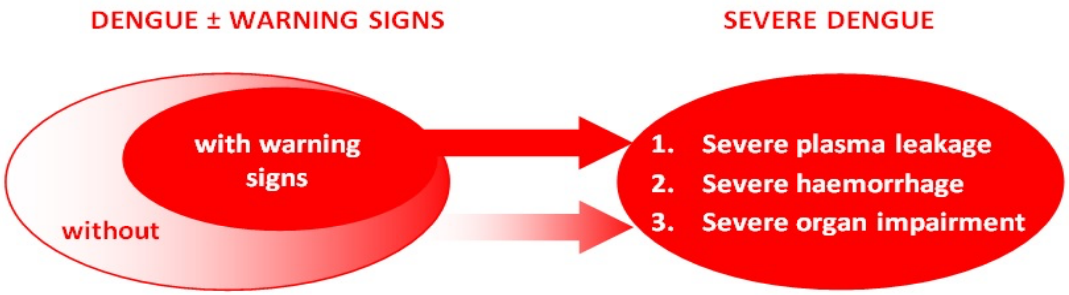


Fig:1.6. Schematic Representation of symptoms of Dengue Fever (source; https://st3.Depositphotos.com/4293685/17809/v/1600/depositphotos)

1.10.1 Classification of Dengue

In 1970s, clinical dengue has been classified according to the World Health Organization guideline as dengue fever and dengue hemorrhagic fever. The classification has been criticized with regard to its usefulness and its applicability and the updated a new guideline that classifies clinical dengue as dengue and severe dengue. Dengue fever without warning signs are classified as the subjects who have fever and any two of the symptoms of nausea, vomiting, rash, aches and pains, leucopenia and positive tourniquet test. Severe dengue is defined as that associated with severe bleeding, severe organ dysfunction, or severe plasma leakage (WHO, 2009).



CRITERIA FOR DENGUE ± WARNING SIGNS

Probable dengue

Live in / travel to dengue endemic area, fever and 2 of the following criteria:

- Nausea, vomiting
- Rash
- Aches and pains
- Tourniquet test positive
- Leukopenia
- Any warning sign

Laboratory-confirmed dengue (important when no sign of plasma leakage)

Warning signs*

- Abdominal pain or tenderness
- Persistent vomiting
- Clinical fluid accumulation
- Mucosal bleed
- Lethargy, restlessness
- Liver enlargement >2cm
- Laboratory: increase in HCT concurrent with rapid decrease in platelet count

*(requiring strict observation and medical intervention)

CRITERIA FOR SEVERE DENGUE

Severe plasma leakage

leading to:

- Shock (DSS)
- Fluid accumulation with respiratory distress

Severe bleeding

as evaluated by clinician

Severe organ involvement

- Liver: AST or ALT $\geq$ 1000
- CNC: Impaired consciousness
- Heart and other organs

Fig: 1.8. Classification of dengue according to severity (WHO, 2009)

1.10.2. Laboratory Diagnosis of Dengue

The current laboratory diagnosis of dengue involves detection of dengue viral component and antibodies that are present in the patient serum at different time of the infection. During the febrile phase, detection of viral nucleic acid in serum is done by means of reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay or detection of the virus-expressed soluble nonstructural protein 1 (NS1) by means of enzyme-linked immunosorbent assay (ELISA)

or the lateral-flow rapid test for a confirmatory diagnosis (Tricou *et al.,*2011) Serologic diagnosis of dengue relies on the detection of high levels of serum IgM that bind dengue virus antigens in an ELISA or a lateral-flow rapid test; IgM can be detected as early as 4 days after the onset of fever (Hunsperger *et al.,*2009). In addition, patients with secondary infections mount rapid anamnestic antibody responses in which dengue virus–reactive IgG may predominate over IgM (Guzman *et al.,* 2010).

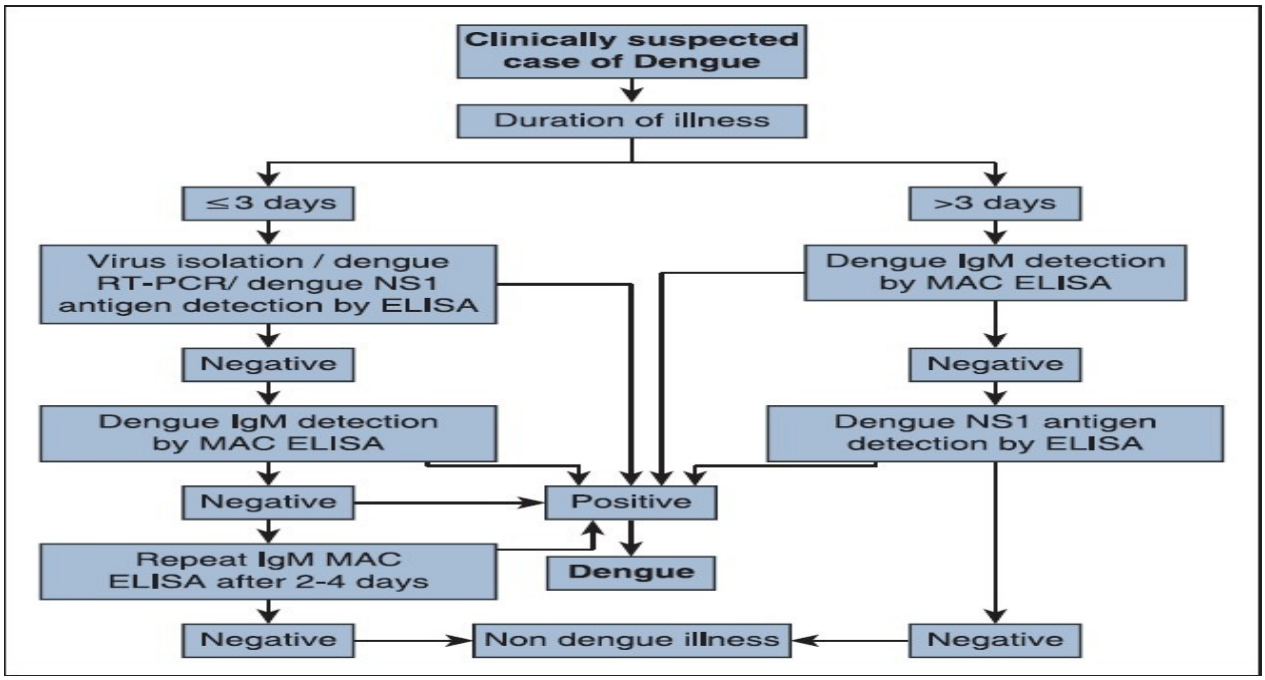


Fig:1.8. Clinical Applicability of Various Dengue Diagnostic Tests in Resource-Limited Endemic

Available from: https://www.researchgate.net/figure/Algorithm-for-laboratory-diagnosis-of-clinically-suspected-dengue-cases-in-endemic-setting_fig2_265393198 [accessed 22 Jan 2019]

1.11. Rationale of the study

Dengue is the most prevalent arthropod-borne virus affecting humans today. Four well-established distinct serotypes of dengue virus, with a fifth one recently discovered in 2013, are responsible for causing a spectrum of disease, ranging from a mild febrile illness to a life-threatening dengue hemorrhagic fever. Compared with nine reporting countries in the 1950s, today the geographic distribution includes more than 100 countries worldwide. Asia, South America and the Pacific Islands are hyper-epidemic regions while currently there is less prevalence in Europe, North America and Australia. The estimated global incidence ranges between 200 and 400 million clinical cases per year. Dengue has been emerging as a major infectious disease in Nepal with three major outbreaks in the year 2010, 2013 and 2016. Rates of dengue infection is increasing and is believed to be due to a combination of unplanned urbanization, population growth, increased international travel, and global warming. Successful vector control programs have also been eliminated, often because of lack of governmental funding.

Dengue has no ethical, geographical, sex, race and age boundary for transmission. The range of infection has extended all over the country. In some of the hospitals in Nepal, only the clinical symptoms have been studied and medications are made according to the nature of fever and symptoms. Besides that, Rapid Diagnostic Kits are distributed in hospitals and dengue is said to be positive or negative based on those kits. IgM, IgG and NS1 kit are available for rapid diagnosis of dengue fever but these kits are not completely reliable as this coincides with other flaviviral infections. Also, there is no certainty that the imported kits will work for Nepal. So, there is strong urge to develop in- house ELISA in order to overcome the need of purchasing the kits from abroad and to make it available all over the endemic regions so that the health workers will diagnose the disease accurately and reliably. This research mainly aims in the survey of antibody titre level against dengue serotypes circulating in Nepalese population by developing serotype specific In-House ELISA and sensitivity analysis of In-house ELISA data with obtained from worldwide widely used commercially available ELISA kit. Study also aims to know whether the infection is acute phase or not based on IgM and IgG titer strength and primary and secondary infection based on IgM to IgG ratio. Furthermore, the serotype of dengue virus keeps on changing in every outbreak, so this study aims to determine prevalent serotype by conventional nested reverse transcriptase PCR.

1.12. Objectives

1.12.1. General Objectives

Survey of antibody titer level against dengue and serotyping by nested RT-PCR circulating in Nepalese population.

1.12.2. Specific Objectives

- Differentiation of primary and secondary infection based on IgG and IgM titre strength.
- Serotype identification by conventional nested reverse transcriptase PCR.
- Optimization of different laboratory conditions for In-House ELISA.
- Define serotype specific antibody titer using purified DENV1- 4 antigen from dengue suspected Nepalese population
- Comparison of antibody titer in Endemic and Non-endemic population for different serotypes of dengue.

1.13. Hypothesis

Null hypothesis:

- There is no difference in antibody titre between endemic and non-endemic samples.
- Prevalence of secondary infection is more than primary infection.
- Multiple serotypes of dengue virus is circulating in the year 2016.

Alternative hypothesis:

- There is significant difference in antibody titre between endemic and non-endemic samples.
- Prevalence of primary infection is more than secondary infection.
- A single serotype of dengue is circulating in the year 2016.

CHAPTER II

LITERATURE REVIEW

2.1. Global Burden of Dengue

The incidence of dengue has grown dramatically around the world in recent decades; 3.6 billion people are estimated to now live in tropical and subtropical areas where the dengue viruses have the potential to be transmitted. Global estimates vary, but regularly approximate 50 million to 200 million dengue infections and over 20,000 dengue related deaths occur annually (Gubler, 2011). Prior to 1970, only 9 countries had experienced severe dengue epidemics. Dengue is now endemic in more than 100 countries in all tropics and subtropics of the world. These include countries in Africa and the Americas in addition to Eastern Mediterranean, South-East Asia and the Western Pacific, which are most seriously affected (WHO, 2018).

A dramatic change in the epidemiology and disease severity was observed since the Second World War, which was followed by massive epidemics of dengue hemorrhagic fever. Prior to the 1980s, severe dengue was rare disease in the Americas where the observed significant expansion of dengue and increased disease severity was clearly associated with the introduction of multiple serotypes (Gubler, 1997).

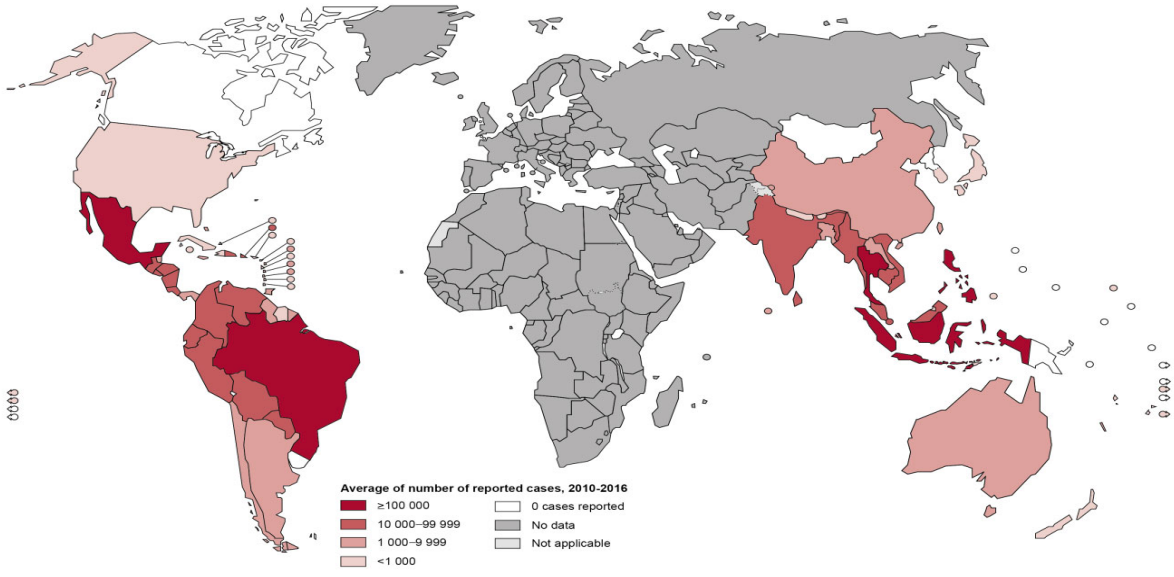


Figure:2.1. Average number of suspected or confirmed dengue cases reported to WHO, 2010-2016.

A combination of increased and unplanned urbanization, changing life styles and lack of effective mosquito control has made most tropical cities highly permissive for efficient dengue transmission. This has led to enhancement of vector breeding and thus increased contact between humans and vectors (i.e. more mosquitoes living closer to more people). Other factors responsible for the emergence of the disease includes increased air travel, inadequate and deteriorating public health infrastructure and changes in vector distribution and density associated with lack of effective mosquito control (Wilder-Smith *et al.,*2008)

2.2. Epidemiology of Dengue in Nepal

Nepal is a Himalayan country surrounded by the dengue-endemic countries India and China. Nepal is a disease-endemic area for many vector-borne diseases, including malaria, kala-azar, Japanese encephalitis, and lymphatic filariasis. Because of the porous border between Nepal and India, social, cultural, and economic activities in cross-border areas are common. In Nepal, the first case of dengue disease was reported in 2004 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). Subsequently, the larger outbreak occurred in nine districts of Terai region in 2006. After four years another significant outbreak occurred in 2010. Although the circulation of all four serotypes was reported during 2006–2007 dengue outbreaks, dengue serotype-1 (DENV-1) strains, closely related to Indian strains, were exclusively identified in the second outbreak in 2010 (Pandey *et al.,* 2013). Similarly, only dengue serotype 2 (DENV-2) was identified during the third outbreak in 2013. Again, during the fourth outbreak in 2016, DENV-1 was predominately reported, with the disease even reaching into the highlands (Gupta *et al.,* 2018). 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).

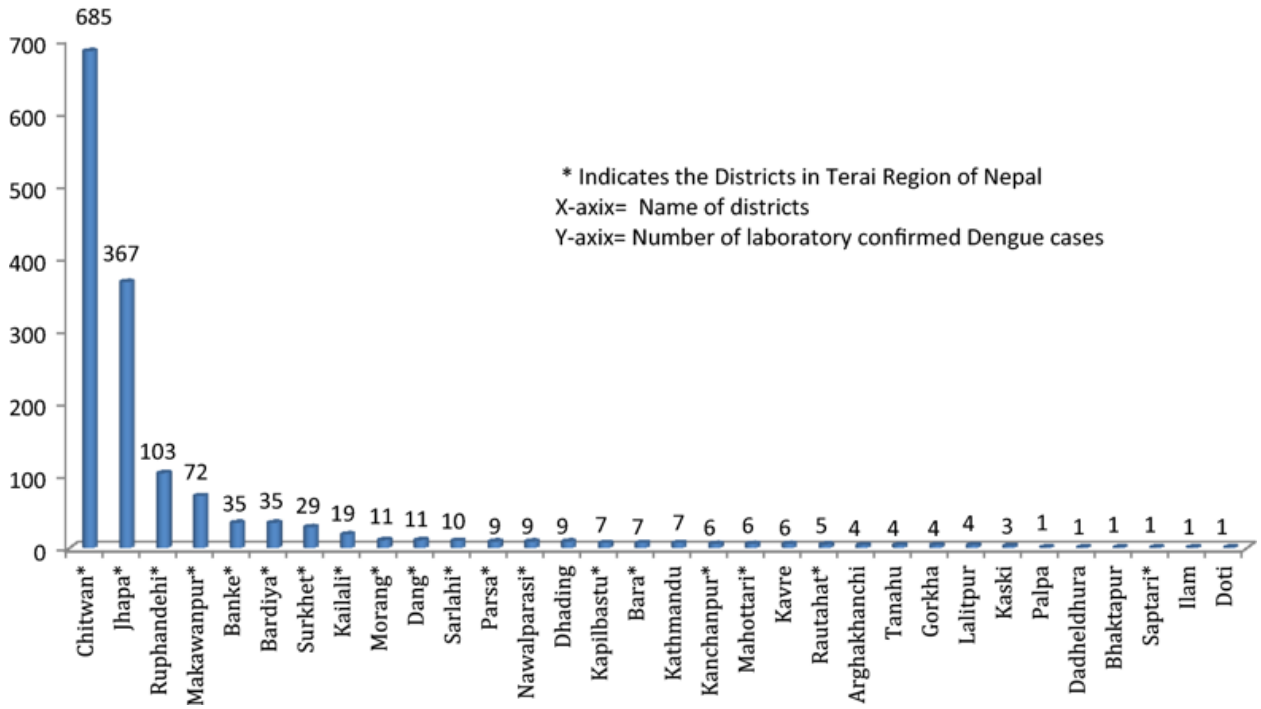


Fig 2.2. Dengue confirmed districts in the year 2016 (Khetan *et al.,* 2018)

2.3. Dengue Virus Evolution

Historically, it has not been determined when DENV first appeared in human populations, mainly because the dis- ease is often asymptomatic and is therefore not diagnosed. The earliest record of dengue found to date is in a Chinese medical encyclopedia in 992 A.D (Gubler,1998). DENV-1 was first isolated by in Japan in 1943 (Kimura and Hotta, 1943) and few months after the first isolation, the same serotype was isolated from Hawaiian and shortly thereafter, DENV- 2 from Papua New Guinean samples (Sabin and Schlesinger, 1945). Viruses similar to DENV-1 and DENV-2 were isolated from Manila patients in 1956 by William Hammond and were called DENV-3 and DENV-4 (Hammond *et al*., 1960). It is now known all four serotypes of dengue virus can cause DHF. Severe dengue (also known as Dengue Haemorrhagic Fever) was first recognized in Manila, the Philippines in 1953 (Quinlos *et al.,* 1954).

2.3.1 Dengue virus serotype and its genotypes

The four dengue virus types (DENV1-4) form a phylogenetic group that is more closely related to one another than to other flaviviruses, despite the close relationship between the four serotypes, they are considered separate flavivirus species based on their antigenic and genetic differences (C.M. Fauquet *et al.,* 2005).

2.3.1.1 DENV1

DENV-1 can be divided into five genotypes based on the complete E gene sequence as described by (Goncalvez *et al.,*2002). Earlier work by Rico- Hesse (1990) also classified DENV-1 into five groups based on the 240- nucleotide E/NS1 junction sequence. The DENV-1 genotypes all have a wide area of distribution apart from genotype III (sylvatic) and genotype II which consists of Thai strains from the 1950s and 1960s. Viruses of genotype I and IV have recently been implicated as causing epidemics in the Pacific between 2000 and 2004 and genotype V viruses are frequently isolated during epidemics in the Americas. However, it is still inconclusive whether any of these three DENV- 1 genotypes can be consistently associated with causing more severe dengue.

2.3.1.2 DENV2

DENV-2 is the most studied serotype among the dengue viruses. The existence of six genotypes of DENV-2 based on the complete E gene sequence was proposed by Twiddy et al. (2002). The sylvatic genotype includes strains from South-East Asia and Africa. The five epidemic genotypes include one with a global distribution (cosmopolitan genotype IV) and one genotype originally described from Central and South America (American genotype IV). Additionally, two lineages of Asiatic origin are separated (Asian genotypes I and II), and one genotype from the Americas genetically associated with the Asiatic viruses, is referred to as the American-Asian genotype, which has been associated with severe disease (Rico- Hesse *et al.,* 1997).

2.3.1.3 DENV3

The current genotype classification for DENV-3 follows the nomenclature proposed by Lanciotti et al. (1994) which recognized four DENV-3 genotypes based on prM/E sequences. These four genotypes are similar to the four groups using a 195- nucleotide region at the 5' terminus of the E gene. Introduced to the Americas via Nicaragua in 1994, genotype III DENV-3 is now widely found in Central and Southern America (Balmaseda *et al.,* 1999) and is considered as the most virulent of the four DENV-3 genotypes. It is worthy of note that genotype IV has never been associated with any DHF epidemics. Although their existence is anticipated through the presence of DENV- 3 antibodies in non-human canopy-dwelling primates, no sylvatic lineage of DENV-3 has been found thus far (Rudnick, 1984).

2.3.1.4 DENV4

DENV-4 is initially separated into two genotypes, I and II, based on the complete E gene sequence (Lanciotti *et al.,* 1997). A further two genotypes were subsequently described, with one found only in non-human primates in Malaysia and another, genotype III, found only in Bangkok, Thailand (Klungthong *et al.,* 2004). Genotype II DENV-4 is the most widespread of the four following an introduction to the Western hemisphere in 1981, possibly via the Pacific islands (Lanciotti *et al.,* 1997; Foster *et al.,* 2003). Although DENV-4 is the least frequently sampled serotype, it is often associated with haemorrhagic fever during secondary infection (Vaughn *et al.,* 2000).

2.3.1.5 Discovery of a new dengue serotype: DENV-5

The recently claimed discovery of a new serotype, DENV-5 in 2013, provides a further challenge to dengue control. This serotype follows the sylvatic cycle unlike the other four serotypes which follow the human cycle. Occurrence of new cases DENV-5 may lead to new challenges in dengue control. The detection of DENV-5 has also raised speculation that there might be more serotypes which have not been identified till date. Phylogenetic evaluation revealed that DENV-5 is genetically similar to the other four serotypes, thereby hinting to a common ancestral origin. Further research is underway to address the unanswered questions on the evolution of DEN (Mustafa *et al.,* 2015).

2.4. Geographical and Seasonal Distribution of Dengue Virus

Dengue viruses have a worldwide distribution in the tropics and subtropical regions. The viruses are endemic in most urban centers of the tropics, with transmission occurring throughout the year. Epidemic transmission occurs periodically in most virus-endemic areas, usually at 3–5year intervals. As surveillance in most endemic countries is poor, cases are not usually reported during interepidemic years, thus potentially downplaying the risk of infection. It is well documented, however, that dengue viruses are maintained during interepidemic periods in most tropical areas and, although risk of infection is lower than during epidemic periods, it is still substantial to unsuspecting visitors. Peak transmission of dengue viruses is usually associated with periods of higher rainfall in most dengue-endemic countries. Factors influencing seasonal transmission patterns of dengue viruses are not well understood, but obviously include mosquito density, which may increase during the rainy season, especially in those areas where the water level in larval habitats is dependent on rainfall. In areas where water storage containers are not influenced by rainfall, however, other factors such as higher humidity and moderate ambient temperatures associated with

the rainy season increase survival of infected mosquitoes, thus increasing the chances of secondary transmission to other persons (Halstead, 2008).

2.5. Diagnostic Approaches

Dengue can be diagnosed by isolation of the virus, by serological tests, or by molecular methods. After the onset of illness, the virus can be detected in serum, plasma, circulating blood cells and other tissues for 4–5 days. During the early stages of the disease, virus isolation, nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis (World Health Organization, 2009)

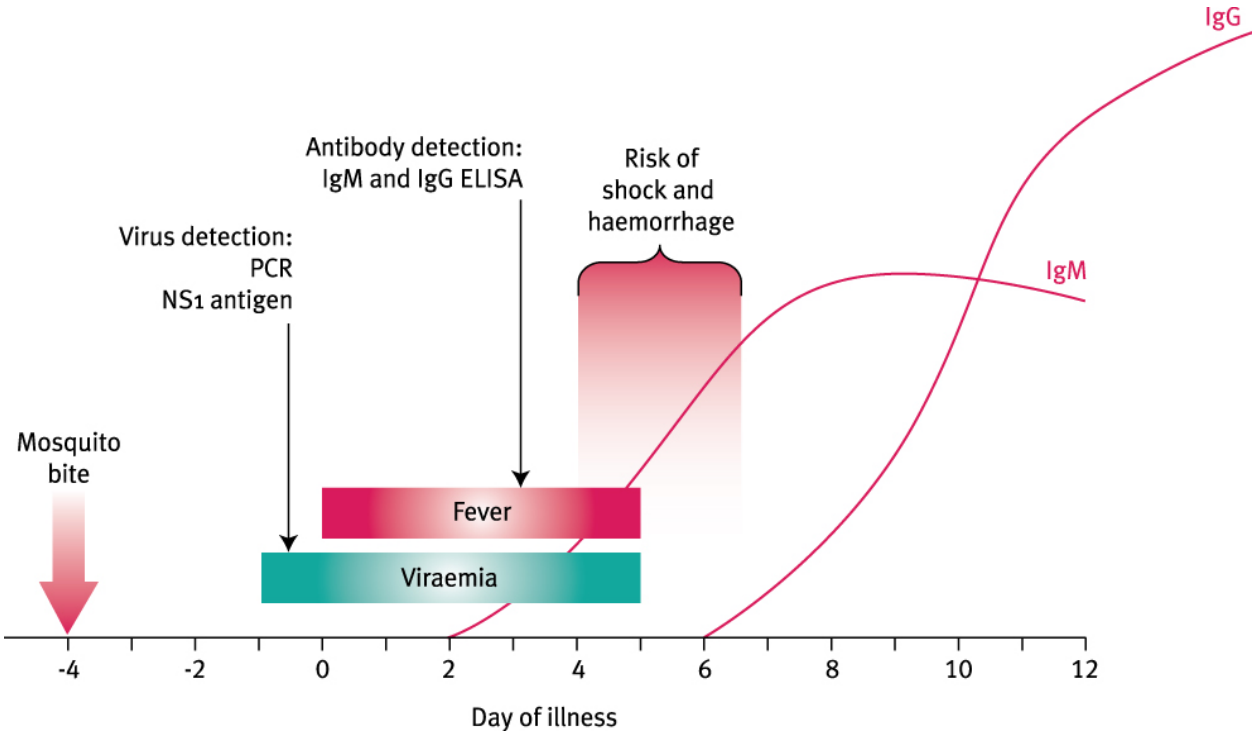


Fig: 2.3. Typical primary dengue infection with timing of diagnostic tests [Source: Tropical Regional Services, Queensland Health]

2.5.1 Virus Isolation

Virus isolation is not commonly used in routine diagnostics but constitutes definitive proof of DENV infection. For isolation of the virus, the blood collection should be performed preferably within the first five to six days of symptoms, during the acute phase. Four isolation systems have routinely been used for dengue viruses; intracerebral inoculation of 1- to 3-day-old baby mice, the use of mammalian cell cultures (primarily LLC-MK_2 cells), intrathoracic inoculation of adult mosquitoes, and the use of mosquito cell cultures (Gubler *et al.,* 1998).

The isolated viruses can be identified by indirect immunofluorescence using monoclonal antibodies against all four serotypes (Henchal *et al.,* 1982).

2.5.2. Nucleic Acid Detection

Molecular diagnosis typically provides more sensitive and rapid detection than traditional virus isolation methods, because it amplifies nucleic acid even for inactivated virus. All the nucleic acid detection assays involve three basic steps; nucleic acid extraction and purification, amplification of nucleic acid and detection and characterization of the amplified product. However, RNA is heat labile and therefore must be handled carefully to avoid RNA degradation.

2.5.2.1 RT-PCR

Polymerase chain reaction (PCR) is a technique of amplification of DNA from a target RNA to produce cDNA through reverse-transcription reaction and hence also known as reverse transcriptase-polymerase chain reaction (RT-PCR). However, several laboratories have published various RT-PCR protocols for dengue virus identification. Among these, the two-step nested RT-PCR protocols originally reported (Lanciotti *et al*., 1992) and later modified to a single-step multiplex RT-PCR for the detection and typing of dengue virus (Harris *et al*., 1998) are well known. These assays used universal dengue primers targeting the C/prM region of the genome for an initial reverse transcription and amplification step, followed by a nested PCR amplification that is serotype-specific (WHO, 2009). Since RT-PCR is highly sensitive to amplicon contamination, without proper controls false-positive results may occur. Improvements in this technology, however, should make it even more useful in the future (Gubler *et al.,* 1998).

2.5.2.2 Real-time RT PCR

The real-time RT-PCR assay is a one-step assay system used to quantitate viral RNA and using primer pairs and probes that are specific to each dengue serotype. The real time RT-PCR uses either non-specific fluorescence dyes (i.e. SYBR green) that binds to any double stranded DNA or specific oligonucleotide probes with fluorescence reporter dye (i.e. TaqMan® Probes) that only allows detection when hybridized to specific DNA targets. The most popular ones use non- specific fluorescence dyes as they are least expensive but is less specific. The use of a fluorescent probe enables the detection of the reaction products in real time without the need for electrophoresis. Real time pcr are either single plex determining one serotype at a time or multiplex, determining all four serotypes. However, the multiplex real-time RT-PCR, although faster, less sensitive than nested RT-PCR but has ability to determine viral titre in a clinical sample, which may be used to study the pathogenesis of dengue (WHO, 2009).

2.5.3 Serological Test

Different serologic tests have been routinely used for diagnosis of dengue infection; hemagglutination-inhibition (HI), complement fixation (CF), neutralization test (NT), immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA), and indirect immunoglobulin G ELISA (Gubler *et al.,* 1998).

Immunological diagnosis detects specific antibodies to the virus and complements the viral diagnosis. The detection of antibodies can be made using Enzyme linked Immuno-Sorbent Assay(ELISA) and immune-chromatographic techniques; test for hemagglutination inhibition (HI) (WHO, 2009).

2.5.3.1 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. Other names, such as enzyme immunoassay (EIA), are also used to describe the same technology. In an ELISA, an antigen must be immobilized on a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction (Lequin,2005).

Types of ELISA

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

2.5.3.1.1. Direct ELISA:

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

2.5.3.1.2. Indirect ELISA:

In this system, initial antigen binding and washing steps are the same as the direct method. The main difference in this case is the use of unconjugated antibody to bind the immobilized antigen upon incubation at optimal temperature (usually 37^{o}C). Following a washing step to remove unbound antibodies, the remaining antigen-bound

antibodies are targeted by a conjugated secondary antibody that will generate the readout signal as described for direct ELISA. This system has been widely applied in diagnostics because it allows large number of samples to be screened with a single conjugated secondary antibody.

2.5.3.1.3. Sandwich ELISA:

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

Development of In-House ELISA

The first step of ELISA is coating. It is a process where a suitably diluted antigen or antibody is incubated until adsorbed to the surface of the well. Coating helps to stabilize the antigen or antibody which is used to coat the ELISA multi-well plate, maximizing adsorption to the plate and optimizing interactions with the detection antibody. The dilution is done on the coating buffer which helps in adsorptive immobilization of proteins and antibodies on plastic surfaces or other binding surfaces. After coating, the plates are incubated at 4◦C overnight. Then the plates are blocked using blocking buffer. Blocking is necessary to prevent the non-specific binding of detection antibodies to the multi-well plate surface itself. The blocking buffers usually contain an unrelated protein or a protein derivative that does not react with any of the antibodies being used in the detection step. The plates are washed

using phosphate-buffer saline which is a water-based salt solution. It is preferred because it is non-toxic to most cells. The washing step helps to remove the unbound materials (Engvall and Perlmann,1971).

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

Fig:2.4 Overview of steps in different types of ELISA

MAC ELISA

IgM antibody capture ELISA (MAC-ELISA) format is most commonly employed in diagnostic laboratories and commercially available diagnostic kits. Dengue-specific antigens, from one to four serotypes (DEN-1, -2, -3, and -4), are bound to the captured anti-dengue IgM antibodies and are detected by monoclonal or polyclonal dengue antibodies directly or indirectly conjugated with an enzyme that will transform a non-colored substrate into coloured products. The optical density is measured by spectrophotometer. Cross-reactivity with other circulating flaviviruses such as Japanese encephalitis, St Louis encephalitis and yellow fever, does not seem to be a problem but some false positives were obtained in sera from patients with malaria, leptospirosis and past dengue infection (Hunsperger EA *et al.,*2009).IgM detection is not useful for dengue serotype determination due to cross-

reactivity of the antibody. MAC-ELISA has good sensitivity and specificity but only when used five or more days after the onset of fever (Vazquez S *et al.,*2005).

IgG ELISA

The IgG ELISA used for the detection of a past dengue infection utilizes the same viral antigens as the MAC ELISA. This assay correlates with the hemagglutination assay (HI) previously used. In general IgG ELISA lacks specificity within the flavivirus serocomplex groups. Primary versus secondary dengue infection can be determined using a simple algorithm. Samples with a negative IgG in the acute phase and a positive IgG in the convalescent phase of the infection are primary dengue infections. Samples with a positive IgG in the acute phase and a 4-fold rise in IgG titer in the convalescent phase (with at least a 7-day interval between the two samples) is a secondary dengue infection (Vazquez S *et al.,*2007).

2.5.3.2 IgM/IgG ratio

The primary and secondary infections was determined by the ratio of the units of dengue virus IgM antibodies to the units of dengue virus IgG antibodies (Innis *et al.,*1989).

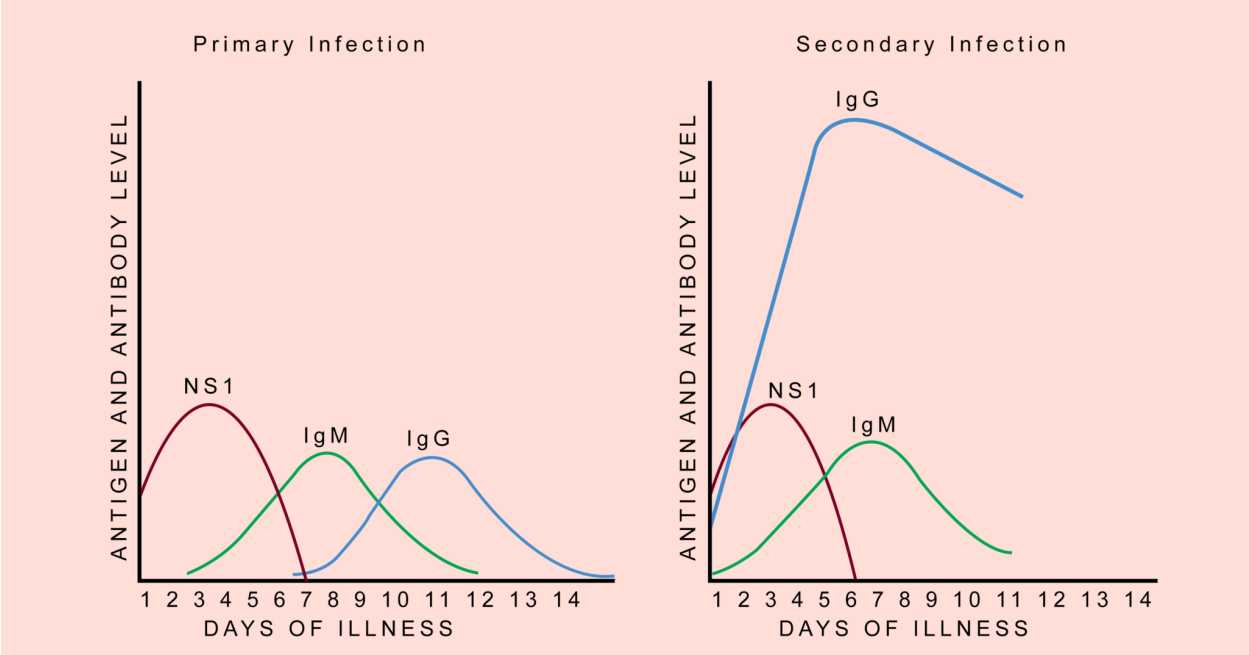


Fig:2.5. Immune response to dengue response (image source: http://philab.com/wp-content/uploads/2016/08/Immunological-response.png)

The acute-phase sera of patients with primary dengue virus infections had higher IgM/IgG ratios, whereas patients with secondary infections had lower IgM/IgG ratios. IgM capture and IgG capture ELISAs are the most common assays for this purpose. In some laboratories, dengue infection is defined as primary if the IgM/IgG OD ratio is greater than 1.2 (using

patient's sera at 1/100 dilution) or 1.4 (using patient's sera at 1/20 dilutions). The infection is secondary if the ratio is less than 1.2 or 1.4. This algorithm has also been adopted by some commercial vendors. However, ratios may vary between laboratories, thus indicating the need for better standardization of test performance (Falconar *et al.,*2006).

2.5.3.3. Haemagglutination-inhibition Test

The HI test are the most commonly used serological techniques for the routine diagnosis of dengue virus infections. It is based on the ability of dengue antigens to agglutinate red blood cells (RBC) of ganders or trypsinized human O RBC. Anti-dengue antibodies in sera can inhibit this agglutination and the potency of this inhibition is measured in an HI test. Serum samples are treated with acetone or kaolin to remove non-specific inhibitors of haemagglutination, and then adsorbed with gander or trypsinized type O human RBC to remove non-specific agglutinins. Each batch of antigens and RBC is optimized. PH optima of each dengue haemagglutinin requires the use of multiple different pH buffers for each serotype. Traditionally, the HI test was used to detect and differentiate primary and secondary dengue virus infections due to its simplicity, sensitivity, and reproducibility. Patients are classified as having secondary dengue virus infections when the HI test titer in their sera is greater than or equal to 1:2,560 and are classified as having primary dengue virus infection if the HI test titer is less than 1:2,560 (WHO, 1997). The HI test has recently become less popular and has gradually been replaced by the E/M-specific capture IgM and IgG ELISA due to the inherent disadvantages of the HI test (Innis *et al.,* 1989).

2.5.4. Antigen Detection

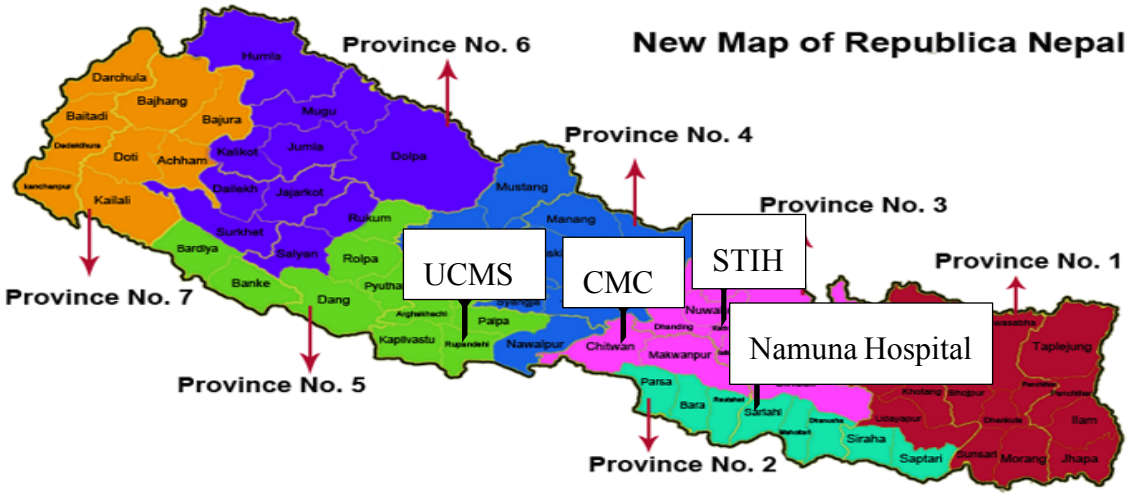
The hexameric form of NS1 protein is highly conserved in all four DENV serotypes and was found circulating in the blood of patients from the first to the ninth day after the onset of fever (Young *et al.,* 2000).Because it is present in the serum during the acute phase of the infection, NS1 antigen is a marker used in the early diagnosis of the disease, with a greater sensitivity in the first five days of disease.) There is also evidence that the sensitivity of NS1 antigen detection is higher in primary infection (> 90%) than in secondary infection (60%–80%). High levels of NS1 antigen were also found to be correlated with higher levels of viraemia and, thus, could potentially be a predictor for severe illness (Hang *et al.,* 2009).

Chapter III

MATERIALS AND METHODS

3.1 Site Selection and Sample Collection

The samples were collected from Sukraraj Tropical and Infectious disease Hospital (STIDH) - Kathmandu, Chitwan Medical College and Teaching Hospital (CMC), Universal College of Medical Science (UCMS), Bhairahawa and Namuna Hospital, Sarlahi, in 2016 and further research activity was carried out at Central Department of Biotechnology, Tribhuvan University, Kritipur.



UCMS: Universal College of Medical Science

CMC: Chitwan Medical College

STIH: Sukraraj Tropical and Infectious Disease Hospital

Fig: 3.1 Sample collection hospital sites

3.2 Ethical approval

For the collection and processing of blood specimens, approval was taken from Nepal Health Research Council (NHRC) (Reg. no. 378/2016) and Research Committee of Central Department of Biotechnology (CDBT). Written consent form was obtained according to NHRC and the privacy of the samples were maintained. In case of children sample was collected after the guardian filled the consent form on the behalf of children.

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 and immediately processed to prevent possible degradation of samples. Serum was separated from blood sample and stored at - 80° until further processing. In case of the samples from STIDH, blood was collected and immediately transferred to CDBT for further processing. In other collection sites, serum was transported to CDBT and further processing was done there.

3.4 Enzyme-linked Immuno-sorbent Assay (ELISA)

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

3.4.1 InBios ELISA

IgM and IgG ELISA was performed based on InBios ELISA protocol.

Preparation of Reagents:

Preparation of 1X wash buffer: The 10X wash buffer was diluted to 1X using high- grade deionized water. To prepare 1X buffer solution, 120 ml 10X wash buffer

was mixed with 1080 ml of deionized water and mixed thoroughly such that the solution was uniform, and no precipitate was present.

Microtitration wells: The required number of coated wells for the assay were selected and the remaining unused wells were repackaged immediately with the supplied dessicant and stored at 4^0C until further use.

3.4.1.1. InBios IgM ELISA:

The experiment was designed as instructed in the InBios protocol and the test was performed. Briefly, 50 µL of 1:100 diluted serum sample were dispensed to the wells coated with anti-human IgM. After 1 hr. incubation at 37^0C the wells were washed with 300 µL wash buffer for 6 times. DENRA and NCA (50 µL) were dispensed to their respective rows and incubated and washed as above. Application of HRP enzyme conjugated anti-human IgG (50 µL), Enwash (150 µL), TMB) substrate (75µL) and stop solution (50µL) were added as similar to the IgG ELISA. The plate was then read at 450nm in ELISA Plate reader for raw OD values.

Test Validation:

Factor (For Assay Verification)	Tolerance
Mean Dengue Negative Control OD in DENRA	<0.30
Mean Dengue IgM Positive Control OD in DENRA	>0.35
Dengue IgM Positive Control Immune Status Ratio (ISR)	>5.0
Dengue Negative Control Immune Status Ratio (ISR)	<1.650

Calculations for Unknown sample analysis:

Calculation of the ISR: The ISR is calculated by dividing the DENRA OD value by the NCA OD value.

Interpretation of Results:

ISR	Result
≤ 1.65	Negative
1.65 < ISR < 2.84	Equivocal
≥ 2.84	Positive

3.4.1.2 InBios IgG ELISA:

The experiment was designed as instructed in the InBios protocol and the test was performed. Briefly, 50 µL of 1:100 diluted serum samples were dispensed, except in the first column, to all the labelled wells for sample in the first 4 rows coated with Dengue Recombinant Antigen (DENRA) and duplicated by dispensing in the remaining last 4 rows coated with Normal Cell Antigen (NCA) while in the first column two wells each of negative and positive sera were dispensed both in DENRA and NCA coated wells making their respective mirror wells. After 1 hr incubation at 37^0C the wells were washed with 300 µL wash buffer for 6 times. HRP enzyme conjugated anti-human IgG (50 µL) was dispensed to all the wells and then incubation and washing were done as above. Enwash (150 µL) was added to all wells followed by 5-minute incubation followed by washing step. Trimethyl Benzidine (TMB) substrate at the rate of 75µL was dispensed to the plate and incubated at ambient

temperature (20-25^{0}C) in dark for 10 minutes and the reaction was stopped by adding 50µL stop solution. The plate was read at 450nm wavelength in ELISA Plate reader (Thermo Electron Corporation Original Multiskan EX) for OD values.

Test Validation:

Factor (For Assay Verification)	Tolerance
Mean Dengue Negative Control OD in DENRA	< 0.300
Mean Dengue IgG Positive Control OD in DENRA	> 0.350
Dengue IgG Positive Control Immune Status Ratio (ISR)	> 5.000
Dengue Negative Control Immune Status Ratio (ISR)	< 2.000

Calculations for unknown sample analysis:

Calculation of the ISR: The ISR is calculated by dividing the DENRA OD value by the NCA OD value.

Interpretation of Results:

ISR	Result
≤ 1.65	Negative
1.65 < ISR < 2.84	Equivocal
≥ 2.84	Positive

Example for Serum Sample Application

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative Control	Sample # 1	Sample # 5	Sample # 9	Sample # 13	Sample # 17	Sample # 21	Sample # 25	Sample # 29	Sample # 33	Sample # 37	Sample # 41
B	Negative Control	Sample # 2	Sample # 6	Sample # 10	Sample # 14	Sample # 18	Sample # 22	Sample # 26	Sample # 30	Sample # 34	Sample # 38	Sample # 42
C	Positive Control	Sample # 3	Sample # 7	Sample # 11	Sample # 15	Sample # 19	Sample # 23	Sample # 27	Sample # 31	Sample # 35	Sample # 39	Sample # 43
D	Positive Control	Sample # 4	Sample # 8	Sample # 12	Sample # 16	Sample # 20	Sample # 24	Sample # 28	Sample # 32	Sample # 36	Sample # 40	Sample # 44
E	Positive Control	Sample # 4	Sample # 8	Sample # 12	Sample # 16	Sample # 20	Sample # 24	Sample # 28	Sample #32	Sample # 36	Sample # 40	Sample # 44
F	Positive Control	Sample #3	Sample # 7	Sample # 11	Sample # 15	Sample # 19	Sample # 23	Sample # 27	Sample # 31	Sample # 35	Sample # 39	Sample # 43
G	Negative Control	Sample # 2	Sample # 6	Sample # 10	Sample # 14	Sample # 18	Sample # 22	Sample # 26	Sample # 30	Sample # 34	Sample # 38	Sample # 42
H	Negative Control	Sample # 1	Sample # 5	Sample # 9	Sample # 13	Sample # 17	Sample # 21	Sample # 25	Sample # 29	Sample # 33	Sample # 37	Sample # 41

Example for DENRA and NCA Application

	1	2	3	4	5	6	7	8	9	10	11	12
A	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
B	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
C	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
D	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA	DENRA
E	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA
F	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA
G	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA
H	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA	NCA

Fig 3.2. Plate design for IgG and IgM

3.4.2. Differentiation of Primary and Secondary DENV infection:

For the dengue samples, IgM: IgG ratio was calculated. The samples with the ratio ≥ 1.2 or 1.4 were said to have primary infection and less than that were said to have secondary infection. The samples with negative ISR ratio in both IgM and IgG were termed as seronegative (Falconar *et al.,* 2006).

3.4.3. In-house ELISA:

Preparation of reagents

Antigen: The antigen used DENV1-4 were provided by Microbix. The antigen contains virus particles that are concentrated from tissue culture supernatants by precipitation and ultracentrifugation.

Human sera: Different suspected human sera samples of dengue were collected from different hospitals of Nepal. The serum samples were from Sukraraj Tropical and Infectious Disease Hospital, Kathmandu, Namuna Hospital, Sarlahi, Universal college of Medical Sciences, Bhairahawa and Chitwan Medical College, Chitwan.

Enzyme conjugated secondary antibody: Goat anti-human IgG Horse radish peroxidase (HRP)conjugated secondary antibody was used. The higher specific enzyme activity of HRP makes it the enzyme of choice. Its small size(40kDa) allows excellent penetration.

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

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

Test controls: For the test controls, the positive and negative control samples were obtained from the In-Bios ELISA kit. Other controls Blank, Primary Antibody absent and Secondary antibody absent were also taken.

Endemic Controls: Endemic controls(n=4) were the serum samples taken from people who had travelled Terai region. Terai region in Nepal is considered as endemic region, where higher dengue cases had been observed. These samples were not included in 32 dengue suspected test samples.

Non-endemic Controls: Non-endemic controls(n=7) were the samples taken from healthy persons of Kathmandu valley. These samples were also not included in 32 dengue suspected test samples.

Procedure

The antigen was diluted in carbonate-bicarbonate buffer (pH 9.6) with different concentration for different antigens. Then four different ELISA micro titer plates were coated with antigen (DENV1-4) diluted in coating buffer (100 µl/well). Plates were then incubated overnight at 4◦C and then washed with wash buffer (1X PBS+0.05% Tween-20) 5 times using automated ELISA plate washer and then blotted dry. The unbound sites were blocked with 1% casein in phosphate buffer solution and again incubated at room temperature for 1 hour. After incubation the plates were again washed and dried as before. Then 50µl of primary antibody diluted in serum diluents (0.1% casein in 1X PBS) in the ratio 1:100 were added to the respective wells and incubated at 37◦C for one hour. The plates should be covered by using paraffin tape. After incubation the plates were washed and dried as before. 25µl of secondary antibody (Goat anti-

human HRP diluted in blocking buffer in the ratio 1:5000) were added to all the wells and again incubated at 37◦C for an hour by covering the plates as before. The plates were washed and dried as before. To all the plates 100µl of TMB substrate were added and incubated in dark for 7 minutes. After that 50µl of stop solution (1N sulfuric acid) was added to each well and incubated for 1 minute. Then optical density reading was taken at 450nm using ELISA plate reader. The absorbance was recorded.

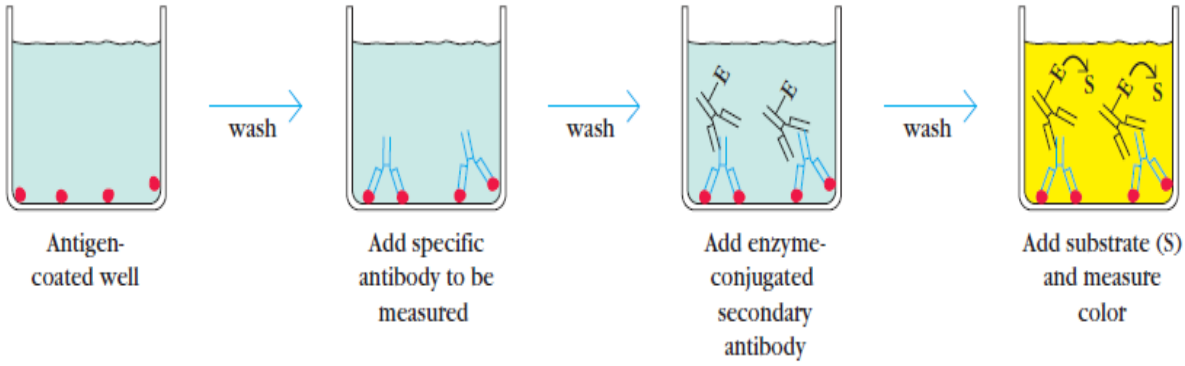


Fig:3.3. Major steps used in ELISA

3.4.3.1. Determination of Cut-off Value:

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

Cut-off OD = Mean OD of negative control + 2 X SD

3.4.3.2. Data Analysis:

Statistical analyses were performed using MS-Excel and Graph-pad Prism8 software. Unpaired T-test was performed to determine the p-value of the data and to test the significance of the result.

3.5. Molecular Assay

3.5.1 Viral RNA Isolation by Trizol

This protocol uses Trizol (also known as TRI REAGENT) for the isolation of total RNA. Trizol is a mixture of guanidine thioacyanate and phenol, which effectively dissolves DNA, RNA and protein on homogenization or lysis of sample. After adding chloroform and centrifuging, the mixture separates into 3 phases with the upper clear aqueous phase containing the RNA. The next steps in the extraction are washes and precipitation of the RNA. The first part of the

protocol – from the homogenized tissue in Trizol to the point of an RNA pellet in 75% ethanol, takes less than 1 hour. The RNA can then be stored for long periods of time, at -20^{0}c.

Materials Required:

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

PROCEDURE

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

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

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

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

5. Redissolving RNA: The RNA pellet was dried. RNA was dissolved in RNase-free water.

3.5.2 cDNA Preparation

BIORAD-iScript^{TM} cDNA Synthesis Kit (catalog no. 1708890) was used for synthesis of cDNA. This kit includes three tubes, which contain all the reagents required for successful reverse transcription. The iScript Reverse Transcriptase is RNase H+, which provides greater sensitivity than RNase H– enzymes in qPCR. iScript is a modified Moloney murine leukemia virus (MMLV) reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided preblended with RNase inhibitor. The unique blend of oligo(dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets <1 kb in length. iScript cDNA Synthesis Kit produces excellent results in both real-time and Kit produces excellent results in both real-time and standard RT-qPCR.

Table:3.1 Components used for cDNA Synthesis

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

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

Table:3.2 Reaction Protocol

Priming	5min at 25^0C
Reverse transcription	20min at 46^0C
Rt- inactivation	1min at 96^0C
Optional step	Hold at 4^0C

3.5.3 Nested RT-PCR:

Nested PCR of the cDNA was performed for partial region of envelope protein using the envelope protein primers (Table 3.3) and Solis Biodyne -5x FIREPol® Master Mix (Cat. No. 04-11-00125). Primers AA6EP_F (10 pm/µl) and AA7EP_R (10 pm/µl) were used for the first round PCR. The PCR conditions were set to initial denaturation at 98^0C for 5 min followed by 35 cycles of the PCR with cycling conditions of denaturation 95^oC, annealing 59^oC and extension 72^oC. The final extension was done at 72^oC for 5 min and the reaction was kept at hold at 4^oC. The PCR product was diluted in the ratio 1:10 and used as template for second round PCR using serotype-specific primers (AA8EP_F & AA9EP_R for DENV1, AA10EP_F & AA11EP_R for DENV2, AA12EP_F & AA13EP_R for DENV3 and AA14EP_F & AA15EP_R for DENV4). The PCR conditions were same as of the first round and only annealing temperature

was different for different serotypes (54.5^{O}C, 53.5^{O}C, 56^{O}C and 53.5^{O}C for DENV1, DENV2, DENV3 and DENV4 respectively). Agarose gel electrophoresis was run in 1.5% agarose and the bands were visualized under UV transilluminator.

Table 3.3. Primers used for dengue virus confirmation and serotype specific PCR

	Primer	Sequence	Location(w.r.t.reference sequence)	Reference sequence (GenBank accession no.)	Expected size of the amplicon (bp)
Universal primer	AA6EP_F	TGGCTGGTGCACAGACAATGGTT	616-638	MF381049.1	600
	AA7EP_R	GCTGTGTCACCCAGAGTGGCCAT	2146-2168	KY849753.1	
DENV-1	AA8EP_F	GGGGCTTCAACATCCCAAGAG	667-687	MH680237.1	500
	AA9EP_R	GCTTAGTTTCAAAGCTTTTTCAC	1170-1148	MG933845.1	
DENV-2	AA10EP_F	ATCCAGATGTCATCAGGAAAC	808-828	MH110734.1	337
	AA11EP_R	CCGGCTCTACTCCTATGATG	1153-1134	MG895167.1	
DENV-3	AA12EP_F	CAATGTGCTTGAATACCTTTGT	893-914	MG895205.1	189
	AA13EP_R	GGACAGGCTCCTCCTTCTTG	1089-1071	MH173166.1	
DENV_4	AA14EP_F	GGACAACAGTGGTGAAAGTCA	953-973	MH178419.1	138
	AA15EP_R	GGTTACACTGTTGGTATTCTCA	1095-1074	MG895393.1	

Table 3.4. Master-mix preparation for PCR

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

Table 3.5. PCR program for envelope protein dengue

PCR Steps	**Temperature for all 4 serotypes**	**Time**
PCR 1		
Initial Denaturation	95^0C	5 min
Denaturation	95^0C	1 min
Annealing	59^0C	45 sec
Extension	72^0C	45 sec
Final Extension	72^0C	5 min
Hold	4^0C	

PCR steps	**Temperature for 4 serotypes**				**Time**
PCR 2	**DENV1**	**DENV2**	**DENV3**	**DENV4**	
Initial Denaturation	95^0C	95^0C	95^0C	95^0C	5 min
Denaturation	95^0C	95^0C	95^0C	95^0C	1 min
Annealing	**54.5^0C**	**53.5^0C**	**56^0C**	**53.5^0C**	**45 sec**
Extension	72^0C	72^0C	72^0C	72^0C	45 sec
Final extension	72^0C	72^0C	72^0C	72^0C	5 min
Hold	4^0C	4^0C	4^0C	4^0C	

Chapter IV

RESULTS

4.1. Study Population

A total of 32 suspected serum samples were taken from different hospitals who presented with symptoms of dengue. Among 32 samples 4 were from Sukraraj Tropical and Infectious Disease Hospital; 4 from Namuna Hospital-Hariwon, Sarlahi; 4 from Universal College of Medical Science (UCMS), Bhairahawa; and the remaining from Chitwan Medical College and Teaching Hospital (CMC); Chitwan.

Fig: Geographical location for the collection of samples

4.2. Gender and Age based Susceptibility

The study consisted of 62.5 % males (n=20) and 37.5% females (n=12) with the ratio of male: female as 1.67:1. The age group ranged from 14 years to 72 years. Higher number of suspected cases were observed in the age group 16- 30(Fig.4.1) followed by 31-45.

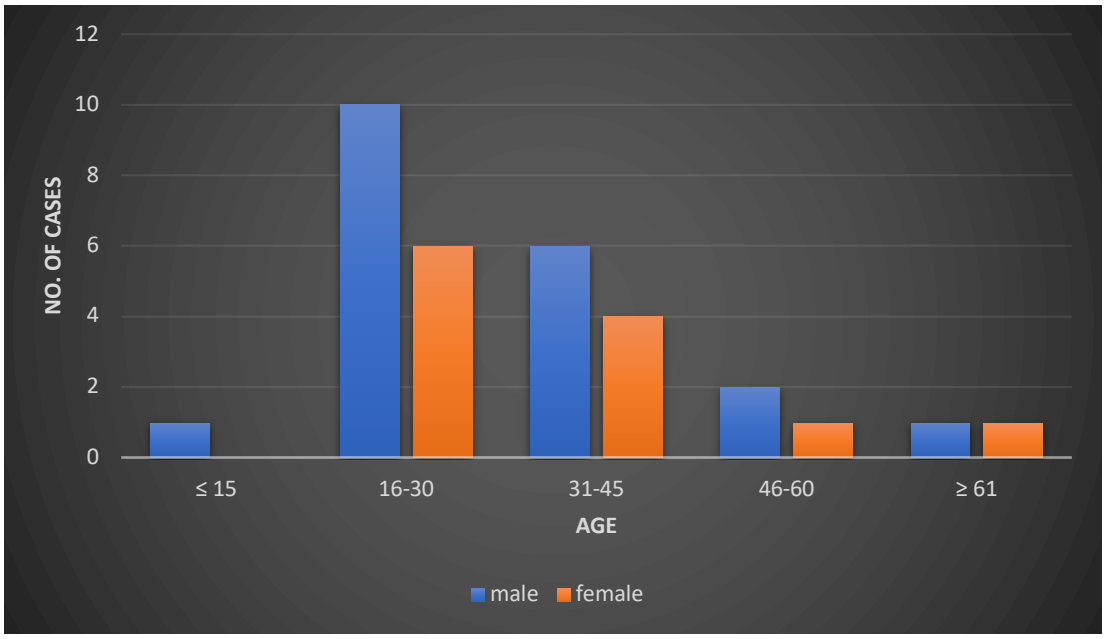


Fig 4.2. Population distribution among different age group for male and female of suspected case (n=32)

4.3. Seroprevalence of Dengue by ELISA

Three different types of ELISA (IgM and IgG detection and In-House,) were performed for all the suspected cases.

4.3.1. Anti- DENV IgM detection by IgM capture ELISA

The optical density of the samples along with the controls were recorded and the ISR was calculated as described in the methodology.

Validation of IgM ELISA

Negative control ISR= 1.512

Positive control ISR= 9.295

The positive control and negative control ISR were in the range provided by the kit. Hence our values were valid. Out of 32 suspected samples, 46.875% (n=15) were positive, 43.75% (n=14) negative and 9.375% (n=3) were equivocal.

Table 4.1. Presentation of Dengue IgM ELISA along with OD values of DENRA and NCA at 450nm, IgM ISR and sero-status i.e. positive or negative

Sample ID	DENRA	NCA	IgM ISR	Status	Sample ID	DENRA	NCA	IgM ISR	Status
CDBT-1	0.231	0.063	3.667	POSITIVE	**CDBT-17**	0.302	0.076	4	POSITIVE
CDBT -2	0.323	0.068	4.75	POSITIVE	**CDBT-18**	0.212	0.073	2.906	POSITIVE
CDBT-3	0.069	0.051	1.353	NEGATIVE	**CDBT-19**	0.084	0.056	1.507	NEGATIVE
CDBT-4	0.461	0.064	7.203	POSITIVE	**CDBT-20**	0.059	0.0403	1.465	NEGATIVE
CDBT-5	0.329	0.08	4.113	POSITIVE	**CDBT-21**	0.157	0.055	2.857	POSITIVE
CDBT-6	0.295	0.052	5.673	POSITIVE	**CDBT-22**	0.212	0.0403	1.794	EQUIVOCAL
CDBT-7	0.104	0.066	1.56	NEGATIVE	**CDBT-23**	0.127	0.043	2.946	POSITIVE
CDBT-8	0.074	0.047	1.581	NEGATIVE	**CDBT-24**	0.067	0.063	0.938	NEGATIVE
CDBT-9	0.279	0.091	3.066	POSITIVE	**CDBT-25**	0.279	0.07	3.986	POSITIVE
CDBT-10	0.407	0.054	7.537	POSITIVE	**CDBT-26**	0.061	0.044	1.386	NEGATIVE
CDBT-11	0.069	0.054	1.296	NEGATIVE	**CDBT-27**	0.053	0.043	1.231	NEGATIVE
CDBT-12	0.289	0.103	2.806	EQUIVOCAL	**CDBT-28**	0.079	0.051	1.55	NEGATIVE
CDBT-13	0.435	0.055	7.769	POSITIVE	**CDBT-29**	0.323	0.075	4.3	POSITIVE
CDBT-14	0.062	0.048	1.286	NEGATIVE	**CDBT-30**	0.856	0.075	11.41	POSITIVE
CDBT-15	0.278	0.143	1.467	NEGATIVE	**CDBT-31**	0.029	0.026	1.115	NEGATIVE
CDBT-16	0.278	0.143	1.947	EQUIVOCAL	**CDBT-32**	0.071	0.054	1.31	NEGATIVE

4.3.2. Anti- DENV IgG Detection by IgG Indirect ELISA

The optical density of the samples along with the controls were recorded and the ISR was calculated as described in the methodology.

Validation of IgG ELISA

Negative control ISR= 0.632

Positive control ISR= 6.459

The positive control and negative control ISR were in the range provided by the kit. Hence our values were valid. Out of 32 suspected samples, 6.25% (n=2) were positive, 81.25% (n=26) negative and 12.5% (n=4) were equivocal.

Table: 4.2. Presentation of Dengue IgG ELISA along with OD values of DENRA and NCA at 450nm, IgG ISR and sero-status i.e. positive or negative

Sample ID	DENRA	NCA	IgG ISR	Status	CDBT-18	0.061	0.053	1.15	NEGATIVE
CDBT-1	0.062	0.041	1.512	NEGATIVE	**CDBT-19**	0.056	0.062	0.91	NEGATIVE
CDBT-2	0.081	0.043	1.884	EQUIVOCAL	**CDBT-20**	0.07	0.053	1.32	NEGATIVE
CDBT-3	0.054	0.042	1.286	NEGATIVE	**CDBT-21**	0.069	0.039	1.769	EQUIVOCAL
CDBT-4	0.092	0.049	1.878	EQUIVOCAL	**CDBT-22**	0.059	0.0403	1.465	NEGATIVE
CDBT-5	0.1	0.043	2.326	EQUIVOCAL	**CDBT-23**	0.174	0.045	3.867	POSITIVE
CDBT-6	0.058	0.042	1.381	NEGATIVE	**CDBT-24**	0.033	0.036	0.917	NEGATIVE
CDBT-7	0.05	0.041	1.219	NEGATIVE	**CDBT-25**	0.076	0.055	1.381	NEGATIVE
CDBT-8	0.109	0.127	0.858	NEGATIVE	**CDBT-26**	0.061	0.057	1.07	NEGATIVE
CDBT-9	0.057	0.042	1.357	NEGATIVE	**CDBT-27**	0.041	0.05	0.82	NEGATIVE
CDBT-10	0.053	0.045	1.178	NEGATIVE	**CDBT-28**	0.258	0.062	4.16	POSITIVE
CDBT-11	0.053	0.044	1.205	NEGATIVE	**CDBT-29**	0.082	0.053	1.55	NEGATIVE
CDBT-12	0.042	0.05	0.84	NEGATIVE	**CDBT-30**	0.042	0.041	1.024	NEGATIVE
CDBT-13	0.061	0.064	0.953	NEGATIVE	**CDBT-31**	0.0539	0.055	0.98	NEGATIVE
CDBT-14	0.072	0.056	1.285	NEGATIVE	**CDBT-32**	0.0471	0.046	1.025	NEGATIVE
CDBT-15	0.071	0.045	1.578	NEGATIVE					
CDBT-16	0.055	0.047	1.17	NEGATIVE					
CDBT-17	0.054	0.051	1.058	NEGATIVE					

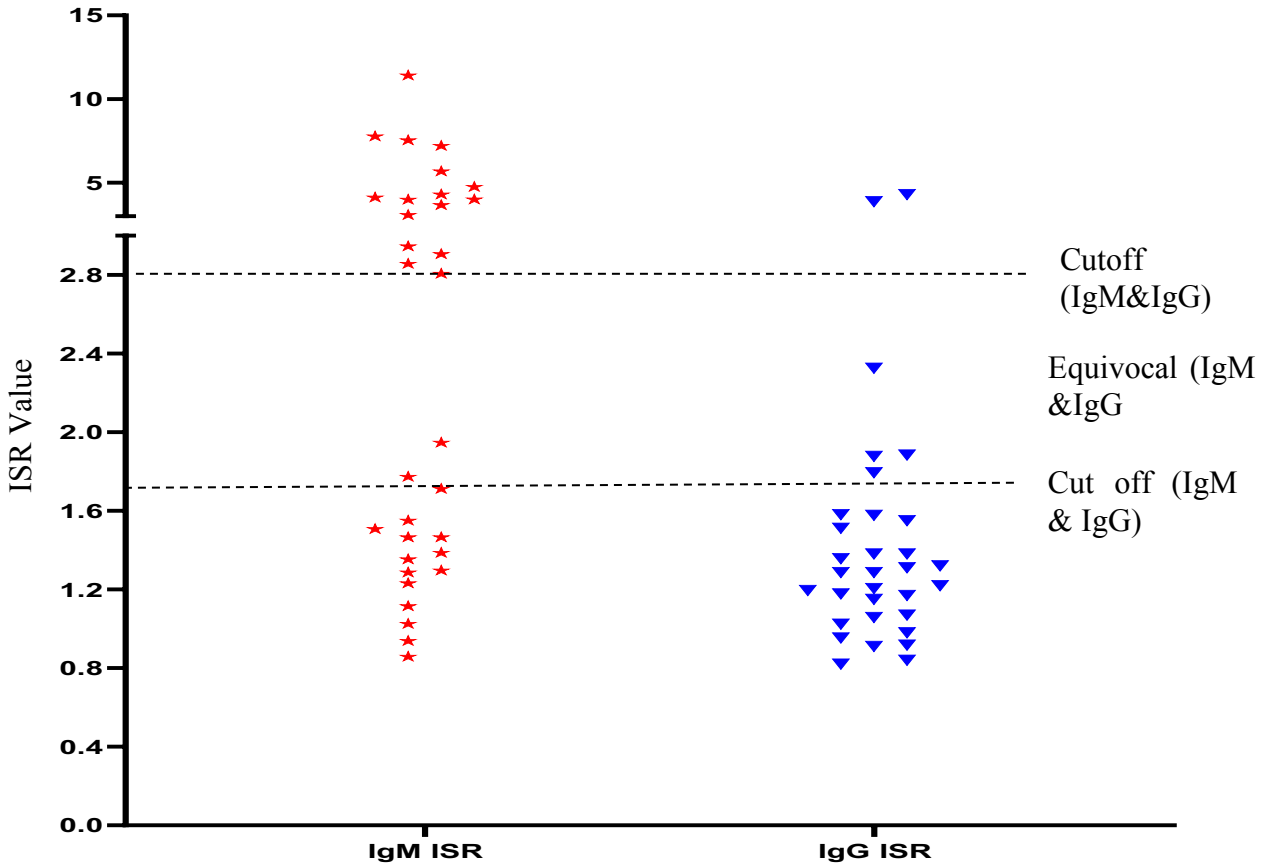


Fig 4.3. Scatter plot of Immune Status Ratio (ISR) of IgM and IgG antibodies of samples 32.

The dotted lines represent the cut off value. The samples that lie above the cut off values are positive and the samples that lie below the cut off value are negative to the test. In case of IgG and IgM the samples that lie between the cut off ranges are equivocal.

4.3.3 Primary and Secondary Dengue Detection by IgM/IgG ratio

The primary and secondary infections was determined, by calculating IgG/IgM ratio among 32 sample only 2 samples were of secondary infection while 30 samples were of primary infection.

Table: 4.3. Presentation of Dengue IgM/IgG ratio and infection-status i.e. positive or negative

Sample ID	IgM ISR	IgG ISR	IgM/IgG Ratio	Status
CDBT-1	3.667	1.512	2.42	Primary Infxn
CDBT-2	4.75	1.884	2.527	Primary Infxn
CDBT-3	1.353	1.286	1.052	Seronegative
CDBT-4	7.203	1.878	3.845	Primary Infxn
CDBT-5	4.113	2.326	1.768	Primary Infxn
CDBT-6	5.673	1.381	4.107	Primary Infxn
CDBT-7	1.56	1.219	1.279	Seronegative
CDBT-8	1.581	0.858	1.165	Seronegative
CDBT-9	3.066	1.357	2.259	Primary Infxn
CDBT-10	7.537	1.178	6.389	Primary Infxn
CDBT-11	1.296	1.205	1.075	Seronegative
CDBT-12	2.806	0.84	3.34	Primary Infxn
CDBT-13	7.769	0.953	8.152	Primary Infxn
CDBT-14	1.286	1.285	1.0007	Seronegative
CDBT-15	1.712	1.578	1.085	Seronegative
CDBT-16	1.947	1.17	1.664	Seronegative
CDBT-17	4	1.058	3.781	Primary Infxn
CDBT-18	2.906	1.15	2.526	Primary Infxn
CDBT-19	1.507	0.91	1.656	Seronegative
CDBT-20	1.465	1.32	1.109	Seronegative
CDBT-21	2.857	1.769	1.615	Primary Infxn
CDBT-22	1.794	1.465	1.224	Seronegative
CDBT-23	2.946	3.867	0.702	Secondary Infxn
CDBT-24	0.938	0.917	1.022	Seronegative
CDBT-25	3.986	1.381	2.886	Primary Infxn
CDBT-26	1.386	1.07	1.295	Seronegative
CDBT-27	1.231	0.82	1.501	Seronegative
CDBT-28	1.55	4.16	0.372	Secondary Infxn

CDBT-29	4.3	1.55	2.774	primary Infxn
CDBT-30	11.41	1.024	11.143	primary Infxn
CDBT-31	1.115	0.98	1.137	Seronegative
CDBT-32	1.31	1.025	1.278	Seronegative

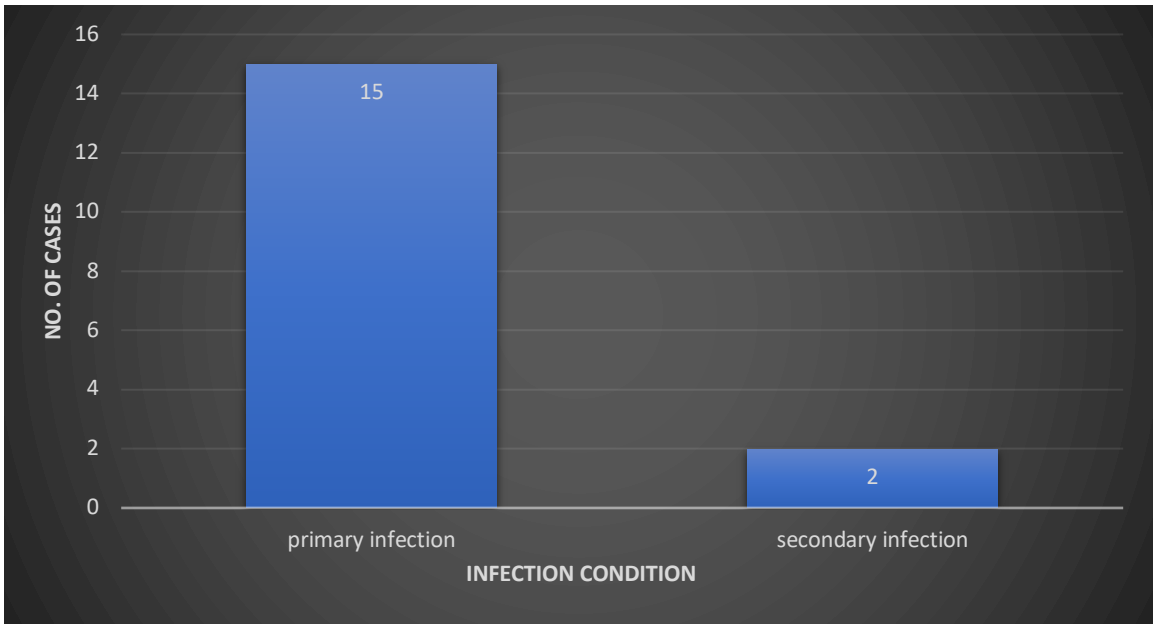
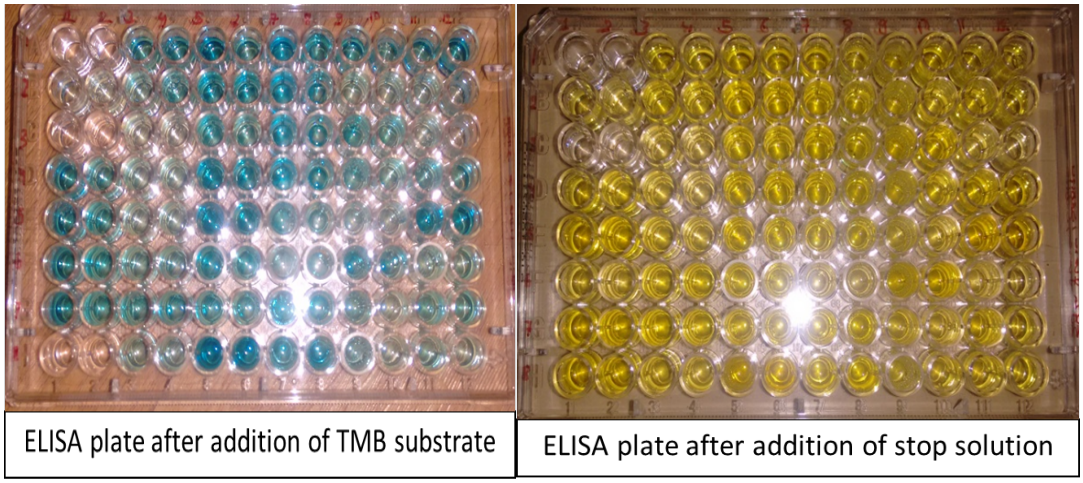


Fig 4.4 Showing no. of cases of primary and secondary infection

The above graph shows the number of cases on Y-axis while primary and secondary infection on the X-axis.

4.3.4 Dengue Detection by In-house ELISA

Dengue suspected serum samples were taken and In-House ELISA for the four antigens of dengue virus was performed. The OD values for all the controls and the samples is shown in the appendix.



ELISA plate after addition of TMB substrate | ELISA plate after addition of stop solution

Fig 4.5 In-House ELISA plate after addition of TMB substrate and after addition of stop solution

The OD readings at 450nm of all the variables are given in Table 4.4. The mean OD of samples is considerably higher than the cut-off value for DEV-1, DENV-2 and DENV-4. In case of DENV-3, the mean OD is relatively lower than cut-off value. Similarly, for endemic control (EC) the mean OD was found near to cut-off value and for Non-endemic control (NEC), the mean OD was found to be considerably lower than cut-off value.

Table 4.4 OD of ELISA at 450nm using different antigens of dengue virus

Antigens	**NEC (n=7)**	**Cut-off value OD**	**EC (n=4)**	**Samples(n=32)**
DENV 1	0.7263± 0.3425	1.4114	1.2565±0.2439	1.7308±0.6795
DENV 2	0.4365±0.1313	0.6991	0.8893± 0.4348	0.9758± 0.5559
DENV 3	0.1479± 0.0332	0.2144	0.115 ± 0.0295	0.13± 0.0414
DENV 4	0.1475±0.0269	0.2013	0.1395± 0.0200	0.3102± 0.2250

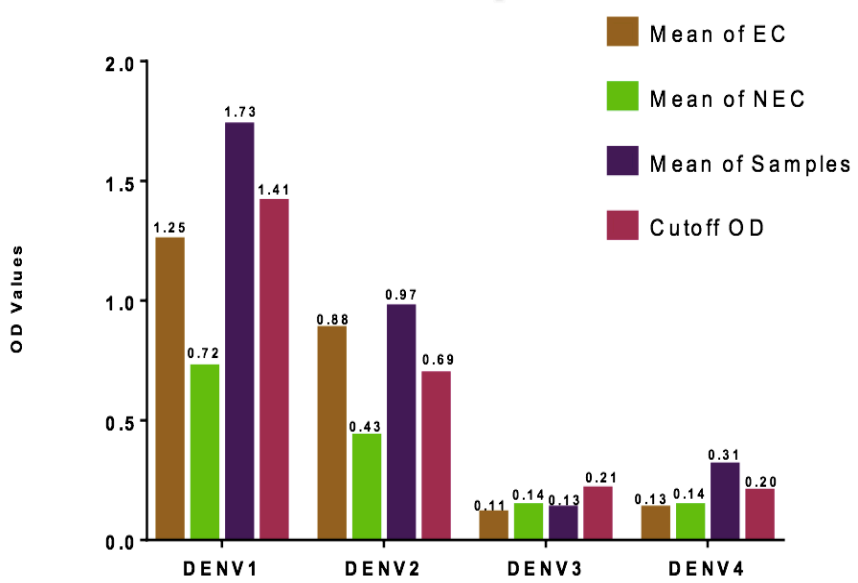


Fig: 4.6 Comparative study of Mean of EC, NEC and samples with cutoff OD for different antigen of dengue

The mean of negative samples was found to be lower than the OD value of positive control. The comparative analysis is shown in Table 4.5. The mean OD for DENV-1 was to be 1.35 times lesser than that of positive control. For DENV-2 it was found to be 1.75 times, for DENV-3 it was 1.69 times and for DENV-4 it was 1.18 times lower than positive control.

Table 4.5 Comparison of Mean of negative samples with positive control

Antigen	Mean of Negative samples	Positive control
DENV-1	1.4506	1.964
DENV-2	0.8976	1.574
DENV-3	0.1246	0.21147
DENV-4	0.2548	0.30175

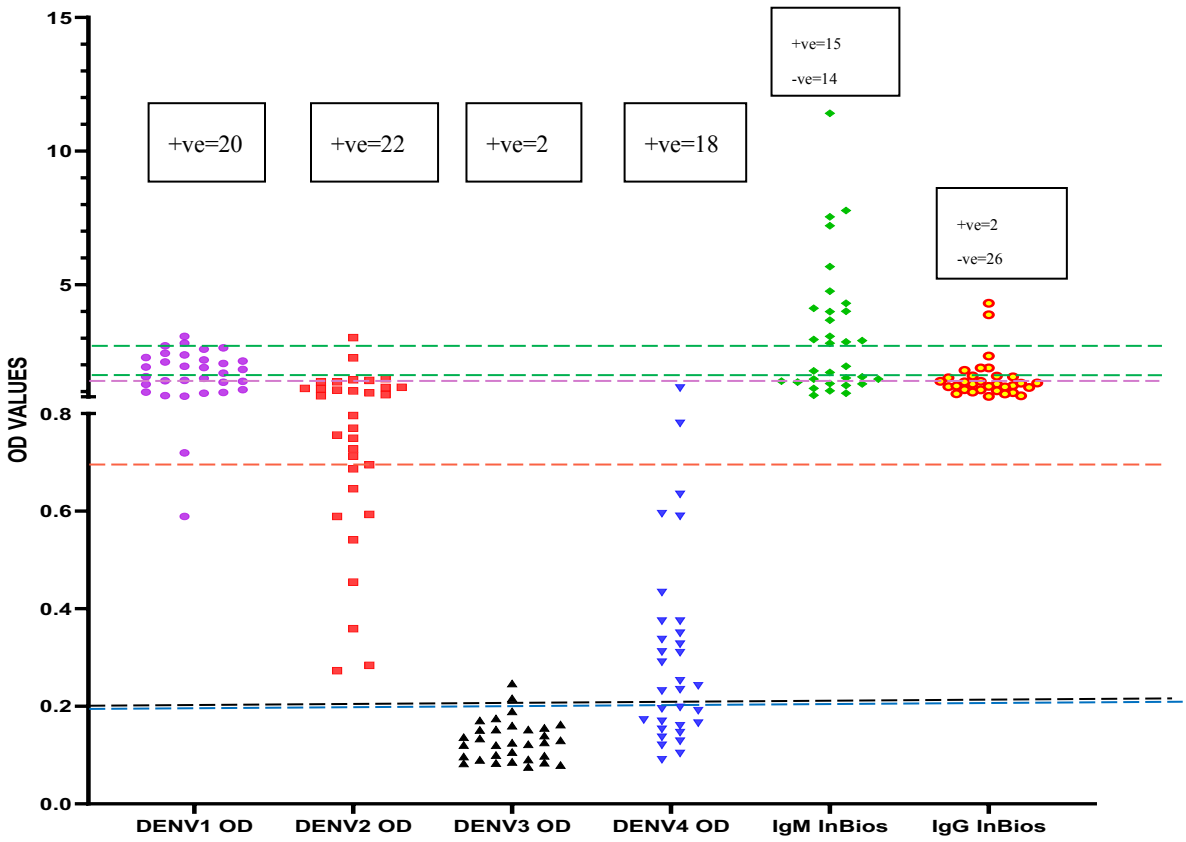
The comparative analysis of Cut-off value means of non-endemic control (NEC), endemic control (EC) and positive samples is shown in Table.4.6. The mean of non-endemic control and endemic control was found to be significantly lower than cut-off value but for endemic control, the antibody titer for DENV-2 was found to be higher than cut-off value. The mean value for positive samples was found to be significantly

higher than cut-off value. Also, there is significant difference between the antibody titer of non-epidemic control with epidemic control. But in case of DENV-3 and 4, there was no discrimination against antibody titer for endemic control and non-endemic control.

Table 4.6 Comparative analysis of antibody titer for positive, endemic and non-endemic samples

Antigen	Cut-off value	Mean of NEC	Mean of EC	Mean of positive samples
DENV-1	1.4114	0.7263	1.2565	2.154
DENV-2	0.6991	0.4365	0.8893	1.187
DENV-3	0.2144	0.1479	0.115	0.1233
DENV-4	0.2013	0.1475	0.1395	0.1521

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



	Cut-off OD for DNEV-1 antibody titer
	Cut-off OD for DNEV-2 antibody titer
	Cut-off OD for DNEV-3 antibody titer
	Cut-off OD for DNEV-4 antibody titer
	Cut off OD for InBios IgM & IgG

Fig: 4.7 Scatter diagram showing the OD values of samples for different antigens of dengue

The above scatter diagram shows the OD values which represents the antibody titer for the antigens of dengue virus. Each dot represents the mean of each sample. The samples that lie above the cut-off value are positive samples. Out of 32 samples, the total no. of positive samples was 20,22,2 and 18 for DENV-1, DENV-2, DENV-3 and DENV-4 respectively and 15 positives for IgM and 2 positives for IgG. The antibody titer for DENV-2 AND DENV-1 were found to be higher and for DENV-3 it was lowest.

4.3.5. Statistical Analysis

The statistical analysis was also performed by unpaired T-test. The p-value was found to be <0.0001 and there was significant difference between the mean of the samples.

4.4. Serotypes Detection by Nested PCR

Serotype specific nested PCR was performed. Out of 32 samples only three samples were found PCR positive among those three PCR positive samples all showed the bands at 500bp when compared with a 100bp ladder, when gel was visualized using UV transilluminator and confirmed DENV1.

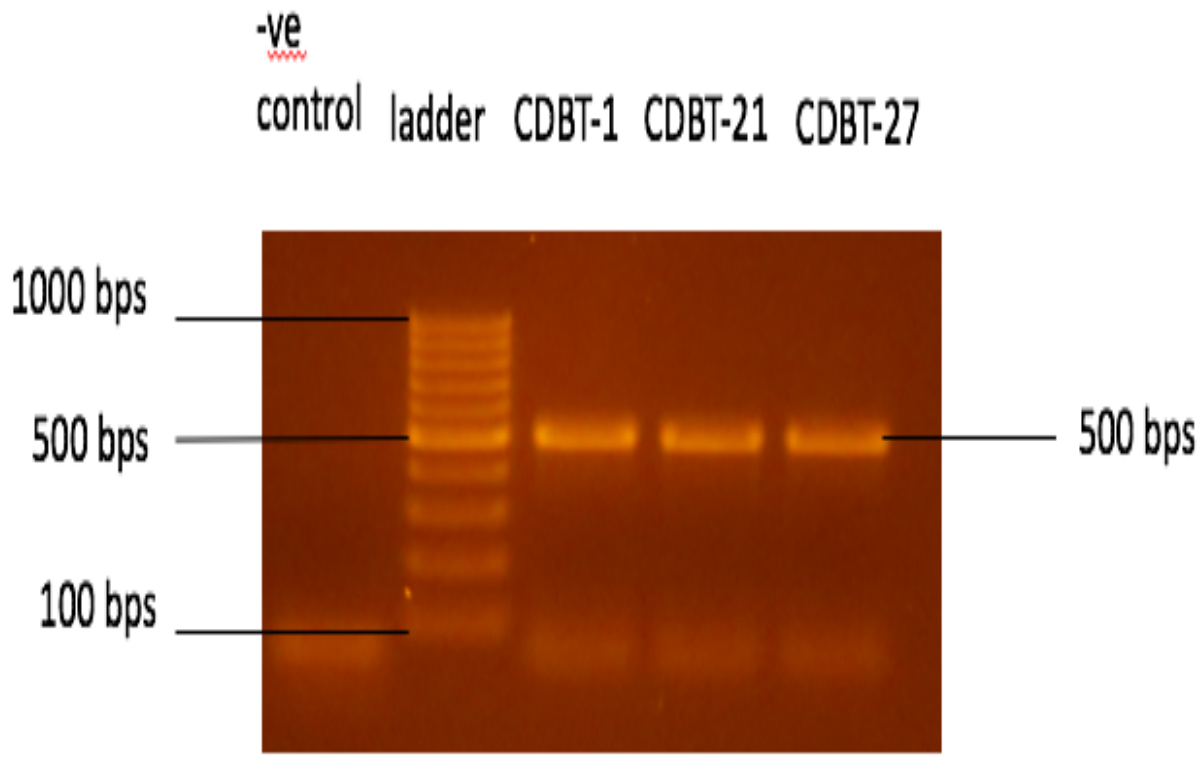


Fig 4.8. Gel image under UV Transilluminator of the second round nested PCR of envelope gene which gave 500 bp. The negative control and 100bp ladder [Thermo Scientific Generuler 100 bp DNA ladder, ready to use (Cat. No. #SM0243)] was run along with the PCR product.

Table: 4.7 Distribution of cases according to ELISA and PCR results

Tests (n =32)	PCR positive	PCR negative	Total
IgG Equivocal	1	3	4
IgM Equivocal	0	3	3
IgG+/IgM+	0	0	0
IgG-/IgM-	1	13	14
IgG+/IgM-	0	1	1
IgM+/IgG-	1	9	10
In-house DENV-1+	1	19	20
In-house DENV-2+	1	21	22
In-house DENV-3+	0	2	2
In- house DENV-4+	0	18	18

From the study, among the 32 samples only 3 samples were found to be PCR positive. For the antibody produced by in-house ELISA against serotype 1 and 2, PCR positive test was given for both by 3.125% while DENV-3 and DENV-4 were found to be PCR negative. None of the samples were found to be positive for both IgM and IgG. IgG positive samples were found to be two. In two, IgG positive and IgM negative were found to be a single. On the other side, out of 15 IgM positive samples, IgM positive and IgG negative was found to be one, which was PCR positive too. Four IgG equivocal samples were obtained in which one IgG equivocal sample was found to be PCR positive while for IgM, three equivocal samples were obtained and all turned to be PCR negative.

Chapter V
DISCUSSION

Dengue disease is becoming a global epidemic, with nearly 40%of the world's population at risk for transmission of one or more of the four dengue virus serotypes (Gubler,1998). It is one of the major public health problems. In the context of Nepal, the dengue epidemic is rising each year. Dengue was reported in 2004 for the first time in Japanese traveler followed by annual reports of indigenous circulation from the year 2006 (Malla *et al.,* 2008). Dengue has infected 32 out of 75 districts of the country spreading rapidly all over the country from the East to the West encompassing even the temperate hilly regions (Khetan *et al.,* 2018). Hence there is a high need to develop rapid and effective tool for the diagnosis of dengue.

Currently, the emerging dengue infection cases are increasing day by day and its transmission in infected area is in increasing trend. Reason for dengue expansion in Nepal may include climate change, virus evolution, rapid urbanization, population growth and poor availability of medical and diagnostic facilities; inadequate mosquito control. DENV infection in Nepal is more common in the Terai region compared to the Hilly and Himalayan region presumably due to its ideal vector environment and the porous border between India and Nepal. Similarly, the number of cases reported in males was significantly higher than in females in our study which is in accordance with our previous report (Gupta *et al.,*2018). This might be because males have a higher vector exposure due to their predominance in outdoor work, especially in fields and forests.

Altogether, 32 samples, 15 samples (46.875%) showed the positive result for IgM capture ELISA while only 2 samples (6.25%) showed the positive to IgG, more people positive with IgM indicates that most of the persons were infected with dengue virus within recent weeks. The primary and secondary infections is determined by the ratio of the units of dengue virus IgM antibodies to the units of dengue virus IgG antibodies, if the ratio is greater than 1.2 were said primary infection less than that were said to have secondary infection (Falconar *et al.,* 2006). Secondary infection is defined as an IgM-negative/IgG-positive or an IgM-positive/IgG-positive result. Negative tests for IgM and/or IgG antibodies may mean that the individual tested does not have a dengue infection and symptoms are due to another cause, or that the level of antibody may be too low to measure. The person may still have a dengue infection, or it may be too soon after initial exposure to the virus to produce a detectable level of antibody (Soo *et al.,* 2016). In our study the prevalence of primary infection was found more than secondary infection in contradiction to previous studies having dominance of secondary

dengue infection in all years except the year 2004 (Gupta *et al*., 2018). Higher the primary infection is a warning ring indicating an increment of new cases, rapid spreading of dengue virus in Nepal and low- immunity profile of Nepalese population against dengue infection.

The study also aims to know whether Nepalese people are capable to fight against dengue virus or not. Significant amount of antibody production has been seen against DENV-1, DENV-2 and DENV-4 serotypes (fig:4.5). But in case of DENV-3, there is lesser antibody production. This suggests that if there is circulation of DENV-3 serotype, antibody production may arise.

There was discrimination against the antibody titer for endemic and non-endemic control in case of DENV-1 and 2. This suggests that the study is able to distinguish between the healthy controls too. But in case of DENV-3 and 4, no such difference was found. This might be because both the population has not been exposed to these serotypes.

On comparing with the commercially available kit (In-Bios), In case of Sample 8, the antibody detection was positive in all the serotypes of dengue, but this sample was shown negative by the kit. This might indicate that the commercially available kit may not be good enough to detect the antibodies circulating in the Nepalese population.

Dengue in Nepal has been emerging as a major infectious disease indicated by repeated outbreaks in the years 2010, 2013 and 2016 in a three-year cyclic pattern (Gupta et al., 2018). DENV1 was responsible for the 2010 and 2016 while it was DENV2 for the year 2013. In our study all the suspected dengue samples were tested for PCR confirmation, a nested RT-PCR was done using universal dengue primers targeting the envelop region of the genome for an initial reverse transcription and amplification step, followed by a nested PCR amplification that is serotype-specific. Among all the 32 samples only three samples were found to be PCR positive and all three PCR positive samples showed the bands at 500bp when compared with a 100bp ladder, when gel was visualized using UV transilluminator and confirmed DENV-1 serotype while the negative samples did not show any bands. A positive PCR result is a definite proof of current infection and it usually confirms the infecting serotype as well. However, a negative result may indicate that no infection is present or that the level of virus is too low to detect. As a previous study already demonstrated DENV-1 circulation in year 2016 (Gupta *et al*., 2018), which is in accordance with our study.

All the result obtained from the test were compared and antibody titer were calculated by In-house ELISA and InBios kit, as well as primary and secondary infection were categorized. Some serologically positive cases have a negative PCR result while some positive PCR have a

negative ELISA result. This can be explained by the fact that PCR diagnosis is useful for early diagnosis for approximately the first 5 days of symptoms while the persistence of antibody after the viremia. This indicates the pitfalls of PCR and serology testing if used singly. Indeed, patients in endemic areas usually give no clear-cut history of history of illness, which makes it hard to estimate the exact timing of the disease. Thus, this study gave information about seroprevalence and serotyping of dengue that will serve as vital data to understand the serological and serotyping importance. A combination of PCR and serological test can be particularly helpful for diagnosis in dengue suspected cases.

The present study also aimed to develop the ELISA without using commercial kits. There is no any confirmation that the available commercial kits will work in the context of Nepalese population or not. Hence it is an initiative to develop a kit for the diagnosis of dengue virus. This study aims to determine the antibody titer produced against the serotypes of dengue virus. In this antigen, the antigens have been provided by Microbix, Canada. Further work can be done by culturing the virus from the serum samples and producing our own antigen. A kit can be developed for the detection of dengue virus which will overcome the need of purchasing the kits from abroad and act according to scenario of Nepal.

Chapter VI

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). It appeared as a new disease in Nepal in 2004 from Japanese traveler with sporadic cases every year and massive outbreaks in 2010, 2013 and 2016. My study was based on 32 suspected dengue samples for which we tried to develop inhouse elisa for determining antibody tire and compared with the commercially available kit (In-Bios) and tried to find out either primary or secondary infection and also the serotype detection by PCR.

Among total of 32 samples, 4 were from Sukraraj Tropical and Infectious Disease Hospital, next 4 were from Namuna Hospital, Sarlahi similarly next 4 were from Universal College of Medical Science (UCMS); Bhairahawa, and the remaining were from Chitwan Medical College and Teaching Hospitals (CMC); Chitwan.

Out of 32 samples 20 (62.5 %) were males and 12 (37.5%) were females with the ratio of male: female as 1.67:1. The age group ranged from 14 years to 72 years. Higher number of suspected cases were observed in the age group 16- 30.

IgG and IgM ELISA were performed for all samples and from ELISA results, recent acute infection or past infection were identified and type of infection whether primary infection or secondary was classified by calculating IgM to IgG ratios. Only a few samples were found of secondary infection while most of them are found to have a primary infection.

Different environmental conditions like temperature, pH, incubation time were monitored, and In-house ELISA was performed, and significant amount of antibody production has been seen against DENV-1, DENV-2 and DENV-4 serotypes. But in case of DENV-3, there is lesser antibody production. This suggests that if there is circulation of DENV-3 serotype, antibody production may arise.

There was discrimination against the antibody titer for endemic and non-endemic control in case of DENV-1 and 2. This suggests that the study is able to distinguish between the healthy controls too. But in case of DENV-3 and 4, no such difference was found. This might be because both the population has not been exposed to these serotypes.

On comparing with the commercially available kit (In-Bios), the sensitivity of antibody detection was seen higher in the undertaken study. In case of Sample 8, the antibody

detection was positive in all the serotypes of dengue, but this sample was shown negative by the kit. This might indicate that the commercially available kit may not be good enough to detect the antibodies circulating in the Nepalese population.

All the 32 suspected samples that were taken for study were tested for molecular confirmation, from all samples only 3 were found PCR positive with Dengue specific primer. Among three dengue positive samples from 2016, all of the samples were found DENV1 positive, which was confirmed by using serotypes specific primer. This study revealed that DENV1 was circulating in Nepalese population in 2016.

CHAPTER VII

CONCLUSION

The developed In-House ELISA was able to determine the antibody produced against the serotypes of dengue virus (DENV 1-4) in Nepalese population. The DENV-1 antibody circulation was found to be higher supporting the circulation of DENV-1 serotype determined by PCR in the year 2016. Few samples were found to be secondary phase infection while most of them were primary phase infection. Primary phase infections are more threatening due to the production of IgM antibodies which fight with dengue virus for short period of time. On the contrary, secondary type infection are characterized by the formation of IgG antibodies which have long term defending immune response against dengue virus. This shows the picture of ongoing dengue infection could increase rapidly in Nepal. Hence, need to be aware of disease and its prevention.

Till now ELISA kits for NS1 antigen and antibody detection (IgG and IgM) kits are available, however, no any serotype specific antibody detection ELISA has been developed. Hence this study might be useful in serotype identification by In-house ELISA, which is important in epidemiological and pathological analysis as well as serotyping by Nested PCR is useful for monitoring dengue virus activity in endemic areas like Nepal, allowing a rapid identification of dengue viruses.

Limitations of the study

1. Sequencing of the isolated dengue virus could not perform because of limited time and budget.

2. The study was carried out in limited number of samples.

Recommendations:

1. Further work can be done by culturing the virus from the serum samples and producing our own antigen.

2. A kit can be developed for the detection of dengue virus which will overcome the need of purchasing the kits from abroad and act according to scenario of Nepal.

3. Along with conventional nested RT-PCR, Real-Time PCR could be performed for serotyping of dengue virus.

4. The significance of this experiment would be improved if the samples were carried out in large number for serotyping of dengue virus.

5. Vector elimination programs should be launched in the endemic areas.

6. Effective epidemiological surveillance for dengue fever must include the ability of routine laboratory confirmation and monitoring of circulating serotypes.

7. Awareness programs regarding dengue should be launched before endemic situation arises.

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Appendices

Appendix:1 Table A: Clinical parameters of dengue samples

Sample ID	Age	Sex	Platelets	Fever	Headache	Joint Pain
CDBT-1	43	M	70000	Yes	Yes	Yes
CDBT-2	18	F	97000	Yes	Yes	No
CDBT-3	29	M	144000	Yes	Yes	Yes
CDBT-4	20	M	96000	Yes	Yes	Yes
CDBT-5	72	M	54000	Yes	Yes	Yes
CDBT-6	36	F	70000	Yes	Yes	Yes
CDBT-7	65	F	90000	Yes	Yes	No
CDBT-8	20	M	70000	Yes	Yes	Yes
CDBT-9	28	M	190000	Yes	Yes	Yes
CDBT-10	21	F	210000	Yes	Yes	Yes
CDBT-11	45	M	340000	Yes	Yes	Yes
CDBT-12	25	F	190000	Yes	Yes	Yes
CDBT-13	25	F	87000	Yes	Yes	Yes
CDBT-14	14	M	88000	Yes	Yes	Yes
CDBT-15	33	F	60000	Yes	Yes	Yes
CDBT-16	18	F	45000	Yes	Yes	Yes
CDBT-17	17	M	56000	Yes	Yes	Yes
CDBT-18	18	M	38000	Yes	Yes	Yes
CDBT-19	21	M	319000	Yes	Yes	Yes
CDBT-20	47	M	75000	Yes	Yes	Yes
CDBT-21	45	F	66000	Yes	Yes	Yes
CDBT-22	34	F	96000	Yes	Yes	Yes
CDBT-23	36	M	79000	Yes	Yes	Yes
CDBT-24	16	M	56000	Yes	Yes	Yes
CDBT-25	24	M	78000	Yes	Yes	Yes
CDBT-26	45	M	57000	Yes	Yes	Yes
CDBT-27	21	F	45000	Yes	Yes	Yes
CDBT-28	41	M	161000	Yes	Yes	No
CDBT-29	34	M	134000	Yes	Yes	Yes
CDBT-30	22	M	98000	Yes	Yes	Yes
CDBT-31	50	M	220000	Yes	Yes	No
CDBT-32	48	F	124000	Yes	Yes	No

Appendix: 2 Table B: Different OD value of controls and samples

SAMPLE ID	SAMPLE ID	DENV1 OD	STATUS	DENV2 OD	STATUS	DENV3 OD	STATUS	DENV4 OD	STATUS	IgM InBios	Status	IgG InBios	
	Blank	0.0255		0.0255		0.0225		0.02845					
	Primary Ab absent	0.4495		0.3695		0.098		0.194					
	Secondary Ab absent	0.043		0.0435		0.01485		0.03375					
	Negative control	0.598		0.5665		0.10675		0.14175					
	Positive control	1.964		1.574		0.30175		0.21147					
	Endemic control 1	1.151		0.3445		0.082		0.12025					
	Endemic control 2	1.2225		1.212		0.15375		0.1475					
	Endemic control 3	1.0465		1.2665		0.11025		0.1263					
	Endemic control 4	1.606		0.7345		0.114		0.164					
	Non-endemic control 1	0.949		0.581		0.1365		0.139					
	Non-endemic control 2	0.5165		0.5365		0.111		0.189					
	Non-endemic control 3	0.837		0.2535		0.1955		0.181					
	Non-endemic control 4	0.792		0.317		0.181		0.125					
	Non-endemic control 5	1.1065		0.39		0.1645		0.1255					
	Non-endemic control 6	0.06265		0.5815		0.111		0.1265					
	Non-endemic control 7	0.8205		0.396		0.136		0.1465					
CDBT-1	Sample 1	2.712	POSITIVE	0.8795	POSITIVE	0.1625	NEGATIV	0.195	NEGATIVE	3.667	POSITIVE	1.512	NEGATIVE
CDBT-2	Sample 2	2.1395	POSITIVE	1.3605	POSITIVE	0.16	NEGATIV	0.78	POSITIVE	4.75	POSITIVE	1.884	EQUIVOCAL
CDBT-3	Sample 3	1.489	POSITIVE	0.646	NEGATIVE	0.12	NEGATIV	0.31	POSITIVE	1.353	NEGATIVE	1.286	NEGATIVE
CDBT-4	Sample 4	2.434	POSITIVE	1.113	POSITIVE	0.1515	NEGATIV	0.6345	POSITIVE	7.203	POSITIVE	1.878	EQUIVOCAL
CDBT-5	Sample 5	2.5865	POSITIVE	1.1505	POSITIVE	0.134	NEGATIV	0.3745	POSITIVE	4.113	POSITIVE	2.326	EQUIVOCAL
CDBT-6	Sample 6	2.2685	POSITIVE	0.796	POSITIVE	0.1365	NEGATIV	0.3745	POSITIVE	5.673	POSITIVE	1.381	NEGATIVE
CDBT-7	Sample 7	1.553	POSITIVE	0.713	POSITIVE	0.097	NEGATIV	0.153	NEGATIVE	1.56	NEGATIVE	1.219	NEGATIVE
CDBT-8	Sample 8	3.062	POSITIVE	3.018	POSITIVE	0.216	POSITIVE	1.1315	POSITIVE	1.581	NEGATIVE	0.858	NEGATIVE
CDBT-9	Sample 9	2.1805	POSITIVE	0.756	POSITIVE	0.175	NEGATIV	0.2315	POSITIVE	3.066	POSITIVE	1.357	NEGATIVE
CDBT-10	Sample 10	1.8985	POSITIVE	0.8355	POSITIVE	0.106	NEGATIV	0.242	POSITIVE	7.537	POSITIVE	1.178	NEGATIVE
CDBT-11	Sample 11	1.9445	POSITIVE	0.9525	POSITIVE	0.126	NEGATIV	0.234	POSITIVE	1.296	NEGATIVE	1.205	NEGATIVE
CDBT-12	Sample 12	2.1065	POSITIVE	1.056	POSITIVE	0.0835	NEGATIV	0.172	NEGATIVE	2.806	EQUIVOCAL	0.84	NEGATIVE
CDBT-13	Sample 13	1.345	NEGATIVE	0.7275	POSITIVE	0.083	NEGATIV	0.1285	NEGATIVE	7.769	POSITIVE	0.953	NEGATIVE
CDBT-14	Sample 14	0.9635	NEGATIVE	0.77	POSITIVE	0.0795	NEGATIV	0.0905	NEGATIVE	1.286	NEGATIVE	1.285	NEGATIVE
CDBT-15	Sample 15	2.047	POSITIVE	1.418	POSITIVE	0.1555	NEGATIV	0.5895	POSITIVE	1.712	EQUIVOCAL	1.578	NEGATIVE
CDBT-16	Sample 16	2.3715	POSITIVE	1.103	POSITIVE	0.122	NEGATIV	0.2905	POSITIVE	1.947	EQUIVOCAL	1.17	NEGATIVE
CDBT-17	Sample 17	1.8265	POSITIVE	1.0205	POSITIVE	0.1895	NEGATIV	0.337	POSITIVE	4	POSITIVE	1.058	NEGATIVE
CDBT-18	Sample 18	0.844	NEGATIVE	0.5935	NEGATIVE	0.09	NEGATIV	0.146	NEGATIVE	2.906	POSITIVE	1.15	NEGATIVE
CDBT-19	Sample 19	1.684	POSITIVE	1.4345	POSITIVE	0.171	NEGATIV	0.4335	POSITIVE	1.507	NEGATIVE	0.91	NEGATIVE
CDBT-20	Sample 20	1.2685	NEGATIVE	0.7495	POSITIVE	0.13	NEGATIV	0.3115	POSITIVE	1.465	NEGATIVE	1.32	NEGATIVE
CDBT-21	Sample 21	0.7195	NEGATIVE	0.359	NEGATIVE	0.1205	NEGATIV	0.1655	NEGATIVE	2.857	POSITIVE	1.769	NEGATIVE
CDBT-22	Sample 22	1.9165	POSITIVE	1.476	POSITIVE	0.0845	NEGATIV	0.35	POSITIVE	1.794	NEGATIVE	1.465	EQUIVOCAL
CDBT-23	Sample 23	1.4125	POSITIVE	1.3665	POSITIVE	0.152	NEGATIV	0.16	NEGATIVE	2.946	POSITIVE	3.867	POSITIVE
CDBT-24	Sample 24	1.0645	NEGATIVE	0.4545	NEGATIVE	0.0985	NEGATIV	0.1695	NEGATIVE	0.938	NEGATIVE	0.917	NEGATIVE
CDBT-25	Sample 25	2.6355	POSITIVE	0.695	NEGATIVE	0.2465	POSITIVE	0.2525	POSITIVE	3.986	POSITIVE	1.381	NEGATIVE
CDBT-26	Sample 26	0.9325	NEGATIVE	0.6865	NEGATIVE	0.1	NEGATIV	0.1365	NEGATIVE	1.386	NEGATIVE	1.07	NEGATIVE
CDBT-27	Sample 27	0.589	NEGATIVE	0.284	NEGATIVE	0.086	NEGATIV	0.1035	NEGATIVE	1.231	NEGATIVE	0.82	NEGATIVE
CDBT-28	Sample 28	1.4035	NEGATIVE	1.157	POSITIVE	0.14	NEGATIV	0.3275	POSITIVE	1.55	NEGATIVE	4.16	POSITIVE
CDBT-29	Sample 29	2.805	POSITIVE	2.254	POSITIVE	0.152	NEGATIV	0.595	POSITIVE	4.3	POSITIVE	1.55	NEGATIVE
CDBT-30	Sample 30	0.9755	NEGATIVE	0.273	NEGATIVE	0.125	NEGATIV	0.191	NEGATIVE	11.41	POSITIVE	1.024	NEGATIVE
CDBT-31	Sample 31	1.3815	NEGATIVE	0.589	NEGATIVE	0.091	NEGATIV	0.198	NEGATIVE	1.115	NEGATIVE	0.98	NEGATIVE
CDBT-32	Sample 32	0.827	NEGATIVE	0.541	NEGATIVE	0.0755	NEGATIV	0.12	NEGATIVE	1.31	NEGATIVE	1.025	NEGATIVE
	SD for non-endemic control	0.342549099		0.131324661		0.033245838		0.026922729					
	SD for endemic control	0.224469931		0.17763516		0.043342627		0.026857649					
	Mean of NEC	0.726307143		0.4365		0.147928571		0.1475					
	Mean of EC	1.2565		0.889375		0.115		0.1395125					
	Cut-off value of NEC	1.411405341		0.699149323		0.214420247		0.201345458					
	Cut-off value of EC	1.705439862		1.24464532		0.201685254		0.193227799					

Appendix:3 Table:3 Details of virus nucleic acid detection and antibody detection status

Sample ID	PCR Status	IgM ISR Status	IgG ISR Status	In-house DENV-1 Status	In-house DENV-2 Status	In-house DENV-3 Status	In-house DENV-4 Status
CDBT-1	+ve	+ve	-	+ve	+ve	-	-
CDBT-2	-	+ve	equivocal	+ve	+ve	-	+ve
CDBT-3	-	-	-	+ve	-	-	+ve
CDBT-4	-	+ve	equivocal	+ve	+ve	-	+ve
CDBT-5	-	+ve	equivocal	+ve	+ve	-	+ve
CDBT-6	-	+ve	-	+ve	+ve	-	+ve
CDBT-7	-	-	-	+ve	+ve	-	-
CDBT-8	-	-	-	+ve	+ve	+ve	+ve
CDBT-9	-	+ve	-	+ve	+ve	-	+ve
CDBT-10	-	+ve	-	+ve	+ve	-	+ve
CDBT-11	-	-	-	+ve	+ve	-	+ve
CDBT-12	-	Equivocal	-	+ve	+ve	-	-
CDBT-13	-	+ve	-	-	+ve	-	-
CDBT-14	-	-	-	-	+ve	-	-
CDBT-15	-	-	-	+ve	+ve	-	+ve
CDBT-16	-	Equivocal	-	+ve	+ve	-	+ve
CDBT-17	-	+ve	-	+ve	+ve	-	+ve
CDBT-18	-	+ve	-	-	-	-	-
CDBT-19	-	-	-	+ve	+ve	-	+ve
CDBT-20	-	-	-	-	+ve	-	+ve
CDBT-21	+ve	+ve	Equivocal	-	-	-	-
CDBT-22	-	Equivocal	-	+ve	+ve	-	+ve
CDBT-23	-	+ve	+ve	+ve	+ve	-	-
CDBT-24	-	-	-	-	-	-	-

CDBT-25	-	+ve	-	+ve	+ve	+ve	+ve
CDBT-26	-	-	-	-	-	-	-
CDBT-27	+ve	-	-	-	-	-	-
CDBT-28	-	-	+ve	-	-	-	+ve
CDBT-29	-	+ve	-	+ve	+ve	-	+ve
CDBT-30	-	+ve	-	-	-	-	-
CDBT-31	-	-	-	-	-	-	-
CDBT-32	-	-	-	-	-	-	-

APPENDIX:4 Ethical approval and Patient's Consent

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 760

10 November 2016

Prof. Krishna Das Manandhar
Principal Investigator
Central Department of Biotechnology
Kirtipur, Kathmandu

Ref: **Approval of Research Proposal** entitled **Dengue and Dengue-like infections in patients visiting selected hospitals in Nepal**

Dear Prof. Manandhar,

It is my pleasure to inform you that the above-mentioned proposal submitted on 23 October 2016 (**Reg.no. 378/2016** please use this Reg. No. during further correspondence) has been approved by NHRC Ethical Review Board on **26 October 2016**.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol before the expiration date of this approval. Expiration date of this study is **July 2020.**

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

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

As per your research proposal, the total research amount is **NRs. 2,500,000.00** and accordingly the processing fee amount to **NRs. 79,950.00**. It is acknowledged that the above-mentioned processing fee has been received at NHRC.

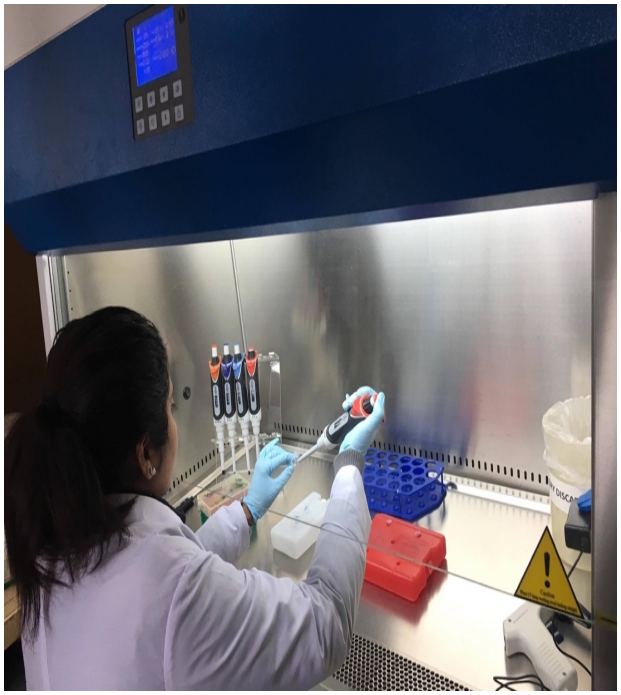
If you have any questions, please contact the Ethical Review M & E section of NHRC.

Thanking you

.....

Dr. Khem Bahadur Karki
Member-Secretary

APPENDIX 5: PHOTOS AND MEMORIES

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