EPIDEMIOLOGY AND IMMUNO-MOLECULAR ANALYSIS OF DENGUE OUTBREAK 2016 IN NEPAL

M.Sc. Thesis

2018

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Institute of Science and Technology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Mahesh Lamsal

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For partial fulfilment of requirement for the degree of two years M.Sc. in Biotechnology

By

Mahesh Lamsal

T.U. Registration No: 5-2-37-973-2009

Supervisor

Prof. Dr Krishna Das Manandhar

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Declaration by the candidate

I hereby, declare that the dissertation entitled "**Epidemiology and immuno-molecular analysis of dengue outbreak 2016 in Nepal"** submitted to Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu for partial fulfilment of requirement for the degree of M.Sc. in Biotechnology is a genuine work performed by me, Mahesh Lamsal (T.U. Registration No: 5-2-37-973-2009) under the guidance and supervision of Prof. Dr Krishna Das Manandhar. No copies of this work have been published or presented previously anywhere or any forms.

Signature of the candidate Date

Dedicated to my PARENTS and the creator NATURE

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I am greatly indebted to a lot of people without whom this thesis would not be completed. They have guided me through several steps of my life and paved me the way to go through. My first gratitude goes to my **Father** and **Mother**, the reason why I am up to here and are a continuous source of encouragement and inspiration to me throughout my life.

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Thank you all.

Mahesh Lamsal

ACRONYMS AND ABBREVIATIONS

bp	Base pairs
CDBT	Central Department of Biotechnology
CDC	Centres for Disease Control and Prevention
cDNA	Complementary DNA
DENRA	Dengue Derived Recombinant Antigen
DENV	Dengue Virus
DENV-1	Dengue Virus type 1
DENV-2	Dengue Virus type 2
DENV-3	Dengue Virus type 3
DENV-4	Dengue Virus type 4
DF	Dengue Fever
DHF	Dengue haemorrhagic fever
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSS	Dengue shock syndrome
E	Envelope protein
ELISA	Enzyme Linked Immunosorbent Assay
HRP	Horse Radish Peroxide
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kb	kilo base pairs
NCA	Normal Cell Antigen
NFW	Nuclease Free Water
NHRC	Nepal Health Research Council
NS	Non-structural
OD	Optical Density
PAb	Polyclonal Antibody
PCR	Polymerase Chain Reaction
PrM	pre-membrane
PRNT	Plaque Reduction Neuralization Test

RDT	Rapid Diagnosis Test
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SDS	Sequence Detection Software
TMB	Tetra methyl benzidine
UTR	Untranslated Region
µg	Microgram

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

ABSTRACT

The emergence and circulation of viral infection has become one of the major public health concerns in the world. Of those emerging diseases, dengue is a mosquito borne flavivirus mainly prevalent in the tropical and sub-tropical countries. Four serotypes of dengue virus (DENV 1- 4) are globally present. Hundreds of dengue cases are being reported annually and the emergence of this virus with yearly shift in serotypes has become the concern of public health in Nepal. No advanced method besides the Rapid Diagnostic kits are applied for diagnosis in most of the hospitals in Nepal. Rapid tests are not reliable as this coincides with the similar flavivirus infections. The detection of dengue virus infections has great importance for the clinical management of patients, surveillance, and clinical trial assessments in the days to come. Two hundred forty (240) Dengue suspected clinical samples were subjected to Dengue IgM, IgG and NS1 capture ELISA. Indirect IgG ELISA for antibody level detection was done for each sample followed by molecular identification. Viral RNA extraction was done, and cDNA was prepared. After the cDNA was made, Reverse transcriptase PCR(RT-PCR) was performed using D1 and DencomR2 primers. Dengue Serotyping was then done using serotype specific primers. Out of 240 acute dengue cases, 64 % of them were male, 36% female and 2% of the total samples were below age of 10. From the study subjects, 60% were NS1 positive, 33% were IgM positive and 7% were IgG positive. Fourteen samples were positive for all NS1, IgM and IgG ELISA. Calculating the ratio between IgM and IgG, it was found that most of the cases were of primary infection. Antibody level for each serum was calculated and categorised as high, medium and low. The maximum antibody level was found to be 43228.70 and the lowest of 953.53. Altogether, 86 samples were found to be PCR positive for dengue virus. While confirming the serotype prevalence, dengue Serotype-1 was found to be prevalent in the year 2016 in Nepal.

Only 37% of the clinically suspected cases were confirmed for dengue virus infection. This implies that confirmatory tests like PCR should be made for the proper diagnosis of the disease and medication to be followed accordingly. To add, ELISA is the preliminary test and RT-PCR is the confirmatory tests for the detection of dengue virus.

Key Words: Dengue virus, RT-PCR, ELISA, Re-emergence, Serotyping

CHAPTER 1

INTRODUCTION

1.1 Background of the Dengue Fever

Dengue is one of the major public health problems in the world. It is a self-limiting acute mosquito-transmitted disease characterized by fever, headache, muscle, joint pains, rash, nausea and vomiting. It is transmitted by the bite of female mosquito of the species *Aedes aegypti* or *A. albopictus* (Gupta et al., 2016).The dengue virus belongs to genus *Flavivirus* (family *Flaviviridae).* Tropical and sub-tropical regions are the main areas where its prevalence is higher. Most common areas are South-East Asia and the Western Pacific Islands. More than 50 million dengue infection cases from more than 100 countries are reported each year in the world(WHO, 2009).Dengue virus infection causes a spectrum of clinical manifestations ranging from normal dengue fever (DF) to fatal haemorrhagic diseases. Dengue fever (DF) is clinically characterised by acute febrile illness with chills, headache, body pain retro-ocular pain and arthralgia followed by nausea and vomiting and a maculopapular rash(WHO, 2009). A severe form of dengue fever, also known as dengue haemorrhagic fever(DHF) can cause severe bleeding, a sudden drop in the blood pressure(shock) and death(Jain & Jain, 2005). Dengue is a climate-sensitive disease. Temperature and precipitation are the most important climate factors in the occurrence and transmission of dengue fever. These climate factors affect mosquito population (survival and development), virus propagation (replication) and vector-host interaction (biting rate). Both the number of cases of dengue fever and the areas of outbreaks within Nepal have increased significantly in recent years. Further expansion and range shift is expected in the future due to global climate change and other associated factors. Due to limited spatially-explicit research in Nepal, there is poor understanding about the present spatial distribution patterns of dengue risk areas and the potential range shift due to future climate change(Acharya et al., 2018).

1.1.1 A short history of Dengue infection

Dengue virus was first isolated by Ren Kimura and Susumu Hotta in 1943.These two scientists were studying blood samples of patients taken during the 1943 dengue epidemic in Nagasaki, Japan. A year later, Albert B. Sabin and Walter Schlesinger independently isolated the dengue virus. Both pairs of scientists had isolated the virus now referred to as dengue virus 1 (DEN-1)(Holmes & Twiddy, 2003) .The first record of a case of probable dengue fever is in a Chinese medical encyclopaedia from the Jinn Dynasty (265–420 AD) which referred to a “water poison” associated with flying insects. The first

recognized dengue epidemics occurred almost simultaneously in Asia, Africa, and North America in the 1780s, shortly after the identification and naming of the disease in 1779. The first confirmed case report dates from 1789 and is by Benjamin Rush, who coined the term "break-bone fever" because of the symptoms of myalgia and arthralgia(Guzman et al., 2010; Wei & Li, 2017).The viral etiology and the transmission by mosquitoes were only deciphered in the 20th century. The socioeconomic impact of World War II resulted in increased spread globally. Nowadays, about 2.5 billion people, or 40% of the world's population, live in areas where there is a risk of dengue transmission. Dengue spread to more than 100 countries in Asia, the Pacific, the Americas, Africa, and the Caribbean(Wei & Li, 2017; Halstead, 2008).

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. Gubler (1997) 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). The sylvatic cycle is presumed to be ancestral because efficient interhuman transmission is thought to require a minimum human population size of 10,000–1 million, which did not exist until about 4000 years ago when urban civilizations arose(Weaver & Vasilakis, 2009).

1.1.3 Epidemiology of Dengue virus

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. In the last 50 years, incidence has increased 30-fold with increasing geographic expansion to new countries and, in the present decade, from urban to rural settings(WHO, 2009). Dengue is widespread throughout the tropics, with risk factors influenced by local spatial variations of rainfall, temperature, relative humidity, degree of urbanization and quality of vector control services in urban areas. Before 1970, only nine countries had experienced severe dengue epidemics. Today, the disease is endemic in more than 100 countries in WHO's African, Americas, Eastern Mediterranean, South-East Asia and Western Pacific regions; the Americas, South-East Asia and Western Pacific regions are the most seriously affected(Bhatt et al., 2013). Over the past four years, epidemic dengue activity has spread to Bhutan and Nepal in the sub-Himalayan foothills(WHO, 2009; Chandele et al., 2016). Major epidemics to date, in 2010, 2013 and 2016 have demonstrated the capacity of infection outbreaks to be explosive and challenging to currently available disease control measures(Subedi & Taylor-Robinson, 2016a).

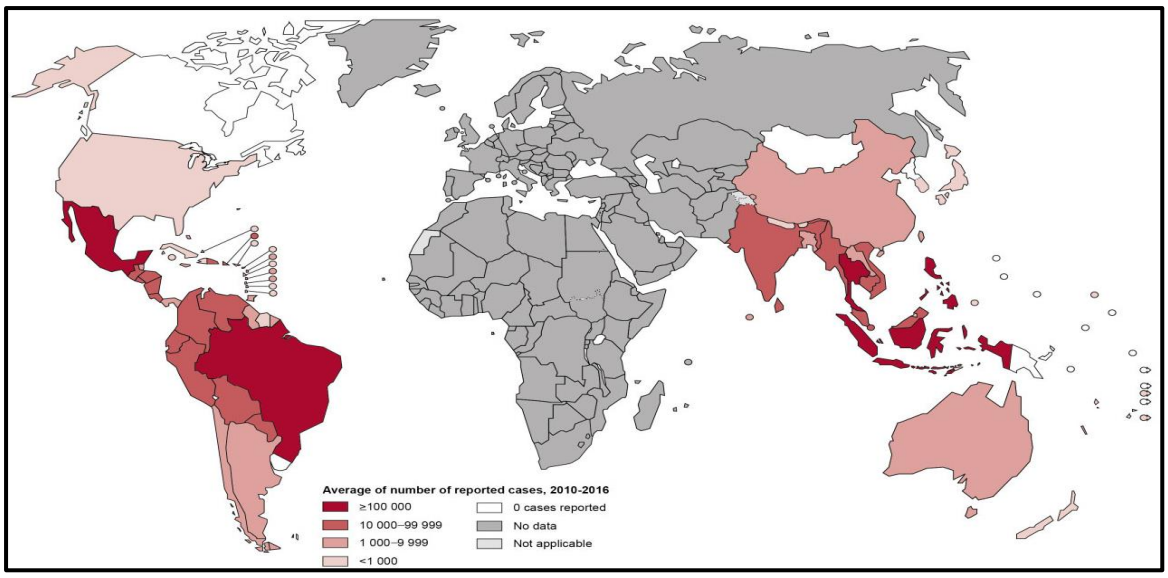

Figure 1. 1 Average number of suspected or confirmed dengue cases reported to WHO, 2010–2016

1.1.4 Dengue virus circulation in Nepal

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

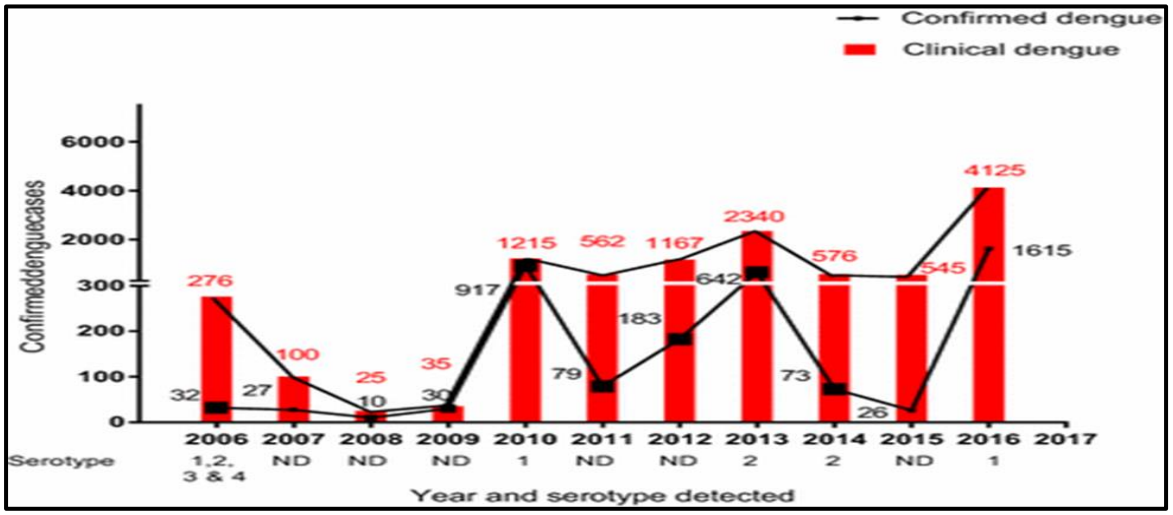

Figure 1. 2 Distribution of dengue cases in different years(Gupta et al., 2018a)

There is an increasing trend of dengue outbreak in every three years in Nepal. Although the first dengue case was reported in 2004 from Japanese traveller, the outbreak of dengue was occurred in the country in 2006 with a remarkable number of 32 confirmed cases. The virus remained almost latent for the three consecutive years from 2007 to 2009 and reoccurred again during a massive outbreak in 2010 Cases of dengue continued to be reported in the subsequent year 2011 and 2012 and two major outbreaks were witnessed in 2013 and 2016 (EDCD, 2016). A clear cyclic 3-year-amplitude demonstrated by major peaks in 2010, 2013 and 2016(Gupta, et al., 2018).

1.1.5 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*

1.1.6 The Virus

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

1.1.7 Structure of Dengue virus

The dengue virus is a positive-stranded RNA virus of 10.7 kilobases in length that encodes a precursor polypeptide for three structural proteins, capsid (C), pre-membrane (prM) and envelop (E), and seven non-structural (NS) proteins. The dengue virus has a roughly spherical shape. Inside the virus is the nucleocapsid, which is made of the viral genome and C proteins.

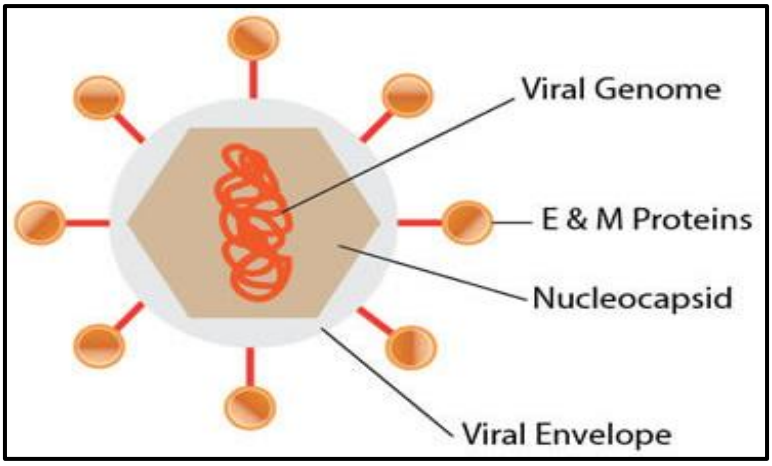

Figure 1. 3 Dengue virus structure (Guzman et al., 2010b).

The nucleocapsid is surrounded by a membrane called the viral envelope, a lipid bilayer that is taken from the host. Embedded in the viral envelope are E and M proteins that span through the lipid bilayer. These proteins form a protective outer layer that controls the entry of the virus into a human.

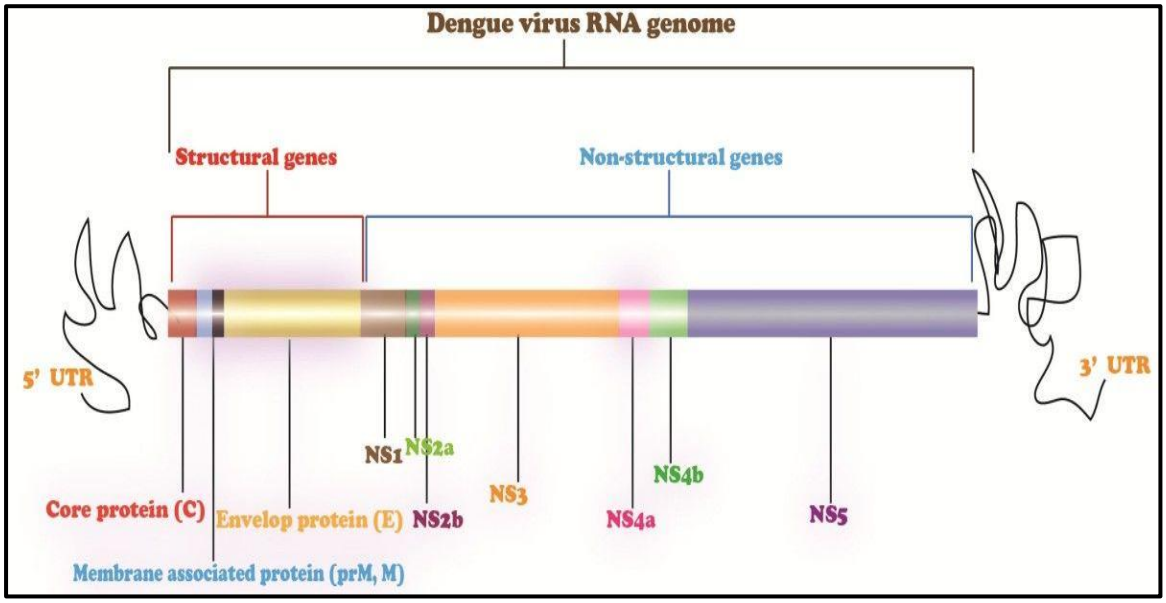

Figure 1. 4 Dengue virus genome (Idrees & Ashfaq, 2012)

The dengue virus genome encodes three structural (capsid [C], membrane [M], and envelope [E]) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Guzman et al., 2010b). There is four distinct dengue virus (DENV) serotypes that share antigenic relationships (DENV-1, DENV-2, DENV-3, and DENV-4). Although infection with one serotype confers lifelong protection against that serotype, it does not necessarily protect against a secondary infection with a heterologous serotype. This is because of the formation of memory cells of the previously attacked serotype of the virus. Within each of the four DENV serotypes, which share nearly 65–70 % amino acid sequence similarity. Indeed, nonprotective but cross-reactive antibodies may enhance disease severity.

Currently, there are no effective vaccines or antiviral drugs against these viruses. This problem thus needs to be addressed as a matter of urgency as failure to develop effective DENV control strategies will inevitably result in a further increase in the number of infected humans. This problem is also exacerbated by the continuing dispersal of these viruses to new geographic areas regardless of the climate and altitudinal factors(Kyle & Harris, 2008; Gregianini et al., 2018).

1.1.8 Transmission cycle

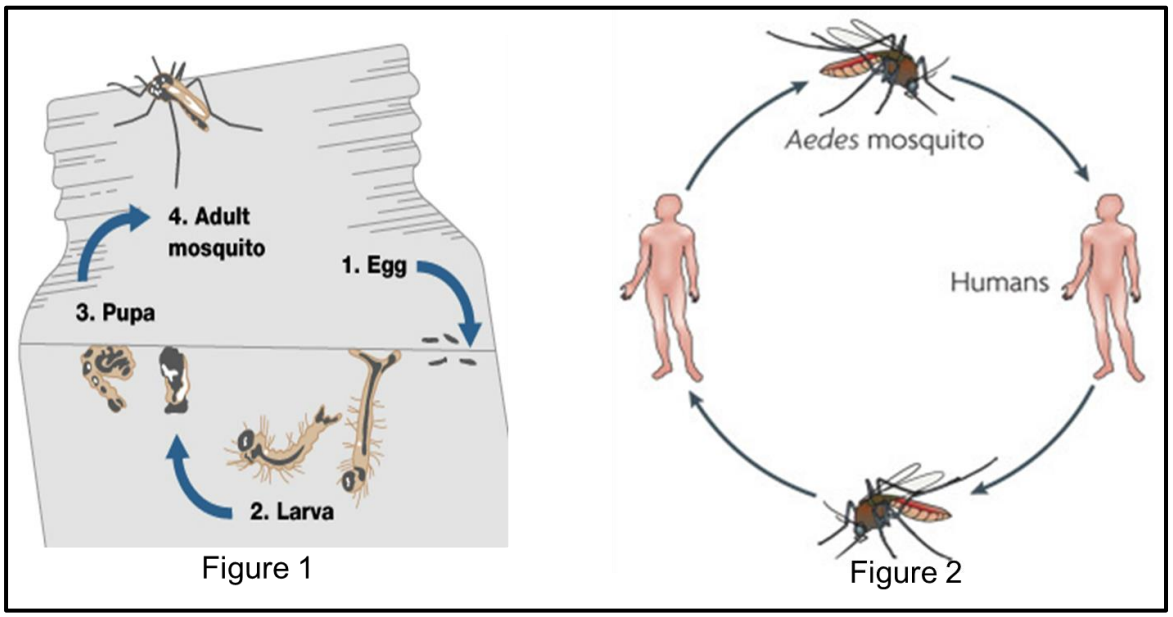

Figure 1. 5 (1) *Aedes aegypti* life cycle(Guzman et al., 2010b) (2) Dengue transmission cycle

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

The various serotypes of the dengue virus are transmitted to humans through the bites of infected *Aedes* mosquitoes, principally *Aedes aegypti*. This mosquito is a tropical and subtropical species widely distributed around the world, mostly between latitudes 35°N and 35°S. These geographical limits correspond approximately to a winter isotherm of 10°C. *Aedes aegypti* has been found as far north as 45°N, but such invasions have occurred during warmer months and the mosquitoes have not survived the winters. Also, because of lower temperatures, *Aedes aegypti* is relatively uncommon above 1000 metres (Holmes & Twiddy, 2003).The immature stages are found in water-filled habitats, mostly in artificial

containers closely associated with human dwellings and often indoors. Studies suggest that most female *Aedes aegypti* may spend their lifetime in or around the houses where they emerge as adults. This means that people, rather than mosquitoes, rapidly move the virus within and between communities. Dengue outbreaks have also been attributed to *Aedes albopictus, Aedes polynesiensis* and several species of the *Aedes scutellaris* complex(WHO , 2009). Each of these species has an ecology, behaviour and geographical distribution. In recent decades *Aedes albopictus* has spread from Asia to Africa, the Americas and Europe, notably aided by the international trade in used tyres in which eggs are deposited when they contain rainwater. The eggs can remain viable for many months in the absence of water(WHO , 2009).

Humans are the main amplifying host of the virus. Dengue virus circulating in the blood of viraemic humans is ingested by female mosquitoes during feeding. The virus then infects the mosquito mid-gut and subsequently spreads systemically over a period of 8-12 days. After this extrinsic incubation period, the virus can be transmitted to other humans during subsequent probing or feeding. The extrinsic incubation period is influenced in part by environmental conditions, especially ambient temperature. Thereafter the mosquito remains infective for the rest of its life. Ae. aegypti is one of the most efficient vectors for arboviruses because it is highly anthropophilic, frequently bites several times before completing oogenesis, and thrives near humans. Vertical transmission (transovarial transmission) of dengue virus has been demonstrated in the laboratory but rarely in the field. The significance of vertical transmission for maintenance of the virus is not well understood. Sylvatic dengue strains in some parts of Africa and Asia may also lead to human infection, causing mild illness. Several factors can influence the dynamics of virus transmission - including environmental and climate factors, host-pathogen interactions and population immunological factors. Climate directly influences the biology of the vectors and thereby their abundance and distribution; it is consequently an important determinant of vector-borne disease epidemics(Amin et al., 2018).

1.1.9 Virus Replication

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

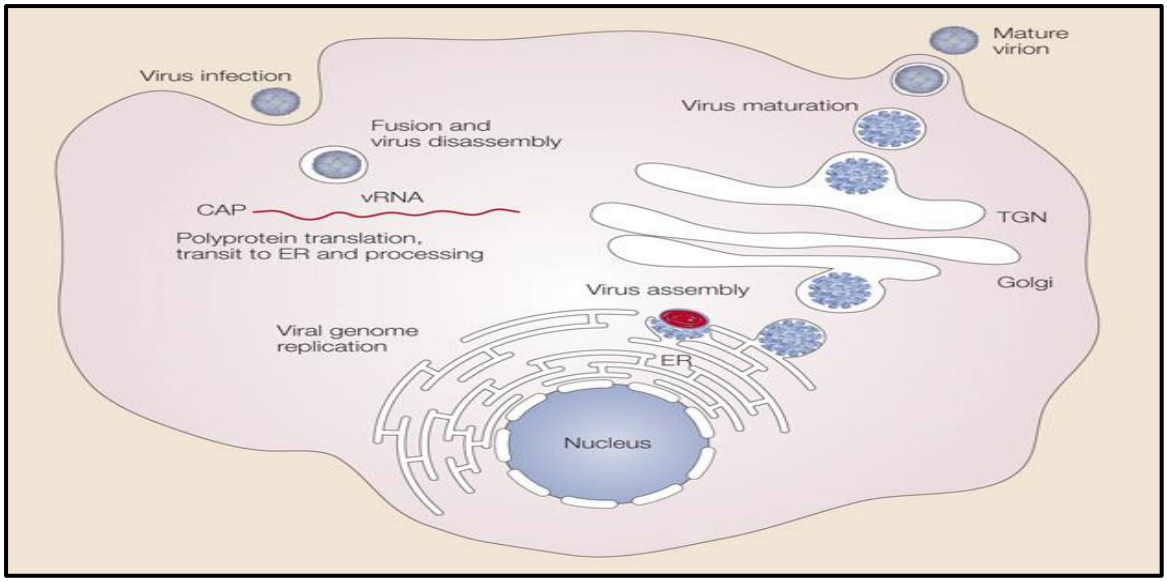

Figure 1. 6 Dengue virus replication (Mukhopadhyay et al., 2005)

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

1.1.10 Host-pathogen interaction

The dengue virus enters via the skin while an infected mosquito is taking a blood meal. During the acute phase of illness, the virus is present in the blood and its clearance from this compartment generally coincides with effervescence.

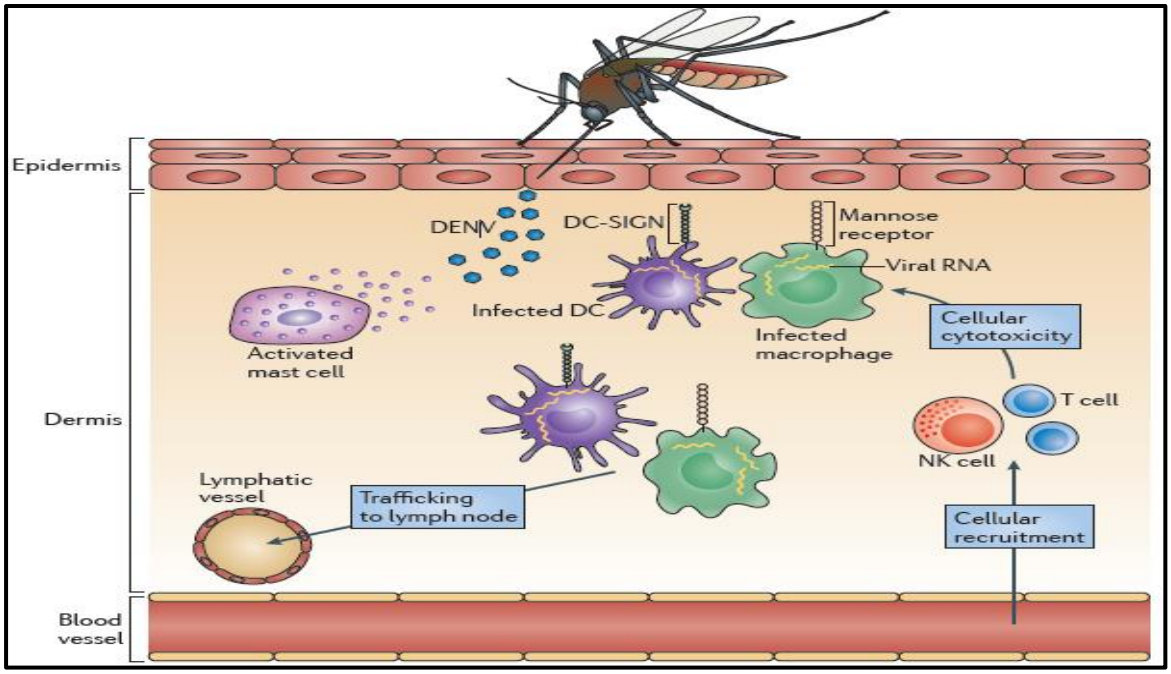

Figure 1. 7 Pathway for the immuno- pathogenesis of DENV(Idrees & Ashfaq, 2012).

Humoral and cellular immune responses are considered to contribute to virus clearance via the generation of neutralizing antibodies and the activation of CD4+ and CD8+ T lymphocytes. In addition, innate host defence may limit infection by the virus. After infection, serotype-specific and cross-reactive antibodies and CD4+ and CD8+ T cells remain measurable for years

It can proceed in two ways, primary infection leads to the direct entry of DENV into immune cells like Macrophages, Monocytes and Dendritic cells. This pathway can further activate DENV specific T cells, cause cytolysis, cytokines production, complement activation and finally leading to plasma leakage. In case of secondary infection, antibody based enhancement occurs which leads to cytokines production and complement activation, high levels of cytokines and complement activation can damage vascular endothelial cells resulting in plasma leakage (Waqar, 2016).

1.1.11 Dengue symptoms and Clinical manifestations

Infection with any of the four serotypes (DENV-1–DENV-4) of dengue virus (DV) can produce a broad spectrum of symptoms, ranging from asymptomatic infection to a severe life-threatening illness. Primary infection with DENV causes an acute febrile illness known as dengue fever (DF) whereas secondary infection with dengue virus may sometimes leads to fatal dengue haemorrhagic fever/ dengue shock syndrome (DHF/DSS) (Whitehead et al ., 2007).Symptomatic dengue illness is typically classified into dengue fever (DF), a self-limiting febrile illness, or a more severe form, dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS)(García et al., 2011).

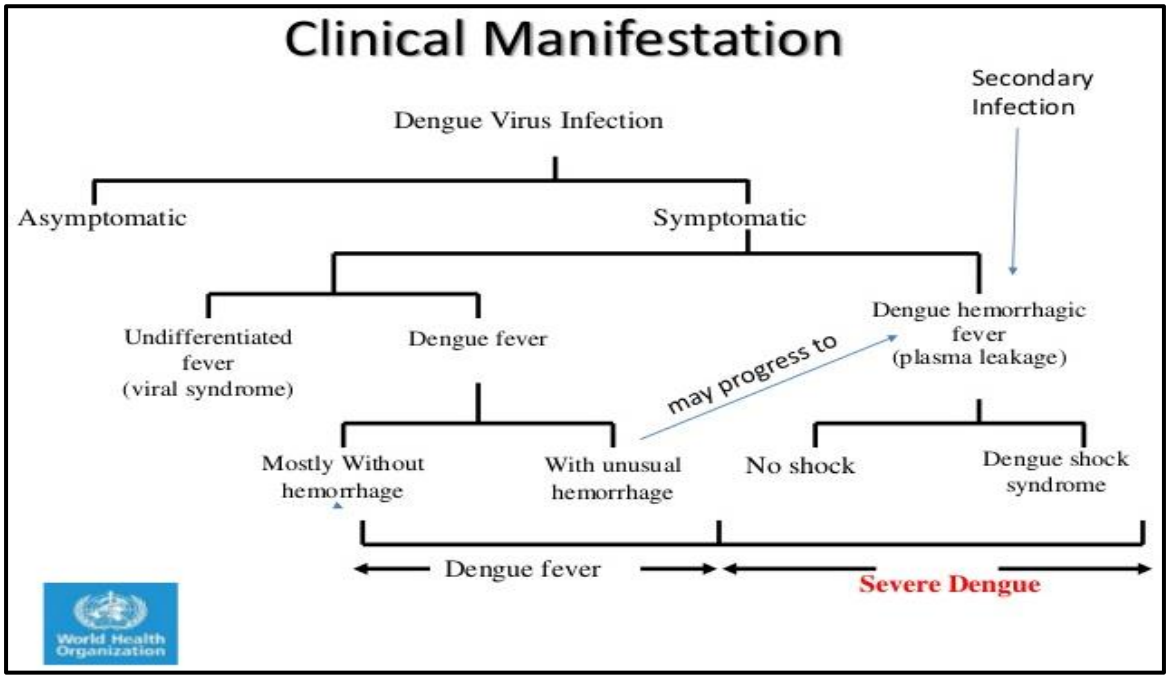

Figure 1. 8 Manifestation of Dengue virus infection

Symptomatic dengue virus infections are grouped into three categories: undifferentiated fever, dengue fever (DF) and dengue haemorrhagic fever (DHF). DHF was further classified into four severity grades, with grades III and IV being defined as dengue shock syndrome (DSS) (WHO, 2009).

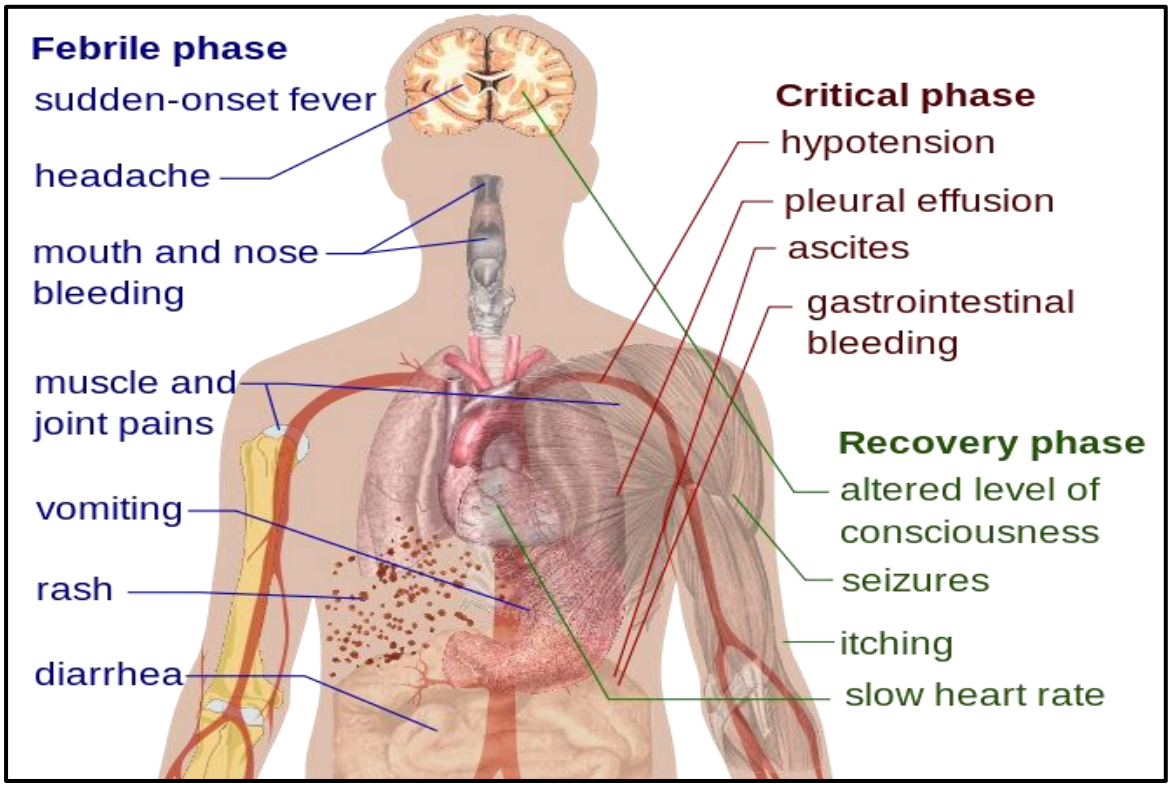

Figure 1. 9 Dengue virus pathogenesis in humans. (WHO, 2009)

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

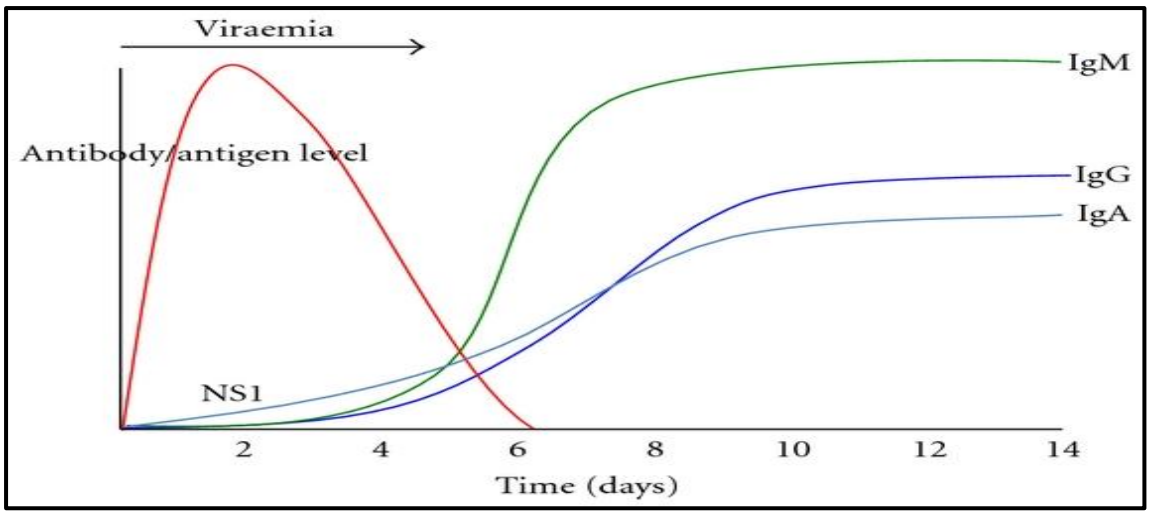

Figure 1. 10 Immune response to dengue infection (Guzman et al., 2010).

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

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

1.1.12 Diagnosis of Dengue virus

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

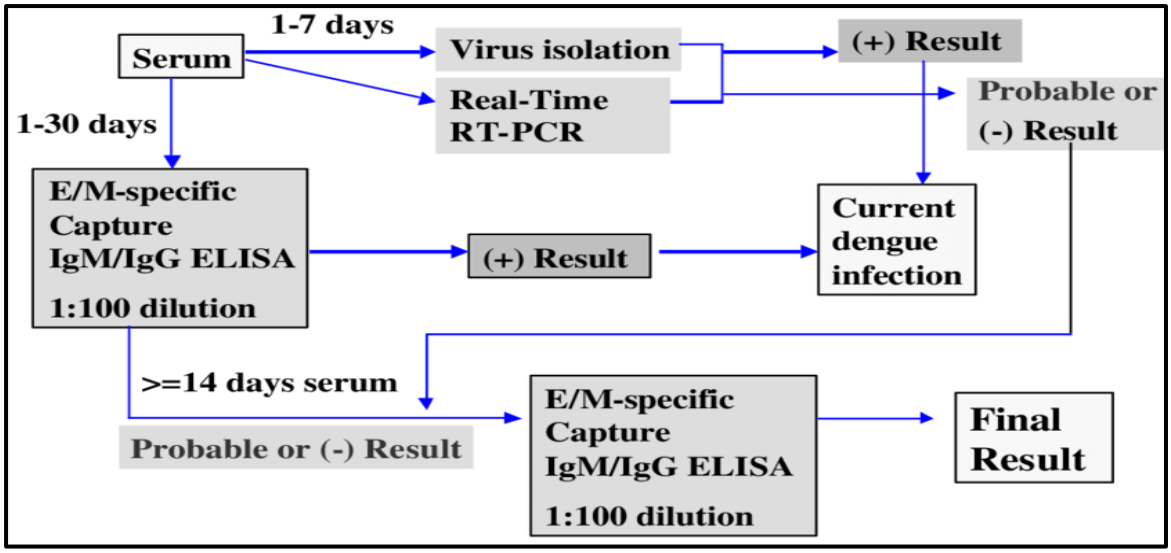

Figure 1. 11 Process for the diagnosis of Dengue (CDC).

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

Detection of IgM antibodies to dengue virus by ELISA is a valuable procedure, particularly in second and subsequent infections where the occurrence of complications is high. Serum IgM antibodies can be detected from dengue patients as early as three to five days after the onset of fever and generally persist for 30 - 90 days, although detectable levels may be present eight months post-infection(Panbio, 2016). IgM antibodies are the first immunoglobulin isotype to appear. These antibodies are detectable in 50% of patients by days 3-5 after onset of illness, increasing to 80% by day 5 and 99% by day 10. IgM levels peak about two weeks after the onset of symptoms and then decline generally to undetectable levels over 2–3 months. Anti-dengue serum IgG is generally detectable at low titres at the end of the first week of illness, increasing slowly thereafter, with serum IgG still detectable after several months, and probably even for life (Tang & Ooi, 2012a).

Detection of a specific antibody of the IgG class to the four dengue serotypes, by ELISA, is valuable for the diagnosis of previous exposure to dengue. The presence of rising levels of IgG in paired sera is suggestive of active dengue infection. Traditionally, hemagglutination-inhibition (HAI) titres have been used to classify infections as primary or secondary. The current definition depends upon an assay of paired serum specimens separated in time by at least 7 days, though any acute specimen with an HAI titre ≥1:2560 is defined as coming from a patient with secondary flavivirus infection. Similarly, a value of > 4 times the cut-off serum value in the Panbio Indirect ELISA can be used to distinguish between primary and secondary dengue infection(E-Deng, 2008).

Primary dengue virus infection is characterized by elevations in specific NS1 antigen levels 0 to 9 days after the onset of symptoms; this generally persists up to 15 days. Earlier diagnosis of Dengue reduces the risk of complication such as DHF or DSS, especially in countries where dengue is endemic. DENGUE NS1 Ag MICROLISA is designed for in vitro qualitative detection of Dengue NS1 antigen in human serum or plasma and is used as a screening test for testing of collected blood samples suspected of DENGUE. The kit detects all four subtypes; DEN1, DEN2, DEN3 & DEN4 of Dengue Virus(Ns & Microlisa).

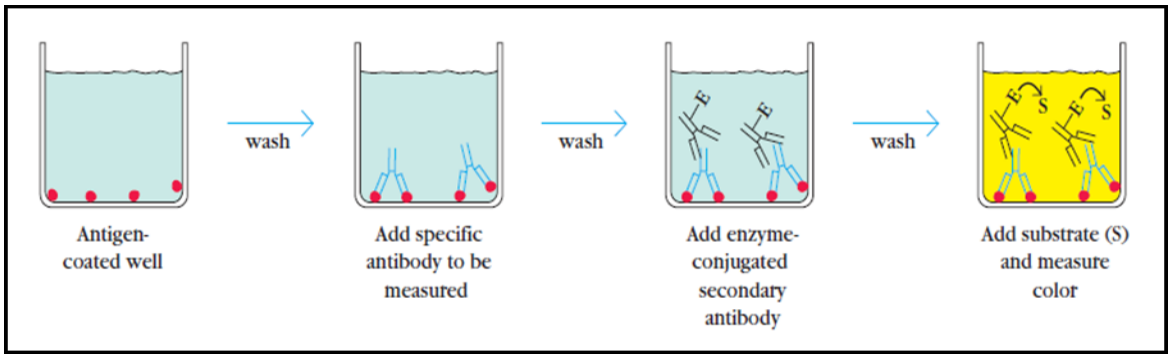

Figure 1. 12 General mechanism for the process of ELISA

In Nepal, serological test, rapid diagnostic tests (RDTs), Enzyme-Linked Immuno-Sorbent Assay (ELISA) have been used as an important tool for the detection of DENV infection during the epidemics. But, due to the less sensitivity and cross-reactivity with other flavivirus infections, we cannot guarantee the DENV (Subedi & Taylor-Robinson, 2016; P. Y. Shu et al., 2009). The correct diagnosis of DENV in preliminary stages is very important to prevent the patients from more severe complications. Non-structural glycoprotein-1 (NS1) has proven to be a useful marker for the early diagnosis of DNEV(P. Shu et al., 2003). Only the clinical observation is insufficient for the diagnosis of dengue infection as the symptoms coincide with Yellow fever, Japanese encephalitis etc. Although serological assays can, in many instances, provide a presumptive diagnosis of recent infection from a single serum specimen, a conclusive diagnosis of acute can be made only when raising level of anti-dengue immunoglobin is detected in the paired sera. The diagnosis of acute dengue infection is possible only on this basis because antibody level is known to rise only for 2-4 weeks following infection. The existing ELISA method is based upon the capture of IgM in the sample with anti-IgM antibodies adsorbed on the polystyrene surface. Unbound immunoglobulins are washed off. Then the antigen labelled with peroxidase react with the IgM captured, and the unbound is eliminated by washing; bound antigen is developed with the aid of a substrate solution (TMB) to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution (Cardosa et al., 1992) . As DENV infections can result in severe and life-threatening illness, identifying which patients are at risk of an outcome that requires supportive interventions is important. Differentiating this group from the thousands of mild cases during outbreaks

is a major medical challenge; simple and inexpensive strategies are urgently needed(Priyamvada et al., 2016).

1.2 The rationale of the study

Dengue has been the most rapidly spreading mosquito-borne viral disease in the world. This infection is considered a major emerging tropical disease and significant public health concern. Since the 1970s, endemic dengue has spread from 9 nations to over 100, moved into urban areas, and explosive outbreaks of disease have become increasingly common. Four different serotypes of DENV (DENV 1-4) have been identified as causes of dengue infection since first recognized in 1943, later accompanied by the fifth serotype (DENV-5) discovered in 2013. Dengue fever and Dengue haemorrhagic fever are now considered major causes of morbidity and mortality in the subtropical and tropical regions of the world. Dengue infection in returned travellers has been increasingly reported from all regions of the world. Most of dengue infections is thought to be sub-clinical and can present atypically as an undifferentiated febrile illness.

There is a pressing need to undertake effective vector surveillance studies supported by the provision of well-equipped diagnostic virology laboratories. Nepal is also one of the vulnerable countries in the world for the emergence and circulation of the diseases. There is a periodic outbreak of Dengue in every three years causing the death of many people. Dengue has no ethical, geographical and sex, race and age boundary for its transmission. The range of infection has extended all over the country and now comprises not only low-lying regions but also hilly locations including the capital city Kathmandu. In most 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 kits are available for the rapid diagnosis of the Dengue fever but the confirmation cannot be made from those Rapid diagnostic kits. So, there comes an urge for the proper diagnosis of the disease using other sophisticated techniques which give the reliable and sensible results. Indirect ELISA method is used for the estimation of antibody level in the suspected samples and this method can play an important role for other studies. Capture ELISA for IgG, IgM and NS1 is another technique for the preliminary diagnosis of the virus. For further confirmation, RNA extraction is done, complementary DNA (cDNA) is made and PCR is done. Using gene-specific primer for Dengue virus can give the confirmatory test for the dengue virus infection. Furthermore, the serotyping method and sequencing methods are the ultimate ways for the proper diagnosis and study of such diseases. This sort of techniques gives a breakthrough in the proper diagnosis of the disease and gives an idea about what serotype and which species of dengue virus was prevalent in the specific year in a certain locality and a country as a whole. Hence, the

immunological and molecular study of the disease plays an important role in the proper diagnosis of the disease and discovery of vaccines targeted to them.

1.3 Objectives

1.3.1 General Objectives

- Epidemiological study of Dengue outbreak 2016 and the immunological and molecular identification of those dengue suspected febrile cases.

1.3.2 Specific Objectives

- Prevalence study of Dengue fever cases.
- Screening of the primary and secondary dengue infection from the study subjects.
- Estimation of mid-point titre value for the detection of antibody level in serum sample.
- Molecular diagnosis and confirmation of dengue virus infection by PCR.
- Serotyping of the dengue cases circulating in the year 2016.

1.4 Research hypothesis

1.4.1 Null hypothesis

- The prevalence of secondary infection is more than primary infection in 2016.
- No new serotype of dengue virus obtained from the patients in the year 2016.

1.4.2 Alternative hypothesis

- The prevalence of primary infection is more than secondary infection in 2016.
- Different serotype of dengue virus from the past years can be seen in the year 2016.

CHAPTER 2

LITERATURE REVIEW

2.1 Dengue

Dengue fever is an acute, mosquito-transmitted viral disease characterized by fever, headache, arthralgia, myalgia, rash, nausea, and vomiting. Infections are caused by any of four virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4). The incidence of dengue is increasing in most tropical areas throughout the world(Rigau-Pérez et al., 1997). Each year, there are around 50 million dengue infections and about 500,000 individuals are hospitalized with dengue haemorrhagic fever, mainly in Southeast Asia, the Pacific and the Americas(Guzman et al., 2010a).Dengue viruses (family Flaviviridae, genus Flavivirus) occur as four antigenically distinct serotypes (DENV 1–4). Infection with any of them generally leads to a mild, self-limiting febrile illness (dengue fever). However, a more severe form of the disease, involving vascular and haemostatic abnormalities (dengue haemorrhagic fever-dengue shock syndrome [DHF-DSS]), is responsible for a high mortality rate, primarily among children. Indeed, DHF-DSS is a leading cause of hospitalization and death among children in Southeast Asia (Lanciotti et al., 1992). A comparison of the prevalence of various signs and symptoms of dengue between age groups of 0 - 54 in California from 1999 to 2001 revealed that a headache, arthralgia, myalgia and retro-orbital pain were present in more than 60 % of children and adults with confirmed dengue. External bleeding and chills were also present in more than 50 % of children. Fever, external haemorrhagic manifestations and rash were present in more than 50 % of infants (Hammond et al., 2005). Recovery from infection by one provides lifelong immunity against that serotype. However, cross-immunity to the other serotypes after recovery is only partial and temporary. Subsequent infections by other serotypes increase the risk of developing severe dengue(Wei & Li, 2017).

2.2 Dengue Virus and its serotype prevalence

In recent years, transmission of Dengue has increased mainly in urban and semi-urban areas and has become a major international public health concern (García et al., 2011). The mosquito species, *Aedes aegypti* is the primary vector of dengue. The virus is transmitted to humans through the bites of infected female *Aedes* mosquitoes. After virus incubation for 4–10 days, an infected mosquito can transmit the virus for the rest of its life. Infected humans are the main carriers and multipliers of the virus, serving as a source of the virus for uninfected mosquitoes. Patients who are already infected with the dengue

virus can transmit the infection (for 4–5 days; maximum 12) via Aedes mosquitoes after their first symptoms appear(WHO, 2009). Unlike many flaviviruses, DENV are highly restricted in their natural vertebrate host range, generally utilizing primates as their amplification and reservoir hosts. They are also among the most widely distributed of the flaviviruses, and all 4 DENV serotypes can be found nearly throughout the tropics and subtropics where the mosquito vector *Aedes aegypti* is abundant, putting at risk of infection nearly a third of the global human population(Livingston et al., 1994). DENV comprise 4 antigenically distinct serotypes (DENV-1–4), which, though epidemiologically nearly identical, are genetically quite distinct. Infection with one DENV serotype leads to life-long protection against homologous challenge, but only brief protection against heterologous infection with a different serotype(Green et al., 1999). 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 generally isolated during epidemics in the Americas (Khetarpal et al., 2016). But it is still inconclusive whether among three DENV-1 genotypes any of these can be consistently associated in causing more severe dengue. DENV-2 is the most studied serotype among the dengue viruses (Martina et al., 2009). The existence of six genotypes of DENV-2 based on the complete E gene sequence following earlier work (Deshwal et al., 2015). Sylvatic DENV-2 strains that are closely related have been isolated from different countries in West Africa and Malaysia, the two locations that are far apart leading to hypothesis that the DENV sylvatic ancestor arose in the Asian-Oceanic region before diverging into today's four DENV serotypes(Abello et al., 2016; JS, 2000).The current genotype classification for DENV-3 follows the nomenclature proposed by which recognised four DENV-3 genotypes based on prM/E sequences(Guzman et al., 2016). These four genotypes are like the four groups described using a 195-nucleotide region at the 5' terminus of the E gene. Genotype III DENV-3 is now widely found in Central and Southern America was introduced to the Americas via Nicaragua in 1994. It is considered as the most virulent of the four DENV-3 genotypes (Kadam et al., 2016; Khetarpal et al., 2016). It is worthy to note that genotype IV has never been associated with any DHF epidemics (Kumar et al., 2016). Initially DENV-4 was separated into two genotypes I and II based on the complete E gene sequence. 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 (Phommanivong et al., 2016). Of the four, Genotype II DENV-4 is the most widespread and was introduced to the Western hemisphere in 1981, possibly via the Pacific islands (Ranjan et al., 2016). Even though DENV-4 is the least frequently sampled serotype but it is often associated with haemorrhagic fever during secondary infection (Shim et al., 2016). Except for the sylvatic genotypes, genotype classification can often unveil the geographical origin of the dengue virus strains (Ong et al., 2010). In Asia, epidemic dengue has expanded geographically from Southeast Asian

countries west to India, Srilanka, the Maldives and Pakistan and east to China (Gubler, 1998, 1998). By the 1980s, the American region was experiencing major epidemics of dengue in countries that had been free of the disease for 35 to 130 years (Gubler, 1987). Before 1980, little was known about the distribution of dengue virus in Africa. Since then, however, major epidemics caused by all four serotypes have occurred in both East and West Africa (Gubler and Trent 1994). In 1997, dengue viruses and *Aedes aegypti* mosquitoes had a worldwide distribution in tropical and subtropical countries of the world. The first virologically proved epidemic of DF in India occurred in Calcutta and eastern coast of India in 1963 - 1964 (Sarkar et al., 1964; Chatterjee et al., 1965; Carey et al., 1966). Then, the dengue infection spread northwards and reached Delhi in 1967 (Balaya et al., 1969). Subsequently, the whole country was involved with widespread epidemics followed by endemic or hyperendemic prevalence of all four serotypes of dengue virus.

2.3 Epidemiological survey of Dengue in Nepal

Nepal is at higher risk of the introduction and establishment of DENV as the country is open bordered (where there is no restriction in travel and trade across the borders) by India and there are high frequencies of travel and trade across the borders(Shrestha et al., 2016). The first case of dengue in Nepal was recorded in a foreigner in Chitwan in 2004 and subsequently, the larger outbreak occurred in 9 districts of Terai region in 2006 with 23 confirmed dengue cases following the Indian epidemic of DF/DHF in September-October 2006. After four years another significant outbreak occurred in 2010 in Chitwan with at least 359 confirmed dengue cases. From the observations above it can be concluded that dengue is a significant problem in the districts of Terai region of Nepal including Chitwan and Dang(Shrestha et al., 2016; Subedi & Taylor-Robinson, 2016).

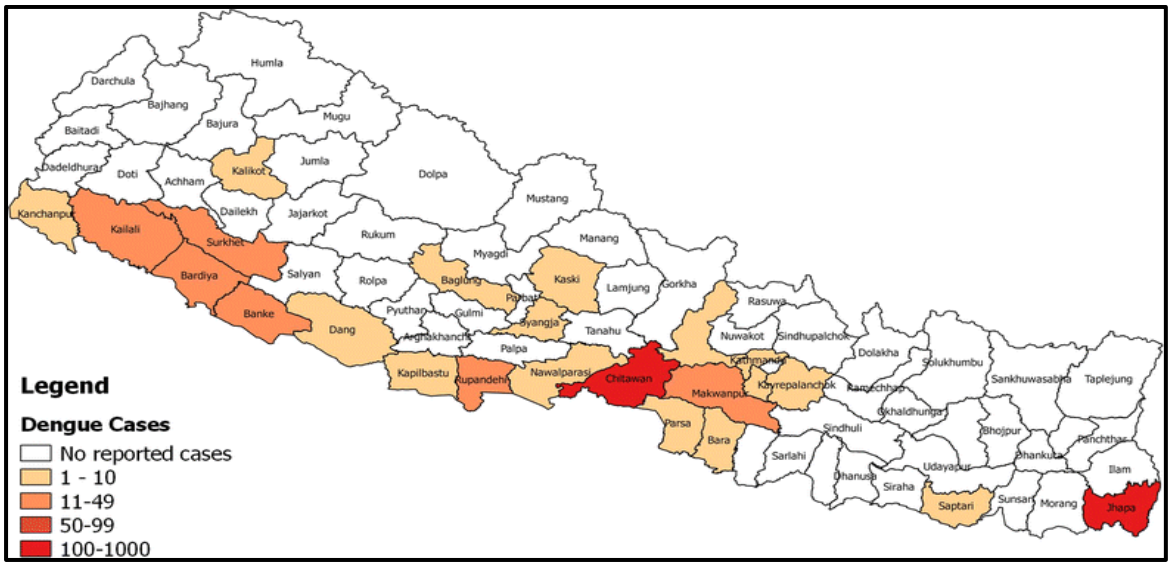

Figure 2. 1 Map of dengue reported districts from 2006 to 2016 in Nepal(Gupta et al., 2018a).

In recent years, GIS (Geographic Information Systems) and spatial statistics were frequently used to characterize spatiotemporal patterns of dengue and other infectious as well as non-infectious diseases(Li et al., 2017). Imported asymptomatic infections of travellers returning from endemic/outbreak areas may occur which are not identified and reported, and thus may serve as a source of localized outbreaks and pose an unknown level of risk to the blood supply(Añez et al., 2016). Dengue is an infectious disease with persistent occurrence, especially in developing countries (Holmes & Twiddy, 2003). Despite the recent economic growth, success in controlling the disease has not been achieved, and dengue has evolved from cyclic epidemic outbreaks to a lack of seasonality, given the increase in dengue cases(Amin et al., 2017).

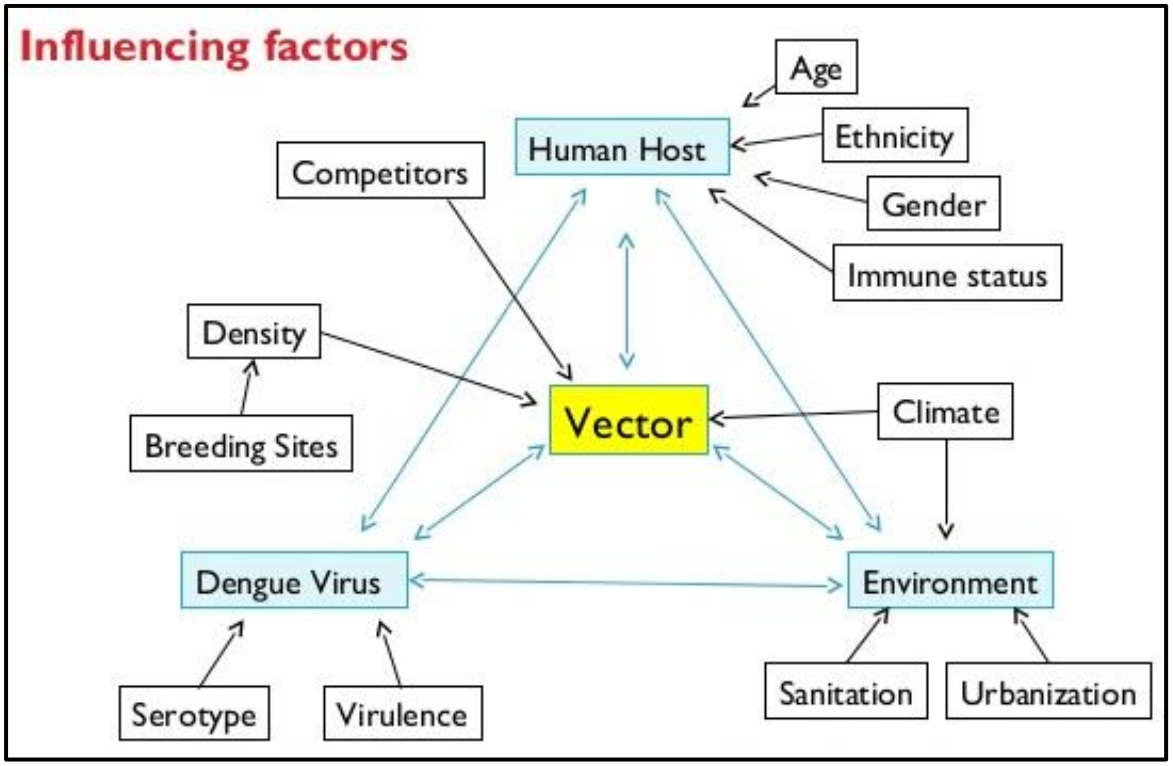

Figure 2.2 Relationship between the environment and mosquito-human relationship(Kyle & Harris, 2008).

The interplay of human, mosquito, and virus biology contributes to the clinical spectrum and geographic distribution of dengue. Each sphere influences and affects the others, all in the context of ecology and against the backdrop of climate and climate change(Kyle & Harris, 2008).Every aspect of dengue viral infection continues to be a challenge; the pathogenesis of severe dengue disease is not yet known, no vaccine is yet available for protection, and the vector control measures are inadequate. *A. aegypti* the primary vector for dengue fever is well adapted to breeding in human-made breeding sites in urban and peri-urban environments(Mistry et al., 2017). Most dengue virus infections in young children are mild and difficult to distinguish from other acute febrile diseases.

Classical dengue fever is most commonly seen in adolescents and adults; but in areas where dengue virus is endemic, resident adults are often immune and overt disease may be limited to arriving susceptible adults such as travellers(World Health Organization, 1997). The first case of DENV2 infection in Nepal was reported in 2004 in a Japanese traveller(B. P. Gupta et al., 2016c). Reports of dengue among travellers worldwide have been increasing, but high-quality data on incidence are lacking. The increase in international travel to dengue-endemic regions, with the associated risk of travel-acquired dengue, poses a serious concern(B. P. Gupta et al., 2016c).Analysing dengue viral strains from outbreaks in various geographical locations adopting molecular approaches has significant epidemiological significance(Kyle & Harris, 2008).

2.4 Sero-diagnosis of dengue infection

A study on dengue outbreak reported in Brazil in 1986 by Nogueira et al., (1989) revealed that 58.2 % of the patients tested by ELISA had IgM antibodies to dengue virus and they were considered as confirmed dengue patients for the further studies. Innis et al., (1989) found that in Jharkhand, anti-dengue IgM appeared in most cases by the 3rd day of febrile illness and declined to an undetectable level after 30 – 60 days. IgM capture ELISA showed 78 % sensitivity in acute serum and 97 % in paired sera. Dengue infections could be classified as primary or secondary by determining the ratio of units of dengue IgM to IgG antibody.

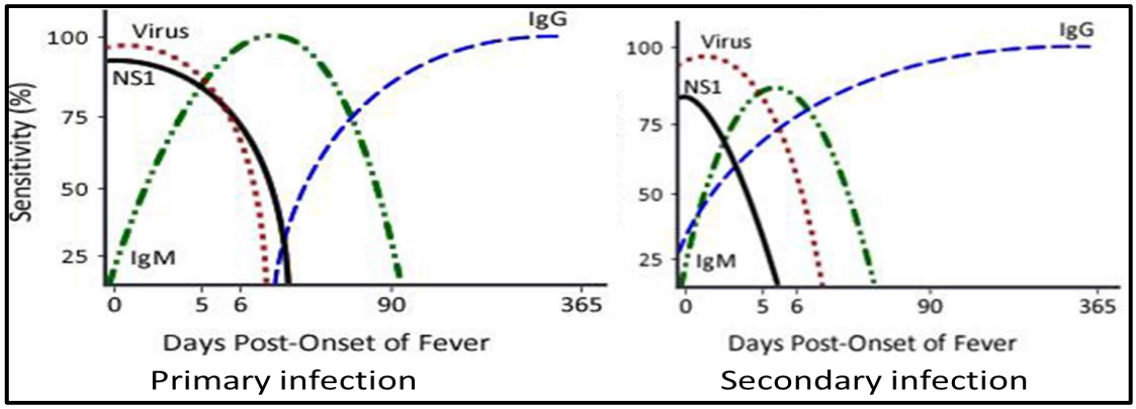

Figure 2. 3 Showing primary and secondary infection and the onset of fever.

According to Henchel and Putnak, (1990), ELISA has been the most widely used method in the past, the extensive cross reaction encountered and the non-availability of results within a short period of time due to requirement of both acute and convalescent sera collected at least seven days apart, have compromised the general applicability of this assay in the diagnosis of dengue. Chouhan et al., (1990) detected IgM antibodies to dengue viruses in 70 % of sera collected in Rajasthan in 1985. A kit for the detection of anti-dengue virus IgM antibody based on detecting dengue virus-specific IgM antibodies

in the test serum by capturing them with an anti-human IgM has been developed by Pellegrino et al., in 1994. This system had 92 % sensitivity, 100 % specificity, 94 % coincidence in single acute-phase serum samples as compared with results for sera from same patients tested by HI. Padbidiri et al., (1995) found virus-specific IgM antibodies in 25 % of sera collected immediately after onset of illness during an epidemic in 1993 in Mangalore, Karnataka. Another most widely used technique till today is ELISA which has been considered as the most useful test for dengue diagnosis due to its high sensitivity the ease of use and there is no use of sophisticated equipment. Moreover, ELISA has been used to detect acute phase (IgM) and convalescent-phase (IgG) antibodies. There have been several reports on the confirmation of dengue infection by using ELISA throughout the world (Guzman and Kouri 1996). Havorth et al., (1999) classified patients as dengue probable based on a positive ELISA test for IgM antibodies. According to them, classification of dengue cases required viral detection either by Polymerised Chain Reaction (PCR) or viral culture. ELISA was performed on serum from 99 patients and was found to be positive in 85 (85%) patients. Lam et al., (2000) evaluated ELISA for combined determination of Immunoglobulin M and Immunoglobulin G antibodies produced during infection by dengue virus. They used commercially available Pan Bio ELISA that utilizes 35 both IgM and IgG capture in the same microtiter well for the diagnosis of dengue infection. Sensitivity in the detection of primary and secondary dengue infections was 95 % and specificity was 94 %. They concluded that PanBio dengue screening ELISA is best suited for routine diagnosis where large numbers of samples are tested, and the cost is an issue and when it is not necessary to distinguish between primary and secondary infections.

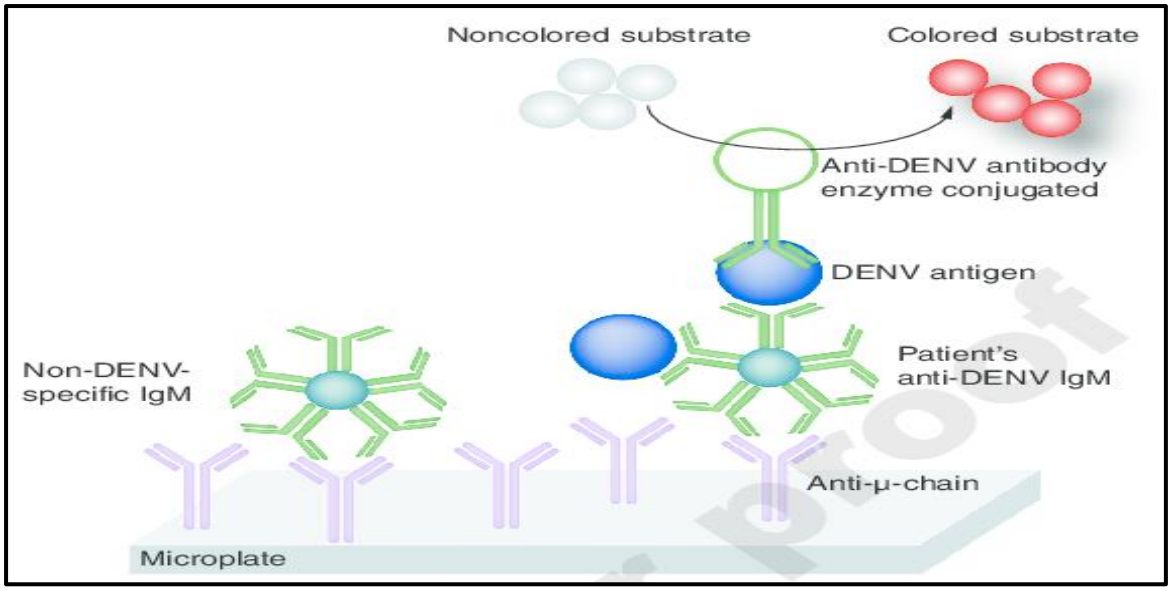

Figure 2. 4 A general mechanism for the Dengue capture ELISA

Antibody tests for DENV are available and used for diagnostic purposes, but they are unsuitable for blood screening because the infectious viremia stage precedes

seroconversion. Moreover, unlike chronic infections, identification of DENV-specific antibodies does not necessarily represent active viral infection. In addition, infection and antibody development can occur in the absence of clinically apparent infection (Añez et al., 2016).

2.5 Dengue IgG Indirect ELISA

Indirect ELISA is a two-step ELISA which involves two binding process of primary antibody and labelled secondary antibody. To calculate antibody titre, a blood serum sample containing antibody is diluted in serial ratios (1:2, 1:4, 1:8, 1:16... and so on). The titre is the amount, or the concentration, of a substance in a solution. In the indirect ELISA test, the sample antibody is sandwiched between the antigen coated on the plate and an enzyme-labelled, anti-species globulin conjugate. The addition of an enzyme substrate-chromogen reagent causes colour to develop. This colour is directly proportional to the amount of bound sample antibody(R., 1995). The more is antibody present in the sample, the stronger the colour development in the test wells. This format of indirect ELISA is suitable for determining total antibody level in samples. Using an appropriate detection method (e.g., colorimetric, chromatographic, etc.), each dilution is tested for the presence of detectable levels of antibody. The assigned titre value is indicative of the last dilution in which the antibody was detected(Chungue et al., 1989).

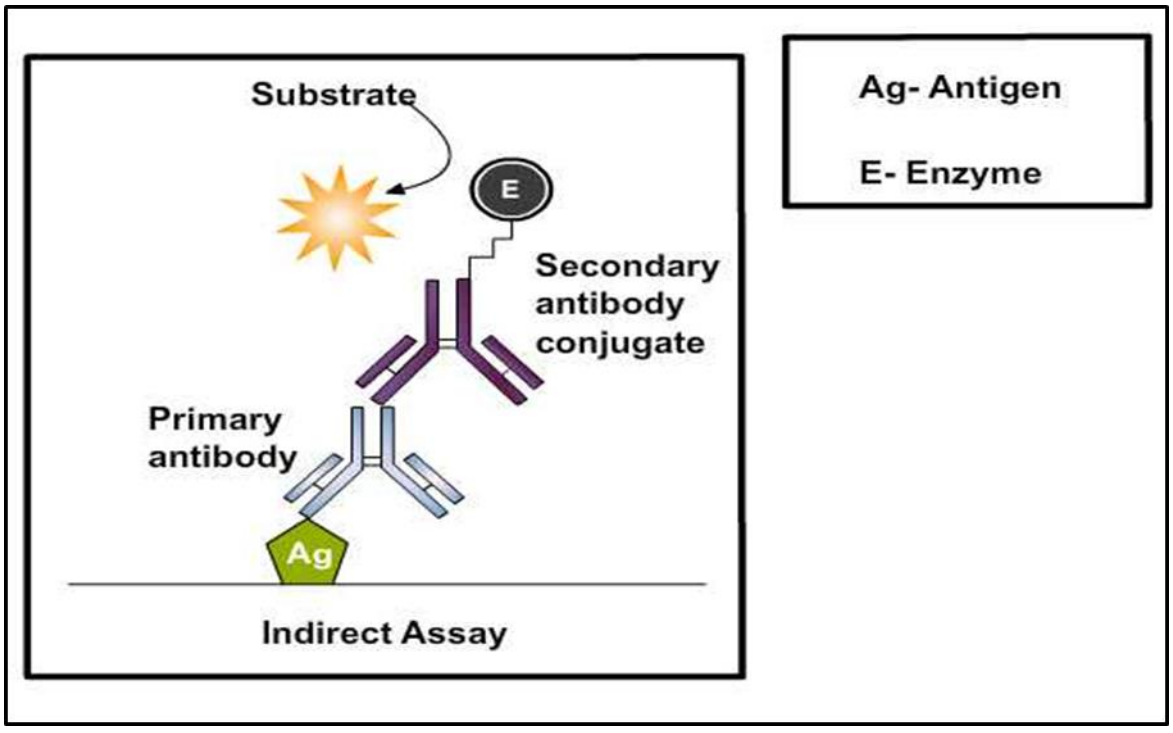

Figure 2. 5 Representation of Dengue indirect ELISA mechanism in diagrammatic version.

The optimum dilution of antigen that might be used as a single dilution to detect and possibly quantify antibodies is best assessed as the dilution (or concentration) that shows

good binding across the whole range of antiserum dilutions. The best way to illustrate this is to draw a graph of the plate data, but this time, plot the dilutions of serum against Use of Indirect ELISA to Titrate Antibodies(Crowther, 1995). The amount of specific antibody in each serum has been titrated over a dilution range. The serum containing the most antibodies will have a higher dilution endpoint (dilution where the OD is the same as the background OD)(N. Gupta et al., 2012, Chungue et al., 1989)

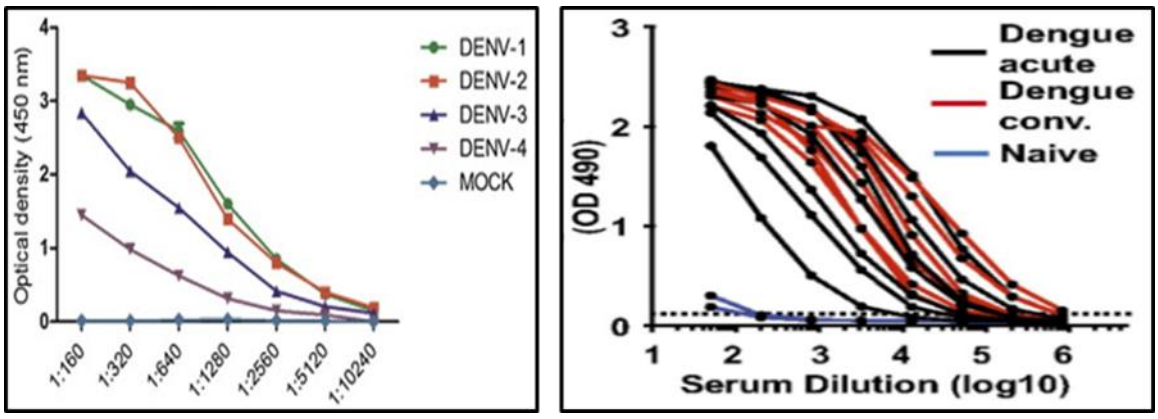

Figure 2. 6 Median endpoint IgG titres for each set of sera are indicated(Priyamvada et al., 2016).

Examination of the serum titration curves for positive and negative sera can tell us which dilution might be suitable to use in the indirect ELISA so that antibodies may be assayed on single wells (or multiple wells using the same dilution). Thus, it was observed that there is low nonspecific activity seen in the negative sera at l/40 and l/80. The positive sera still show high OD values at these dilutions, so that the relative sensitivity of the assay (detection of specific antibodies) can be made at such dilutions(Dowall et al., 2012).No literatures regarding the estimation of the antibody level in dengue samples have been reported from Nepal.

2.6 Molecular Approaches

For the control and prevention of Dengue virus, it is important in a country like Nepal where there is a rapid increase in the burden of dengue and frequent epidemiological transition between serotypes (Gupta et al., 2015). With the advancement in molecular diagnostics over the past two decades, several conventional nested RT-PCR, as well as real-time RT-PCR (rRT-PCR) methods, have been developed for the identification of DENV serotypes (Ahamed et al., 2017). Although rRT-PCR methods have relatively higher sensitivity and lower risk of contamination, conventional RT-PCR methods still serve as a method of choice for DENV surveillance in many laboratories worldwide because of its relatively low cost (Sittivicharpinyo et al., 2017).There are other studies using various RT-PCR protocols which have reported a wide range of DENV detection rates (5% to 84%) among serologically and/or clinical confirmed dengue patients(Ahamed et al., 2017). In

our current study, the modified method CprM654 showed an increased detection rate of 75.2% in dengue serology-positive samples mainly due to increased detection of DENV-2 serotype (21/121). The CprM654 method uses a degenerate reverse primer 'DencomR2 which was designed using a mixture of identical oligonucleotide sequences in which some positions enable binding and amplification of all common viral variants. This is likely to have contributed towards the higher rate of DENV-2 detection seen in our study (Ahamed et al., 2017; Anoop et al., 2010). RT-PCR using degenerate primers can be a sensitive and specific method for the detection of DENV(Klungthong et al., 2007; Ahamed et al., 2017).This phenomenon was also observed in the sequence and phylogenetic analysis of selected strains based on detection approach in which the RT-PCR method using CprM564 alone or in combination with Env641 or CprM511 was able to detect highly divergent DENV strains among all the four serotypes (Livingston et al., 1994). A recent report reviewed the performance differences and advantages of the four most commonly used conventional RT-PCR assays for detecting dengue viral RNA in clinical specimens. Those authors concluded that the heminested protocol using amplimers located at the junction region of the capsid and pre-membrane genes (C-prM) of DENV was the most sensitive method among them.

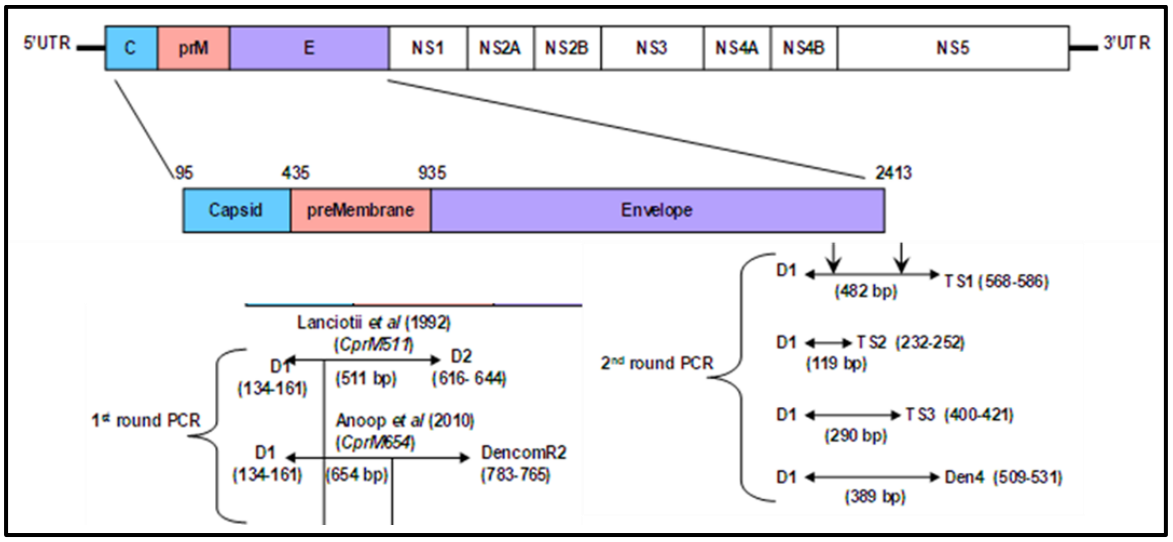

Figure 2. 7 DENV genome organization, gene regions and primer positions used in this study for serotyping

The C-prM protocol utilizes a DENV consensus sequence for outer amplimers D1 and D2 in an initial RT-PCR, followed by a subsequent serotype-specific heminested PCR, combining D1 with one or more of the following serotype-specific internal amplimers: TS1, TS2, TS3, and TS4 (Chien et al., 2006). In spite of this, several authors have reported false-negative PCR results using this protocol due to a mismatch between the dengue viral RNA sequence and the D1, D2, or TS sequence (Chandele et al., 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Site selection and sample collection

Dengue samples were collected from the suspected patients with febrile cases from different parts of Nepal where the cases were seen either or not as an outbreak. Collection sites were the hospitals where the febrile cases with suspect of dengue were reported; especially from especially Damak Hospital-Damak, Namuna Hospital-Hariwan, Bakular Hospital-Chitwan, Chitwan Medical College and Research centre-Chitwan, Universal College of Medical Sciences-Bhairahwa and Sukraraj Tropical Hospital-Teku. These samples were collected mainly in the months of October to December in the Year 2016. Nausea, Vomiting, Headache, Muscle pain and joint pain, Rashes, swelling of the glands and pain behind the eyes (orbital pain) were the common symptoms for the enrolment of the subject. Blood (6-10 ml) was drawn from each patient and serum was isolated from the blood by centrifugation (2500 rpm 10 minutes at ambient temperature) and aliquoted into different cryovials (150 for PCR, 75 µL for ELISA) at -20°C in the local hospital. The samples were transported to Central Department of Biotechnology, Kirtipur Kathmandu maintaining cold chain and stored at -80°c freezer until used All the processing of samples like a centrifuge, serum separation, an aliquot was done maintaining the sterile condition inside the laminar hood and all the discards were disposed of after autoclaving.

Figure 3. 1 Geographical location for the collection of samples.

3.1.1 Sample Size

240 clinically suspected febrile cases were taken as the samples for the study. These samples were the febrile cases visiting different hospitals in Nepal.

3.2 Ethical Approval

The ethical approval was taken from Nepal Health Research Council (NHRC). The samples were collected only after taking the consent of the patients. The consent was taken in the written form. Each record of the samples was taken, and the privacy is maintained for those clinical details.

3.3 Outline of research

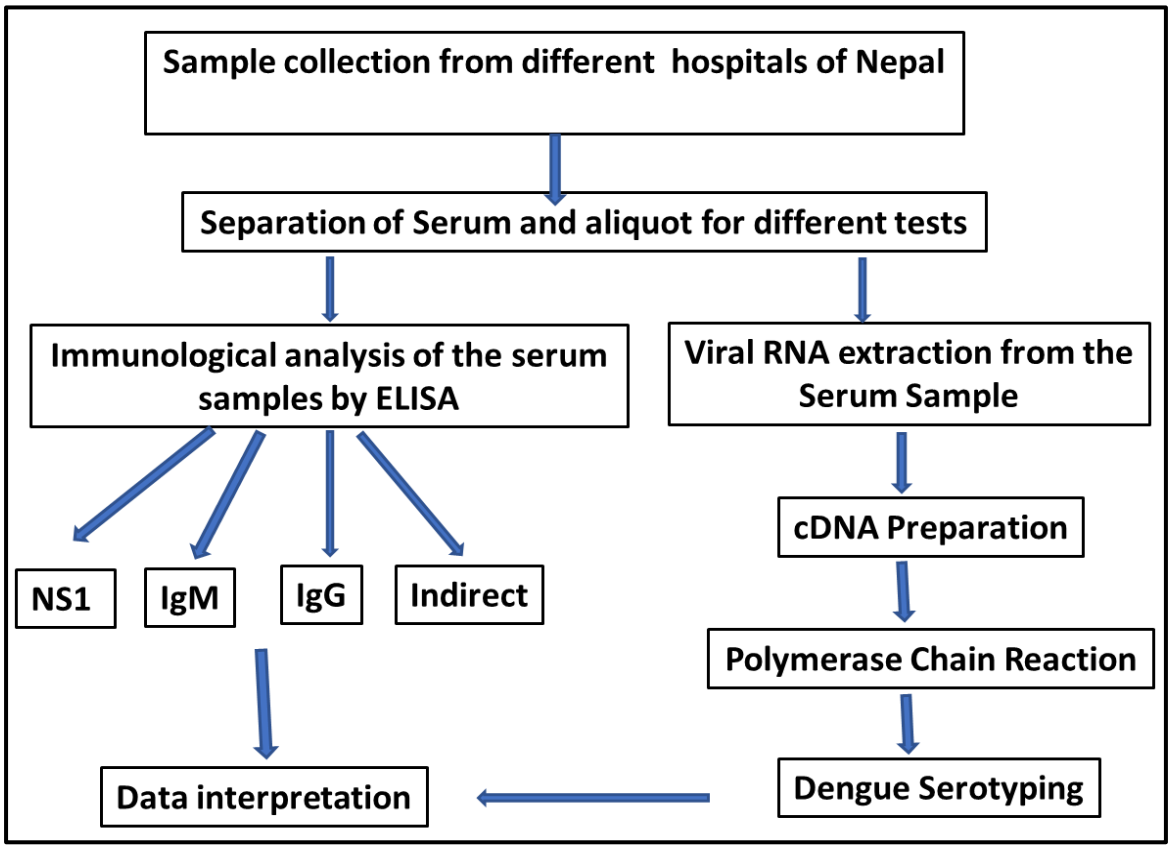

Figure 3. 2 General outline of the research work

3.4 Enzyme-linked Immuno-Sorbent Assay (ELISA)

Humoral immune responses by the titre level of IgG/IgM antibodies and antigen of dengue by NS1 antigen detection in serum were carried in all the suspected 240 samples. ELISA, to detect NS1 Ag in the serum of the suspected patients, were done by Dengue NS1 Ag MICROLISA kit (J. Mitra & Co. Pvt. Ltd, Cat no. EDA081116). Panbio Dengue IgM capture ELISA kit (Cat no.01PE20/01PE21) was used for the IgM test. IgG capture ELISA was done by using Inbios IgG capture ELISA kit (Cat no.01PE10) (3.1.2).

3.4.1.a Reconstitution conjugate and substrate

The conjugate (monoclonal anti-dengue NS1 antibodies linked to Horseradish Peroxidase (HRP) concentrate was diluted in the ratio 1:50 in diluent provided and according to the manufacturer's instruction. For the preparation of working substrate solution, TMB substrate and TMB Diluent were mixed in the ratio 1:1.

3.4.1.b Dengue NS1 Ag using MICROLISA kit

The provided microwells coated with Anti-dengue NS1 antibodies with high reactivity to Dengue NS1 Ag were dispensed with 50µL of Diluent (kit provided). Then, 50µL of positive and negative control were added in order and 50µL of Calibrator was added in triplicate. Samples (50µL) were kept in the remaining wells. After the addition of samples, 100µL of working conjugate solution was added in each well. and thoroughly mixed. The plate was covered with a seal and incubated at 37°c for 90 minutes. The plate was washed six times with working wash buffer (Kit provided 25× and made 1% in distilled water). The working substrate at the rate of 150 µL per well was added to each well and again incubated at 20°*c* -30°c for 30 min in dark. The reaction was stopped by 100µL of stop solution and the absorbance was taken at 450 nm ELISA reader.

The samples were added in the wells followed by addition of enzyme conjugate (monoclonal anti-dengue NS1 antibodies linked to Horseradish Peroxidase (HRP)). A sandwich complex was formed in the well wherein dengue NS1 (from serum sample) was "trapped" or "sandwiched" between the antibody and antibody HRP conjugate. Unbound conjugate was then washed off with wash buffer. The amount of bound peroxidase is proportional to the concentration of dengue NS1 antigen present in the sample. Upon addition of the substrate buffer and chromogen, a blue colour was developed. The intensity of the developed blue colour is proportional to the concentration of dengue NS1 antigen in the sample. To limit the enzyme-substrate reaction, stop solution was added and a yellow colour developed which was finally read at 450nm spectrophotometrically. (Ns & Microlisa, n.d.)

Test Validation

NC O.D must be < 0.3

PC O.D must be > 1.0

Mean calibrator O.D must be ≥ 0.35

Cut off value must be ≥1.5 ×NC O.D

The ratio of PC O.D /cut off must be > 1.1

Calculation of Cut-off Value, Sample OD Ratio and NS1 Ag unit were done as below

OD of Calibrator = Average of Triplicate readings

Calibration Factor = 0.4 (Provided in the kit)

Cut-off Value of the ELISA = Average of Triplicate Readings X Calibration Factor

Sample OD Ratio= (OD of sample)/ (Cut-Off value.

Dengue NS1 Ag units = sample OD ratio × 10.

3.4.2 Dengue IgM Capture ELISA using Panbio kit

First, the antigen was diluted in 1/250 using the Antigen Diluent (Kit provided). The required volume of diluted antigen was taken and mixed with equal volume of MAb tracer in a clean polypropylene tube and the antigen-MAb tracer solution was left at room temperature until required. Within 10 minutes after mixing the MAb Tracer and diluted antigen, 100µl diluted serum sample and controls were pipetted into their respective microwells of the assay plate. The plate was covered and incubated at 37°c for 1 hour. After incubation, the plate was washed six times with wash buffer and 100µl of the antigen-MAb complex from the antigen vial was pipetted to the appropriate wells of the plate. It was again covered and incubated for 1 hour at 37°c.After washing, 100µl of TMB was added and incubated for 10 minutes in the dark at room temperature. The reaction was stopped by 100µl of stop solution and absorbance reading was taken at 450nm.

For the interpretation of results, the average absorption of the triplicates of the calibrator was calculated and multiplied by the calibration factor. This gives the Cut-off value. The Calibration factor is kit specific. (0.80 provided). An index value was calculated by dividing the sample absorbance by Cut-off value calculated. Panbio units = Index Value × 10. Interpretation of results was done finally.

Test Validation

NC O.D must be < 0.400

Cut off value must be ≥1.5 ×NC O.D

The ratio of PC O.D /cut off must be > 1.1

Calculation of Cut-off Value, Sample OD Ratio and IgM Ag unit were done as below

Cut-off OD value

OD of Calibrator = Average of Triplicate readings

Calibration Factor = 0.80 (Provided in the kit)

Cut-off Value of the ELISA = Average of Triplicate Readings X Calibration Factor

Sample OD Ratio= (OD of sample)/ (Cut-Off value.

Dengue IgM Ag units = sample OD ratio × 10.

3.4.3 Panbio Dengue IgG Capture ELISA

For the Dengue IgG Capture ELISA same protocol was followed as for Dengue IgM Capture ELISA. After the absorbance taken, the average absorption of the triplicates of the calibrator was calculated and multiplied by the calibration factor. This gives the Cut-off value. The Calibration factor was kit specific. (0.79 provided). An index value was calculated by dividing the sample absorbance by Cut-off value calculated. Panbio units = Index Value × 10. Interpretation of results was then done.

Test Validation

NC O.D must be < 0.400

Cut off value must be ≥1.5 ×NC O.D

The ratio of PC O.D /cut off must be > 1.1

Calculation of Cut-off Value, Sample OD Ratio and IgG Ag unit were done as below

Cut-off OD value

OD of Calibrator = Average of Triplicate readings

Calibration Factor = 0.79 (Provided in the kit)

Cut-off Value of the ELISA = Average of Triplicate Readings X Calibration Factor

Sample OD Ratio= (OD of sample)/ (Cut-Off value.

Dengue IgG Ag units = sample OD ratio × 10.

3.4.4 IgG Mid-Point Titrations (DENV-2 Indirect ELISA)

Plates were coated with Den-2 antigen (3 µg/ml) Microbix, (100µl/well) in Carbonate-bicarbonate coating buffer(PH-9.2) and incubated at 4°c overnight. Next day, the plates were washed three times with 1× PBS-T (Tween-0.1%). After washing the plates were blocked with 200µl blocking buffer (1×PBS-T Tween-0.05% +10% FBS) and kept in the rocker at room temperature. Plasma dilutions: 1:500, 1:1k, 1:2k, 1:4k, 1:8k, 1:16k, 1:32k, 1:64k, 1:128k, 1:256k, 1:512k, 1:1024k was done. The plates were then again washed three times with wash buffer, 1oo µl IgG-HRP peroxidase (1: 20,000) and incubated for 90 minutes in the rocker. The plates were kept in the last wash and in the meantime OPD (o-phenylenediamine dihydrochloride) substrate solution (4 mg of OPD in 10 ml citrate phosphate buffer, pH 5.0 +10 µl of H_2O_2 added just before use) was prepared and this solution was filtered through 0.22µm syringe filter and 100µl of it was added to the respective wells and incubated in the dark for 5 minutes. After incubation, it was stopped

with 100µl of 2N HCl and the reading was taken in the ELISA plate reader at 490nm wavelength and the results were analysed by Graph pad prism.

3.5 Viral RNA Isolation

Viral RNA was extracted from 140 µL of serum for each sample using QIAamp viral RNA kit using spin protocol (Qiagen, Germany) according to the manufacturer's instructions.

3.5.1 Preparation of reagents

Carrier RNA was added to Buffer AVL. 310 µL of Buffer AVE was added to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µL. The carrier RNA was dissolved thoroughly and divided into required sized aliquots and stored at -20°C. 130 ml of 96-100 % ethanol was added to buffer AW1 and 160 ml to buffer AW2 as indicated in the bottle to make buffer AW1 and AW2 complete.

3.5.2 RNA isolation by using Spin protocol

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

3.6 RNA Quantification by Nanodrop

The quantification of the viral RNA was done by nanodrop reading. 1 µl of the RNA sample was loaded on NanoDrop™ 8000 Spectrophotometer and the value of RNA concentration of the sample in ng/µL was noted.

3.7 cDNA Preparation

cDNA conversion was carried out in a 20 µL reverse transcription (RT) reaction mix containing 5 µL RNA, 5x RT buffer, 10mM dNTP mix (Fermentas), Random Primers (Promega), RNase Inhibitor (1:4) (Fermentas), RevertAid M-MuLV RT (Fermentas) and diethylpyrocarbonate (DEPC) treated water. Here, we used random primers for the RT step, since this allowed us to use the synthesized cDNA for the different RT-PCR methods. The CDNA reaction set up was designed as 5× Buffer-4µl, DNTPS-2µl, RNAse-1µl, DEPC-6µL, Random Hexamer-1µl, Ribolock-1µl and template-5µl. The RT mixture was incubated at 25^0C for 10 min, 42^0C for 60 min followed by 70^0C for 15 min to inactivate the enzyme. The resulting cDNA was used as template for DENV PCR confirmation and serotyping methods.

3.8 PCR Amplification

PCR was performed by using 5x FIREPol® Master Mix contain containing FIREPol® DNA Ploymerase, 5x Reaction Bufffer B, 12.5 mM MgCl2 and 1 mM dNTPs. The PCR Reaction set up was prepared with Mastermix- 4µl, Forward Primer (D1: 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3')- 1µl, Reverse Primer (Den.com.R2: 5-GCNCCTTCDGMNGACATCC-3)- 1µl, NFW-13µL and template-1µl. The PCR amplification was carried out with initial denaturation at 94^0C for 5 min followed by 35 cycles of 94^0C 30 sec, 60^0C for 1 min and 68^0C for 1 min and a final step of 68^0C for 10 min. Dengue-specific universal PCR was done using D1 & DencomR2 primers targeting 654 bp of CprM region.

3.9 Agarose gel electrophoresis and gel documentation

The amplified PCR products were analysed using 2% agarose gel stained with ethidium bromide (0.5µg/ml), run at 100 v and visualized under UV light using a gel documentation system (Biorad).

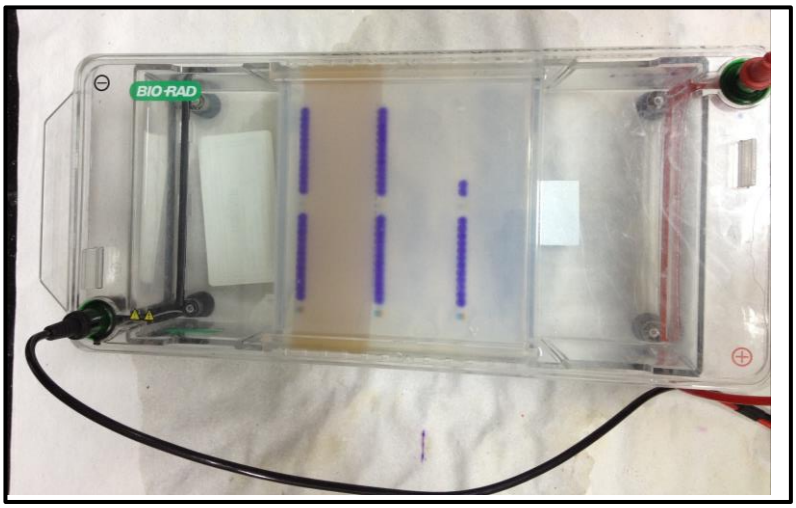

Figure 3. 3 Gel run apparatus and the gel for visualisation under UV transilluminator

3.10 Dengue Serotyping

The serotyping of the Dengue virus was performed only for the PCR positive samples. The serotyping was performed by using the different set of primers. 15 picomoles each of D1 (forward primer) and four DENV serotype-specific reverse primers TS1, TS2, TS3 and Den4 for DENV-1, DENV-2, DENV-3 and DENV-4, respectively were used in serotyping. The PCR reaction was done for 35 cycles under the following conditions: 1 min at 94 ◦C, 1 min at 55 ◦C, and 1 min at 72 ◦C, with a final extension for 10 min at 72 ◦C. Amplified PCR products were then analysed in 2% agarose gel. The DENV serotypes were identified as DENV-1, DENV-2, DENV-3 and DENV-4 based on the band sizes 482 bp, 119 bp, 290 bp and 389 bp, respectively.

Table 1. Showing dengue specific primers for different serotypes.

Second round PCR using D1 & following dengue serotype-specific primers		
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	511 bp
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	482 bp
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	119 bp
TS3	5'-TAACATCATCATGAGACAGAGC-3'	290 bp
Den4	5'-TGTTGTCTTAAACAAGAGAGGTC-3'	389 bp

CHAPTER 4

RESULTS

4.1 Epidemiological analysis of Dengue virus

The distribution of the dengue samples taken for experiment showed that the maximum number of the patients were of the age group 15-45. This might be due to the fact that these population are mostly migratory and are attacked with Dengue in different regions.

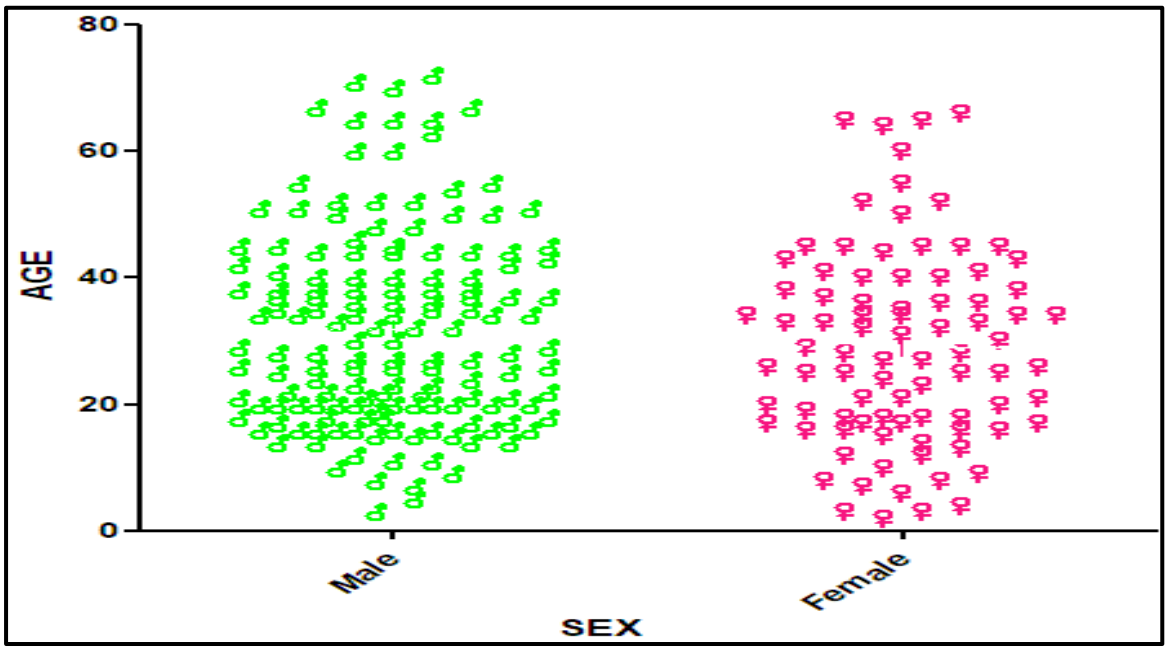

Figure 4. 1 Age and Sex wise representation of samples in Graph Pad Prism.

Out of total 240 samples, there were 153(63.75%) males and 87(36.25%) female patients with Dengue infection. Most of the patients were in the age range of 15-45 as shown in the figure. The average age of all patients was found to be 30. Some of the patients (about 2%) were below the age of 10.

4.2 ELISA

Three different types of ELISA (IgM, IgG, NS1 capture ELISA) performed for all 240 samples and the interpretation of analysed results have been represented in this result section.

4.2.1 Dengue NS1 response of study subjects by MICROLISA

The optical density of the Positive control and negative control along with the calibrator was obtained as 2.531, 0.040 and 0.569 respectively. Also, the cut off value was calculated to be 0.227.

Validation of NS1 Ag ELISA test

Negative control O.D =0.040

Positive Control O.D =2.531

Calibrator mean =0.569

Cut off value for the test =0.227

Table 2 Presentation of the Dengue NS1 Ag MICROLISA data along with OD ratio, NS1 Ag units and sero-status i.e., Positive or Negative.

Sample ID	NS1 OD Value	NS1 OD Ratio	NS1 Ag Unit	Result		Sample ID	NS1 OD Value	NS1 OD Ratio	NS1 Ag Unit	Result
Nep-1	3.8292	16.812	168.124	POSITIVE		Nep-125	3.4045	14.9478	149.478	POSITIVE
Nep-2	3.826	16.798	167.984	POSITIVE		Nep-126	0.0357	0.15674	1.56744	NEGATIVE
Nep-3	3.6691	16.11	161.095	POSITIVE		Nep-127	0.0578	0.25378	2.53776	NEGATIVE
Nep-4	3.7121	16.298	162.983	POSITIVE		Nep-128	0.0667	0.29285	2.92852	NEGATIVE
Nep-5	3.9528	17.355	173.551	POSITIVE		Nep-129	0.0459	0.20153	2.01528	NEGATIVE
Nep-6	3.9295	17.253	172.528	POSITIVE		Nep-130	0.6041	2.65235	26.5235	POSITIVE
Nep-8	4	17.562	175.623	POSITIVE		Nep-131	3.8258	16.7975	167.975	POSITIVE
Nep-9	3.8435	16.875	168.752	POSITIVE		Nep-132	0.0492	0.21602	2.16017	NEGATIVE
Nep-10	3.8681	16.983	169.832	POSITIVE		Nep-133	0.0426	0.18704	1.87039	NEGATIVE

Out of 240 samples, 142 samples were NS1 positive (NS1 Ag Units > 11), 97 samples were NS1 negative (NS1 Ag Units < 9) and only one sample was found equivocal (NS1 Antigen units between 9 – 11). The equivocal sample was further confirmed with other ELISA results and by PCR. The positive control OD, Negative control OD and Calibrator mean OD were in the range provided by Kit. Hence our all values were valid. There were 112 samples that showed the NS1 units greater than 100. Hence, they are supposed to be strongly NS1 positive samples. The data were presented in the excel sheet and analysed as shown in the appendix (table 1).

4.2.2 Dengue IgM response of study subjects by Panbio Capture ELISA

Validation of NS1 Ag ELISA test

Negative control O.D =0.072

Positive Control O.D =1.269

Calibrator mean =0.388

Cut off value for the test =0.310

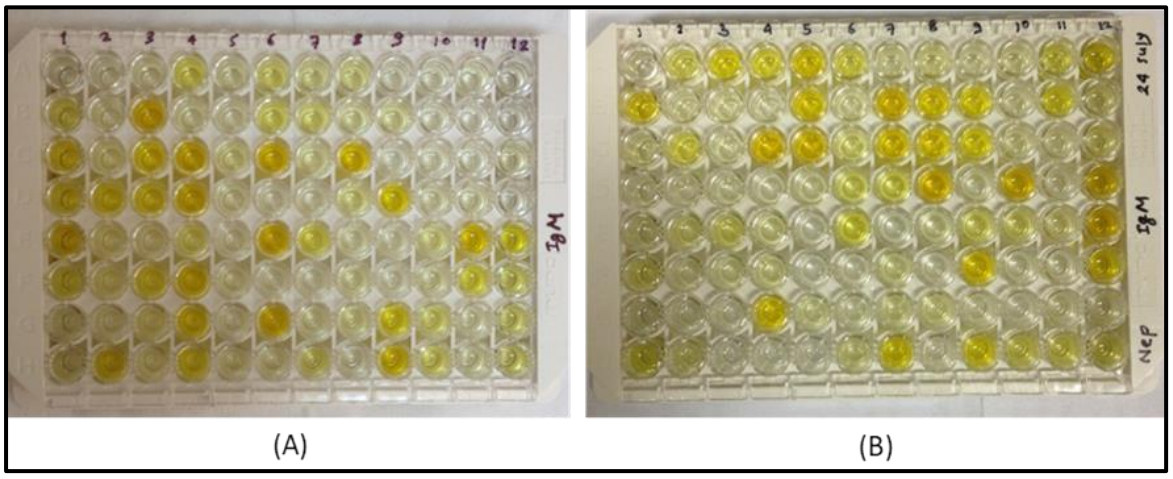

Figure 4. 2 Yellow colour developed after the addition of stop solution.

Yellow colour was developed after the addition of stop solution in some wells while the rest of them were not. The yellow coloured wells were supposed to have positive samples in them. The difference in intensity in the colour might be due to the level of dengue antibody in the samples.

Table 3 Presentation of the Panbio Dengue IgM Capture ELISA data along with OD ratio, IgM Index Values and sero-status i.e., Positive or Negative.

Sample ID	IgM OD Value	IgM Index Value	PanBio Units	IgM Sero Status	Sample ID	IgM OD Value	IgM Index Value	PanBio Units	IgM Sero Status
Nep-1	0.09	0.304	3.042	NEGATIVE	Nep-125	0.42	1.464	14.637	POSITIVE
Nep-2	0.47	1.511	15.11	POSITIVE	Nep-126	0.071	0.246	2.4633	NEGATIVE
Nep-3	1.61	5.175	51.75	POSITIVE	Nep-127	0.092	0.32	3.2019	NEGATIVE
Nep-4	0.9	2.908	29.08	POSITIVE	Nep-128	0.159	0.555	5.5468	NEGATIVE
Nep-5	2.09	6.729	67.29	POSITIVE	Nep-129	0.09	0.315	3.1462	NEGATIVE
Nep-6	0.57	1.828	18.28	POSITIVE	Nep-130	0.245	0.853	8.5292	NEGATIVE
Nep-8	0.14	0.454	4.542	NEGATIVE	Nep-131	0.427	1.487	14.867	POSITIVE
Nep-9	0.18	0.595	5.952	NEGATIVE	Nep-132	0.164	0.572	5.7245	NEGATIVE
Nep-10	0.18	0.573	5.733	NEGATIVE	Nep-133	0.133	0.462	4.6165	NEGATIVE

After the calculation of IgM index value, we found 78 out of 240 samples were Panbio Dengue IgM Capture ELISA Positive. The index value above1.1 were considered as positive. The index value below 0.9 was considered as negative and between 0.9-1.1 were considered as equivocal. 9 samples fall into the category of equivocal and the remaining

153 samples were calculated as negative. All the control values were in the range provided by the kit, so, our test was valid. The equivocal samples were further confirmed by PCR.

4.2.3 Dengue IgG response of study subjects by Panbio Capture ELISA

Validation of IgG Ag ELISA test

Negative control O.D =0.048

Positive Control O.D =1.316

Calibrator mean =0.597

Cut off value for the test =0.471

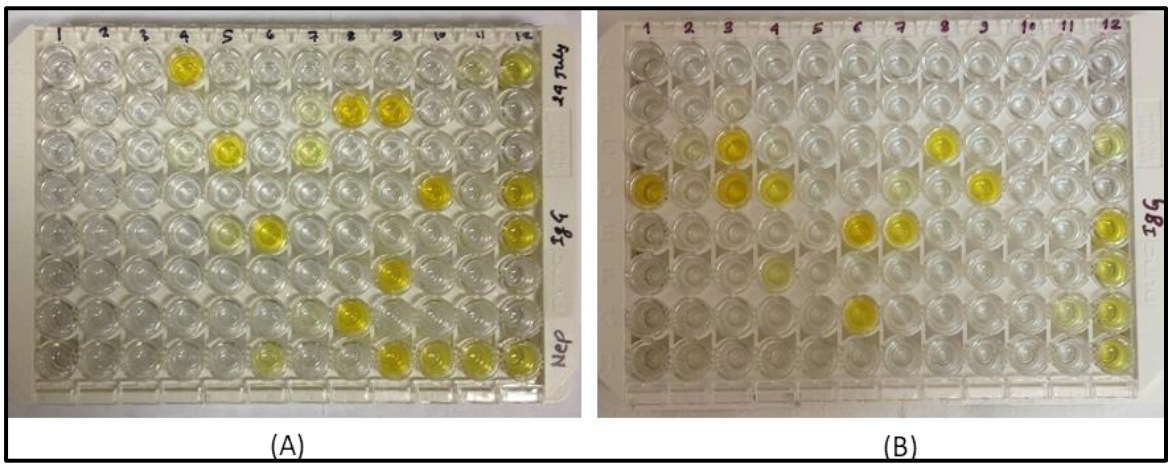

Figure 4. 3 Development of yellow colour after the addition of stop solution. The yellow coloured wells showing the positive cases of Dengue IgG test while the others are negative.

Yellow colour was developed after the addition of the stop solution. The intensity of the yellow colour showed the level of positivity while the non-coloured parts showed the negative regions fir IgG capture ELISA.

Table 4 Presentation of the Panbio Dengue IgG Capture ELISA data along with OD ratio, IgG Index Values and sero-status i.e., Positive or Negative.

Sample ID	IgG OD Value	IgG Index Value	PanBio Units	IgG Sero Status		Sample ID	IgG OD Value	IgG Index Value	PanBio Units	IgG Sero Status
Nep-1	0.0522	0.1106	1.1062	NEGATIVE		Nep-125	1.41	3.90407	39.041	POSITIVE
Nep-2	0.0486	0.103	1.0299	NEGATIVE		Nep-126	0.0486	0.13457	1.3457	NEGATIVE
Nep-3	0.1668	0.3535	3.5347	NEGATIVE		Nep-127	0.0619	0.17139	1.7139	NEGATIVE
Nep-4	1.9426	4.1166	41.166	POSITIVE		Nep-128	0.0504	0.13955	1.3955	NEGATIVE
Nep-5	0.0506	0.1072	1.0723	NEGATIVE		Nep-129	0.0474	0.13124	1.3124	NEGATIVE
Nep-6	0.0553	0.1172	1.1719	NEGATIVE		Nep-130	0.2103	0.58229	5.8229	NEGATIVE
Nep-8	0.0536	0.1136	1.1358	NEGATIVE		Nep-131	0.049	0.13567	1.3567	NEGATIVE
Nep-9	0.0486	0.103	1.0299	NEGATIVE		Nep-132	0.0478	0.13235	1.3235	NEGATIVE
Nep-10	0.0578	0.1225	1.2249	NEGATIVE		Nep-133	0.8718	2.41388	24.139	POSITIVE

From the IgG Capture ELISA test, we found only 19 samples to be positive, only three samples were equivocal and remaining 218 samples were found to be negative. Only a few samples from 240 samples turned out to be positive. All the test controls were in the range as provided by the kit. Hence our test was valid.

Table 5 Plot showing the control values for ELISA test along with the sample tests.

TEST	NS1		IgM		IgG	
Status	Standard	Sample(240)	Standard	Sample(240)	Standard	Sample(240)
Positive	> 11	142(59.16%)	>1.1	78(32.5%)	> 2.2	19(7.91%)
Negative	< 9	97(40.41%)	<0.9	153(67.75%)	< 1.8	218(90.8%)
Equivocal	9 - 11	1(0.41%)	0.9-1.1	9(3.75%)	1.8 - 2.2	3(1.25%)

As shown in the table above, 59.16% of the samples were NS1 positive, 32.5% IgM positive and 7.19 % IgG positive. Very few samples were Equivocal.

4.3 Antibody level detection of study subjects by IgG Mid-Point Titrations (DEN -2 Indirect ELISA)

Half of the maximum OD value of all samples calculated using Microsoft Excel and Graph-Pad software showed the titre value at what specific dilutions the sample gave.

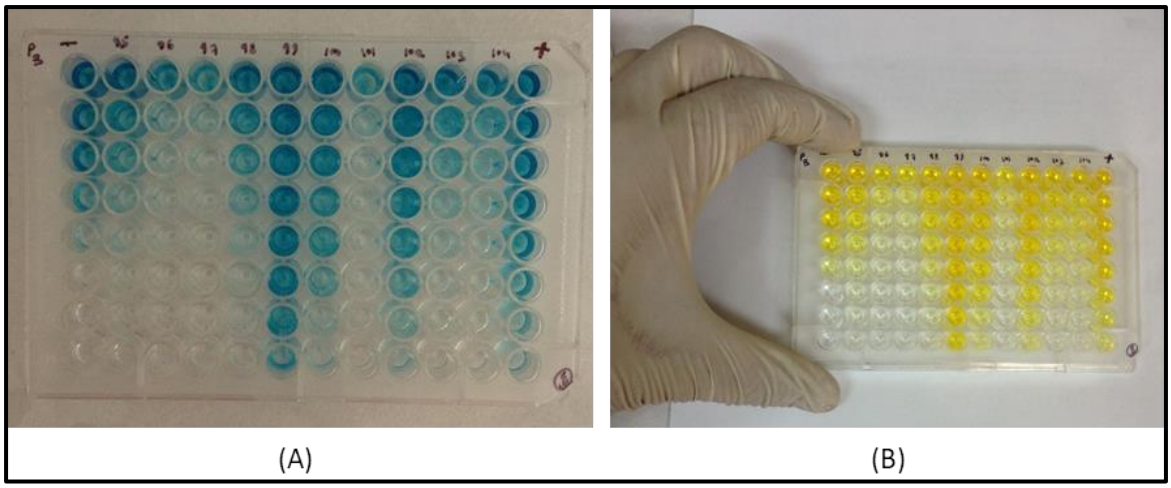

Figure 4. 4 (A) Development of green colour after the addition of substrate, (B) yellow colour after the addition of Stop solution.

The green colour was developed after the addition of the TMB and the wells which showed the intense colour was the positive samples having higher antibody level and the intensity decreased as we go down as the wells were done with serial dilutions method.

All the possible mid-point values calculated are shown in the table in the appendices.

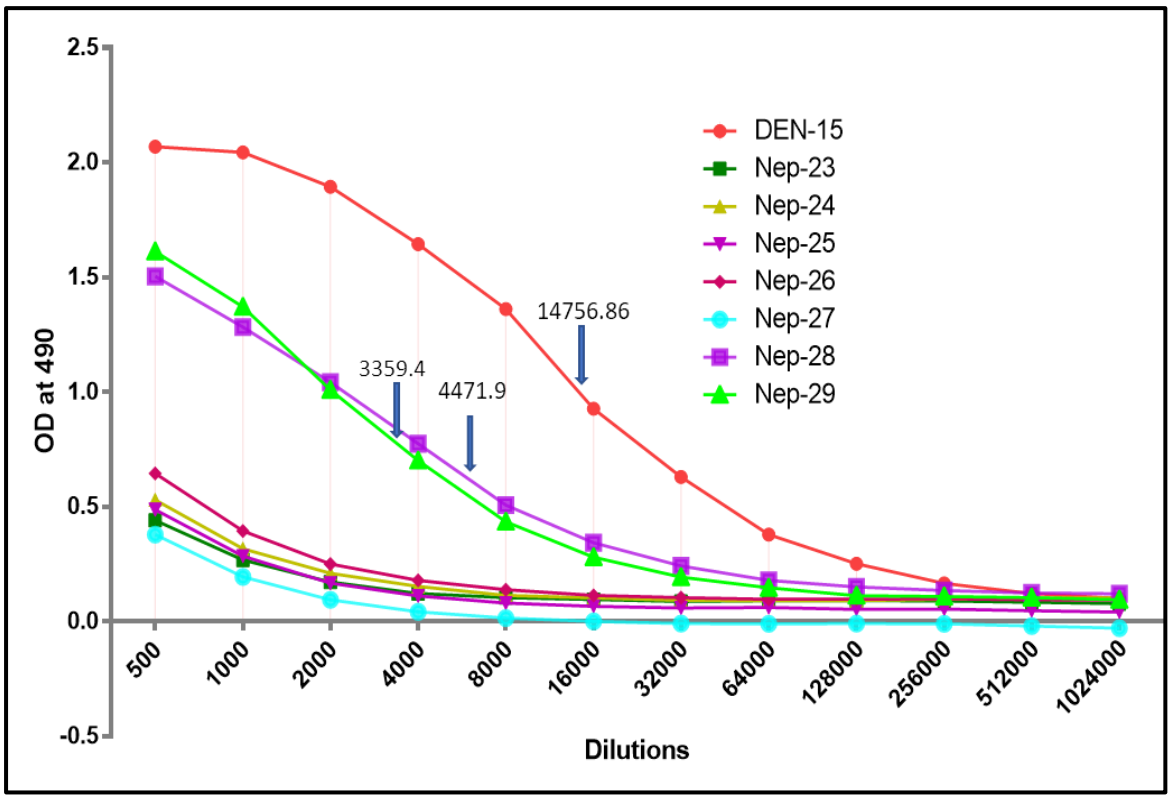

Figure 4. 5 Graphical representation of mid-point titrations of eight Samples along with the positive control.

The dilutions are presented in the x-axis and the OD value at 490 nm on Y-axis. Each dot in the figure shows the dilutions. The dilutions were of two-fold up to 12 dilutions. The red-bold curve at the uppermost region is the Positive control and other curves are the curves of the samples. Each point in the graph shows the dilution factor. DEN-15 (14756.86) is the positive control as shown in the graph above. Nep-28 and Nep-29 were found to have high level of antibody titre as 4471.9 and 3359.4 respectively. Similarly, Nep-23, Nep-24, Nep-25, Nep-26 and Nep-27 were found to have low level antibody in them. They all have the antibody level in the range of 1500. The high value of antibody titre means that the serum can work effectively for neutralisation tests even at higher dilutions. The lower the value, lower is the range of dilutions up to which they can show their effect of neutralisation.

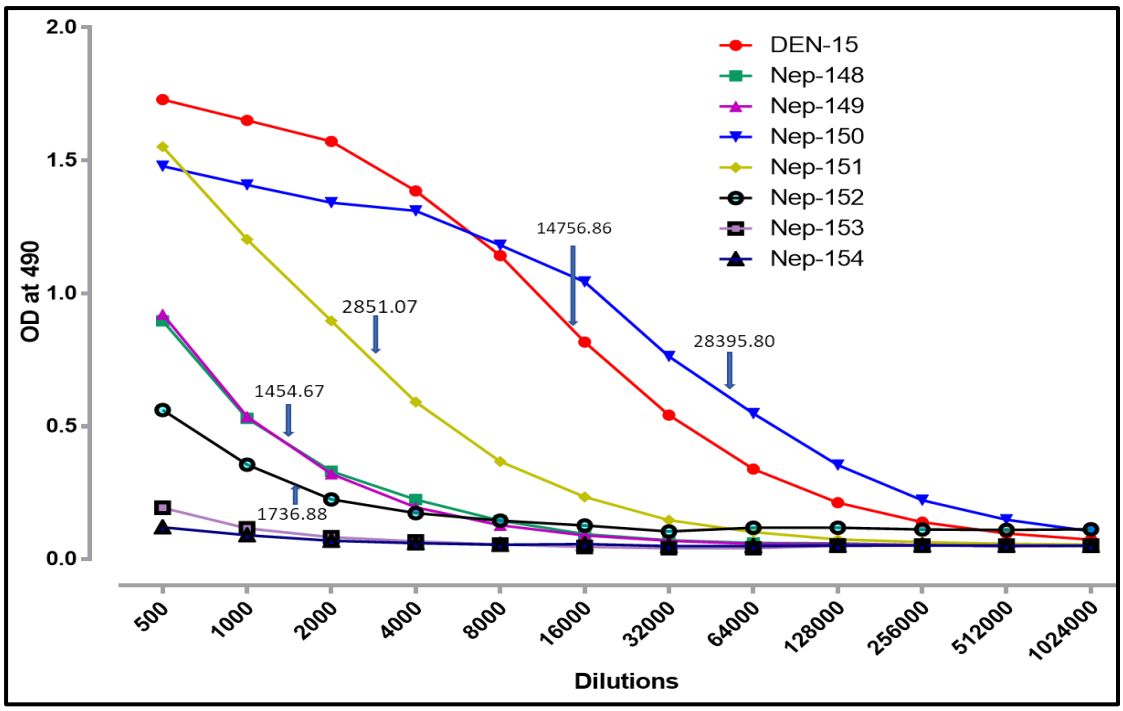

Figure 4. 6 Graphical representation of mid-point titrations of different samples.

The dilutions are presented in the x-axis and the OD value at 490 nm on Y-axis. Each dot in the figure shows the dilutions. The red-bold curve at the uppermost region is a Positive control and other curves are the curves of the samples.

From the graph above, Nep-150 was found to have high antibody level (28935.80) while Nep-151 had medium level of antibody. Nep-148 and Nep-149 had nearly equal amount of antibody level as shown in the figure 4.6. Remaining all had low level of antibody as shown above.

Similarly, estimation of antibody level in all 240 samples showed that 50 samples had mid-point titre value greater than 2000. Nep-198 had the highest level of antibody which value was found to be 43228.70. Nep-64 is the sample having the lowest antibody level of 953.53.

Table 6 Classification of samples according to the different range of titre values.

Range of titre values	Number of samples
High(>5,000)	15
Medium(2,000-5,000)	35
Low(<2000)	176

Out of 240 samples, few samples did not give the titre values. This might be due to the low level of antigen to detect. Besides that, 15 samples (Nep-

4,21,31,46,48,99,123,125,150,158,179,191,198,199,216) showed the high values for antibody level. Thirty-five samples showed the minimum antibody level while 176 showed the low level of antibody for Dengue IgG in them as shown in the table. The calculation of the antibody level for all samples were made and were presented in the graph and table as shown in the appendices.

4.4 Viral RNA quantification

Viral RNA successfully isolated from all 240 samples from 140 µl of the serum sample were of high-quality which were eluted in a special RNAse- free buffer. This confirmation was done by nanodrop reading.

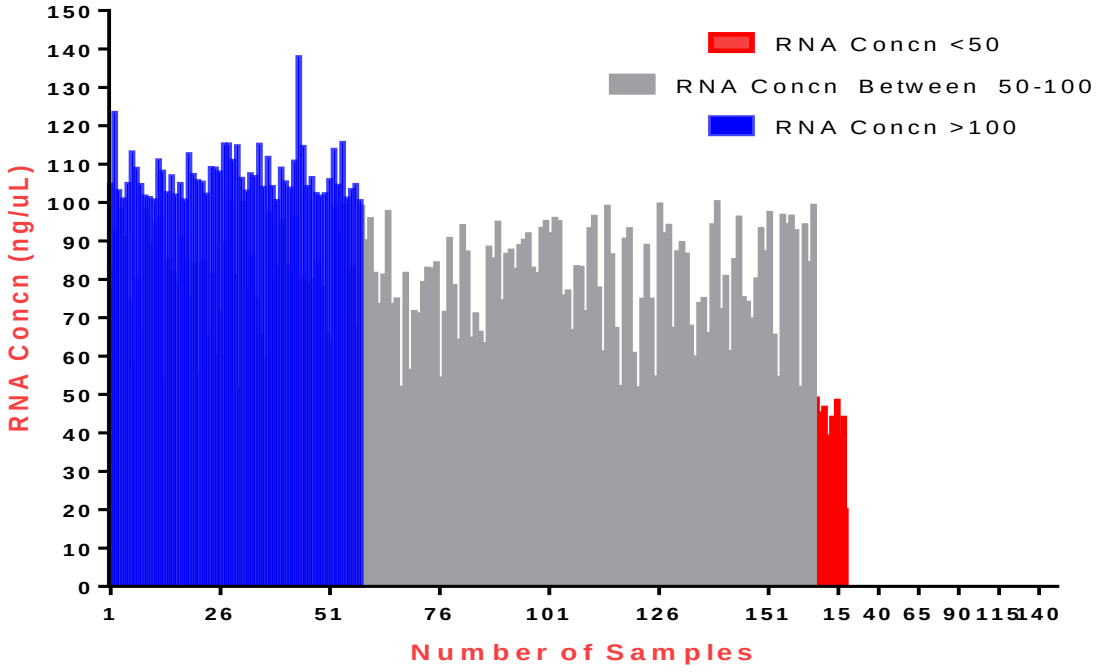

Figure 4. 7 Graphical representation of Nanodrop reading for all samples.

Hence, the quantification of RNA was done only by nanodrop readings using the only 1µl of the isolated RNA. The quantified RNA value is shown in the table in the appendix. The Nanodrop reading was taken in the unit ng/µl. The highest nanodrop reading was found to be 137.8 ng/µL and the lowest was 6.3ng/µL. There were only 19 samples whose reading was less than 50ng/µL and in between 50-100ng/µL, there were 162 samples. Greater than 100ng/µL of RNA concentration, there were 59 samples altogether. The isolated RNA was made cDNA and finally, PCR was done.

4.5 cDNA of Dengue Virus

cDNA prepared for all 240 samples using the Revert Aid M-MuLV RT (Fermentas) Kit maintaining equal concentration of RNA gave the better PCR results. 50ng/µl

concentration of RNA was maintained for all samples and cDNA was prepared. 20µl of cDNA was prepared and stored for PCR reaction.

4.6 Amplification of the Virus by PCR

PCR was done for all samples using Dengue specific universal primer D1(5-TCAATATGCTGAAACGCGCGAGAAACCG-3) and DencomR2(5-GCNCCTTCDGMNGACATCC-3). The expected size of the primer set is 654bp. Total number of 86 samples were found to be PCR positive using the DENV specific primers for C-prM region. 35.83% of the whole samples were PCR positive for Dengue virus.

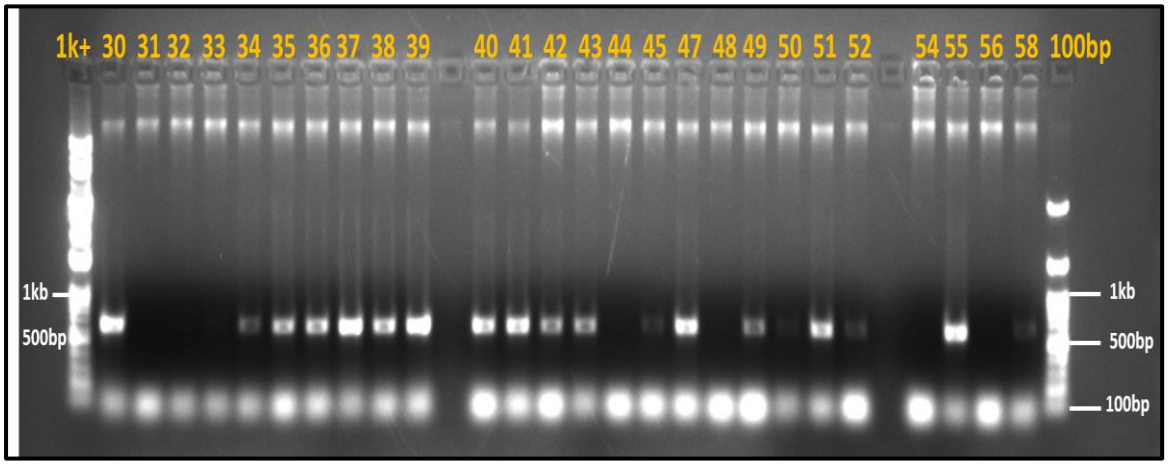

Figure 4. 8 Agarose gel (2%) showing PCR amplification of Dengue virus isolated from patient's serum.

The first lanes on the left side contain the 1k+ ladder and samples simultaneously up to the final band on the extreme right of 100kb DNA ladder. The samples which are showing the white band at the level of 654bp are PCR positive and the rest are negative. Sample number Nep-30, 34, 35, 36, 37,38 ,39, 40, 41, 42, 43, 45, 47, 49, 50, 51, 52, 55, 58 are positive and the rest are negative.

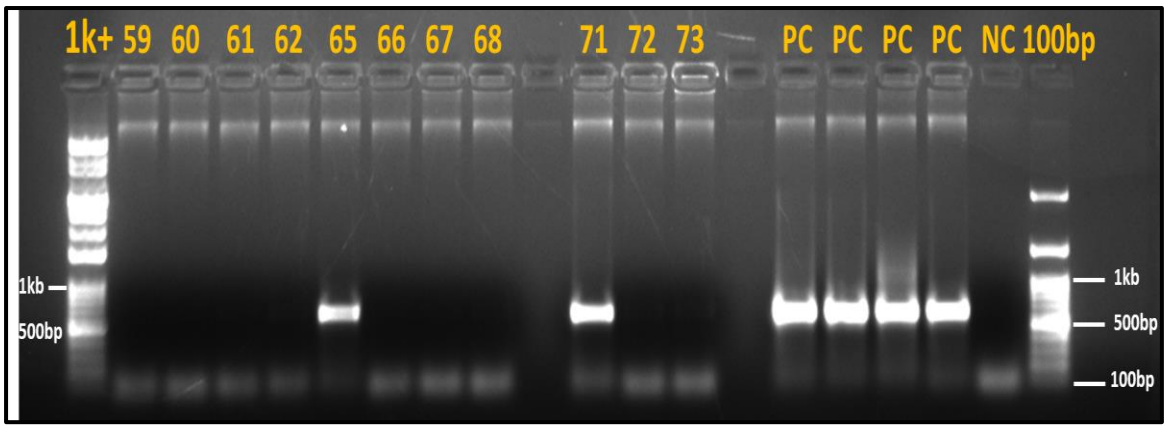

Figure 4. 9 Agarose gel (2%) showing PCR amplification of Dengue virus isolated from patient's serum.

The first lanes on the left side contain the 1k+ ladder and samples in between and a band on the extreme right of 100kb DNA ladder. Four positive control and one negative control are included and marked as PC for positive control and NC as a negative control in the latter wells. The samples which are showing the white band at the level of 654bp are supposed to be PCR positive and the rest are negative. Sample no 65 and 71 are PCR positive and the rest are negative.

Similarly, out of total 240 samples, 86 samples were found to be PCR positive for Dengue virus and all the results are shown in the appendices.

4.7 Correlation between NS1, IgM, IgG and PCR results

The representation of NS1, IgM, IgG results along with PCR confirmation is shown below.

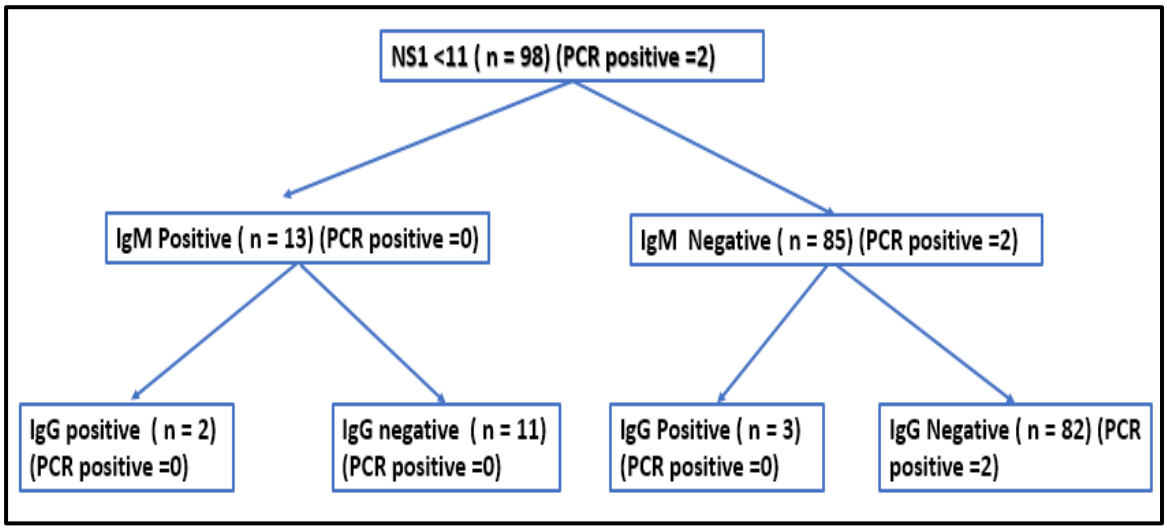

Figure 4. 10 Numerical representation of IgM, IgG and NS1 results.

From the study, it was found that 98 samples were below 11 NS1 units and considered as negative. From the NS1 negative samples, two samples were found to be PCR positive.There were 13 IgM positive and 85 negative samples. Of those 13 IgM positives, two were IgG positive and 11 were IgG negative. On the other side, out of 85 IgM negatives, 3 were IgG positive and 82 were IgG negative. Two PCR positives had come from both IgM and IgG negative samples.

While calculating the Dengue NS1 units in the increasing order and the compared with IgM and IgG values showed the variation in the positive and negative cases.

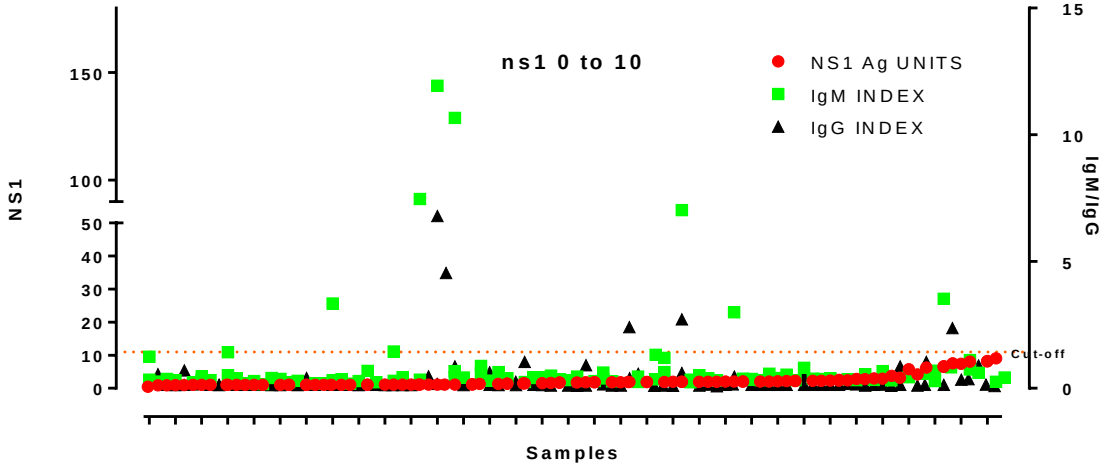

Figure 4. 11 Comparison of Dengue IgM and IgG capture ELISA Indexes with the NS1 Ag units in the increasing order.

The NS1 units below 10 are considered as negative.The red dots represent the NS1 Ag units, black dots represent the IgG Index values and green dots represent the IgM Index. Each dot in the graph represents the sample tested.The left bar represents the NS1 units and the right bar represent the IgG and IgM units.

Very few samples showed IgM and IgG positive for NS1 index between 0-10. Only 13 samples were IgM positive for NS1 negative and 11 samples were IgG positive.

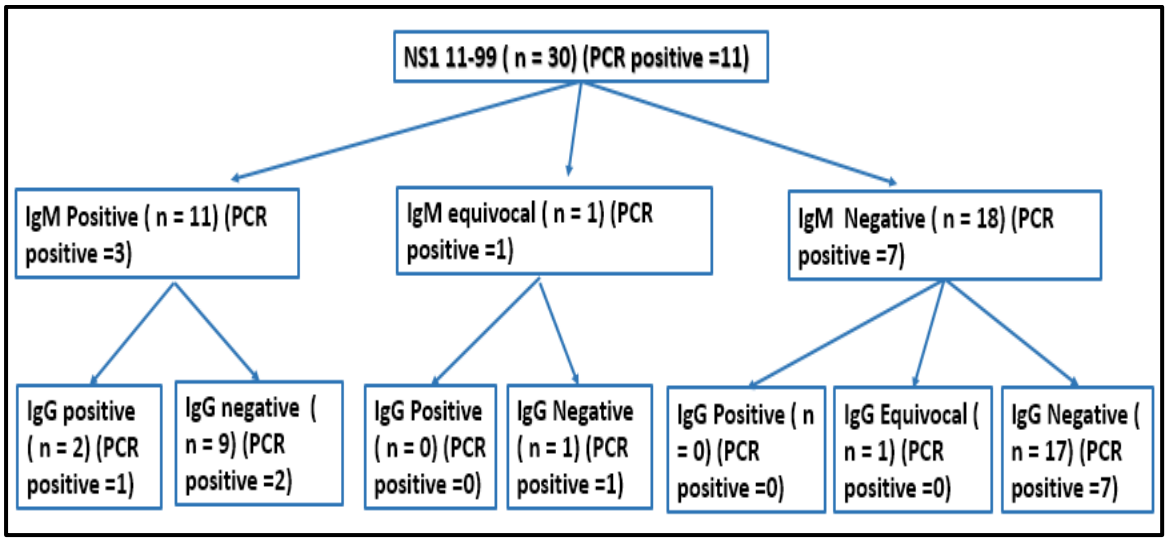

Figure 4. 12 Numerical representation of IgM, IgG and NS1results.

30 samples were in between 11-99 NS1 units and considered as positive. From the NS1 positives of range 11-99 samples, 11 samples were found to be PCR positive.There were 11 IgM positive and 18 negative samples and 1 equivocal. Of those 11 IgM positives, two were IgG positive and 9 were IgG negative. On the other side, out of 18 IgM negatives, 0 were IgG positive 1 equivocal and 17 were IgG negative. From 1 IgM equivocal sample, it was found to be IgG negative but PCR positive.

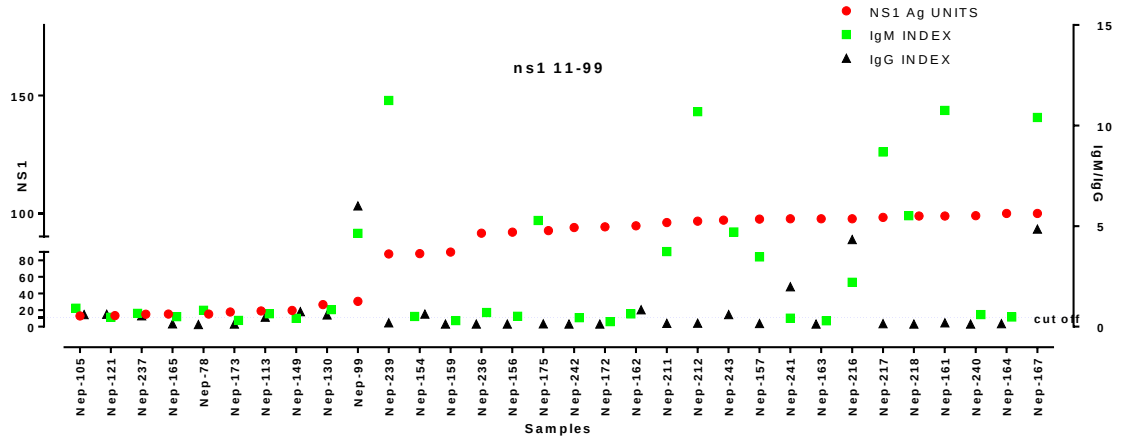

Figure 4. 13 Comparison of Dengue IgM and IgG capture ELISA Indexes with the NS1 Ag units in the increasing order.

The red dots represent the NS1 Ag units, black dots represent the IgG Index values and green dots represent the IgM Index. Each dot in the graph represents the sample tested.The left bar represents the NS1 units and the right bar represent the IgG and IgM units.

The NS1 units above 11 were considered positive.But, the results obtained for the values between 11-100 and above 100 were compared. This was done as the positive cases for NS1 greater than 100 units gave the better PCR results as compared to other. Many samples were IgM Positive in comparison to IgG. It was found that 6 samples to have IgM index values greater than NS1 units and only one sample had index value equal to NS1 units.

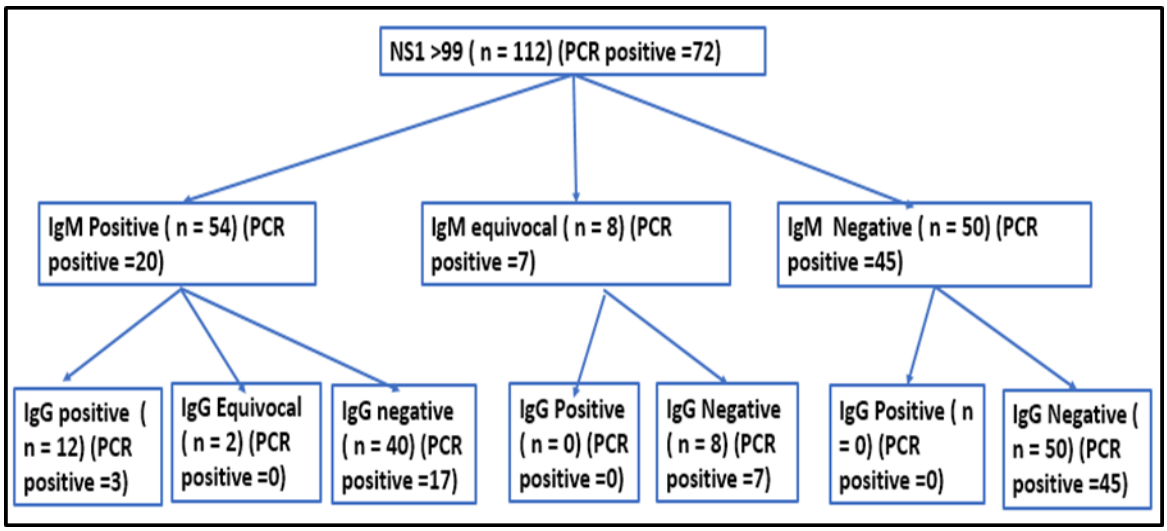

Figure 4. 14 Numerical representation of IgM, IgG and NS1results.

Altogether, 112 samples were above 99 NS1 units and considered as positive. From the NS1 positive samples, 72 samples were found to be PCR positive.There were 54 IgM

positive, 50 negative and 8 equivocal samples. Of those 54 IgM positives, 12 were IgG positive and 40 were IgG negative. Also, 2 samples were found Equivocal. On the other side, out of 50 IgM negatives, 0 IgG positive and 50 samples were IgG negative. Out of 8 IgM equivocal samples, all 8 were IgG negatives but 7 of them were PCR positive.

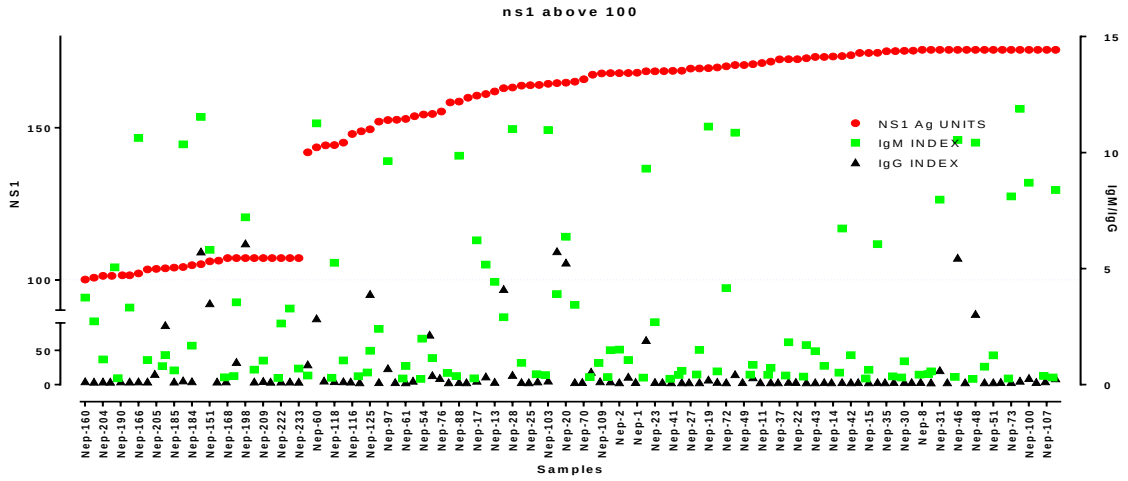

Figure 4. 15 Comparison of Dengue IgM and IgG capture ELISA Indexes with the NS1 Ag units in the increasing order.

The NS1 units above 100 are presented in the graph.The red dots represent the NS1 Ag units, black dots represent the IgG Index values and green dots represent the IgM Index. Each dot in the graph represents the sample tested.The left bar represents the NS1 units and the right bar represent the IgG/IgM units.

Many IgM values were found scattered over the plot as compared to IgG values.when the NS1 units are larger, there are many IgM positive cases but the IgG values showing positive results are very low.

Venn-diagram showing the relation between the IgM, IgG and NS1 positive samples.

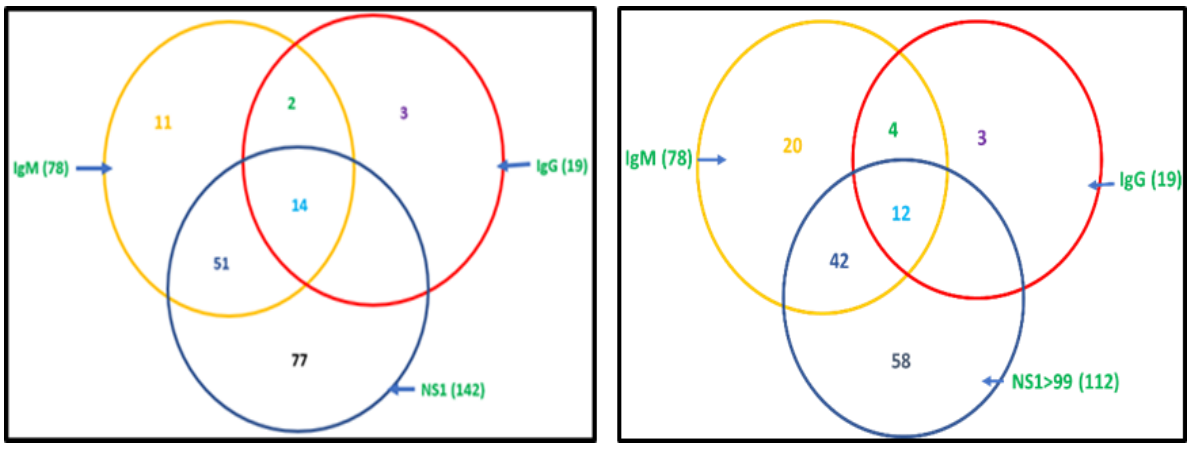

Figure 4. 16 Venn-diagram showing the relation between the IgM, IgG and NS1 positive samples.

The NS1 unit below 100 showed 142 NS1 positive samples. 14 samples showed all IgM, IgG and NS1 positive. 16 samples were both IgG and IgM positive. 65 samples were both IgM and NS1 positive. 14 samples were both IgG and NS1 positive.

The NS1 unit greater than 99 shows 112 NS1 positive samples. 12 samples were showing all IgM, IgG and NS1 positive.16 samples were both IgG and IgM positive. 54 samples were both IgM and NS1 positive. 12 samples were both IgG and NS1 positive.

Table 7 Table shows the summary of the comparison of all data with the IgM, IgG and NS1 units and their possible combinations.

SN	TESTS (n=240)	POSITIVE	NEGATIVE	EQUIVOCAL
1	IgM	78	153	9
2	IgG	19	218	3
3	NS1	142	97	1
4	IgM + NS1	65	175	-
5	IgG + NS1	14	226	-
6	IgM +IgG +NS1	14	226	-
7	IgM +IgG	16	224	-

There are different numerical values that show different combinations of all three IgM, IgG and NS1 values. There were altogether 14 samples that showed all three tests positive.78 samples showed IgM positive while 19 showed IgG positive.142 samples were NS1 positive. 9 samples were equivocal for IgM test, 3 samples were for IgG and 1 sample was equivocal for NS1. These samples were further confirmed by PCR.

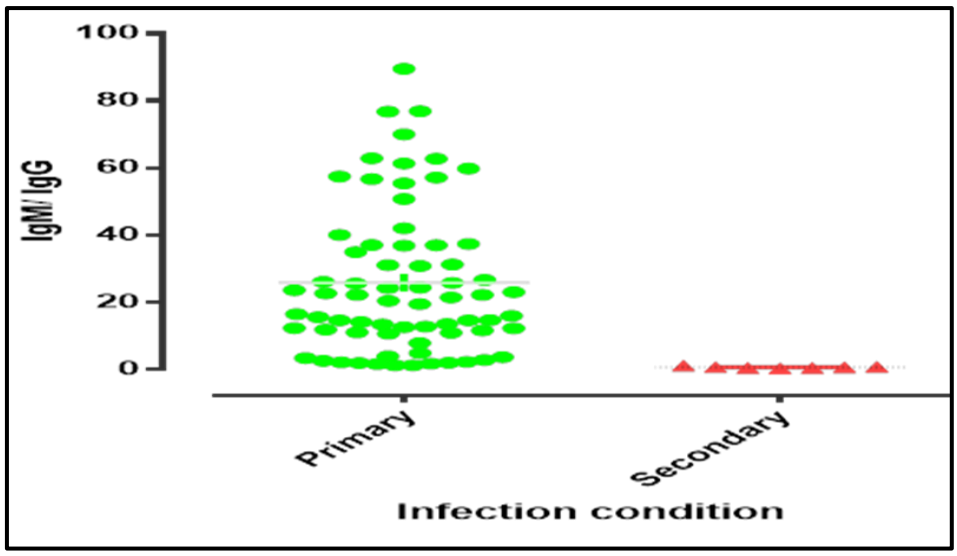

Figure 4. 17 Showing the comparison of primary Dengue infection with the secondary infections.

The above graph shows the IgM and IgG number on the left Y-axis while primary or secondary infection condition on the X-axis.

Only 7 samples were of the secondary infection while 70 samples are of Primary Dengue infection. The primary and secondary infection was calculated based on the ratio between IgM and IgG.

Table 8 Distribution of samples according to different age groups along with the sero status and PCR results.

Age group(Yrs)	Total Number	NS1 Positive	IgM Positive	IgG Positive	PCR Positive
0-10	15(6.25)	5(3.52)	6(7.69)	2(10.52)	3(3.48)
11-20	60(25)	33(23.23)	23(29.48)	6(31.57)	17(19.76)
21-30	50(20.83)	31(21.83)	12(15.38)	1(5.26)	21(24.41)
31-40	56(23.33)	37(26.05)	18(23.07)	6(31.57)	26(30.230
41-50	31(12.91)	23(16.19)	13(16.66)	3(15.78)	10(11.62)
51-60	15(6.25)	8(5.63)	4(5.12)	0(0)	7(8.13)
>60	13(5.41)	5(3.52)	2(2.56)	1(5.26)	2(2.32)
Total	240(100%)	142(100%)	78(100%)	19(100%)	86(100%)

Table: Comparison of platelets count of the dengue patients with the ELISA results and PCR.

While the samples were presented according to different age groups along with the ELISA and molecular results, it was found that the age group between 31-40 had the larger number of samples and the NS1, IgM and IgG along with the PCR results were higher in that age group. 6.25% of the samples were below the age of 10 and 5.41% were above 60 years.

Table 9 Representation of test results with the platelets count

Sero Status	Platelets count less than 50,000	Platelets count 50,000 - 100000/ml	Platelets count 100000 -200000/ml	Platelets count > than 2000000/ml	Total
NS1 Positive	9(0.64%)	69(49.28%)	52(37.14%)	10(7.14%)	140
IgM Positive	5(6.75%)	35(47.29%)	29(39.18%)	5(6.75%)	74
IgG Positive	2(10.52%)	10(52.63%)	6(31.75%)	1(5.26%)	19
PCR Positive	6(6.9%)	44(51.16%)	29(33.72%)	7(8.13%)	86
Total	17(7.45%)	103(45.17%)	85(37.28%)	23(10.08%)	228

The count in the platelets number versus tests performed showed that most of the patients having the platelets count in the range of 50,000- 1 lakh per ml were PCR positive (51.16 %) out of 103 patients. The number of patients were also more in this platelet count range.

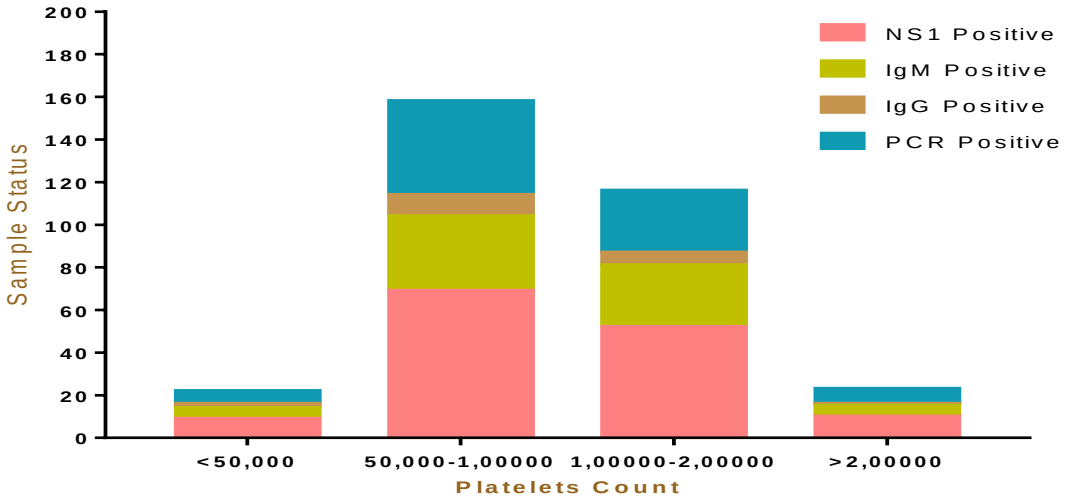

Figure 4. 18 Graphical representation of the platelets counts of the patients versus test results.

From the graph, it was calculated that higher is the platelets count, lower is the chance to have positive sample. In the range of Platelets count between 50,000 and 1,00000, there were the maximum number of platelets count. Similarly, below 50,000 of platelets count, there were less sample and lesser number of positive cases.

4.8 Dengue Serotyping

Serotyping of the dengue virus was performed by using serotype-specific primers. Almost all the PCR positive samples were found to be DENV 1 serotype. Those results are shown in the figures below.

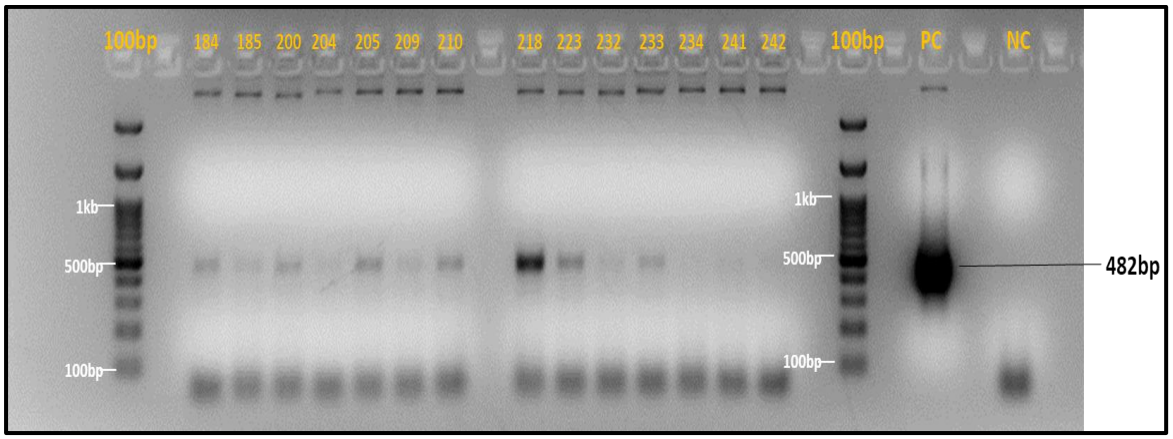

Figure 4. 19 Agarose gel (2%) showing the positive bands for DENV 1 serotype of length 482 bp from Dengue positive samples.

The base pair of 482 was seen while compared with the 100bp ladder. Multiplex PCR was performed for the serotyping but could not get the good results. Hence serotyping was done one by one using the different set of primers for each serotype

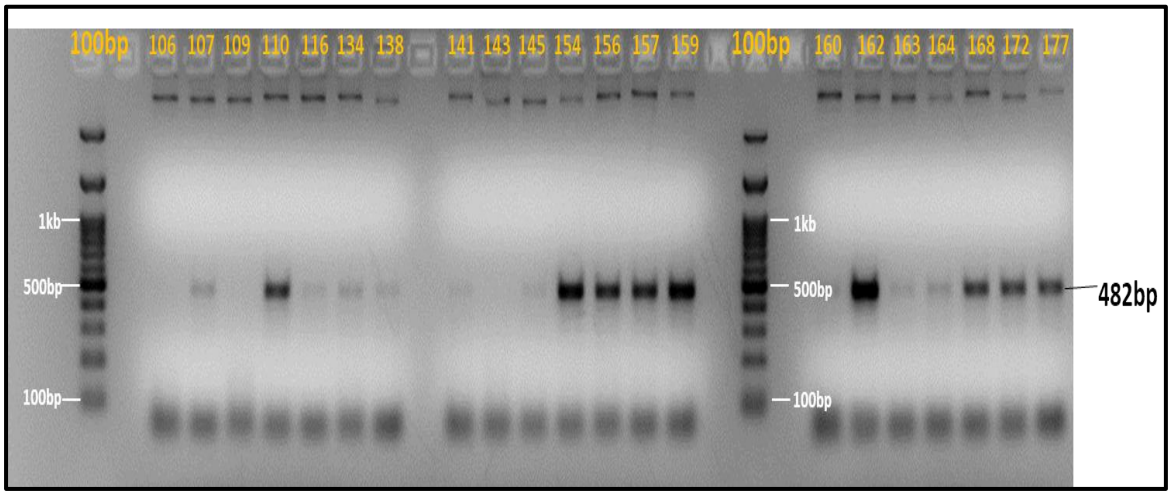

Figure 4. 20 Agarose gel (2%) showing the positive bands for DENV 1 serotype of length 482 bp from Dengue positive sample.

All the 86 PCR confirmed cases were tested for dengue serotype and showed the band size of 482bp when compared to the marker size. Hence the samples tested were of the serotype 1. This confirms that dengue serotype 1 was prevalent in the year 2016. Other figures showing the serotype positive for Dengue 1 are attached in the appendices.

CHAPTER 5

DISCUSSION

5.1 Dengue epidemiology

Dengue fever has become one of the major public health problems in Nepal with hundreds of cases each year since 2004(Gupta, et al., 2018). Some studies have reported that an epidemiological shift in dengue viruses and climate change might be responsible for the observed increase in dengue burden(Mutheneni et al., 2017). Reasons for the currently observed and predicted expansion are multifactorial. They may include climate change, virus evolution, and societal factors such as rapid urbanization, population growth and development, socioeconomic factors, as well as global travel and trade(Murray et al., 2013). (Gupta et al., 2018). In 2012, dengue was classified by (WHO) as the 'most important mosquito-borne viral disease in the world' due to the significant geographic spread of the virus and its vector into previously unaffected areas and the subsequent costly burden of disease it brings(Murray et al., 2013). The first case of dengue in Nepal was reported from a Japanese traveller after returning to his country in 2004. Although several cases of dengue fever were previously suspected in Nepal, a scientific documentation as case report of dengue from the indigenous Nepali population was only published after 2006(Gupta et al., 2018c). The Chitwan, Sarlahi, Jhapa and Rupandehi districts in the Terai region and Kathmandu of Nepal were focal epidemics during the outbreak in 2010, 2013 and 2016. There is an increasing trend in the number of reported dengue cases, which have steadily increased from 32 laboratory-confirmed case in 2006 to 4000 dengue suspected cases in 2016 according to the National Epidemiology and Disease control division, Ministry of Health, Government of Nepal(Gupta et al., 2018).There has become a trend of outbreak in every three years and as a consequence, so many dengue suspected cases were reported from Nepal in 2016. The infection of dengue has crossed its boundary from its localised geographical regions to other high lands also. The circulation of multiple serotypes in the country was identified during the 2006 outbreak. However, a particular serotypes predominated every year with DENV-1 in 2010 and 2016, DENV-2 in 2013(Gupta et al., 2018c).

5.2 Dengue IgM, IgG and NS1 ELISA

The ratio of IgM and IgG is the indicator of the prevalence of primary or secondary infection of Dengue virus(Schilling et al., 2004). The categorization is done based on the ratio of IgM and IgG tests for dengue antibodies and if the ratio is greater than 1.2, it is

likely that the person became infected with dengue virus within recent weeks. If the IgG is positive but the IgM is low or negative (ratio below 1.2), then it is likely that the person had an infection sometime in the past. If the dengue IgG antibody titre increases four-fold or greater between an initial sample and one taken 2 to 4 weeks later, then it is likely that a person has had a recent infection(Tang & Ooi, 2012). Analysis of all 240 Dengue samples from the year 2016 showed that the prevalence of primary infection is more than that of secondary infection. But there is a controversy regarding the previous study made and this study as the previous study showed that there was the prevalence of secondary dengue infection in all years besides the year 2004 (Gupta et al., 2018a). This implies that new dengue cases might have seen in the year 2016. Study made by Khalid et al., explained that higher number of secondary dengue infections occurs only in Dengue-endemic countries. But, this statement also contradicts our results(Soo et al., 2016). ELISA based tests to differentiate secondary dengue are convenient techniques as they are cheaper and more easily available in resource poor countries which bear the brunt of the disease. Altogether, 78 samples (33%) showed the positive results for IgM capture ELISA while only 19 samples (7%) showed positive to IgG. By calculating the ratio between IgM and IgG values, all samples were categorised as primary and secondary infection which were 21 % and 2.9 % respectively. 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 just be that it is too soon after initial exposure to the virus to produce a detectable level of antibody(Soo et al., 2016).

Moreover, from the study, it was found that 60% of the cases were found to be NS1 positive. NS1 antigen test (non-structural protein 1), is a test for dengue, introduced in 2006. It allows rapid detection on the first day of fever, before antibodies which appear some 5 or more days later. The presence of dengue NS1antigen is consistent with acute-phase infection with dengue virus. The NS1 antigen is typically detectable within 1 to 2 days following infection and up to 9 days following symptom onset. The NS1 antigen may also be detectable during secondary dengue virus infection, but for a shorter duration of time (1-4 days following symptom onset)(Holmes & Twiddy, 2003).

The dengue virus non-structural antigen non-structural protein 1 (NS1) that develops right at the beginning of the feverish period and before the appearance of dengue IgM and/or IgG is emerging as a suitable option for dengue diagnosis. Consequent to a multi-country evaluation of two commercially available NS1 enzyme-linked immunosorbent assay (ELISA) assays, a combination of NS1 and IgM detection in samples during the first few days of illness was recommended to increase overall dengue diagnostic sensitivity. Considering this new epidemiological situation, it is expected that dengue and flavivirus

diagnostic guide- lines will change with new algorithms according to the epidemiological situation and more-sensitive and specific as well as better evaluated commercial kits for serology(Rowe et al., 2018).

5.3 Dengue Indirect ELISA (Mid-point titrations)

Dengue Indirect ELISA was performed to find out the mid-point titre value of all samples using the serial dilution techniques. By analysing the peak, it was found the sample to have different antibody level. It was found that 6.25% of the samples had very high level of antibody level while 14.6% had medium and 73.3% had low level of antibody. Indirect ELISA is an appropriate technique for measuring the titre of the antisera generated. The calculation of antibody level helps in finding out the results of other tests like PRNT as the level of antibody in the sample is known and easily selected for other immunological tests. The optimum dilution of antigen that might be used as a single dilution to detect and possibly quantify antibodies is best assessed as the dilution (or concentration) that shows good binding across the whole range of antiserum dilutions. At the first four concentrations (dilutions) of antigen (IgG), there is little difference in the end point detection for the dilutions of antiserum. After this, the OD readings and the end point detections are reduced. At the extreme, in column 10, hardly any antibody is detectable even where the serum is most concentrated. The higher values in row A, B, and C correspond to the nonspecific binding to the wells seen in row 12. Thus, the dilution of antigen up to 3^{rd} and 4^{th} dilutions is optimal to detect antibodies(R., 1995). The serum containing the most antibodies will have a higher dilution end point (dilution where the OD is the same as the back- ground OD). Thus, the estimation of Dengue antibody level by using indirect ELISA can be a better way for the estimation and calculation of the titre of all the samples for other diagnosis purposes(Priyamvada et al., 2016).

5.4 PCR confirmation and Dengue Serotyping

After performing the ELISA tests, all the samples were tested for PCR confirmation. When PCR was done using the dengue specific universal primer, 37 % of the clinically suspected cases were found to be PCR positive. PCR was done by using the primers D1 and DencomR2.when the gel was visualised using UV transilluminator, the positive samples showed the DNA bands at 654bp when compared with a 100bp ladder. The negative samples did not show any bands. PCR test that detects the presence of the virus itself is generally considered the most reliable means of diagnosis, but the test is not widely available. A positive result from a PCR is considered conclusive. A negative result on a PCR test may indicate that no infection is present or that the level of virus is too low to detect, as may happen if the test was performed after the 5-day window during which the virus is present in the sample collected for this test. Many laboratories utilize a nested RT-PCR assay, using universal dengue primers targeting the C/prM region of the genome for an

initial reverse transcription and amplification step, followed by a nested PCR amplification that is serotype-specific. A combination of the four serotype-specific oligonucleotide primers in a single reaction tube (one-step multiplex RT-PCR) is an interesting alternative to the nested RT-PCR. The products of these reactions are separated by electrophoresis on an agarose gel, and the amplification products are visualized as bands of different molecular weights using ethidium bromide dye and compared with standard molecular weight markers. In this assay design, dengue serotypes are identified by the size of their bands(Ahamed et al., 2017).

RT-PCR for detecting and typing dengue viruses was carried out according to Lanciotti et al., (1992). The CprM654 method was used for the detection of serotype among dengue positive samples. Comparison studies of the efficacies of some of the RT-PCR methods revealed that primers targeting C-prM region and Env region were more sensitive and had been recommended for DENV surveillance and research(Ahamed et al., 2017).In our study, used the serotype-specific set of primers as D1 and four primers TS1, TS2, TS3 and Den4 respectively for all four serotypes. D1 5’-TCAATATGCTGAAACGCGCGAGAAACCG-3’ (511 Dengue consensus) and TS1 5’-CGTCTCAGTGATCCGGGGG-3’ 482 (Dl and TS1) were used for the analysis of DENV1. We did all four set of reactions for all samples and almost all PCR positive samples gave the DENV 1 serotype. These were confirmed by observing the base length at 482bp compared to the ladder of size 100bp.

From our study, it was confirmed that so many samples from Nepal in the year 2016 were infected with dengue. Also, all the data were categorised as positive and negative or primary or secondary infection. we compared all the results obtained from the tests performed. Moreover, there are some valuable observations arising from this study. There is a discrepancy in the diagnosis of dengue infection. Some serologically positive cases have a negative PCR result while some PCR positive cases have a negative ELISA result. This can be explained by the fact that in the latter situation the usefulness of the PCR testing for early diagnosis at the time of presentation to the physician is confirmed, while the former case demonstrates the persistence of antibody after the viremia stage. This indicates the pitfalls of PCR and serology testing if used singly. Indeed, patients in endemic areas usually give no clear-cut history of illness, which makes it hard to estimate the exact timing of the disease. A combination of PCR and serology testing can be particularly helpful for diagnosis in such cases. Moreover, effective dengue control is not possible if control efforts are limited to one country or a few countries. It requires the adoption of a regional approach through collaboration among countries and sustained partnerships to enable countries to implement evidence-based interventions and the use of best practices.

CHAPTER 6

SUMMARY

Dengue prevalence

Among all other viral diseases, Dengue has become one of the fast emerging and growing diseases in Nepal. Since its history is not so long, more than 100 cases have been reported every year causing the death of many people This disease was first seen in Nepal in 2006 and since than it has been spreading year by year increasing both the burden and severity(Acharya et.al., 2016).It is emerging and re-emerging with a periodicity of every three years like 2010, 2013, 2016 in a massive way. Similarly, more than four thousand suspected cases were reported, and 1600 cases were dengue confirmed in the year 2016. My study was based on 240 dengue cases and I analysed those samples as positive or negative along with the type of serotype present in that year.

Of all the samples included for study, 153(64%) of the patients were male and 87(36%) of them were female. About 2 % of the patients were children below 10 years of age and most of the patients were in the age range of 15-45. The study from the eleven years shows that most of the cases are found in adults of ages 20-45. Secondary infection cases were mainly found in the year 2008 to 2015, but primary cases were found to be predominant in the year 2016. There is the change in the occurrence of dengue cases also. Previously dengue cases were mainly concentrated in the months of August and September but now those cases are even seen by the last of December also. Hence, we can say that there is no seasonal limitation to spread the dengue virus in Nepal as they are found to spread through a wide range of areas through different seasons.

Immunological status

Different immunological and molecular tests were performed for all 240 samples. At first, IgG, IgM and NS1 ELISA were performed for all those samples. From ELISA results, positives and negatives were identified and type of infection whether primary or secondary was identified. Only a few samples were found of secondary infection while most of them are found to have a primary infection. Only 7 samples were of secondary while 70 samples were of primary infection.NS1 ELISA was performed as it gives results with the Dengue non-structural proteins. Besides capture ELISA, indirect ELISA was also performed for each sample to calculate the amount of antibody present in that sample. The mid-point titrations value for each sample was calculated. This mid-point value gives the indication at which point the titre value of the sample reduces to half. This was done

by serial dilutions at different concentrations. Fifteen samples gave the high level of antibody, 35 samples had medium and 176 samples had low level of antibody in them. The highest amount of antibody was 43228.70(Nep-198) and Nep-64 was the sample having the lowest antibody level of 953.53. This antibody level detection method can be an important part for the study of similar immunological techniques like PRNT, FRNT, ELISPOT etc.

Molecular Analysis

All the 240 samples that were taken for study were tested for molecular confirmation. Altogether 142 samples were screened for dengue by NS1 ELISA. From all samples, only 86 samples were found PCR positive with Dengue specific universal primer. The positive and negative of those samples were confirmed by seeing the band length at 654bp as compared to the marker. The negative ones did not show any bands. Some sample (2) even they were NS1 negative, they showed the PCR positive results. This may be due to the fact that the antibody level is too low to detect by NS1 ELISA. From samples having NS1 units 10- 100 there were only 11 samples positive for PCR but there were 72 samples PCR positive for NS1 units greater than 100. Finally, all the PCR positive samples (86) were then tested for serotype identification. All the PCR positive samples showed the Serotype 1, and which was confirmed by using serotype specific primer.

Hence, epidemiological study along with the immunological and molecular techniques helps in the correct diagnosis and management of the disease. Also, such studies should be available in every part of Nepal for proper management of Dengue cases each year.

CHAPTER 7

CONCLUSION

This epidemiological, immunological and molecular study on Dengue virus is a very important part for the control of the virus. This study mainly focusses on the actual detection of the virus using immunological and molecular techniques. Till date, no dengue vaccine has been developed. In this scenario, the prevalence and type of Dengue virus in different locations may be a key component for the study of the viruses from different geographical areas.

Mostly secondary dengue causes are more severe disease than the primary. Early on, it is important to differentiate the two. ELISA based methods can differentiate between them in lower cost and effectively. RT-PCR is especially useful for monitoring dengue virus activity in endemic areas like in Nepal, allowing a rapid identification of dengue viruses

Nepal has become the victim of dengue in each year starting from the year 2006 and has a massive outbreak in the year 2010, 2013 and 2016.The infection in Nepal has increased its territory towards hilly region also thought it was prevalent in the Terai region only. Nepal reported the death of three people in the year 2016 due to dengue. There has been the shift of DENV serotype each year. There is the shift of serotype from DENV 1 from the year 2010 to DENV 2 in the year 2013. Again, we found that the serotype shifted from DENV 2 to DENV 1 while coming to the year 2016.

Efficient and accurate diagnosis of dengue is of primary importance for clinical care (i.e. early detection of severe cases, case confirmation and differential diagnosis with other infectious diseases), surveillance activities, outbreak control, pathogenesis, academic research, vaccine development, and clinical trials. Laboratory diagnosis methods for confirming dengue virus infection may involve detection of the virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques. After the onset of illness, e 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.

Recommendations/ Future perspectives:

1. Dengue has become a global health problem and these days it has been seen that there is no any geographical boundary for its transmission as they have been reported from hilly regions as well up to the height of 2500 metres.
2. Ecological and epidemiological surveillance coupling with the clinical records play an important role in the correct diagnosis and management of dengue virus infection.
3. A combined effect of immunological and molecular approaches can be a conclusive in most of the cases.
4. Government of Nepal should focus on providing the Rapid diagnostic kits to all endemic areas as soon as earlier before the outbreak of the disease.
5. There is a high chance of cross reactivity with other flavivirus infections and may lead to complications if not checked at right time.
6. Each region should have a well-equipped lab for the proper diagnosis and management of the Dengue. People should be made aware about the spread of the disease and there should be a well-equipped lab for the virus isolation, molecular basis of diagnosis with RT-PCR machine for rapid and effective diagnosis.

CHAPTER 8

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APPENDICES

Appendix 1: Preparation of reagents

cDNA Preparation

5X Reaction Buffer	=4 µL
Ribo-Lock RNase Inhibitor (20 U/µL)	=1 µL
10 mM dNTP Mix	=2 µL
Revert Aid H Minus M-MuLV Reverse Transcriptase (200 U/µL)	=1 µL
Water, nuclease-free	=7 µL
RNA	=5 µL
Total volume	= 20 µL

PCR Mix

Master mix	= 4µL
Forward primer	= 1µL
Reverse primer	= 1µL
NFW	= 13µL
Template	= 1µL
Total	= 20µL

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 s	35
Annealing	58	30 s	
Extension	72	45 s	

Appendix 2: Presentation of Dengue ELISA results

Table A: Presentation of the Dengue NS1 Ag MICROLISA data along with OD ratio, NS1 Ag units and sero-status i.e., Positive or Negative.

Sample ID	NS1 OD Value	NS1 OD Ratio	NS1 Ag Unit	Result		Sample ID	NS1 OD Value	NS1 OD Ratio	NS1 Ag Unit	Result
Nep-1	3.8292	16.812	168.124	POSITIVE		Nep-125	3.4045	14.9478	149.478	POSITIVE
Nep-2	3.826	16.798	167.984	POSITIVE		Nep-126	0.0357	0.15674	1.56744	NEGATIVE
Nep-3	3.6691	16.11	161.095	POSITIVE		Nep-127	0.0578	0.25378	2.53776	NEGATIVE
Nep-4	3.7121	16.298	162.983	POSITIVE		Nep-128	0.0667	0.29285	2.92852	NEGATIVE
Nep-5	3.9528	17.355	173.551	POSITIVE		Nep-129	0.0459	0.20153	2.01528	NEGATIVE
Nep-6	3.9295	17.253	172.528	POSITIVE		Nep-130	0.6041	2.65235	26.5235	POSITIVE
Nep-8	4	17.562	175.623	POSITIVE		Nep-131	3.8258	16.7975	167.975	POSITIVE
Nep-9	3.8435	16.875	168.752	POSITIVE		Nep-132	0.0492	0.21602	2.16017	NEGATIVE
Nep-10	3.8681	16.983	169.832	POSITIVE		Nep-133	0.0426	0.18704	1.87039	NEGATIVE
Nep-11	3.9016	17.13	171.303	POSITIVE		Nep-134	3.503	15.3802	153.802	POSITIVE
Nep-12	3.8134	16.743	167.431	POSITIVE		Nep-135	0.0463	0.20328	2.03284	NEGATIVE
Nep-13	3.6879	16.192	161.92	POSITIVE		Nep-136	0.0477	0.20943	2.09431	NEGATIVE
Nep-14	3.9499	17.342	173.424	POSITIVE		Nep-137	0.0525	0.23051	2.30506	NEGATIVE
Nep-15	3.9766	17.46	174.596	POSITIVE		Nep-138	3.5204	15.4566	154.566	POSITIVE

Nep-16	3.9901	17.519	175.189	POSITIVE		Nep-139	0.054	0.23709	2.37092	NEGATIVE
Nep-17	3.6575	16.059	160.586	POSITIVE		Nep-140	0.0453	0.19889	1.98894	NEGATIVE
Nep-18	3.9119	17.176	171.755	POSITIVE		Nep-141	3.2846	14.4213	144.213	POSITIVE
Nep-19	3.8629	16.96	169.604	POSITIVE		Nep-142	0.0326	0.14313	1.43133	NEGATIVE
Nep-20	3.754	16.482	164.823	POSITIVE		Nep-143	3.2328	14.1939	141.939	POSITIVE
Nep-21	3.7514	16.471	164.708	POSITIVE		Nep-144	0.0393	0.10525	1.05249	NEGATIVE
Nep-22	3.9298	17.254	172.541	POSITIVE		Nep-145	3.861	10.3401	103.401	POSITIVE
Nep-23	3.8394	16.857	168.572	POSITIVE		Nep-146	0.0532	0.14247	1.42475	NEGATIVE
Nep-24	4	17.562	175.623	POSITIVE		Nep-147	0.0394	0.10552	1.05517	NEGATIVE
Nep-25	3.7354	16.401	164.006	POSITIVE		Nep-148	0.0479	0.12828	1.28281	NEGATIVE
Nep-26	3.9372	17.287	172.866	POSITIVE		Nep-149	0.727	1.94697	19.4697	POSITIVE
Nep-27	3.8595	16.945	169.455	POSITIVE		Nep-150	3.926	10.5142	105.142	POSITIVE
Nep-28	3.7174	16.322	163.216	POSITIVE		Nep-151	3.9611	10.6082	106.082	POSITIVE
Nep-29	3.8393	16.857	168.568	POSITIVE		Nep-152	0.0502	0.13444	1.3444	NEGATIVE
Nep-30	3.9926	17.53	175.299	POSITIVE		Nep-153	0.0387	0.10364	1.03642	NEGATIVE
Nep-31	4	17.562	175.623	POSITIVE		Nep-154	3.2848	8.797	87.97	POSITIVE
Nep-32	3.978	17.466	174.658	POSITIVE		Nep-155	0.0422	0.11302	1.13016	NEGATIVE
Nep-33	3.7621	16.518	165.178	POSITIVE		Nep-156	3.4304	9.18693	91.8693	POSITIVE
Nep-34	3.9468	17.329	173.288	POSITIVE		Nep-157	3.638	9.7429	97.429	POSITIVE
Nep-35	3.9896	17.517	175.167	POSITIVE		Nep-158	0.0418	0.11194	1.11944	NEGATIVE
Nep-36	3.8265	16.801	168.006	POSITIVE		Nep-159	3.3579	8.99277	89.9277	POSITIVE
Nep-37	3.9291	17.251	172.511	POSITIVE		Nep-160	3.7363	10.0062	100.062	POSITIVE
Nep-38	4	17.562	175.623	POSITIVE		Nep-161	3.6879	9.87654	98.7654	POSITIVE
Nep-39	3.994	17.536	175.36	POSITIVE		Nep-162	3.5309	9.45608	94.5608	POSITIVE
Nep-40	3.9764	17.459	174.587	POSITIVE		Nep-163	3.6439	9.7587	97.587	POSITIVE
Nep-41	3.8424	16.87	168.704	POSITIVE		Nep-164	3.7264	9.97965	99.7965	POSITIVE
Nep-42	3.9599	17.386	173.863	POSITIVE		Nep-165	0.5653	1.51393	15.1393	POSITIVE
Nep-43	3.9467	17.328	173.283	POSITIVE		Nep-166	3.8122	10.2094	102.094	POSITIVE
Nep-44	3.8857	17.061	170.605	POSITIVE		Nep-167	3.7264	9.97965	99.7965	POSITIVE
Nep-45	3.84	16.86	168.599	POSITIVE		Nep-168	4	10.7124	107.124	POSITIVE
Nep-46	4	17.562	175.623	POSITIVE		Nep-169	0.0391	0.10471	1.04713	NEGATIVE
Nep-47	4	17.562	175.623	POSITIVE		Nep-170	0.0836	0.22389	2.23889	NEGATIVE
Nep-48	4	17.562	175.623	POSITIVE		Nep-171	0.0322	0.08623	0.86235	NEGATIVE
Nep-49	3.8859	17.061	170.614	POSITIVE		Nep-172	3.5153	9.4143	94.143	POSITIVE
Nep-50	4	17.562	175.623	POSITIVE		Nep-173	0.6609	1.76995	17.6995	POSITIVE
Nep-51	4	17.562	175.623	POSITIVE		Nep-174	0.0525	0.1406	1.406	NEGATIVE
Nep-52	3.8922	17.089	170.89	POSITIVE		Nep-175	3.4549	9.25254	92.5254	POSITIVE
Nep-53	0.0429	0.1884	1.88356	NEGATIVE		Nep-176	0.0402	0.10766	1.07659	NEGATIVE
Nep-54	3.5151	15.433	154.334	POSITIVE		Nep-177	0.0602	0.16122	1.61221	NEGATIVE
Nep-55	3.6415	15.988	159.883	POSITIVE		Nep-178	0.0489	0.13096	1.30959	NEGATIVE
Nep-56	0.0388	0.1704	1.70355	NEGATIVE		Nep-179	0.0421	0.11275	1.12748	NEGATIVE
Nep-57	3.3049	14.51	145.104	POSITIVE		Nep-180	0.0397	0.10632	1.0632	NEGATIVE
Nep-58	3.7321	16.386	163.861	POSITIVE		Nep-181	0.3088	0.827	8.26995	NEGATIVE
Nep-59	0.1304	0.5725	5.72532	NEGATIVE		Nep-182	0.0425	0.11382	1.13819	NEGATIVE
Nep-60	3.27	14.357	143.572	POSITIVE		Nep-183	0.0413	0.11061	1.10605	NEGATIVE

Nep-61	3.4827	15.291	152.911	POSITIVE	Nep-184	3.9129	10.4791	104.791	POSITIVE
Nep-62	0.042	0.1844	1.84405	NEGATIVE	Nep-185	3.8838	10.4012	104.012	POSITIVE
Nep-63	3.3901	14.885	148.845	POSITIVE	Nep-186	0.0394	0.10552	1.05517	NEGATIVE
Nep-64	0.0444	0.1949	1.94942	NEGATIVE	Nep-187	0.0395	0.10578	1.05785	NEGATIVE
Nep-65	3.476	15.262	152.617	POSITIVE	Nep-188	0.0392	0.10498	1.04981	NEGATIVE
Nep-66	0.0637	0.2797	2.7968	NEGATIVE	Nep-189	0.3019	0.80852	8.08516	NEGATIVE
Nep-67	0.0719	0.3157	3.15683	NEGATIVE	Nep-190	3.7884	10.1457	101.457	POSITIVE
Nep-68	0.1835	0.8057	8.05673	NEGATIVE	Nep-191	0.0411	0.11007	1.1007	NEGATIVE
Nep-69	0.0444	0.1949	1.94942	NEGATIVE	Nep-192	0.04	0.10712	1.07124	NEGATIVE
Nep-70	3.7793	16.593	165.933	POSITIVE	Nep-193	4	10.7124	107.124	POSITIVE
Nep-71	4	17.562	175.623	POSITIVE	Nep-194	0.039	0.10445	1.04446	NEGATIVE
Nep-72	3.877	17.022	170.223	POSITIVE	Nep-195	0.0385	0.10311	1.03107	NEGATIVE
Nep-73	4	17.562	175.623	POSITIVE	Nep-196	0.0403	0.10793	1.07927	NEGATIVE
Nep-74	0.0404	0.1774	1.7738	NEGATIVE	Nep-197	0.2808	0.75201	7.52009	NEGATIVE
Nep-75	0.0411	0.1805	1.80453	NEGATIVE	Nep-198	4	10.7124	107.124	POSITIVE
Nep-76	3.538	15.534	155.339	POSITIVE	Nep-199	0.0407	0.109	1.08998	NEGATIVE
Nep-77	3.6061	15.833	158.329	POSITIVE	Nep-200	4	10.7124	107.124	POSITIVE
Nep-78	0.3466	1.5218	15.2178	POSITIVE	Nep-201	0.0392	0.10498	1.04981	NEGATIVE
Nep-79	0.2064	0.9062	9.06217	EQUIVOCAL	Nep-202	0.0388	0.10391	1.0391	NEGATIVE
Nep-80	0.0443	0.1945	1.94503	NEGATIVE	Nep-203	0.0387	0.10364	1.03642	NEGATIVE
Nep-81	0.0439	0.1927	1.92747	NEGATIVE	Nep-204	3.7825	10.1299	101.299	POSITIVE
Nep-82	3.8608	16.951	169.512	POSITIVE	Nep-205	3.8674	10.3573	103.573	POSITIVE
Nep-83	0.0448	0.1967	1.96698	NEGATIVE	Nep-206	3.8904	10.4189	104.189	POSITIVE
Nep-84	0.0397	0.1743	1.74306	NEGATIVE	Nep-207	0.0395	0.10578	1.05785	NEGATIVE
Nep-86	0.0458	0.2011	2.01089	NEGATIVE	Nep-208	0.0385	0.10311	1.03107	NEGATIVE
Nep-88	3.6122	15.86	158.597	POSITIVE	Nep-209	4	10.7124	107.124	POSITIVE
Nep-89	3.4622	15.201	152.011	POSITIVE	Nep-210	4	10.7124	107.124	POSITIVE
Nep-90	0.0468	0.2055	2.05479	NEGATIVE	Nep-211	3.5825	9.59427	95.9427	POSITIVE
Nep-91	0.0411	0.1805	1.80453	NEGATIVE	Nep-212	3.6038	9.65131	96.5131	POSITIVE
Nep-92	0.0449	0.1971	1.97137	NEGATIVE	Nep-213	0.2777	0.74371	7.43706	NEGATIVE
Nep-93	0.0425	0.1866	1.866	NEGATIVE	Nep-214	0.0409	0.10953	1.09534	NEGATIVE
Nep-94	0.0412	0.1809	1.80892	NEGATIVE	Nep-215	0.0401	0.10739	1.07392	NEGATIVE
Nep-95	0.0413	0.1813	1.81331	NEGATIVE	Nep-216	3.6441	9.75924	97.5924	POSITIVE
Nep-96	0.052	0.2283	2.28311	NEGATIVE	Nep-217	3.6661	9.81816	98.1816	POSITIVE
Nep-97	3.4736	15.251	152.511	POSITIVE	Nep-218	3.6859	9.87118	98.7118	POSITIVE
Nep-98	4	17.562	175.623	POSITIVE	Nep-219	0.0355	0.09507	0.95072	NEGATIVE
Nep-99	0.6931	3.0431	30.4312	POSITIVE	Nep-220	0.0409	0.10953	1.09534	NEGATIVE
Nep-100	4	17.562	175.623	POSITIVE	Nep-221	0.242	0.6481	6.48099	NEGATIVE
Nep-101	4	17.562	175.623	POSITIVE	Nep-222	4	10.7124	107.124	POSITIVE
Nep-102	0.0953	0.4184	4.18423	NEGATIVE	Nep-223	3.9685	10.628	106.28	POSITIVE
Nep-103	3.7456	16.445	164.454	POSITIVE	Nep-224	4	10.7124	107.124	POSITIVE
Nep-104	0.0493	0.2165	2.16456	NEGATIVE	Nep-225	0.0379	0.1015	1.015	NEGATIVE
Nep-105	0.2929	1.286	12.86	POSITIVE	Nep-226	0.0386	0.10337	1.03374	NEGATIVE
Nep-106	3.7368	16.407	164.067	POSITIVE	Nep-227	0.0334	0.08945	0.89448	NEGATIVE
Nep-107	4	17.562	175.623	POSITIVE	Nep-228	3.7603	10.0704	100.704	POSITIVE

Nep-108	0.0626	0.2749	2.74851	NEGATIVE	Nep-229	0.0174	0.0466	0.46599	NEGATIVE
Nep-109	3.8234	16.787	167.87	POSITIVE	Nep-230	3.7896	10.1489	101.489	POSITIVE
Nep-110	4	17.562	175.623	POSITIVE	Nep-231	0.0399	0.10686	1.06856	NEGATIVE
Nep-111	0.0469	0.2059	2.05919	NEGATIVE	Nep-232	3.7826	10.1302	101.302	POSITIVE
Nep-112	0.1422	0.6243	6.24341	NEGATIVE	Nep-233	4	10.7124	107.124	POSITIVE
Nep-113	0.4332	1.902	19.02	POSITIVE	Nep-234	3.8741	10.3752	103.752	POSITIVE
Nep-114	0.0526	0.2309	2.30945	NEGATIVE	Nep-235	0.2509	0.67193	6.71934	NEGATIVE
Nep-115	0.0516	0.2266	2.26554	NEGATIVE	Nep-236	3.4136	9.14194	91.4194	POSITIVE
Nep-116	3.3699	14.796	147.958	POSITIVE	Nep-237	0.5624	1.50616	15.0616	POSITIVE
Nep-117	0.0326	0.1431	1.43133	NEGATIVE	Nep-238	0.0393	0.10525	1.05249	NEGATIVE
Nep-118	3.2863	14.429	144.288	POSITIVE	Nep-239	3.2682	8.75254	87.5254	POSITIVE
Nep-119	0.0855	0.3754	3.75395	NEGATIVE	Nep-240	3.6929	9.88993	98.8993	POSITIVE
Nep-120	0.0438	0.1923	1.92308	NEGATIVE	Nep-241	3.6416	9.75254	97.5254	POSITIVE
Nep-121	0.3031	1.3308	13.3079	POSITIVE	Nep-242	3.5031	9.38163	93.8163	POSITIVE
Nep-122	0.0669	0.2937	2.9373	NEGATIVE	Nep-243	3.6199	9.69443	96.9443	POSITIVE
Nep-123	0.0469	0.2059	2.05919	NEGATIVE	Nep-244	0.0359	0.09614	0.96144	NEGATIVE

Table B: Presentation of the Panbio Dengue IgM Capture ELISA data along with OD ratio, IgM Index Values and sero-status i.e., Positive or Negative.

Sample ID	IgM OD Value	IgM Index Value	PanBio Units	IgM Sero Status	Sample ID	IgM OD Value	IgM Index Value	PanBio Units	IgM Sero Status
Nep-1	0.09	0.304	3.042	NEGATIVE	Nep-125	0.42	1.464	14.637	POSITIVE
Nep-2	0.47	1.511	15.11	POSITIVE	Nep-126	0.071	0.246	2.4633	NEGATIVE
Nep-3	1.61	5.175	51.75	POSITIVE	Nep-127	0.092	0.32	3.2019	NEGATIVE
Nep-4	0.9	2.908	29.08	POSITIVE	Nep-128	0.159	0.555	5.5468	NEGATIVE
Nep-5	2.09	6.729	67.29	POSITIVE	Nep-129	0.09	0.315	3.1462	NEGATIVE
Nep-6	0.57	1.828	18.28	POSITIVE	Nep-130	0.245	0.853	8.5292	NEGATIVE
Nep-8	0.14	0.454	4.542	NEGATIVE	Nep-131	0.427	1.487	14.867	POSITIVE
Nep-9	0.18	0.595	5.952	NEGATIVE	Nep-132	0.164	0.572	5.7245	NEGATIVE
Nep-10	0.18	0.573	5.733	NEGATIVE	Nep-133	0.133	0.462	4.6165	NEGATIVE
Nep-11	0.13	0.427	4.271	NEGATIVE	Nep-134	0.071	0.247	2.4738	NEGATIVE
Nep-12	0.29	0.933	9.328	EQIVOCAL	Nep-135	0.86	2.995	29.95	POSITIVE
Nep-13	1.38	4.433	44.33	POSITIVE	Nep-136	0.094	0.326	3.2577	NEGATIVE
Nep-14	0.16	0.516	5.157	NEGATIVE	Nep-137	0.109	0.38	3.8047	NEGATIVE
Nep-15	0.2	0.64	6.402	NEGATIVE	Nep-138	0.329	1.148	11.477	POSITIVE
Nep-16	0.1	0.309	3.093	NEGATIVE	Nep-139	0.11	0.385	3.8465	NEGATIVE
Nep-17	1.93	6.219	62.19	POSITIVE	Nep-140	0.145	0.505	5.0451	NEGATIVE
Nep-18	0.22	0.724	7.239	NEGATIVE	Nep-141	0.085	0.297	2.965	NEGATIVE
Nep-19	3.45	11.12	111.2	POSITIVE	Nep-142	0.123	0.43	4.296	NEGATIVE
Nep-20	1.98	6.375	63.75	POSITIVE	Nep-143	0.114	0.397	3.9685	NEGATIVE

Nep-21	1.21	3.902	39.02	POSITIVE	Nep-144	0.107	0.371	3.7141	NEGATIVE
Nep-22	0.11	0.349	3.489	NEGATIVE	Nep-145	0.305	1.061	10.609	EQIVOCAL
Nep-23	0.84	2.69	26.9	POSITIVE	Nep-146	0.185	0.643	6.4283	NEGATIVE
Nep-24	0.18	0.573	5.73	NEGATIVE	Nep-147	0.084	0.293	2.9302	NEGATIVE
Nep-25	0.14	0.453	4.526	NEGATIVE	Nep-148	0.122	0.425	4.2507	NEGATIVE
Nep-26	0.53	1.705	17.05	POSITIVE	Nep-149	0.118	0.412	4.1183	NEGATIVE
Nep-27	0.14	0.437	4.368	NEGATIVE	Nep-150	3.311	11.54	115.37	POSITIVE
Nep-28	3.42	11.02	110.2	POSITIVE	Nep-151	1.668	5.811	58.105	POSITIVE
Nep-29	2.89	9.307	93.07	POSITIVE	Nep-152	0.174	0.606	6.0624	NEGATIVE
Nep-30	0.31	1.006	10.06	EQIVOCAL	Nep-153	0.408	1.42	14.198	POSITIVE
Nep-31	2.48	7.973	79.73	POSITIVE	Nep-154	0.147	0.512	5.1182	NEGATIVE
Nep-32	1.88	6.054	60.54	POSITIVE	Nep-155	3.06	10.66	106.63	POSITIVE
Nep-33	1.07	3.436	34.36	POSITIVE	Nep-156	0.149	0.517	5.174	NEGATIVE
Nep-34	0.25	0.813	8.134	NEGATIVE	Nep-157	0.997	3.472	34.72	POSITIVE
Nep-35	0.11	0.359	3.589	NEGATIVE	Nep-158	3.427	11.94	119.4	POSITIVE
Nep-36	0.33	1.061	10.61	EQIVOCAL	Nep-159	0.087	0.304	3.0417	NEGATIVE
Nep-37	0.12	0.392	3.924	NEGATIVE	Nep-160	1.076	3.75	37.5	POSITIVE
Nep-38	0.1	0.333	3.335	NEGATIVE	Nep-161	3.086	10.75	107.51	POSITIVE
Nep-39	0.13	0.434	4.336	NEGATIVE	Nep-162	0.182	0.633	6.3342	NEGATIVE
Nep-40	0.08	0.266	2.656	NEGATIVE	Nep-163	0.083	0.291	2.9058	NEGATIVE
Nep-41	0.13	0.422	4.22	NEGATIVE	Nep-164	0.139	0.483	4.829	NEGATIVE
Nep-42	0.39	1.268	12.68	POSITIVE	Nep-165	0.143	0.497	4.9719	NEGATIVE
Nep-43	0.45	1.442	14.42	POSITIVE	Nep-166	3.051	10.63	106.29	POSITIVE
Nep-44	3.37	10.86	108.6	POSITIVE	Nep-167	2.987	10.41	104.06	POSITIVE
Nep-45	0.08	0.254	2.54	NEGATIVE	Nep-168	0.107	0.373	3.7315	NEGATIVE
Nep-46	3.27	10.54	105.4	POSITIVE	Nep-169	0.083	0.287	2.8744	NEGATIVE
Nep-47	0.08	0.248	2.482	NEGATIVE	Nep-170	0.155	0.539	5.3935	NEGATIVE
Nep-48	3.24	10.42	104.2	POSITIVE	Nep-171	0.098	0.342	3.4249	NEGATIVE
Nep-49	0.13	0.433	4.329	NEGATIVE	Nep-172	0.071	0.248	2.4807	NEGATIVE
Nep-50	0.24	0.773	7.735	NEGATIVE	Nep-173	0.09	0.313	3.1288	NEGATIVE
Nep-51	0.39	1.266	12.66	POSITIVE	Nep-174	0.088	0.305	3.0486	NEGATIVE
Nep-52	0.27	0.856	8.559	NEGATIVE	Nep-175	1.516	5.283	52.83	POSITIVE
Nep-53	0.08	0.247	2.472	NEGATIVE	Nep-176	0.198	0.69	6.9021	NEGATIVE
Nep-54	0.62	1.982	19.82	POSITIVE	Nep-177	0.116	0.405	4.0486	NEGATIVE
Nep-55	0.09	0.275	2.749	NEGATIVE	Nep-178	0.253	0.882	8.8219	NEGATIVE
Nep-56	0.13	0.431	4.313	NEGATIVE	Nep-179	0.194	0.677	6.7697	NEGATIVE
Nep-57	0.33	1.047	10.47	EQIVOCAL	Nep-180	0.091	0.316	3.1566	NEGATIVE
Nep-58	0.29	0.937	9.373	EQIVOCAL	Nep-181	0.074	0.257	2.5678	NEGATIVE
Nep-59	0.18	0.586	5.862	NEGATIVE	Nep-182	0.125	0.434	4.3413	NEGATIVE
Nep-60	3.5	11.26	112.6	POSITIVE	Nep-183	0.099	0.344	3.4389	NEGATIVE
Nep-61	0.25	0.803	8.034	NEGATIVE	Nep-184	0.482	1.68	16.804	POSITIVE
Nep-62	0.12	0.389	3.892	NEGATIVE	Nep-185	0.177	0.615	6.153	NEGATIVE
Nep-63	0.16	0.522	5.218	NEGATIVE	Nep-186	0.054	0.187	1.8745	NEGATIVE
Nep-64	0.2	0.637	6.37	NEGATIVE	Nep-187	0.061	0.204	2.039	NEGATIVE
Nep-65	0.08	0.264	2.643	NEGATIVE	Nep-188	0.05	0.167	1.674	NEGATIVE

Nep-66	0.1	0.33	3.296	NEGATIVE	Nep-189	0.333	1.116	11.162	POSITIVE
Nep-67	0.21	0.674	6.744	NEGATIVE	Nep-190	1.51	5.056	50.562	POSITIVE
Nep-68	0.19	0.608	6.084	NEGATIVE	Nep-191	2.23	7.466	74.655	POSITIVE
Nep-69	2.18	7.029	70.29	POSITIVE	Nep-192	0.055	0.183	1.8347	NEGATIVE
Nep-70	0.1	0.33	3.296	NEGATIVE	Nep-193	1.06	3.55	35.503	POSITIVE
Nep-71	0.08	0.264	2.643	NEGATIVE	Nep-194	0.057	0.191	1.905	NEGATIVE
Nep-72	1.29	4.161	41.61	POSITIVE	Nep-195	0.068	0.227	2.2733	NEGATIVE
Nep-73	2.52	8.114	81.14	POSITIVE	Nep-196	0.072	0.242	2.424	NEGATIVE
Nep-74	0.11	0.348	3.48	NEGATIVE	Nep-197	0.221	0.738	7.3825	NEGATIVE
Nep-75	0.1	0.323	3.235	NEGATIVE	Nep-198	2.155	7.214	72.137	POSITIVE
Nep-76	0.16	0.507	5.07	NEGATIVE	Nep-199	0.432	1.447	14.47	POSITIVE
Nep-77	0.11	0.368	3.682	NEGATIVE	Nep-200	0.193	0.646	6.4551	NEGATIVE
Nep-78	0.25	0.815	8.147	NEGATIVE	Nep-201	0.121	0.406	4.0579	NEGATIVE
Nep-79	0.13	0.421	4.214	NEGATIVE	Nep-202	0.114	0.383	3.8268	NEGATIVE
Nep-80	0.37	1.2	12	POSITIVE	Nep-203	0.155	0.519	5.1862	NEGATIVE
Nep-81	0.41	1.316	13.16	POSITIVE	Nep-204	0.325	1.087	10.868	EQIVOCAL
Nep-82	0.47	1.5	15	POSITIVE	Nep-205	0.239	0.8	8.0019	NEGATIVE
Nep-83	0.07	0.236	2.356	NEGATIVE	Nep-206	3.093	10.35	103.55	POSITIVE
Nep-84	0.16	0.499	4.989	NEGATIVE	Nep-207	0.999	3.344	33.437	POSITIVE
Nep-86	0.12	0.398	3.985	NEGATIVE	Nep-208	0.14	0.467	4.6706	NEGATIVE
Nep-88	3.06	9.865	98.65	POSITIVE	Nep-209	0.31	1.038	10.382	EQIVOCAL
Nep-89	0.75	2.403	24.03	POSITIVE	Nep-210	0.088	0.294	2.9363	NEGATIVE
Nep-90	0.11	0.366	3.66	NEGATIVE	Nep-211	1.115	3.731	37.314	POSITIVE
Nep-91	0.14	0.46	4.603	NEGATIVE	Nep-212	3.195	10.7	106.96	POSITIVE
Nep-92	0.11	0.343	3.435	NEGATIVE	Nep-213	0.247	0.826	8.2563	NEGATIVE
Nep-93	0.09	0.277	2.765	NEGATIVE	Nep-214	0.087	0.291	2.9095	NEGATIVE
Nep-94	0.19	0.623	6.232	NEGATIVE	Nep-215	0.085	0.285	2.8459	NEGATIVE
Nep-95	0.08	0.289	2.888	NEGATIVE	Nep-216	0.659	2.207	22.07	POSITIVE
Nep-96	0.23	0.811	8.111	NEGATIVE	Nep-217	2.596	8.691	86.909	POSITIVE
Nep-97	2.76	9.631	96.31	POSITIVE	Nep-218	1.649	5.52	55.203	POSITIVE
Nep-98	3.41	11.89	118.9	POSITIVE	Nep-219	0.111	0.371	3.713	NEGATIVE
Nep-99	1.33	4.643	46.43	POSITIVE	Nep-220	0.129	0.43	4.3023	NEGATIVE
Nep-100	2.5	8.698	86.98	POSITIVE	Nep-221	0.209	0.699	6.9874	NEGATIVE
Nep-101	0.11	0.377	3.773	NEGATIVE	Nep-222	0.788	2.637	26.366	POSITIVE
Nep-102	0.13	0.447	4.47	NEGATIVE	Nep-223	0.094	0.315	3.1472	NEGATIVE
Nep-103	3.15	10.97	109.7	POSITIVE	Nep-224	0.98	3.28	32.798	POSITIVE
Nep-104	0.12	0.404	4.038	NEGATIVE	Nep-225	0.1	0.334	3.3447	NEGATIVE
Nep-105	0.26	0.918	9.181	EQIVOCAL	Nep-226	0.096	0.32	3.2041	NEGATIVE
Nep-106	0.12	0.412	4.118	NEGATIVE	Nep-227	0.08	0.266	2.6617	NEGATIVE
Nep-107	0.09	0.312	3.118	NEGATIVE	Nep-228	0.814	2.727	27.267	POSITIVE
Nep-108	0.1	0.362	3.624	NEGATIVE	Nep-229	0.372	1.246	12.465	POSITIVE
Nep-109	0.09	0.325	3.251	NEGATIVE	Nep-230	0.993	3.323	33.233	POSITIVE
Nep-110	2.41	8.39	83.9	POSITIVE	Nep-231	0.107	0.357	3.5724	NEGATIVE
Nep-111	0.11	0.379	3.791	NEGATIVE	Nep-232	0.084	0.281	2.8124	NEGATIVE
Nep-112	0.08	0.282	2.822	NEGATIVE	Nep-233	0.208	0.698	6.9774	NEGATIVE

Nep-113	0.19	0.647	6.467	NEGATIVE	Nep-234	0.381	1.275	12.749	POSITIVE
Nep-114	0.11	0.367	3.669	NEGATIVE	Nep-235	1.056	3.534	35.339	POSITIVE
Nep-115	0.09	0.321	3.205	NEGATIVE	Nep-236	0.211	0.705	7.0544	NEGATIVE
Nep-116	0.11	0.367	3.665	NEGATIVE	Nep-237	0.196	0.658	6.5756	NEGATIVE
Nep-117	0.11	0.397	3.968	NEGATIVE	Nep-238	0.07	0.235	2.347	NEGATIVE
Nep-118	1.51	5.249	52.49	POSITIVE	Nep-239	3.359	11.25	112.47	POSITIVE
Nep-119	0.13	0.445	4.449	NEGATIVE	Nep-240	0.179	0.599	5.9863	NEGATIVE
Nep-120	0.1	0.354	3.54	NEGATIVE	Nep-241	0.125	0.42	4.1951	NEGATIVE
Nep-121	0.13	0.468	4.683	NEGATIVE	Nep-242	0.134	0.449	4.4931	NEGATIVE
Nep-122	0.09	0.308	3.083	NEGATIVE	Nep-243	1.407	4.71	47.101	POSITIVE
Nep-123	0.1	0.332	3.324	NEGATIVE	Nep-244	0.096	0.321	3.2141	NEGATIVE

Table C: Presentation of the Panbio Dengue IgG Capture ELISA data along with OD ratio, IgG Index Values and sero-status i.e., Positive or Negative.

Sample ID	IgG OD Value	IgG Index Value	PanBio Units	IgG Sero Status		Sample ID	IgG OD Value	IgG Index Value	PanBio Units	IgG Sero Status
Nep-1	0.0522	0.1106	1.1062	NEGATIVE		Nep-125	1.41	3.90407	39.041	POSITIVE
Nep-2	0.0486	0.103	1.0299	NEGATIVE		Nep-126	0.0486	0.13457	1.3457	NEGATIVE
Nep-3	0.1668	0.3535	3.5347	NEGATIVE		Nep-127	0.0619	0.17139	1.7139	NEGATIVE
Nep-4	1.9426	4.1166	41.166	POSITIVE		Nep-128	0.0504	0.13955	1.3955	NEGATIVE
Nep-5	0.0506	0.1072	1.0723	NEGATIVE		Nep-129	0.0474	0.13124	1.3124	NEGATIVE
Nep-6	0.0553	0.1172	1.1719	NEGATIVE		Nep-130	0.2103	0.58229	5.8229	NEGATIVE
Nep-8	0.0536	0.1136	1.1358	NEGATIVE		Nep-131	0.049	0.13567	1.3567	NEGATIVE
Nep-9	0.0486	0.103	1.0299	NEGATIVE		Nep-132	0.0478	0.13235	1.3235	NEGATIVE
Nep-10	0.0578	0.1225	1.2249	NEGATIVE		Nep-133	0.8718	2.41388	24.139	POSITIVE
Nep-11	0.049	0.1038	1.0384	NEGATIVE		Nep-134	0.061	0.1689	1.689	NEGATIVE
Nep-12	0.2626	0.5565	5.5648	NEGATIVE		Nep-135	0.0556	0.15395	1.5395	NEGATIVE
Nep-13	0.0567	0.1202	1.2015	NEGATIVE		Nep-136	0.0474	0.13124	1.3124	NEGATIVE
Nep-14	0.0488	0.1034	1.0341	NEGATIVE		Nep-137	0.0479	0.13263	1.3263	NEGATIVE
Nep-15	0.0486	0.103	1.0299	NEGATIVE		Nep-138	0.147	0.40702	4.0702	NEGATIVE
Nep-16	0.0541	0.1146	1.1464	NEGATIVE		Nep-139	0.0504	0.13955	1.3955	NEGATIVE
Nep-17	0.0785	0.1664	1.6635	NEGATIVE		Nep-140	0.0507	0.14038	1.4038	NEGATIVE
Nep-18	0.0488	0.1034	1.0341	NEGATIVE		Nep-141	0.0623	0.1725	1.725	NEGATIVE
Nep-19	0.1034	0.2191	2.1912	NEGATIVE		Nep-142	0.3752	1.03887	10.389	NEGATIVE
Nep-20	2.4826	5.2609	52.609	POSITIVE		Nep-143	0.3157	0.87412	8.7412	NEGATIVE
Nep-21	2.7127	5.7485	57.485	POSITIVE		Nep-144	0.0487	0.13484	1.3484	NEGATIVE
Nep-22	0.0549	0.1163	1.1634	NEGATIVE		Nep-145	0.0471	0.13041	1.3041	NEGATIVE
Nep-23	0.0538	0.114	1.1401	NEGATIVE		Nep-146	0.0914	0.25307	2.5307	NEGATIVE
Nep-24	0.0453	0.096	0.96	NEGATIVE		Nep-147	0.0472	0.13069	1.3069	NEGATIVE
Nep-25	0.0486	0.103	1.0299	NEGATIVE		Nep-148	0.1209	0.33475	3.3475	NEGATIVE
Nep-26	0.0489	0.1036	1.0363	NEGATIVE		Nep-149	0.2707	0.74953	7.4953	NEGATIVE
Nep-27	0.0487	0.1032	1.032	NEGATIVE		Nep-150	2.0723	5.73787	57.379	POSITIVE
Nep-28	0.199	0.4217	4.2171	NEGATIVE		Nep-151	1.2673	3.50895	35.09	POSITIVE
Nep-29	0.9065	1.921	19.21	EQIVOVAL		Nep-152	0.0499	0.13817	1.3817	NEGATIVE

Nep-30	0.0505	0.107	1.0702	NEGATIVE		Nep-153	0.0481	0.13318	1.3318	NEGATIVE
Nep-31	0.2938	0.6226	6.226	NEGATIVE		Nep-154	0.2254	0.6241	6.241	NEGATIVE
Nep-32	0.05	0.106	1.0596	NEGATIVE		Nep-155	0.313	0.86665	8.6665	NEGATIVE
Nep-33	0.0519	0.11	1.0998	NEGATIVE		Nep-156	0.0477	0.13207	1.3207	NEGATIVE
Nep-34	0.0511	0.1083	1.0829	NEGATIVE		Nep-157	0.0564	0.15616	1.5616	NEGATIVE
Nep-35	0.0486	0.103	1.0299	NEGATIVE		Nep-158	2.4553	6.79834	67.983	POSITIVE
Nep-36	0.1615	0.3422	3.4224	NEGATIVE		Nep-159	0.0479	0.13263	1.3263	NEGATIVE
Nep-37	0.0491	0.104	1.0405	NEGATIVE		Nep-160	0.0529	0.14647	1.4647	NEGATIVE
Nep-38	0.0483	0.1024	1.0235	NEGATIVE		Nep-161	0.0701	0.1941	1.941	NEGATIVE
Nep-39	0.0494	0.1047	1.0468	NEGATIVE		Nep-162	0.3029	0.83868	8.3868	NEGATIVE
Nep-40	0.0513	0.1087	1.0871	NEGATIVE		Nep-163	0.048	0.1329	1.329	NEGATIVE
Nep-41	0.0488	0.1034	1.0341	NEGATIVE		Nep-164	0.0527	0.14592	1.4592	NEGATIVE
Nep-42	0.0488	0.1034	1.0341	NEGATIVE		Nep-165	0.0495	0.13706	1.3706	NEGATIVE
Nep-43	0.0482	0.1021	1.0214	NEGATIVE		Nep-166	0.05	0.13844	1.3844	NEGATIVE
Nep-44	0.2113	0.4478	4.4777	NEGATIVE		Nep-167	1.7534	4.85489	48.549	POSITIVE
Nep-45	0.0499	0.1057	1.0574	NEGATIVE		Nep-168	0.0488	0.13512	1.3512	NEGATIVE
Nep-46	2.5802	5.4678	54.678	POSITIVE		Nep-169	0.0509	0.14093	1.4093	NEGATIVE
Nep-47	0.0483	0.1024	1.0235	NEGATIVE		Nep-170	0.1798	0.49784	4.9784	NEGATIVE
Nep-48	1.4373	3.0458	30.458	POSITIVE		Nep-171	0.048	0.1329	1.329	NEGATIVE
Nep-49	0.0512	0.1085	1.085	NEGATIVE		Nep-172	0.0475	0.13152	1.3152	NEGATIVE
Nep-50	0.0486	0.103	1.0299	NEGATIVE		Nep-173	0.0475	0.13152	1.3152	NEGATIVE
Nep-51	0.0475	0.1007	1.0066	NEGATIVE		Nep-174	0.0466	0.12903	1.2903	NEGATIVE
Nep-52	0.1482	0.3141	3.1405	NEGATIVE		Nep-175	0.0516	0.14287	1.4287	NEGATIVE
Nep-53	0.186	0.3942	3.9416	NEGATIVE		Nep-176	0.0481	0.13318	1.3318	NEGATIVE
Nep-54	1.0156	2.1522	21.522	EQIVOVAL		Nep-177	0.0467	0.1293	1.293	NEGATIVE
Nep-55	0.0474	0.1004	1.0045	NEGATIVE		Nep-178	0.229	0.63407	6.3407	NEGATIVE
Nep-56	0.0505	0.107	1.0702	NEGATIVE		Nep-179	1.6423	4.54727	45.473	POSITIVE
Nep-57	0.067	0.142	1.4198	NEGATIVE		Nep-180	0.0492	0.13623	1.3623	NEGATIVE
Nep-58	0.0493	0.1045	1.0447	NEGATIVE		Nep-181	0.051	0.14121	1.4121	NEGATIVE
Nep-59	0.0489	0.1036	1.0363	NEGATIVE		Nep-182	0.0458	0.12681	1.2681	NEGATIVE
Nep-60	1.3477	2.8559	28.559	POSITIVE		Nep-183	0.0624	0.17278	1.7278	NEGATIVE
Nep-61	0.0481	0.1019	1.0193	NEGATIVE		Nep-184	0.0511	0.14149	1.4149	NEGATIVE
Nep-62	0.0499	0.1057	1.0574	NEGATIVE		Nep-185	0.0476	0.1318	1.318	NEGATIVE
Nep-63	0.048	0.1017	1.0172	NEGATIVE		Nep-186	0.1466	0.40591	4.0591	NEGATIVE
Nep-64	0.0483	0.1024	1.0235	NEGATIVE		Nep-187	0.0516	0.13364	1.3364	NEGATIVE
Nep-65	0.0492	0.1043	1.0426	NEGATIVE		Nep-188	0.049	0.12691	1.2691	NEGATIVE
Nep-66	0.0498	0.1055	1.0553	NEGATIVE		Nep-189	0.3467	0.89792	8.9792	NEGATIVE
Nep-67	0.0484	0.1026	1.0257	NEGATIVE		Nep-190	0.0527	0.13649	1.3649	NEGATIVE
Nep-68	0.1713	0.363	3.6301	NEGATIVE		Nep-191	0.1804	0.46722	4.6722	NEGATIVE
Nep-69	1.285	2.7231	27.231	POSITIVE		Nep-192	0.048	0.12432	1.2432	NEGATIVE
Nep-70	0.0529	0.1121	1.121	NEGATIVE		Nep-193	0.3764	0.97485	9.7485	NEGATIVE
Nep-71	0.0539	0.1142	1.1422	NEGATIVE		Nep-194	0.0508	0.13157	1.3157	NEGATIVE
Nep-72	0.049	0.1038	1.0384	NEGATIVE		Nep-195	0.0511	0.13234	1.3234	NEGATIVE
Nep-73	0.0498	0.1055	1.0553	NEGATIVE		Nep-196	0.0504	0.13053	1.3053	NEGATIVE
Nep-74	0.0486	0.103	1.0299	NEGATIVE		Nep-197	0.1332	0.34498	3.4498	NEGATIVE

Nep-75	0.0477	0.1011	1.0108	NEGATIVE		Nep-198	2.3505	6.0876	60.876	POSITIVE
Nep-76	0.1337	0.2833	2.8333	NEGATIVE		Nep-199	0.0504	0.13053	1.3053	NEGATIVE
Nep-77	0.048	0.1017	1.0172	NEGATIVE		Nep-200	0.0517	0.1339	1.339	NEGATIVE
Nep-78	0.0499	0.1057	1.0574	NEGATIVE		Nep-201	0.0494	0.12794	1.2794	NEGATIVE
Nep-79	0.04	0.0848	0.8476	NEGATIVE		Nep-202	0.052	0.13468	1.3468	NEGATIVE
Nep-80	0.0488	0.1034	1.0341	NEGATIVE		Nep-203	0.0531	0.13752	1.3752	NEGATIVE
Nep-81	0.0462	0.0979	0.979	NEGATIVE		Nep-204	0.0511	0.13234	1.3234	NEGATIVE
Nep-82	0.0483	0.1024	1.0235	NEGATIVE		Nep-205	0.178	0.46101	4.6101	NEGATIVE
Nep-83	0.2858	0.6056	6.0565	NEGATIVE		Nep-206	0.0705	0.18259	1.8259	NEGATIVE
Nep-84	0.142	0.3009	3.0092	NEGATIVE		Nep-207	0.0561	0.14529	1.4529	NEGATIVE
Nep-86	0.037	0.0784	0.7841	NEGATIVE		Nep-208	0.0506	0.13105	1.3105	NEGATIVE
Nep-88	0.052	0.1102	1.1019	NEGATIVE		Nep-209	0.0598	0.15488	1.5488	NEGATIVE
Nep-89	0.051	0.1081	1.0808	NEGATIVE		Nep-210	0.049	0.12691	1.2691	NEGATIVE
Nep-90	0.2172	0.4603	4.6027	NEGATIVE		Nep-211	0.0636	0.16472	1.6472	NEGATIVE
Nep-91	0.0486	0.103	1.0299	NEGATIVE		Nep-212	0.0673	0.1743	1.743	NEGATIVE
Nep-92	0.0499	0.1057	1.0574	NEGATIVE		Nep-213	0.9152	2.37029	23.703	POSITIVE
Nep-93	0.0492	0.1043	1.0426	NEGATIVE		Nep-214	0.0495	0.1282	1.282	NEGATIVE
Nep-94	0.4273	0.9055	9.055	NEGATIVE		Nep-215	0.0552	0.14296	1.4296	NEGATIVE
Nep-95	0.0541	0.1498	1.4979	NEGATIVE		Nep-216	1.6732	4.33345	43.335	POSITIVE
Nep-96	0.0505	0.1398	1.3983	NEGATIVE		Nep-217	0.0584	0.15125	1.5125	NEGATIVE
Nep-97	0.2564	0.7099	7.0993	NEGATIVE		Nep-218	0.0507	0.13131	1.3131	NEGATIVE
Nep-98	0.0613	0.1697	1.6973	NEGATIVE		Nep-219	0.0519	0.13442	1.3442	NEGATIVE
Nep-99	2.1699	6.0081	60.081	POSITIVE		Nep-220	0.0493	0.12768	1.2768	NEGATIVE
Nep-100	0.1019	0.2821	2.8215	NEGATIVE		Nep-221	0.4012	1.03908	10.391	NEGATIVE
Nep-101	0.0414	0.1146	1.1463	NEGATIVE		Nep-222	0.0499	0.12924	1.2924	NEGATIVE
Nep-102	0.3101	0.8586	8.5862	NEGATIVE		Nep-223	0.0516	0.13364	1.3364	NEGATIVE
Nep-103	0.063	0.1744	1.7444	NEGATIVE		Nep-224	0.0519	0.13442	1.3442	NEGATIVE
Nep-104	0.0497	0.1376	1.3761	NEGATIVE		Nep-225	0.2707	0.70109	7.0109	NEGATIVE
Nep-105	0.2184	0.6047	6.0472	NEGATIVE		Nep-226	0.0501	0.12975	1.2975	NEGATIVE
Nep-106	0.0505	0.1398	1.3983	NEGATIVE		Nep-227	0.05	0.1295	1.295	NEGATIVE
Nep-107	0.0528	0.1462	1.4619	NEGATIVE		Nep-228	0.0491	0.12717	1.2717	NEGATIVE
Nep-108	0.0556	0.1539	1.5395	NEGATIVE		Nep-229	0.2155	0.55813	5.5813	NEGATIVE
Nep-109	0.0513	0.142	1.4204	NEGATIVE		Nep-230	0.0498	0.12898	1.2898	NEGATIVE
Nep-110	0.0976	0.2702	2.7024	NEGATIVE		Nep-231	0.0515	0.13338	1.3338	NEGATIVE
Nep-111	0.0499	0.1382	1.3817	NEGATIVE		Nep-232	0.049	0.12691	1.2691	NEGATIVE
Nep-112	0.0472	0.1307	1.3069	NEGATIVE		Nep-233	0.0484	0.12535	1.2535	NEGATIVE
Nep-113	0.1701	0.471	4.7098	NEGATIVE		Nep-234	0.9896	2.56298	25.63	POSITIVE
Nep-114	0.0489	0.1354	1.354	NEGATIVE		Nep-235	0.0512	0.1326	1.326	NEGATIVE
Nep-115	0.0482	0.1335	1.3346	NEGATIVE		Nep-236	0.0522	0.13519	1.3519	NEGATIVE
Nep-116	0.0469	0.1299	1.2986	NEGATIVE		Nep-237	0.2077	0.53793	5.3793	NEGATIVE
Nep-117	0.047	0.1301	1.3014	NEGATIVE		Nep-238	0.0498	0.12898	1.2898	NEGATIVE
Nep-118	0.0542	0.1501	1.5007	NEGATIVE		Nep-239	0.0726	0.18803	1.8803	NEGATIVE
Nep-119	0.0479	0.1326	1.3263	NEGATIVE		Nep-240	0.05	0.1295	1.295	NEGATIVE
Nep-120	0.2064	0.5715	5.7149	NEGATIVE		Nep-241	0.768	1.98906	19.891	EQIVOVAL
Nep-121	0.2247	0.6222	6.2216	NEGATIVE		Nep-242	0.0496	0.12846	1.2846	NEGATIVE

Nep-122	0.0528	0.1462	1.4619	NEGATIVE		Nep-243	0.231	0.59827	5.9827	NEGATIVE
Nep-123	0.0492	0.1362	1.3623	NEGATIVE		Nep-244	0.0583	0.15099	1.5099	NEGATIVE

Table D: Comparison of NS1, IgM, IgG and PCR results

SAMPLE ID	AGE	SEX	NS1 Ag UNITS	STATUS	IgM INDEX	STATUS	IgG INDEX	STATUS	IgM/IgG (RATIO)	SERO STATUS	IgM/IgG(RATIO)	IgG MID POINT	RNA CONC^N	PCR RESULT	Platelets count
Nep-1	30	F	168.12	+VE	0.30	-VE	0.11	-VE	2.75		2.75	1,712.6	77.2	+VE	94000
Nep-2	22	M	167.98	+VE	1.51	+VE	0.10	-VE	14.67	1°	14.67	1,687.7	81.8	+VE	210000
Nep-3	17	M	161.10	+VE	5.17	+VE	0.35	-VE	14.64	1°	14.64	1,705.8	92.5	-VE	56000
Nep-4	36	M	162.98	+VE	2.91	+VE	4.12	+VE	0.71	2°	0.71	8,798.8	98	-VE	79000
Nep-5	18	M	173.55	+VE	6.73	+VE	0.11	-VE	62.76	1°	62.76	1,734.7	90.8	-VE	38000
Nep-6	25	F	172.53	+VE	1.83	+VE	0.12	-VE	15.60	1°	15.60	1,425.3	74	-VE	87000
Nep-8	24	M	175.62	+VE	0.45	-VE	0.11	-VE	4.00		4.00	NA	57.5	-VE	78000
Nep-9	45	M	168.75	+VE	0.60	-VE	0.10	-VE	5.78		5.78	2,033.1	48.9	-VE	57,000
Nep-10	22	M	169.83	+VE	0.57	-VE	0.12	-VE	4.68		4.68	1,623.3	10.4	-VE	98,000
Nep-11	34	M	171.30	+VE	0.43	-VE	0.10	-VE	4.11		4.11	1,541.3	79.8	+VE	186000
Nep-12	41	M	167.43	+VE	0.93	EQU	0.56	-VE	1.68		1.68	NA	65.8	+VE	161000
Nep-13	27	M	161.92	+VE	4.43	+VE	0.12	-VE	36.89	1°	36.89	1,509.7	110.3	-VE	67000
Nep-14	21	M	173.42	+VE	0.52	-VE	0.10	-VE	4.99		4.99	NA	104.6	+VE	319000
Nep-15	45	M	174.60	+VE	0.64	-VE	0.10	-VE	6.22		6.22	1,575.1	123.3	+VE	75000
Nep-16	28	F	175.19	+VE	0.31	-VE	0.11	-VE	2.70		2.70	2,126.4	97.9	+VE	140000
Nep-17	33	F	160.59	+VE	6.22	+VE	0.17	-VE	37.38	1°	37.38	1,756.6	88.5	+VE	60000
Nep-18	16	F	171.76	+VE	0.72	-VE	0.10	-VE	7.00		7.00	1,665.2	102.9	+VE	85000
Nep-19	16	M	169.60	+VE	11.12	+VE	0.22	-VE	50.74	1°	50.74	1,645.7	94.2	-VE	88000
Nep-20	34	M	164.82	+VE	6.37	+VE	5.26	+VE	1.21	1°	1.21	1,876.1	95.9	+VE	134000
Nep-21	32	M	164.71	+VE	3.90	+VE	5.75	+VE	0.68	2°	0.68	5,195.8	53.4	-VE	96000
Nep-22	44	M	172.54	+VE	0.35	-VE	0.12	-VE	3.00		3.00	1,580.0	84.7	+VE	56000
Nep-23	16	F	168.57	+VE	2.69	+VE	0.11	-VE	23.60	1°	23.60	1,496.4	45	-VE	115000
Nep-24	34	M	175.62	+VE	0.57	-VE	0.10	-VE	5.97		5.97	1,557.2	81.6	+VE	80000
Nep-25	21	M	164.01	+VE	0.45	-VE	0.10	-VE	4.39		4.39	1,400.8	78.7	-VE	49000
Nep-26	16	M	172.87	+VE	1.70	+VE	0.10	-VE	16.45	1°	16.45	1,538.9	90.8	-VE	56000
Nep-27	18	F	169.45	+VE	0.44	-VE	0.10	-VE	4.23		4.23	2,439.0	84.9	+VE	45000
Nep-28	34	F	163.22	+VE	11.02	+VE	0.42	-VE	26.12	1°	26.12	4,471.9	75.1	-VE	96000
Nep-29	26	F	168.57	+VE	9.31	+VE	1.92	EQU	4.85	1°	4.85	3,359.4	83.7	-VE	78000
Nep-30	17	F	175.30	+VE	1.01	EQU	0.11	-VE	9.40		9.40	1,535.4	55.2	+VE	37000
Nep-31	19	M	175.62	+VE	7.97	+VE	0.62	-VE	12.81	1°	12.81	5,719.0	84.5	-VE	81000
Nep-32	27	M	174.66	+VE	6.05	+VE	0.11	-VE	57.14	1°	57.14	1,401.7	64.7	-VE	56000
Nep-33	34	M	165.18	+VE	3.44	+VE	0.11	-VE	31.25	1°	31.25	1,949.4	81.2	+VE	98000
Nep-34	35	M	173.29	+VE	0.81	-VE	0.11	-VE	7.51		7.51	1,452.6	59.9	+VE	66000
Nep-35	22	M	175.17	+VE	0.36	-VE	0.10	-VE	3.48		3.48	1,565.8	71.5	+VE	67000
Nep-36	33	M	168.01	+VE	1.06	EQU	0.34	-VE	3.10		3.10	2,624.3	89.4	+VE	82000
Nep-37	16	M	172.51	+VE	0.39	-VE	0.10	-VE	3.77		3.77	4,303.2	99.9	+VE	86400
Nep-38	23	M	175.62	+VE	0.33	-VE	0.10	-VE	3.26		3.26	1,764.7	100.8	+VE	90000

Nep-39	29	F	175.36	+VE	0.43	-VE	0.10	-VE	4.14		4.14	1,574.6	81	+VE	94000
Nep-40	27	M	174.59	+VE	0.27	-VE	0.11	-VE	2.44		2.44	1,581.2	104.8	+VE	99000
Nep-41	38	F	168.70	+VE	0.42	-VE	0.10	-VE	4.08		4.08	1,473.8	51.1	+VE	103200
Nep-42	34	M	173.86	+VE	1.27	+VE	0.10	-VE	12.26	1°	12.26	2,420.5	99.9	+VE	107400
Nep-43	45	F	173.28	+VE	1.44	+VE	0.10	-VE	14.12	1°	14.12	1,555.2	113	+VE	66000
Nep-44	34	M	170.61	+VE	10.86	+VE	0.45	-VE	24.25	1°	24.25	1,505.4	77.4	-VE	86000
Nep-45	32	F	168.60	+VE	0.25	-VE	0.11	-VE	2.40		2.40	NA	108.7	+VE	96000
Nep-46	44	M	175.62	+VE	10.54	+VE	5.47	+VE	1.93	1°	1.93	15,240.6	11.7	-VE	78000
Nep-47	16	M	175.62	+VE	0.25	-VE	0.10	-VE	2.42		2.42	2,971.3	85.7	+VE	167000
Nep-48	34	F	175.62	+VE	10.42	+VE	3.05	+VE	3.42	1°	3.42	13,582.2	74.9	-VE	123000
Nep-49	21	F	170.61	+VE	0.43	-VE	0.11	-VE	3.99		3.99	1,039.8	65.1	+VE	45000
Nep-50	16	F	175.62	+VE	0.77	-VE	0.10	-VE	7.51		7.51	1,276.7	59	+VE	56000
Nep-51	45	F	175.62	+VE	1.27	+VE	0.10	-VE	12.58	1°	12.58	1,172.2	104.5	+VE	86000
Nep-52	34	F	170.89	+VE	0.86	-VE	0.31	-VE	2.73		2.73	1,423.6	96.8	+VE	93000
Nep-53	38	M	1.88	-VE	0.25	-VE	0.39	-VE	0.63		0.63	1,327.8	79.2	-VE	44000
Nep-54	32	M	154.33	+VE	1.98	+VE	2.15	EQU	0.92		0.92	4,837.6	101.5	-VE	88000
Nep-55	40	M	159.88	+VE	0.27	-VE	0.10	-VE	2.74		2.74	2,163.9	83.2	+VE	65000
Nep-56	16	M	1.70	-VE	0.43	-VE	0.11	-VE	4.03		4.03	1,385.6	95.3	-VE	76000
Nep-57	36	M	145.10	+VE	1.05	EQU	0.14	-VE	7.37		7.37	1,371.2	83.3	-VE	87000
Nep-58	52	M	163.86	+VE	0.94	EQU	0.10	-VE	8.97		8.97	2,087.7	81.4	+VE	66000
Nep-59	40	M	5.73	-VE	0.59	-VE	0.10	-VE	5.66		5.66	3,910.2	101.1	-VE	45000
Nep-60	23	F	143.57	+VE	11.26	+VE	2.86	+VE	3.94	1°	3.94	1,128.2	95.9	-VE	78000
Nep-61	29	M	152.91	+VE	0.80	-VE	0.10	-VE	7.88		7.88	1,128.2	80.5	-VE	66000
Nep-62	27	F	1.84	-VE	0.39	-VE	0.11	-VE	3.68		3.68	1,224.4	100.5	+VE	61000
Nep-63	38	M	148.85	+VE	0.52	-VE	0.10	-VE	5.13		5.13	1,333.6	6.3	-VE	78000
Nep-64	20	F	1.95	-VE	0.64	-VE	0.10	-VE	6.22		6.22	953.5	79.5	-VE	56000
Nep-65	17	F	152.62	+VE	0.26	-VE	0.10	-VE	2.53		2.53	1,129.0	110.9	+VE	86000
Nep-66	14	M	2.80	-VE	0.33	-VE	0.11	-VE	3.12		3.12	1,165.2	46.5	-VE	67000
Nep-67	26	M	3.16	-VE	0.67	-VE	0.10	-VE	6.57		6.57	2,101.5	108	-VE	66000
Nep-68	20	M	8.06	-VE	0.61	-VE	0.36	-VE	1.68		1.68	NA	102.4	-VE	80000
Nep-69	18	M	1.95	-VE	7.03	+VE	2.72	+VE	2.58	1°	2.58	4,400.6	78.4	-VE	49000
Nep-70	43	F	165.93	+VE	0.33	-VE	0.11	-VE	2.94		2.94	1,871.6	79.8	+VE	66000
Nep-71	37	F	175.62	+VE	0.26	-VE	0.11	-VE	2.31		2.31	1,791.5	106.8	+VE	86000
Nep-72	18	F	170.22	+VE	4.16	+VE	0.10	-VE	40.07	1°	40.07	1,840.6	101.8	+VE	96000
Nep-73	44	M	175.62	+VE	8.11	+VE	0.11	-VE	76.88	1°	76.88	1,461.6	104.8	+VE	209,000
Nep-74	27	F	1.77	-VE	0.35	-VE	0.10	-VE	3.38		3.38	1,015.8	84	-VE	144,000
Nep-75	64	F	1.80	-VE	0.32	-VE	0.10	-VE	3.20		3.20	1,254.7	78	-VE	88,000
Nep-76	20	M	155.34	+VE	0.51	-VE	0.28	-VE	1.79		1.79	1,138.9	100.5	+VE	93,000
Nep-77	60	M	158.33	+VE	0.37	-VE	0.10	-VE	3.62		3.62	1,721.8	65.7	+VE	167,000
Nep-78	63	M	15.22	+VE	0.81	-VE	0.11	-VE	7.70		7.70	990.1	16.5	-VE	145,000
Nep-79	51	M	9.06	EQU	0.42	-VE	0.08	-VE	4.97		4.97	1,028.9	34.1	-VE	167,000
Nep-80	12	F	1.95	-VE	1.20	+VE	0.10	-VE	11.60	1°	11.60	1,969.3	112.5	-VE	88,000
Nep-81	45	M	1.93	-VE	1.32	+VE	0.10	-VE	13.44	1°	13.44	995.5	39	-VE	107,000
Nep-82	55	M	169.51	+VE	1.50	+VE	0.10	-VE	14.65	1°	14.65	1,394.8	63.4	+VE	62,000
Nep-83	8	M	1.97	-VE	0.24	-VE	0.61	-VE	0.39		0.39	3,874.5	107.1	-VE	274,000

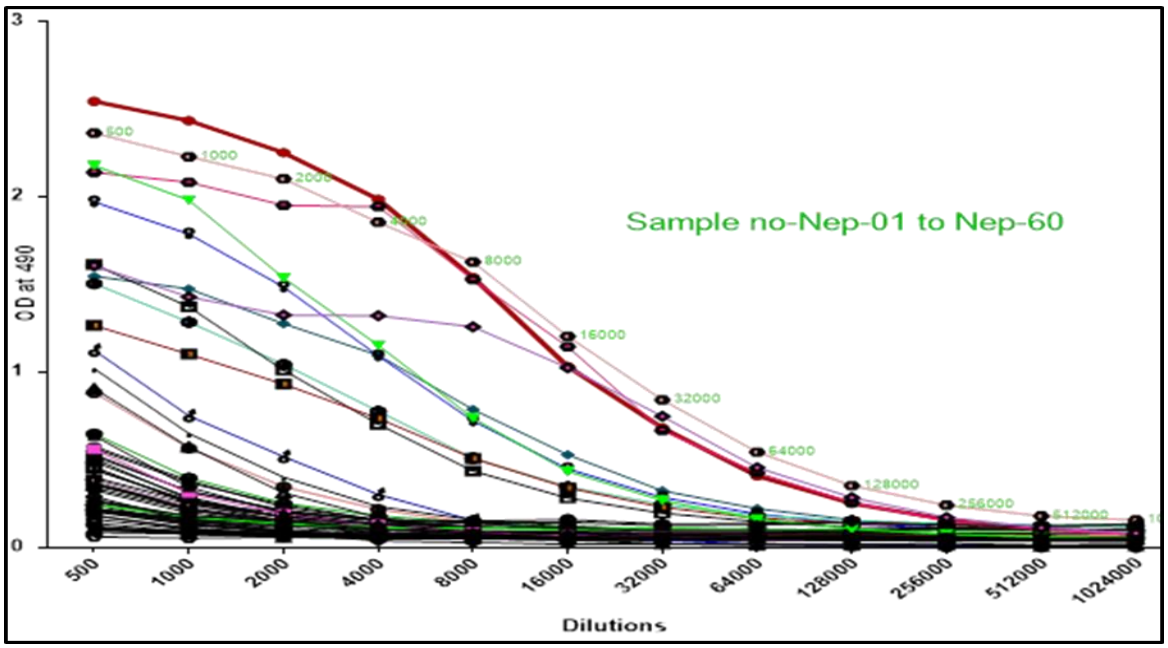
Nep-84	65	M	1.74	-VE	0.50	-VE	0.30	-VE	1.66		1.66	972.5	98.4	-VE	178,000
Nep-86	25	F	2.01	-VE	0.40	-VE	0.08	-VE	5.08		5.08	NA	91.7	-VE	130,000
Nep-88	26	M	158.60	+VE	9.87	+VE	0.11	-VE	89.53	1°	89.53	1,388.3	99.1	-VE	154,000
Nep-89	24	M	152.01	+VE	2.40	+VE	0.11	-VE	22.23	1°	22.23	992.7	71.1	+VE	85,000
Nep-90	20	M	2.05	-VE	0.37	-VE	0.46	-VE	0.80		0.80	1,485.1	82.7	-VE	81,000
Nep-91	35	M	1.80	-VE	0.46	-VE	0.10	-VE	4.47		4.47	1,665.4	67.6	-VE	156,000
Nep-92	38	F	1.97	-VE	0.34	-VE	0.11	-VE	3.25		3.25	2,324.2	98.8	-VE	198,000
Nep-93	24	F	1.87	-VE	0.28	-VE	0.10	-VE	2.65		2.65	1,018.1	89.9	-VE	212,000
Nep-94	34	F	1.81	-VE	0.62	-VE	0.91	-VE	0.69		0.69	1,472.8	105.5	-VE	108,000
Nep-95	15	F	1.81	-VE	0.29	-VE	0.15	-VE	1.93		1.93	965.2	95.6	-VE	179,000
Nep-96	33	F	2.28	-VE	0.81	-VE	0.14	-VE	5.80		5.80	1,092.6	81.4	-VE	122,000
Nep-97	25	F	152.51	+VE	9.63	+VE	0.71	-VE	13.57	1°	13.57	1,860.1	73.3	-VE	167,000
Nep-98	16	M	175.62	+VE	11.89	+VE	0.17	-VE	70.03	1°	70.03	1,373.3	80.9	-VE	123,000
Nep-99	20	M	30.43	+VE	4.64	+VE	6.01	+VE	0.77	2°	0.77	6,202.5	97.5	+VE	122,000
Nep-100	25	F	175.62	+VE	8.70	+VE	0.28	-VE	30.83	1°	30.83	1,740.7	73.3	+VE	190,000
Nep-101	26	M	175.62	+VE	0.38	-VE	0.11	-VE	3.29		3.29	1,422.9	74.7	+VE	145,000
Nep-102	71	M	4.18	-VE	0.45	-VE	0.86	-VE	0.52		0.52	1,637.9	51.7	-VE	203,000
Nep-103	38	M	164.45	+VE	10.97	+VE	0.17	-VE	62.90	1°	62.90	1,966.7	81.4	+VE	111,000
Nep-104	15	M	2.16	-VE	0.40	-VE	0.14	-VE	2.93		2.93	1,599.3	56.1	-VE	141,000
Nep-105	22	M	12.86	+VE	0.92	EQU	0.60	-VE	1.52		1.52	1,426.5	71.4	+VE	136,000
Nep-106	38	M	164.07	+VE	0.41	-VE	0.14	-VE	2.95		2.95	1,413.7	70.8	+VE	102,000
Nep-107	31	F	175.62	+VE	0.31	-VE	0.15	-VE	2.13		2.13	1,437.2	79	+VE	232,000
Nep-108	15	M	2.75	-VE	0.36	-VE	0.15	-VE	2.35		2.35	2,152.1	27.3	-VE	87,000
Nep-109	48	M	167.87	+VE	0.33	-VE	0.14	-VE	2.29		2.29	1,764.6	82.7	+VE	67,000
Nep-110	51	M	175.62	+VE	8.39	+VE	0.27	-VE	31.05	1°	31.05	1,457.1	82.5	+VE	125,000
Nep-111	35	M	2.06	-VE	0.38	-VE	0.14	-VE	2.74		2.74	1,220.3	84.1	-VE	169,000
Nep-112	52	F	6.24	-VE	0.28	-VE	0.13	-VE	2.16		2.16	1,471.6	54.1	-VE	398,000
Nep-113	20	M	19.02	+VE	0.65	-VE	0.47	-VE	1.37		1.37	1,023.6	71.2	-VE	143000
Nep-114	48	M	2.31	-VE	0.37	-VE	0.14	-VE	2.71		2.71	NA	90.5	-VE	51,000
Nep-115	30	M	2.27	-VE	0.32	-VE	0.13	-VE	2.40		2.40	1,084.2	78.2	-VE	265,000
Nep-116	34	F	147.96	+VE	0.37	-VE	0.13	-VE	2.82		2.82	1,612.1	64	+VE	121,000
Nep-117	55	F	1.43	-VE	0.40	-VE	0.13	-VE	3.05		3.05	1,772.2	93.8	-VE	143,000
Nep-118	43	F	144.29	+VE	5.25	+VE	0.15	-VE	34.98	1°	34.98	1,385.4	86.9	-VE	146,000
Nep-119	14	M	3.75	-VE	0.44	-VE	0.13	-VE	3.35		3.35	1,763.6	64.5	-VE	186,000
Nep-120	67	M	1.92	-VE	0.35	-VE	0.57	-VE	0.62		0.62	1,426.4	70.8	-VE	116,000
Nep-121	50	M	13.31	+VE	0.47	-VE	0.62	-VE	0.75		0.75	1,280.9	66	-VE	95,000
Nep-122	16	F	2.94	-VE	0.31	-VE	0.15	-VE	2.11		2.11	1,532.8	63.1	-VE	144,000
Nep-123	27	M	2.06	-VE	0.33	-VE	0.14	-VE	2.44		2.44	128,952.5	88.2	-VE	250,000
Nep-125	65	M	149.48	+VE	1.46	+VE	3.90	+VE	0.37	2°	0.37	6,383.8	85.1	-VE	145,000
Nep-126	42	M	1.57	-VE	0.25	-VE	0.13	-VE	1.83		1.83	1,509.2	94.7	-VE	207,000
Nep-127	38	M	2.54	-VE	0.32	-VE	0.17	-VE	1.87		1.87	1,379.2	74.2	-VE	133,000
Nep-128	28	F	2.93	-VE	0.55	-VE	0.14	-VE	3.97		3.97	1,185.4	86.3	-VE	158,000
Nep-129	7	M	2.02	-VE	0.31	-VE	0.13	-VE	2.40		2.40	1,405.5	87.4	-VE	161,000
Nep-130	26	M	26.52	+VE	0.85	-VE	0.58	-VE	1.46		1.46	1,860.2	105.1	-VE	353,000
Nep-131	38	M	167.98	+VE	1.49	+VE	0.14	-VE	10.96	1°	10.96	1,608.5	82.4	-VE	164,000

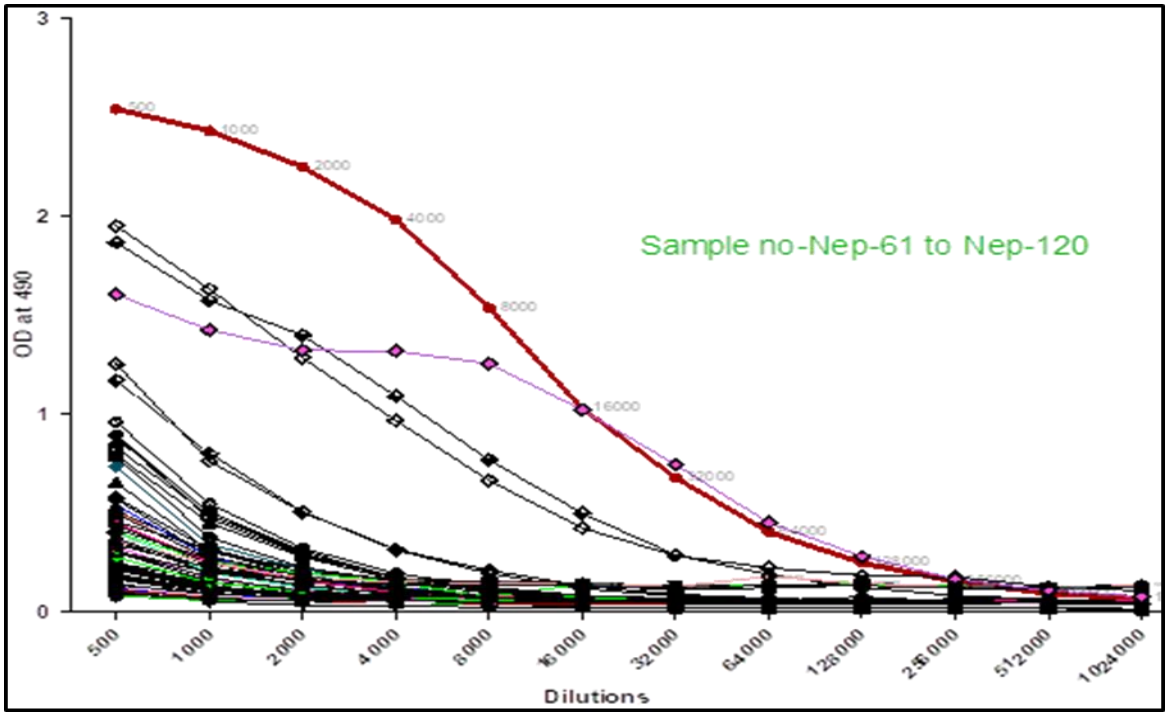
Nep-132	35	F	2.16	-VE	0.57	-VE	0.13	-VE	4.33		4.33	1,284.0	88.6	-VE	127,000
Nep-133	16	M	1.87	-VE	0.46	-VE	2.41	+VE	0.19		0.19	1,662.9	90	-VE	236,000
Nep-134	70	M	153.80	+VE	0.25	-VE	0.17	-VE	1.46		1.46	1,402.9	91.7	+VE	252,000
Nep-135	28	M	2.03	-VE	2.99	+VE	0.15	-VE	19.45	1°	19.45	1,193.3	82.7	-VE	30,000
Nep-136	19	F	2.09	-VE	0.33	-VE	0.13	-VE	2.48		2.48	2,189.3	81.3	-VE	120,000
Nep-137	36	F	2.31	-VE	0.38	-VE	0.13	-VE	2.87		2.87	1,427.0	93.1	-VE	209,000
Nep-138	10 m	M	154.57	+VE	1.15	+VE	0.41	-VE	2.82	1°	2.82	1,400.9	94.9	+VE	208,000
Nep-139	20	M	2.37	-VE	0.38	-VE	0.14	-VE	2.76		2.76	NA	91.7	-VE	362,000
Nep-140	23	M	1.99	-VE	0.50	-VE	0.14	-VE	3.59		3.59	1,556.2	95.7	-VE	216,000
Nep-141	45	F	144.21	+VE	0.30	-VE	0.17	-VE	1.72		1.72	1,199.0	94.8	+VE	122,000
Nep-142	46	M	1.43	-VE	0.43	-VE	1.04	-VE	0.41		0.41	1,361.1	75.5	-VE	138,000
Nep-143	9	F	141.94	+VE	0.40	-VE	0.87	-VE	0.45		0.45	1,403.4	76.8	+VE	83,000
Nep-144	52	F	1.05	-VE	0.37	-VE	0.13	-VE	2.75		2.75	1,444.6	66.5	-VE	151,000
Nep-145	66	F	103.40	+VE	1.06	EQU	0.13	-VE	8.14		8.14	1,526.7	83.1	+VE	87000
Nep-146	21	F	1.42	-VE	0.64	-VE	0.25	-VE	2.54		2.54	1,507.0	82.9	-VE	102000
Nep-147	44	M	1.06	-VE	0.29	-VE	0.13	-VE	2.24		2.24	3,769.1	102	-VE	133,000
Nep-148	38	M	1.28	-VE	0.43	-VE	0.33	-VE	1.27		1.27	1,512.3	71.4	-VE	111,000
Nep-149	35	M	19.47	+VE	0.41	-VE	0.75	-VE	0.55		0.55	1,454.7	108.9	-VE	130,000
Nep-150	11	M	105.14	+VE	11.54	+VE	5.74	+VE	2.01	1°	2.01	28,395.8	93	-VE	147,000
Nep-151	50	F	106.08	+VE	5.81	+VE	3.51	+VE	1.66	1°	1.66	2,851.1	108.8	-VE	156,000
Nep-152	40	F	1.34	-VE	0.61	-VE	0.14	-VE	4.39		4.39	1,539.5	107.8	-VE	173,000
Nep-153	9	M	1.04	-VE	1.42	+VE	0.13	-VE	10.66	1°	10.66	1,577.8	115.1	-VE	189,000
Nep-154	37	M	87.97	+VE	0.51	-VE	0.62	-VE	0.82		0.82	2,721.1	96.2	+VE	197,000
Nep-155	28	M	1.13	-VE	10.66	+VE	0.87	-VE	12.30	1°	12.30	1,736.9	77.6	-VE	161,000
Nep-156	16	M	91.87	+VE	0.52	-VE	0.13	-VE	3.92		3.92	1,590.9	115.1	+VE	64,000
Nep-157	18	M	97.43	+VE	3.47	+VE	0.16	-VE	22.23	1°	22.23	1,397.4	110.8	+VE	92,000
Nep-158	36	F	1.12	-VE	11.94	+VE	6.80	+VE	1.76	1°	1.76	7,816.8	114.6	-VE	134,000
Nep-159	45	F	89.93	+VE	0.30	-VE	0.13	-VE	2.29		2.29	1,974.4	106.1	+VE	157,000
Nep-160	32	F	100.06	+VE	3.75	+VE	0.15	-VE	25.60	1°	25.60	1,676.3	102.8	+VE	182,000
Nep-161	44	F	98.77	+VE	10.75	+VE	0.19	-VE	55.39	1°	55.39	1,685.5	107.3	-VE	330,000
Nep-162	30	M	94.56	+VE	0.63	-VE	0.84	-VE	0.76		0.76	1,410.7	106.6	+VE	127,000
Nep-163	25	M	97.59	+VE	0.29	-VE	0.13	-VE	2.19		2.19	1,414.4	115	+VE	87,000
Nep-164	25	M	99.80	+VE	0.48	-VE	0.15	-VE	3.31		3.31	1,418.1	60.9	+VE	141,000
Nep-165	50	M	15.14	+VE	0.50	-VE	0.14	-VE	3.63		3.63	1,051.8	98.8	-VE	141,000
Nep-166	60	M	102.09	+VE	10.63	+VE	0.14	-VE	76.78	1°	76.78	2,381.2	103.8	-VE	167,000
Nep-167	42	M	99.80	+VE	10.41	+VE	4.85	+VE	2.14	1°	2.14	NA	86.2	-VE	67,000
Nep-168	21	M	107.12	+VE	0.37	-VE	0.14	-VE	2.76		2.76	1,450.7	111.6	+VE	124000
Nep-169	32	M	1.05	-VE	0.29	-VE	0.14	-VE	2.04		2.04	1,442.2	67	-VE	86000
Nep-170	37	M	2.24	-VE	0.54	-VE	0.50	-VE	1.08		1.08	1,799.2	104	-VE	96000
Nep-171	22	M	0.86	-VE	0.34	-VE	0.13	-VE	2.58		2.58	1,494.5	51.9	-VE	78000
Nep-172	37	M	94.14	+VE	0.25	-VE	0.13	-VE	1.89		1.89	1,504.1	100.3	+VE	167000
Nep-173	20	M	17.70	+VE	0.31	-VE	0.13	-VE	2.38		2.38	1,388.5	90.3	-VE	123000
Nep-174	65	M	1.41	-VE	0.30	-VE	0.13	-VE	2.36		2.36	2,113.2	93	-VE	45000
Nep-175	67	M	92.53	+VE	5.28	+VE	0.14	-VE	36.98	1°	36.98	1,454.2	60.5	-VE	170000
Nep-176	12	F	1.08	-VE	0.69	-VE	0.13	-VE	5.18		5.18	2,004.3	51.6	-VE	65000

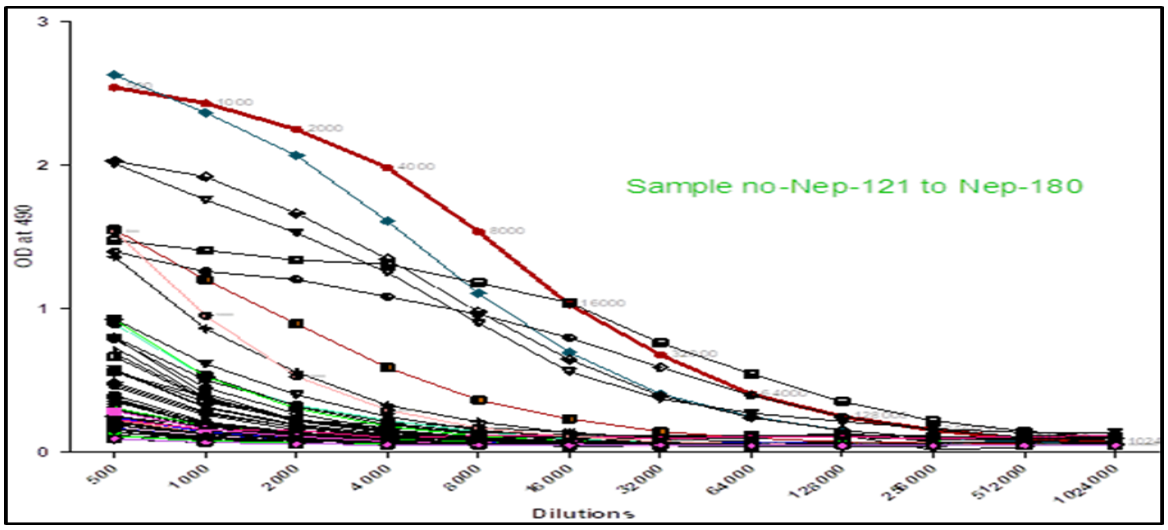
Nep-177	8	F	1.61	-VE	0.40	-VE	0.13	-VE	3.13		3.13	NA	74.6	+VE	47000
Nep-178	17	F	1.31	-VE	0.88	-VE	0.63	-VE	1.39		1.39	1,563.2	108.8	-VE	88,000
Nep-179	7	F	1.13	-VE	0.68	-VE	4.55	+VE	0.15		0.15	6,884.6	88.7	-VE	80000
Nep-180	51	M	1.06	-VE	0.32	-VE	0.14	-VE	2.32		2.32	NA	74.6	-VE	145000
Nep-181	14	M	8.27	-VE	0.26	-VE	0.14	-VE	1.82		1.82	1,602.0	105.2	-VE	36000
Nep-182	65	F	1.14	-VE	0.43	-VE	0.13	-VE	3.42		3.42	NA	103.6	-VE	76000
Nep-183	26	F	1.11	-VE	0.34	-VE	0.17	-VE	1.99		1.99	NA	110.6	-VE	70000
Nep-184	55	M	104.79	+VE	1.68	+VE	0.14	-VE	11.88	1°	11.88	1,946.1	137.8	+VE	41000
Nep-185	23	M	104.01	+VE	0.62	-VE	0.13	-VE	4.67		4.67	1,305.6	114.4	+VE	209,000
Nep-186	72	M	1.06	-VE	0.19	-VE	0.41	-VE	0.46		0.46	1,870.9	54.4	-VE	144,000
Nep-187	36	F	1.06	-VE	0.20	-VE	0.13	-VE	1.53		1.53	1,374.1	99.4	-VE	NA
Nep-188	65	F	1.05	-VE	0.17	-VE	0.13	-VE	1.32		1.32	1,970.6	104	-VE	NA
Nep-189	40	M	8.09	-VE	1.12	+VE	0.90	-VE	1.24	1°	1.24	1,450.4	91.7	-VE	NA
Nep-190	43	M	101.46	+VE	5.06	+VE	0.14	-VE	37.05	1°	37.05	1,243.4	106.3	-VE	NA
Nep-191	18	F	1.10	-VE	7.47	+VE	0.47	-VE	15.98	1°	15.98	8,820.8	102.2	-VE	NA
Nep-192	29	M	1.07	-VE	0.18	-VE	0.12	-VE	1.48		1.48	1,841.4	101.5	-VE	NA
Nep-193	20	M	107.12	+VE	3.55	+VE	0.97	-VE	3.64	1°	3.64	4,087.8	102.1	-VE	NA
Nep-194	28	M	1.04	-VE	0.19	-VE	0.13	-VE	1.45		1.45	1,534.1	105.8	-VE	NA
Nep-195	21	F	1.03	-VE	0.23	-VE	0.13	-VE	1.72		1.72	1,822.2	93.9	-VE	NA
Nep-196	45	F	1.08	-VE	0.24	-VE	0.13	-VE	1.86		1.86	1,091.0	67.1	-VE	NA
Nep-197	45	M	7.52	-VE	0.74	-VE	0.34	-VE	2.14		2.14	1,203.9	113.7	-VE	NA
Nep-198	40	M	107.12	+VE	7.21	+VE	6.09	+VE	1.18	2°	1.18	43,228.7	43.9	-VE	37000
Nep-199	6	F	1.09	-VE	1.45	+VE	0.13	-VE	11.09	1°	11.09	5,228.0	87	-VE	81000
Nep-200	36	M	107.12	+VE	0.65	-VE	0.13	-VE	4.82		4.82	NA	32	+VE	56000
Nep-201	52	M	1.05	-VE	0.41	-VE	0.13	-VE	3.17		3.17	1,982.6	104.3	-VE	98000
Nep-202	40	M	1.04	-VE	0.38	-VE	0.13	-VE	2.84		2.84	2,090.5	115.5	-VE	66000
Nep-203	8	F	1.04	-VE	0.52	-VE	0.14	-VE	3.77		3.77	NA	89.4	-VE	67000
Nep-204	40	F	101.30	+VE	1.09	EQU	0.13	-VE	8.21		8.21	1,870.3	86.4	+VE	88000
Nep-205	37	M	103.57	+VE	0.80	-VE	0.46	-VE	1.74		1.74	NA	67.6	+VE	174000
Nep-206	44	M	104.19	+VE	10.35	+VE	0.18	-VE	56.71	1°	56.71	1,743.3	59.6	-VE	189000
Nep-207	17	M	1.06	-VE	3.34	+VE	0.15	-VE	23.01	1°	23.01	4,797.4	73.5	-VE	90000
Nep-208	16	M	1.03	-VE	0.47	-VE	0.13	-VE	3.56		3.56	1,539.1	18.8	-VE	78000
Nep-209	3	F	107.12	+VE	1.04	EQU	0.15	-VE	6.70		6.70	1,915.3	74.8	+VE	67000
Nep-210	52	M	107.12	+VE	0.29	-VE	0.13	-VE	2.31		2.31	2,545.8	65.7	+VE	101000
Nep-211	2	F	95.94	+VE	3.73	+VE	0.16	-VE	22.65	1°	22.65	1,445.3	94	-VE	120000
Nep-212	44	M	96.51	+VE	10.70	+VE	0.17	-VE	61.37	1°	61.37	1,727.8	100	-VE	180000
Nep-213	18	M	7.44	-VE	0.83	-VE	2.37	+VE	0.35		0.35	1,864.8	71.9	-VE	55000
Nep-214	5	M	1.10	-VE	0.29	-VE	0.13	-VE	2.27		2.27	NA	80.6	-VE	240000
Nep-215	21	M	1.07	-VE	0.28	-VE	0.14	-VE	1.99		1.99	1,541.7	61	-VE	43000
Nep-216	10	F	97.59	+VE	2.21	+VE	4.33	+VE	0.51	2°	0.51	8,554.4	84.9	-VE	340000
Nep-217	3	M	98.18	+VE	8.69	+VE	0.15	-VE	57.46	1°	57.46	1,829.3	48.3	-VE	121000
Nep-218	15	M	98.71	+VE	5.52	+VE	0.13	-VE	42.04	1°	42.04	1,024.1	96	+VE	74000
Nep-219	50	M	0.95	-VE	0.37	-VE	0.13	-VE	2.76		2.76	1,109.6	34.6	-VE	80000
Nep-220	60	F	1.10	-VE	0.43	-VE	0.13	-VE	3.37		3.37	1,273.8	75	-VE	63000
Nep-221	4	F	6.48	-VE	0.70	-VE	1.04	-VE	0.67		0.67	1,546.4	73.8	-VE	230000

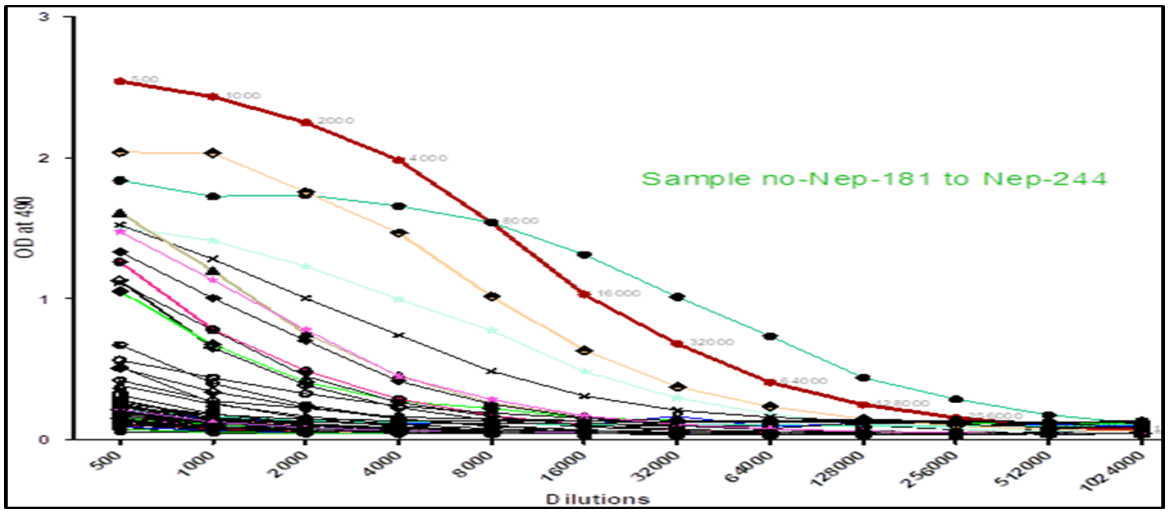
Nep-222	41	F	107.12	+VE	2.64	+VE	0.13	-VE	20.40	1°	20.40	1,011.1	69.5	-VE	88000
Nep-223	20	M	106.28	+VE	0.31	-VE	0.13	-VE	2.35		2.35	1,459.8	32.1	+VE	160000
Nep-224	17	M	107.12	+VE	3.28	+VE	0.13	-VE	24.40	1°	24.40	1,031.7	79.9	-VE	99000
Nep-225	14	M	1.01	-VE	0.33	-VE	0.70	-VE	0.48		0.48	1,583.5	93	-VE	160000
Nep-226	26	M	1.03	-VE	0.32	-VE	0.13	-VE	2.47		2.47	1,510.4	39.1	-VE	93000
Nep-227	20	M	0.89	-VE	0.27	-VE	0.13	-VE	2.06		2.06	1,172.0	87	-VE	68000
Nep-228	18	F	100.70	+VE	2.73	+VE	0.13	-VE	21.44	1°	21.44	2,244.0	97.2	-VE	60000
Nep-229	3	F	0.47	-VE	1.25	+VE	0.56	-VE	2.23	1°	2.23	1,106.9	65.3	-VE	178000
Nep-230	37	M	101.49	+VE	3.32	+VE	0.13	-VE	25.77	1°	25.77	1,437.9	101	-VE	134000
Nep-231	18	M	1.07	-VE	0.36	-VE	0.13	-VE	2.68		2.68	1,920.0	43.9	-VE	80000
Nep-232	17	F	101.30	+VE	0.28	-VE	0.13	-VE	2.22		2.22	1,678.9	19.9	+VE	49000
Nep-233	20	M	107.12	+VE	0.70	-VE	0.13	-VE	5.57		5.57	1,900.1	54.3	+VE	66000
Nep-234	20	F	103.75	+VE	1.27	+VE	2.56	+VE	0.50	2°	0.50	2,333.0	103.2	+VE	56000
Nep-235	12	M	6.72	-VE	3.53	+VE	0.13	-VE	26.65	1°	26.65	2,541.8	96.5	-VE	86000
Nep-236	41	F	91.42	+VE	0.71	-VE	0.14	-VE	5.22		5.22	1,827.9	94.1	-VE	93000
Nep-237	26	M	15.06	+VE	0.66	-VE	0.54	-VE	1.22		1.22	1,183.0	96.3	-VE	44000
Nep-238	40	F	1.05	-VE	0.23	-VE	0.13	-VE	1.82		1.82	1,903.1	92.5	-VE	88000
Nep-239	20	M	87.53	+VE	11.25	+VE	0.19	-VE	59.82	1°	59.82	1,544.2	51.7	-VE	65000
Nep-240	11	M	98.90	+VE	0.60	-VE	0.13	-VE	4.62		4.62	2,207.4	94	-VE	110000
Nep-241	33	F	97.53	+VE	0.42	-VE	1.99	EQU	0.21		0.21	2,304.5	84.2	+VE	114000
Nep-242	54	M	93.82	+VE	0.45	-VE	0.13	-VE	3.50		3.50	NA	104.5	+VE	147000
Nep-243	13	F	96.94	+VE	4.71	+VE	0.60	-VE	7.87	1°	7.87	1,621.0	99.1	-VE	67000
Nep-244	14	F	0.96	-VE	0.32	-VE	0.15	-VE	2.13		2.13	2,380.5	100.3	-VE	88000

Appendix 3: Representation of Indirect ELISA results by Graph Pad Prism




Sample no-Nep-61 to Nep-120
OD at 490
Dilutions
500
1000
2000
4000
8000
16000
32000
64000
128000
256000
512000
1024000

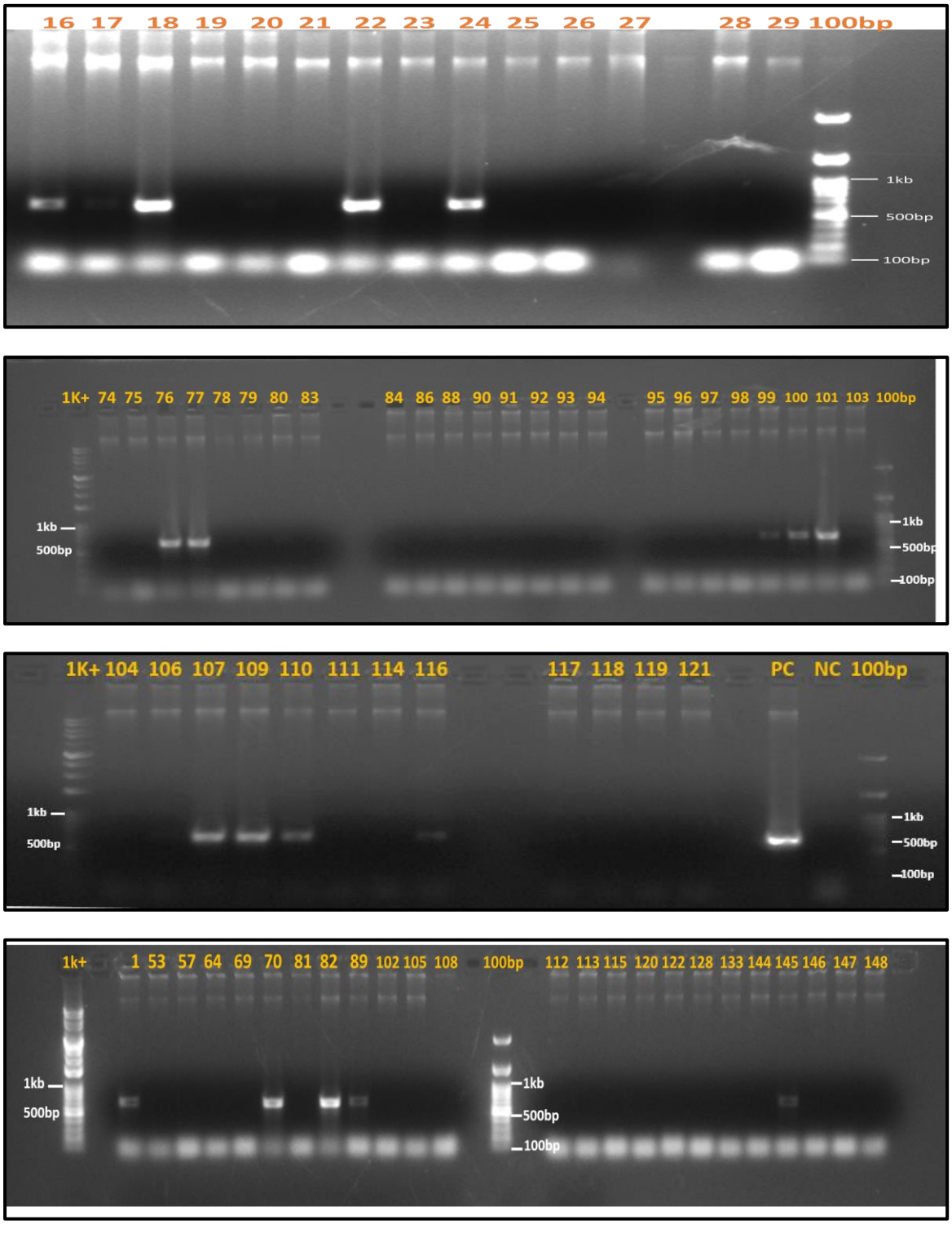

Sample no-Nep-121 to Nep-180
OD at 490
Dilutions
500
1000
2000
4000
8000
16000
32000
64000
128000
256000
512000
1024000

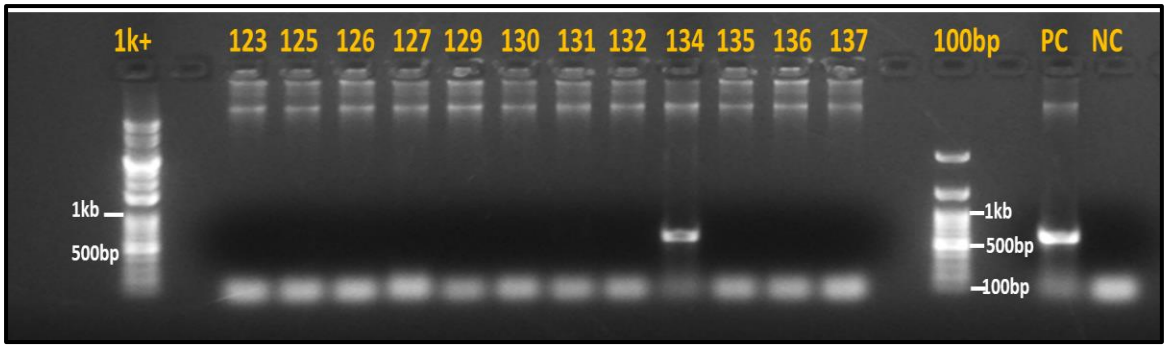

Sample no-Nep-181 to Nep-244
OD at 490
Dilutions
500
1000
2000
4000
8000
16000
32000
64000
128000
256000
512000
1024000

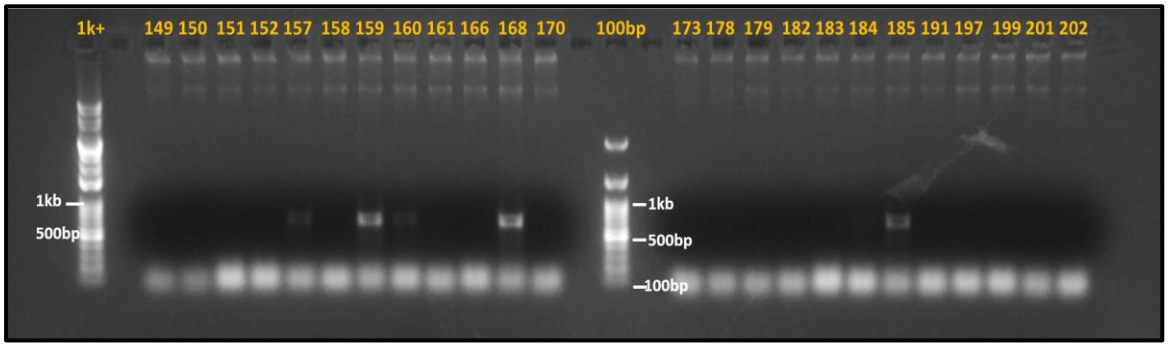
Graphical representation of mid-point titrations of all samples. The dilutions are presented in the x-axis and the OD value at 490 nm on Y-axis. Each dot in the figure shows the dilutions. The red-bold curve at the uppermost region is the Positive control and other curves are the curves of the samples.

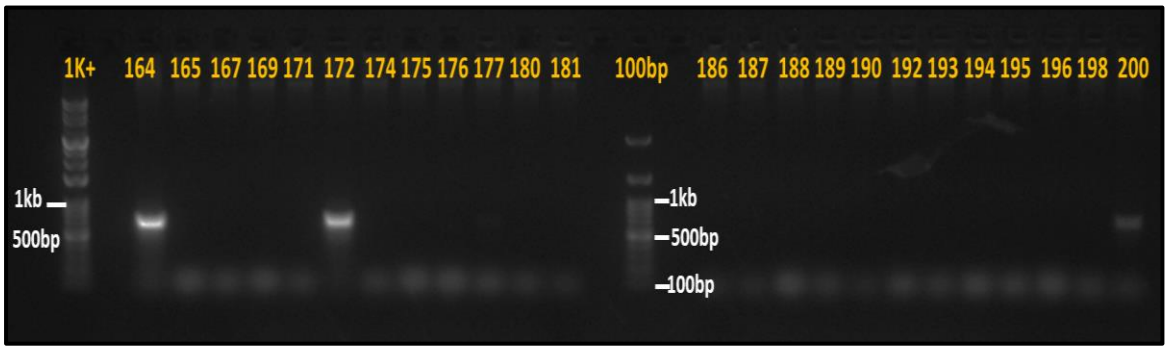
Appendix 4: Dengue PCR results

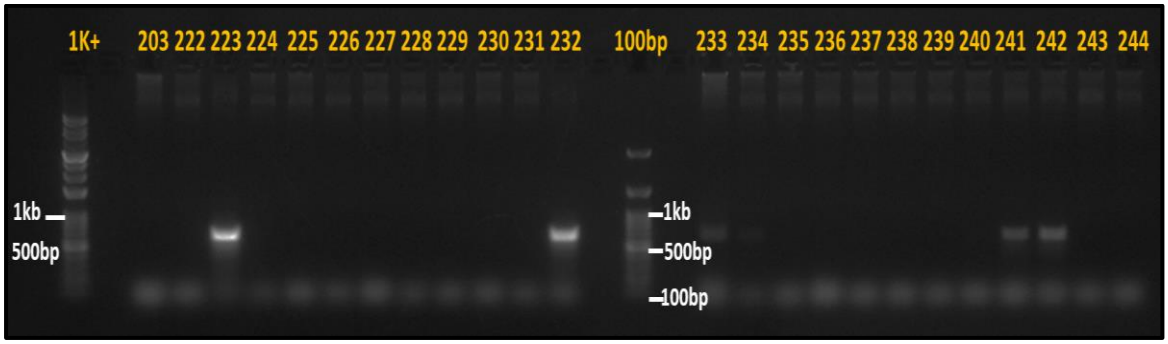
Agarose gel (2%) showing PCR amplification of Dengue virus isolated from patient's serum.

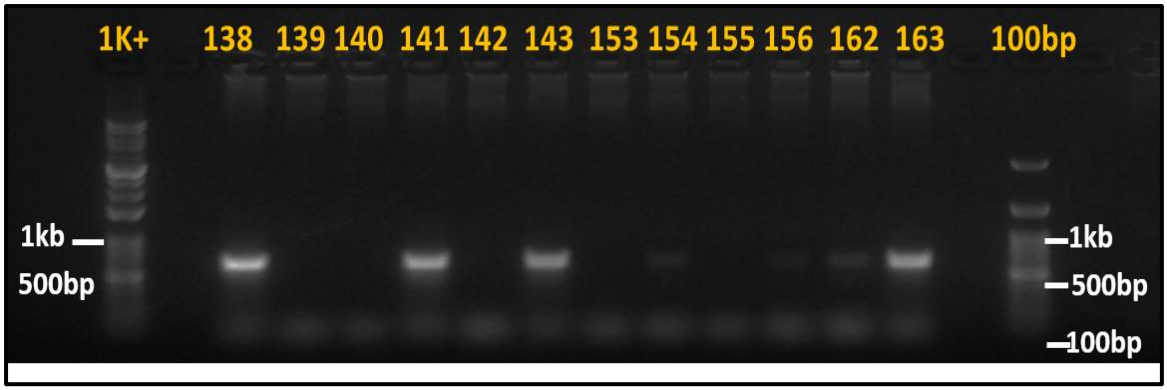



1k+
123 125 126 127 129 130 131 132 134 135 136 137
100bp
PC NC
1kb
500bp
1kb
500bp
100bp


1k+
149 150 151 152 157 158 159 160 161 166 168 170
100bp
173 178 179 182 183 184 185 191 197 199 201 202
1kb
500bp
1kb
500bp
100bp


1K+
164 165 167 169 171 172 174 175 176 177 180 181
100bp
186 187 188 189 190 192 193 194 195 196 198 200
1kb
500bp
1kb
500bp
100bp

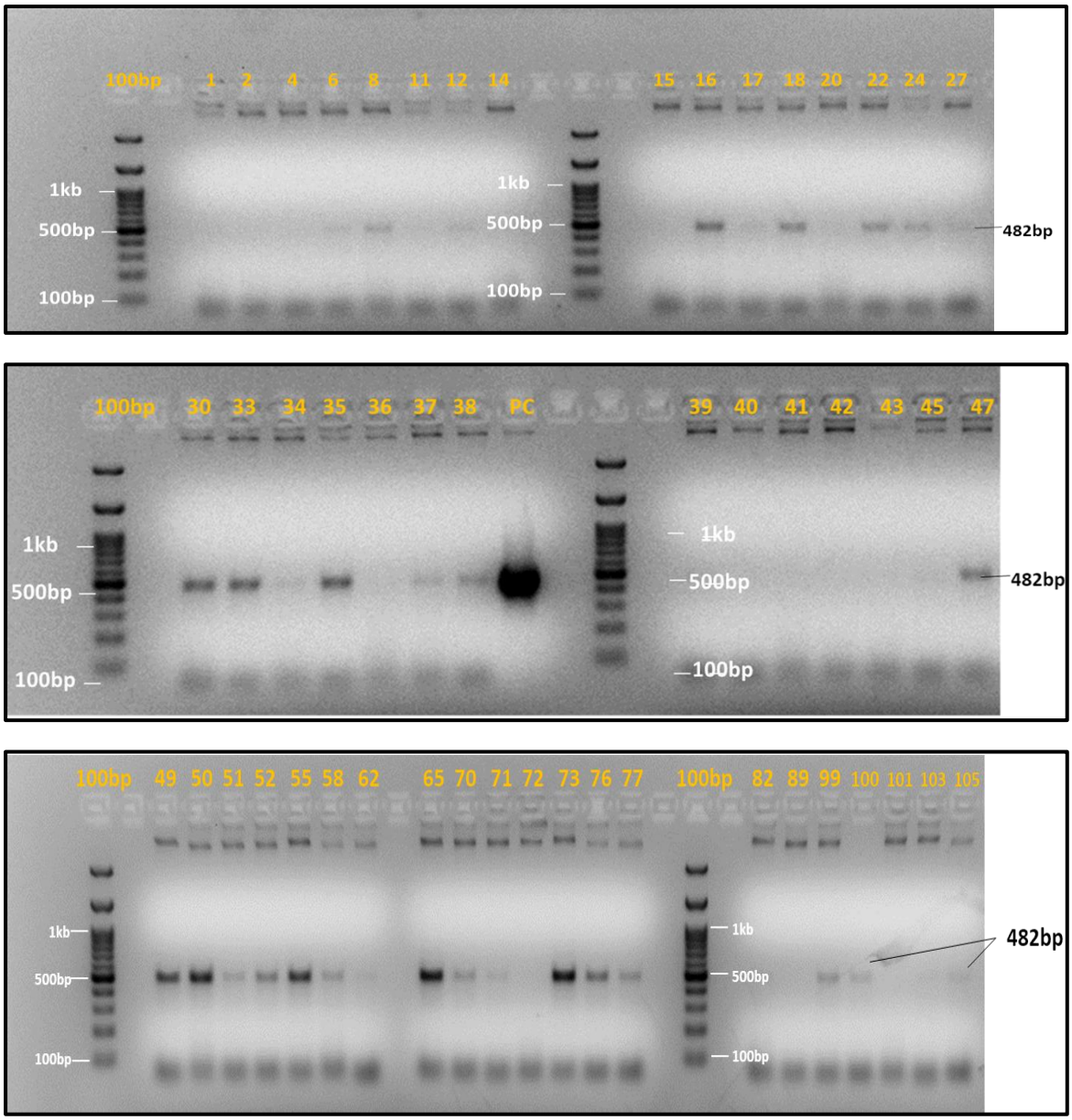

1K+
203 222 223 224 225 226 227 228 229 230 231 232
100bp
233 234 235 236 237 238 239 240 241 242 243 244
1kb
500bp
1kb
500bp
100bp


1K+
138 139 140 141 142 143 153 154 155 156 162 163
100bp
1kb
500bp
1kb
500bp
100bp

The first lanes on the left side contain the 1k+ ladder and samples in between and a band on the extreme right of 100kb DNA ladder. Four positive control and one negative control are included and marked as PC for positive control and NC as negative control in the latter wells. The samples which are showing the white band at the level of 654bp are supposed to be PCR positive and the rest are negative.

Appendix 5: Dengue serotyping results

Agarose gel (2%) showing the positive bands for DENV 1 serotype of length 482 bp from Dengue positive samples



Appendix 6: Ethical approval and Patient's Consent

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 1968

18 May 2017

Prof. Krishna Das Manandhar
Principal Investigator
Central Department of Biotechnology, Tribhuvan University
Kirtipur, Nepal

Subject: Approval of requested amendment on proposal entitled **Dengue and Dengue-like infections in patients visiting the hospitals in Nepal**

Dear Prof. Manandhar,

The Ethical Review Board meeting held on 17 May 2017 discussed on the amendment requested by you on 13 April 2017 and has decided to approve the following changes in your proposal.

- Inclusion of all patients who visit for dengue and dengue like fever.
- Collaboration extension with Genetic Engineering and Biotechnology, (ICGEB), New Delhi, India
- Collection of 50 samples per year of control population from dengue endemic and non-endemic regions.

If you have any queries, please feel free to contact the Ethical Review M & E section of NHRC.

Thanking you,

Prof. Dr. Anjani Kumar Jha
Executive Chairman

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

Consent to Participate in a Research Study

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

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

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

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

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

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

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

18 years or older during the acute phase of the illness or during the first visit will be sent to Karius for testing.

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

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

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

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

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

Part 2: Additional Consent for Genetic Research

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

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

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

If you agree, the data will entered into external scientific databases so that it can be broadly shared with other researchers performing other genomic studies. For example, the National Institutes of Health (NIH, an agency of the US federal government) maintains a database called "dbGaP." Databases like this serve as a repository of all kinds of genomic data from studies funded by the NIH and conducted in the US and around the world. The aim of collecting this information in a repository is to allow qualified researchers to look for genetic connections for a range of topics in the future. The information may be used to learn if certain genes are associated with certain traits, diseases and /or treatment effects. Making data broadly available in this way, means that your contribution and the data generated in this study could be helpful in advancing other areas of scientific research.

Confidentiality will be maintained in the following manner. Traditionally used identifying information about you (such as name, phone number, address) will NOT be included in these databases or shared with others. De-identified genomic data generated in this study may be deposited in databases that will be publicly accessible via the Internet. Researches with an approved study may access and utilize your de-identified genetic, genomic and/or health information deposited in the database (dbGAP) after approval by the regulatory authority (NIH). Strict safety measures are in place to protect the privacy of your information. However, because your genetic information is unique to you, there is a small chance that someone could trace it back to you or your family. The risk of this happening is very small, but may grow in the future. Researchers will always have a duty to protect your privacy and keep your information confidential.

You may withdraw consent for research use of genomic data or health information at any time. In this event, data will be withdrawn from any repository, if possible, but data already distributed for research use will not be retrieved.

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

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

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

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

If you do not wish your data to be shared in external scientific databases, you may still take part in this study and your data will not be submitted to an external database. Please indicate below whether you consent to the sharing of your data in this way.

☐ I **DO AGREE** to consent to my or my child's genetic, genomic and/or health information being submitted to an external database and broadly shared with other researchers

☐ I **DO NOT AGREE** to consent to my or my child's genetic, genomic and/or health information being submitted to an external database and broadly shared with other researchers.

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

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

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

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

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

Do you have any questions?

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

_____ _____

Name of Subject Study code

_____

Name of Parent or Guardian (if applicable)

_____ _____

Signature of Subject/Parent or Guardian Date

An assent process HAS _____ or WILL NOT BE _____ completed

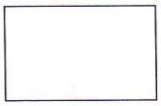
If unable to read and/or write:

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

_____
Name of Witness

_____ _____
Signature Date

Thumb print of subject/parent



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

[_____]
Person conducting the Consent

_____ _____
Signature Date

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

Appendix 7. Photos and publication