SERO-EPIDEMIOLOGICAL STUDY OF DENGUE VIRUS IN SOME HOSPITALS OF NEPAL

A

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(Environment and Public Health)

By

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LETTER OF RECOMMENDATION

The dissertation entitled **"Sero-epidemiological Study of Dengue Virus in Some Hospitals of Nepal"** has been completed by **Mr. Dharma Prasad Kuikel** under our supervision. We hereby recommend this dissertation for examination as a partial fulfillment for the Degree of Master of Science in Microbiology (Environment and Public Health).

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ABSTRACT

Dengue is an emerging mosquito-borne viral disease in the world and is the serious public health problem of Nepal. This study was designed to determine sero-epidemiology of dengue virus infection during the period (June-Nov) of 2010 among suspected patients with fever visiting Koshi Zonal Hospital (KZH), Biratnagar, Narayani sub-regional Hospital (NSH), Birgunj, Sukraraj Tropical and Infections Disease Hospital (STIDH), Kathmandu and Dhading District Hospital (DDH), Dhadingbeshi. The sero-prevalence of anti-dengue IgM antibody was determined by enzyme linked immunosorbent assay (ELISA). Among 271 serum samples tested, the anti-dengue IgM positivity was 14.4%. Sero-positivity in male was 10.7% of total and that in female was 3.7%. Among different age groups, the highest positive cases (11.8%) were from age group 15-50 years and found least among the age group above 50 years (0.4%). Out of 4 different hospitals, the highest positive cases were from STIDH with 9.2% and the least positive cases were from DDH (0.4%). The distribution of dengue positive cases among different occupation group was highest in students (5.9%) having least positivity among others and labour groups (0.4%). RT-PCR showed 4.7% positivity of 21 samples tested.

Key words: dengue fever, epidemiology, IgM Capture ELISA, RT-PCR.

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LIST OF ABBREVIATIONS

ADCC:	Antibody-dependent Cellular Cytotoxicity
ADE:	Antibody-dependent Enhancement
cDNA:	Complementary DNA
CFT:	Complement Fixation Test
DENV (-1, -2, -3 & -4):	Dengue Virus (-1, -2,-3 & -4)
DF:	Dengue Fever
DHF:	Dengue Hemorrhagic Fever
DNA:	Deoxyribonucleic Acid
DSS:	Dengue Shock Syndrome
DENV:	Dengue Virus
DVI:	Dengue Virus Infection
ELISA:	Enzyme Linked Immunosorbent Assay
HI:	Hemagglutination Inhibition
HRP:	Horse Reddish Peroxidase
IFA:	Immunofluorescence Assay
IFN- :	Interferon-
MA:	Microneutralization Assay
NT:	Neutralization Test
PA:	Particle Agglutination
PAHO:	Pan American Health Organization
PCR:	Polymerase Chain Reaction
PRNT:	Plaque Reduction and Neutralization Test
RNA:	Ribonucleic Acid
RT-PCR:	Reverse Transcriptase PCR
SEARO:	South East Asian Regional Office
TAE:	Tris-acetate-EDTA
TMB:	Tetramethylbenzidine
UTR:	Untranslated Region

CHAPTER – I

1. INTRODUCTION

Dengue is an endemic disease affecting tropical and subtropical regions worldwide. It is a vector borne disease and transmitted to humans primarily by *Aedes aegypti* and *Aedes albopictus* mosquitoes. Four dengue virus (DENV) serotypes viz DENV-1, DENV-2, DENV-3 and DENV-4 are responsible for the disease (Gubler, 1998). Dengue can be asymptomatic or lead to an undifferentiated fever, dengue fever (DF) or dengue hemorrhagic fever (DHF) with plasma leakage which possibly can develop into hypovolemic shock (Sirinavin *et al*., 2004). DF is a self-limited febrile illness, which usually accompanied with retro orbital or frontal headache. A transient macular rash that blanches under pressure, nausea, vomiting, lymphadenopathy and taste aberrations can develop (Kurane, 2007). DHF is a life threatening complication of dengue characterized by high fever of 2-7 days, hemorrhagic phenomena, thrombocytopenia and sometimes circulatory failure some patients progress to shock known as dengue shock syndrome (DSS) (Shah *et al*., 2006).

An estimated 50 million dengue infections occur annually and approximately 2.5 billion people live in dengue endemic countries. Some 1.8 billion (more than 70%) of the population at risk for dengue worldwide live in the South-East Asia Region and Western Pacific Region, which bear nearly 75% of the current global disease burden due to dengue (WHO, 2009).

DENV infection was recorded since 90's in Nepal and the first case of dengue was reported in 2004. Then a confirmed outbreak was observed in nine districts of Terai region in Nepal in 2006 (Sah *et al*., 2009). Although DF has been already observed in foreign visitor earlier (Pandey *et al*., 2004) the outbreak occurred in Nepal following the Indian epidemic of DF/DHF in September-October 2006. The occurrence of DENV-1, DENV-2, DENV-3 and DENV-4 serotypes (WHO/SEARO, 2006, Takasaki *et al*., 2008 and Pandey *et al*., 2008) in the territory of Nepal augment the chances for the epidemic DF/DHF to be flourished in the country.

DENV infection is diagnosed by four basic methods which include virus isolation and characterization, detection of DENV specific antibodies, detection of dengue antigen and detection of viral nucleic acid by nucleic acid amplification technique (WHO, 2009). In the early stages of infection, isolation and identification of dengue virus is traditionally the only way to diagnose a current dengue infection (Buchy *et al*., 2006). Molecular diagnosis based on reverse transcription polymerase chain reaction (RT-PCR) such as one step or nested. RT-PCR, nucleic acid sequence-based amplification (NASBA), or real time RT-PCR, has gradually replaced the virus isolation method as the new standard for the detection of DENV in acute phase serum samples (Shu and Hugng 2004). At the end of the acute phase of DENV infection, serology is the method of choice for diagnosis serologically; infection can be diagnosed by IgM/IgG Capture enzyme linked immune-sorbent assay (ELISA), Hemagglutination inhibition test, complement fixation test, neutralization test, particle agglutination test and rapid immunochromatic test. The choice of diagnostic method depends on the purpose for which the testing is done (e.g. clinical diagnosis, epidemiological survey, vaccine development), the type of laboratory facilities and technical expertise available, costs, and the time of sample collection (WHO, 2009).

Nepal has no dengue surveillance programs, and health professionals do not usually consider dengue as a differential diagnosis (Pandey *et al*., 2008). Nepal is bordered by India in the eastern western and southern belt. As the risk of dengue virus infection increases in India and other neighboring countries. Nepal will surely be pushed towards the increased risk of dengue virus infection. So proper management of disease is required to prevent the increased threat of dengue virus infection (DVI) in Nepal. More and more studies for the sero-prevalence of the disease are required for the proper management of disease. This study would initiate in establishing sero-epidemiological studies and its implications for crafting appropriate future interventions of dengue in Nepal. The information generated through this study would be important by providing necessary information to the concerned authority for implementing, prophylactic measures monitoring and planning for surveillance and disease management.

CHAPTER- II

2. OBJECTIVES

2.1 General Objectives

To study the prevalence and distribution of dengue virus in some hospitals of Nepal.

2.2 Specific Objectives

1. To detect the anti-dengue IgM in the serum samples of the clinically suspected patients.
2. To describe the demographic characteristics of the patients.
3. To detect dengue virus among seronegative febrile cases.

CHAPTER- III

3. LITERATURE REVIEW

3.1 Dengue Virus

3.1.1 The Virus: Morphology and Structure

DENV is a mosquito-borne member of the genus *Flavivirus* of family Flaviviridae. There are four antigenically related serotypes (DENV-1, DENV-2, DENV-3 and DENV-4), which co-circulate in tropical and subtropical regions around the world between their vectors, the mosquitoes *Ae. aegypti* and *Ae. albopictus* and the vertebrate hosts (Acosta *et al*., 2008).

Dengue virion is spherical, lipid enveloped agents of 40-50 nm in diameter. The Dengue virus contains a single-stranded positive-sense RNA genome of approximately 11 kb which is translated into a large poly-protein during the infections life-cycle. This polyprotein is processed by cellular and viral proteases into three mature, structural proteins (C, pr M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The envelope protein (E) plays a key role in several important processes including receptor binding, blood cell hemagglutination, induction of a protective immune response, membrane fusion and virion assembly NS3, a large multifunctional protein of 618 amino acids endowed with protease, heliase, nucleoside 5 -triphosphatase (NTPase), as well as 5 terminal RNA-triphosphatase activities, plays an important role in viral poly-protein processing and genome replication (Guzman *et al*., 2004; Xu *et al*., 2005).

The gene order in dengue virus genome is 5 -c-PrM (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3 which is expressed as a single poly-protein. The coding sequence is flanked by 5 and 3 untranslated regions (UTRs), which contain C is-acting RNA elements that control viral translation, RNA synthesis, and encapsidation. The viral RNA has a type I cap at the 5 end. The 5 UTR is about 100 nucleotides long and shows high sequence conservation among different DENV serotypes specific nucleotides at the

3 end of the viral genome also plays a crucial role in viral RMA synthesis. The approximately 450-nucleotide-long DENV 3 UTR Lacks a poly (A) tail but ends in a very conserved 3 stem loop (3 SL) structure. A detailed functional analysis of the 3 SL revealed its absolute requirement for viral replication (Lodeiro *et al*., 2008; Zhang *et al*., 2004).

3.1.2 Replication

DENV replicates in a wide variety of culture cells of both vertebrate and arthropod origins. Virus practices enter the cell by receptor-mediated endocytosis. In secondary infection with a heterologous serotype which reportedly read to more sever disease, Antibody dependent enhancement (ADE) can mediate virus attachment and uptake. The ADE model postulates that non-neutralizing antibodies can interact with the DENV and facilitate viral entry into monocytes and macrophages via Fc receptors (Watowich *et al*., 2009, Rothwel *et al*., 2009). However DENV-2 may enter human peripheral blood monocytes by direct fusion with the plasma membrane. DENV has been shown to infect numerous cell lines in vitro including endothelial cells, B and T cells, and hepatocytes. However one important target cell type for DVI in vivo is cell of monocyte lineage (Wati *et al*., 2007). Upon acidification of the endocytic vesicle, the nucleocapsid enters into the cytoplasm where the virus genome is released. The genome is translated into a single poly-protein, and undergoes co-translational and post-translational processing by host and viral proteases to produce the individual proteins required for viral replication and packaging. Replication takes place on intercellular membranes and assembly occurs on the endoplasmic reticulum (ER) membrane. Newly assembled virus particles are transported through the trans-golgi network and are released from the cell by exocytosis (Watowich *et al*., 2009).

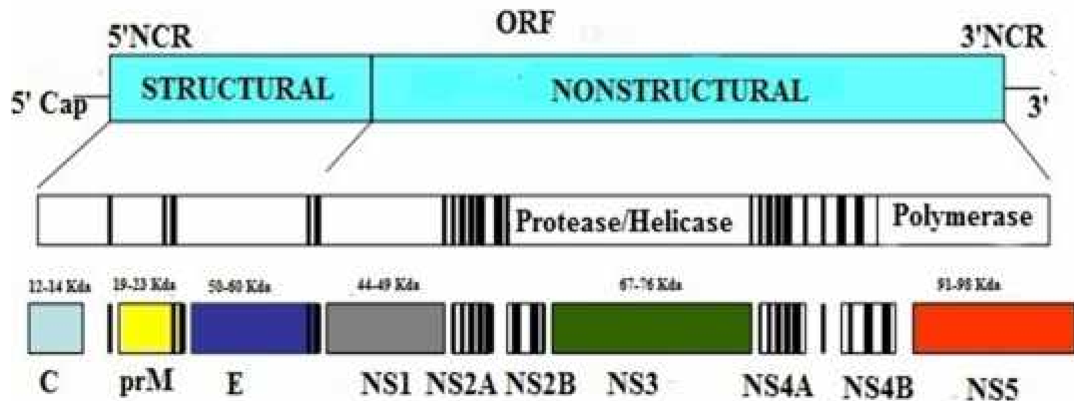


Fig.1 Mature protein generated by proteolytic processing cascade

(Source: Fields Virology, 2001)

3.1.3 The Vector

Ae. aegypti is the principal vector for dengue viruses. This vector is able to maintain the four serotypes of dengue viruses (DENV 1, 2, 3 and 4) in an urban transmission cycle (Herrera *et al*., 2006). *Ae. aegypti* is a tropical mosquito that lays its eggs on the walls of containers commonly found in and around homes. Female mosquitoes remain infections for their entive lives and have the potential to transmit virus during each human feeding (Vilela *et al*., 2010).

Dengue outbreaks have also been attributed to *Ae. albopictus*, *Ae. polynesiensis* and several species of the *Ae. scutellaris* complex. Each of these species has its own particular geographical distribution; however *Ae. aegypti* is the most efficient epidemic vector than others. *Ae. aegypti* is a tropical and subtropical species of mosquito found around the globe, usually between latitudes 35^0 N and 35^0S. *Ae. aegypti* is believed have originated in the jungles of Africa and was most likely spread throughout the rest of the world via slave and trading ships during the seventeenth to nineteenth century. It is an anthropophilic species that has adapted extremely well to the urban environment, which is found both indoors and outdoors in close proximity to human dwellings. Their ability to breed in artificial containers facilitated their passive spread in the last decades through

main transportation routes. These mosquito species coexist in man-made containers in urban, suburban and rural settlements in tropical and subtropical regions (WHO, 1997, Gubler, 1998; Vezzani and Carbajo, 2008). *Ae. aegypti* has a relatively short flight range, the majority remaining within 100 meters of where they emerged. They feed almost entirely on humans, mainly during daylight hours and both indoors and outdoors, because of low temperature it is usually not found above 1000 meters but has been reported at 2121m in India, at 2200m in Colombia, where the mean annual temperature is 17^0C, out at 2400m in Eritrea, (WHO, 1997; WHO, 2009).

Entomological study of mosquitoes carried out during eighties revealed the presence of *Ae. albopictus* in Terai plains of Nepal which has been reported regularly *Ae Albopictus* is considered as an inefficient vector for DF transmission. Cross-sectional entomological survey after the 2006 outbreak identified *Ae. Aegypti* in 5 major urban areas of Terai region bordering with India, i.e. Biratnagar (Morang), Birgunj (Parsa), Bharatpur (Chitwan), Tulsipur (Dang) and Nepalganj (Banke) (WHO/SEARO, 2006).

3.1.4 The Host

In humans, each of the four DENV serotypes has been associated with DF and with DHF. DSS occurs with higher frequency in two immunologically defined groups: children who have experienced a previous dengue infection and infants with waning levels of maternal antibody. The first infection produces life-long immunity to the infecting serotypes but only temporary and partial protection against the other three serotypes and secondary or sequential infections are possible after a short time. Transmission of dengue virus from infected humans to feeding mosquitoes is determined by the magnitude and duration of viraemia in the human host; persons with high viraemia provide a higher infectious dose of virus to the feeding mosquito, normally leading to a greater percentage of feeding mosquitoes becoming infected, although even very low levels of virus in blood may be infectious to some vector mosquitoes (WHO, 1997). Classic DHF/ DSS are almost totally confined to children. Shock cases and deaths occur more frequently in female than male children. Black people are less susceptible to shock syndrome than write and Asian people (Kouri *et al*., 1989; Halstead *et al*., 1970; Guzman *et at*., 1990).

3.1.5 Transmission of Dengue Virus

DENV is transmitted to humans through the bite of infected *Aedes* mosquitoes, principally *Ae. aegypti*. Once infected, a mosquito remains infected for life, transmitting the virus to susceptible individuals during probing and feeding. Infected female mosquitoes may also pass the virus to the next generation of mosquitoes by transovarian transmission, but this occurs infrequently and probably does not contribute significantly to human transmission. Humans are the main amplifying host of the virus, although studies have shown that men keys in some parts of the world may become infected and perhaps serve as a source of virus for feeding mosquitoes (WHO, 1997). Symptoms of DVI usually begin 4-7 days after the mosquito bite and typically last 3-10 days. In order for transmission to occur the mosquito must feed on a person during a 5-day period when large amount of virus circulate in the blood; this period usually begins a little before the person become symptomatic. Some people never have significant symptoms but can still infect mosquitoes. After entering the virus in the blood meal, it requires an additional 8-12 days incubation before it can then be transmitted to another human. In rare cases dengue can be transmitted inorgan transplants or blood transfusions from infected donors, and there is evidence of transmission from an infected pregnant mother to her fetus. But in the vast majority of infections, a mosquito bite is responsible (CDC, 2010).

The primitive enzootic transmission cycle of dengue viruses involves canopy-dwelling *Aedes* mosquitoes and lower primates in the rain forests of Asia and Africa. Current evidence suggests that these viruses do not regularly move out of the forest to urban areas. An epidemic transmission cycle may occur in rural villages or island where the human population is small introduced viruses quickly infect the majority of susceptible individuals in these areas and increasing heard immunity causes the virus to disappear from the population. The most important transmission cycle from a public health standpoint is the urban endemic/epidemic cycle in large urban centers of the tropic. The viruses are maintained in an *Ae. aegyti-human-Ae. aegypti* cycle with periodic epidemics. Often multiple virus serotypes co-circulate in the same city. Because of high viraemia resulting from dengue infection of humans, the viruses are efficiently transmitted

between mosquitoes and humans without the need for an enzootic amplification host (Gubler, 1998).

Climate factors influence the transmission of dengue virus. Dengue transmission is largely confined to tropical and subtropical regions because freezing temperatures kill overwintering larvae and eggs of *Ae. Aegypti* mosquitoe. Also, temperature strongly affects pathogen replication, maturation, and period of infectivity, as laboratory data suggest that the extrinsic incubation period shortens nonlinearly with higher temperatures, increasing the proportion of mosquitoes that become infections at a given time. Also elevated temperatures can shorten insect survival time or disrupt pathogen development (Patz *et al*., 1998).

3.2 Epidemiology

DF and DHF are increasingly important public health problems in the tropics and subtropics. Dengue occurs principally in the tropical areas of Asia, Oceania, Africa and the Americas. The distribution is constrained only by the range of the principal vector *Ae. aegypti*. The first reported epidemics of dengue fever occurred in 1779-1780 in Asia, Africa and North America, the near simultaneous occurrence of outbreaks on three continents indicates that these viruses and their mosquito vector have had a worldwide distribution in the tropics for more than 200 years. A global pandemic of dengue began in Southeast Asia after World War II. Epidemics caused by multiple serotypes (hyperendemicity) are more frequent, the geographic distribution of dengue viruses has expanded, and DHF has emerged in the pacific region and the Americas. In Southeast Asia epidemic DHF first appeared in the 1950s, but by 1975 it had become a leading cause of hospitalization and death among children in many countries (Gubler and clark, 1995; McBride *et al*., 2000; Guzman and Kouri, 2001).

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 setting. An estimated 50 million dengue infections occurs annually and approximately 2.5 billion people live in dengue endemic countries. The 2005 world Health Assembly resolution WHA 58.3 on

the revision of the International Health Regulations (IHR) includes dengue as an example of a disease that may constitute a public health emergency of international concern with implications for health security due to disruption and rapid epidemic spread beyond national borders (WHO, 2009).

Dengue Fever (DF) is an old disease that became distributed worldwide in the tropics during 18th and 19th centuries when the shipping industry and commerce were expanding. Both the principal mosquito vector *Ae. aegypti*, and the virus responsible for DF were spread via sailing ships because the mosquito used the stored water on the ships as a breeding site and could maintain the transmission cycle, even on long voyage. When such a ship called at a port, often both the mosquito and the virus were introduced. Because of the slow mode of transportation, epidemics were infrequent, with intervals of 10 to 40 years. When a new DENV was introduced, however, it frequently resulted in major epidemics that affected numerous countries in that region (Gubler, 2002).

Demographic and societal changes such as population growth, urbanization and modern transportation contributed greatly to the increased incidence and geographical spread of dengue activity. Increased epidemic activity caused by multiple virus serotypes increased the rate of genetic changes in the viruses, and thus increased the probability of the emergence of virus strains or genotypes with greater epidemic potential and/or virulence an important risk factor for DHF. In the past 20 years, new virus strains have been detected with increasing frequency in new geographical areas, some resulting in epidemic transmission and other resulting in silent transmission. This change in the transmission dynamics of dengue viruses also increased the probability of secondary DVI, another principal risk factor for DHF (Gubler, 2002).

3.2.1 Molecular Epidemiology

Molecular epidemiology is one of the emerging sciences and is useful tool to study the origin, genetic relationship and classification of the viruses. Nucleotide sequence analysis of dengue viruses can reveal genetic variation among strains, within the same serotype, and geographical movement of strains (Pandey *et al*., 2000). The traditional techniques of fingerprinting, RNA-DNA hybridization, antigenic analysis, short RNA oligonucleotide

finger printing for determining the origin and spread of dengue epidemics has been replaced by nucleotide sequencing of the entire E gene. With a single geographic area, genetic changes in the virus population may be found over time with the appearance of new variants by mutation and selection (Chang *et al*., 1994; Walker *et al*., 1988).

Based on sequences of the complete envelope (E) gene or the E-NS1, DENV-1 is currently divided into four to five genotypes including a sylvatic clade (Zhang *et al*., 2005; Holmes and Twiddy, 2003). In the Americas, it was found that one or two genotypes of DENV-1 were maintained at an endemic level causing the classical DF and occasional sporadic DHF (Osman *et al*., 2009). DENV-2, have been classified within six genotypes, five of which are present in humans with differing geographical distributions (Twiddy *et al*., 2002a). Two genotypes 'Asian I' and 'Asian II', are currently restricted to South-East Asia. An 'American' genotype is now only found in the Americas. A 'cosmopolitan' genotype has a wide distribution across the tropical and subtropical world (Rico-Hesse *et al*., 1997; Twiddy *et al*., 2002a). DENV-3 has been divided into four genotypes (I-IV). Sometimes including a genotype V (Tung *et al*., 2008; Holmes and Twiddy, 2003; Messer *et al*., 2003). DENV-4 is divided into endemic genotypes (I-II) and one sylvatic genotype and shows the least genetic diversity among the serotypes, at least among available strains (Klungthong *et al*., 2004; Holmes and Twiddy, 2003; Foster *et al*., 2003).

To determine the molecular evolution of DEN-2 in Thailand, 105 isolates of the E (envelope) gene and 10 complete genomes samples over a 27 year period were sequenced. Phylogenetic analysis of these data revealed the three genotypes of DENV-2 have circulated in Thailand, (Zhang *et al*., 2006). Among three genotypes, subtype I virus seems to be more closely associated with severe clinical manifestations, suggesting that some pathogenic virus factors, may directly correlate with the occurrence of DHF (Pandey and Igarashi; 2000). Phylogenetic study demonstrated that the 2002, Taiwan isolates were of the cosmopolitan genotype, which is different from the Asian 1 and Asian 2 genotypes of Taiwan DEN-2 isolates from 1981 to 1998 and the American/Asian genotype of 2005 Taiwan isolates (Tung *et al*., 2008 b).

Previous phylogenetic studies revealed that the DENV-3 circulating during the 1960s Latin American outbreak was a genotype V virus. Sequence analysis of the envelope (E) gene of 15 Venezuelan DENV-3 viruses isolated during 2000 and 2001 from patients presenting with different disease severity, revealed that the strain circulating in Venezuela is closely related to isolates that were previously present in Panama and Nicaragua in 1994 and since then have spread through Central American countries and Mexico. DENV-3 strain currently circulating in the Americas is related to the strain that caused DHF epidemics in Sri Lanka and India in 1989-1991 (genotype III) (Uzcategui, *et al*., 2003). To date, three major genotypes of DENV-4 virus have been described. Extensive molecular epidemiological studies have documented the spread of DENV-4 in the Caribbean, most notably Puerto Rico, where genotype II seems to have become established since the early 1980s after having been imported from South-East Asia (Foster, *et al*., 2003; Klungthong *et al*., 2004).

3.2.2 Sero-epidemiology

Dengue is endemic in all WHO regions except the European Region. Dengue has been hyperendemic in Southeast Asia since World War II and in many other tropical regions since the 1970s (Gubler, 2006). A global pandemic of dengue begun in South-East Asia after World War II. All the serotypes have been found to be co-circulated in many parts of tropical and subtropical world (Gubler and Clark, 1995).

Serological evidence of dengue infection has been reported in different countries or Islands of the Eastern African Costal region. DENV-2 was the first serotype formally identified in the Southwest Indian Ocean Island. Subsequently circulation of DENV-3 subtype III between the East-Africa coastal region, Southeast Asia and Latin America has been well characterized. In addition DENV-1 circulation was reported during the outbreak of dengue both in the Comoros in 1993 and Reunion in 2004. A cross-sectional survey conducted in November-December 2006 in Mayotte among 1,154 inhabitants aged 2 years determined 22.73%, prevalence of DENV-specific IgG antibodies (Sissoko *et al*., 2010).

In the dengue outbreak of Bangladesh in 2002, 6132 clinical cases of dengue was reported. Of the total 200 clinically diagnosed dengue cases, virus isolation and IgM-Capture ELISA confirmed that 100 (50%) cases were dengue (Islam *et al*., 2006).

The descriptive cross-sectional study during April-October 2009 was carried out in Pakistan to determine the sero-prevalence of Dengue viral Infection in healthy population residing in rural areas of district Rawalpindi. ELISA test was done to detect the presence of IgG anti-dengue antibodies. Out of 96 healthy individuals (48 males and 48 females), 13.5% were found to be positive for IgG anti-dengue antibodies, of which 16.6% and 10.4% were males and female respectively (Zafar *et al*., 2010).

Serological study performed in Thailand from 1973 to 1999 showed the DEN-3 was the most frequent serotype in primary (49% of all isolates), DEN-2 in secondary and DHF (37% and 35% respectively) (Ananda *et al*., 2003).

Dengue was first reported in Indonesia in 1968 and since then the number of reports in the literature and the number of DENV infected cases reported by the Indonesian health authorities have increased. Over time, the morbidity and mortality of dengue disease have increased and DHF epidemics occur throughout all the 29 provinces. All dengue serotypes are circulating, although severe disease is predominantly attributed to DENV-3. However DEN-4 has been isolated in almost all epidemics (Setiati *et al*., 2006; Sukri *et al*., 2003).

A total of 279 samples (35 CSF and 244 Serum) were collected between July 1999 and September 2000 during rainy and post rainy season in Southwestern Nepal. ELISA was applied to differentiate Japanese encephalitis (JE) and DVI by detecting specific IgM antibodies. The results showed that 28% were positive for JE-IgM antibodies and 10.4% positive for DENV-IgM antibodies. ELISA was found highly specific because only 10.7% DENV cross reacted with JEV antigen (Sherchand *et al*., 2004).

Singapore experienced the Dengue epidemic in the year 2005. A total of 14,006 cases of DF/DHF comprising 13,625 cases of DF and 381 cases of DHF, including 27 deaths were reported, giving an incidence rate of 322.6 per 100,000 and a case fatality rate of 0.19%.

The predominant dengue serotype was DENV-1 comprising 71.2% of all cases followed by DENV-3 (19.0%), DENV-2 (9.2%) and DEN-4 (0.6%). Gene sequencing carried out by the Environmental Health Institute showed that the same DENV-1 had been circulating in Singapore since 2002 (Koh *et al*., 2008).

3.2.3 Disease Burden: Global Scenario

3.2.3.1 Dengue Situation in Nepal

Dengue is an emerging disease in Nepal; presumably transmission is moving into north from Terai. DENV infection was recorded since 90's and the first case of dengue was reported in October, 2004 from a dengue patient returning to Japan from Nepal (Takasaki *et al*., 2008; Malla *et al*., 2008; Sah *et al*., 2009).

A confirmed outbreak of DF was reported in nine districts of Terai region in Nepal in 2006 (Pandey *et al*., 2008). Initially there were reports of suspected DF outbreaks in Banke district. The clinical observation, Pathological and laboratory investigation results proved introduction of DF in Banke, Bardiya, Dang, Kapilbastu, Parsa, Rupandehi and Jhapa districts. Two suspected, 7 probable and 23 confirmed DF cases were recorded during 2006 outbreaks. Ninety four percent patients were adults and male to female ratio was 4:1. All the four serotypes have been found in Nepal indicating the possibility of severe form of disease i.e. DHF during outbreaks (WHO/SEARO, 2006; Malla *et al*., 2008). The first DENV-2 strain of Nepal origin was isolated from a Japanese traveler who visited Nepal and in which DF developed after the patient returned to Japan. Isolated DENV-2 (Gen Bank accession no. AB 194882) was 98% homologous with DENV-2 isolated in India (Pandey *et al*., 2008).

3.2.3.2 Dengue in Asia and the Pacific

Dengue is described as 'endemic' in many countries in South-East Asia region. In 2003, eight SEA Region Countries, Bangladesh, India, Indonesia, Maldives, Myanmar, Sri Lanka, Thailand and Timor-Leste reported dengue cases. In 2004, Bhutan reported the country's first dengue outbreak. At present, the Democratic Peoples' Republic of Korea is the only country in the South-East Asia Region that has no reports of indigenous

dengue cases. Reported case fatality rates for the region are approximately 1% but in India, Indonesia and Myanmar, focal outbreaks away from the urban areas have reported case fatality rates of 3.5% (WHO, 2009).

Epidemic dengue is a major public health problem in Indonesia, Myanmar, Sri Lanka, Thailand and Timor-Leste which are in the tropical monsoon and equatorial zone where *Ae. aegypti* is widespread in both urban and rural areas, where multiple virus serotypes are circulating, and dengue is a leading cause of hospitalization and death in children. Cyclic epidemics are increasing in frequency and in country geographic expansion is occurring in Bangladesh, India Maldives-Counties in the deciduous dry and wet climatic zone with multiple virus serotypes circulating. Over the past four years epidemic dengue activity has spread to Bhutan and Nepal in the Sub-Himalayan foothills (WHO, 2009).

Dengue has emerged as a serious public health problem in the Western Pacific Region. Since the last major pandemic in 1998, epidemics have recurred in much of the area. Between 2001 and 2008, the four countries in the Western Pacific Region-Cambodia, Malaysia, Philippines, and Viet Nam reported 1,020,333 cases with the highest numbers of cases and deaths (WHO, 2009).

3.2.3.3 Dengue in the Americas

In the 1950s, 1960s, and most of the 1970s epidemic dengue was rare in the American region because the principal mosquito vector, *Ae. aegypti*, had been eradicated from most of Central and South American. The eradication program was discontinued in the early 1970s, and this species then began to reinvade the countries from which it had been eradicated. Epidemic dengue invariably followed re-infestation of a country by *Ae. aegypti*. 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. New DENV strains and serotypes were introduced (DENV-1 in 1977, a new strain of DENV-2 in 1981, DENV-4 in 1981, and a new strain of DENV-3 in 1994). Moreover, many countries of the region evolved from nonendemicity or hypoendemicity to hyperendemicity, and epidemic DHF emerged much as it had in South-East Asia 25 years earlier (Gubler; 1998).

In the Caribbean, and in Central and South America, DF has spread with cyclical outbreaks occurring every 3-5 years. The biggest outbreak occurred in 2002 with more than 1 million reported cases. From 2001 to 2007, more than 30 countries of the Americas notified a total of 4332731 cases of dengue. The number of cases of DHF in the same period was 106037. The total number of dengue deaths from 2001 to 2007 was 1299, with a DHF case fatality rate of 1.2%. All four serotypes of the DENV circulate in the region (WHO, 2009).

3.2.3.4 Dengue in Africa

Although there is poor documentation of dengue and dengue outbreak in the African region, it is known that dengue has been on the continent since the start of the 20th Century. Dengue in Africa existed as far back as 1926-1927, when it caused an epidemic in Durban, South Africa. All four serotypes of DENV have caused outbreaks in the region. For Eastern Africa, the available evidence so far indicates that DENV-1, DENV-2 and DENV-3 appear to be common causes of acute fever. In Western Africa in the 1960s DENV-1, DENV-2 and DENV-3 were isolated for the first time from samples taken from humans in Nigeria (Sang, 2006; WHO, 2009)

3.2.3.5 Dengue in the WHO Eastern Mediterranean and Other Region

Outbreaks of dengue have been documented in the Eastern Mediterranean Region possibly as early as 1799 in Egypt. Recent outbreaks of suspected dengue have been recorded in Pakistan, Saudi Arabia, Sudan and Yemen, 2005-2006. Dengue is now endemic in all WHO regions except the WHO European Region. Globally, reporting on dengue cases shows cyclical variation with high epidemic years and nonepidemic years (WHO, 2009).

3.2.3.6 Dengue in International Travel

Travelers play an essential role in the global epidemiology of dengue infections, as viraemic travelers carry various dengue serotypes and strains into areas with mosquitoes that can transmit infection. Travelers often transport the DENV from areas in tropical developing countries, where limited laboratory facilities exist, to developed countries

with laboratories that can identity virus serotypes. Travel related dengue demonstrated a defined seasonality for multiple regions (South-East Asia, South Central Asia, Caribbean, South America). An increase in cases in travelers could be due to increased travel activity to dengue endemic areas, for instance (WHO, 2009).

3.3 Factors Responsible for the Increased Incidence

The factors responsible for the dramatic resurgence and emergence of epidemic DF and DHF respectively as a global public health problem are complex and not fully understood (Gubler, 1987; Gubler and Tient, 1994). Two major factors have been the unprecedented global population growth and the associated unplanned and uncontrolled urbanization, especially in tropical developing countries. The substandard housing, crowding, and deterioration in water, sewer, and waste management systems associated with unplanned urbanization have created ideal conditions for increased transmission of mosquito-borne diseases in tropical urban centers. Decreasing resources for vector borne infections disease control and prevention, and changes in public health policy have all contributed to increased epidemic dengue activity, the development of hyperendemicity, and the emergence of epidemic DHF (Gubler, 1998).

There is increasing scientific interest in the potential effects on health of global climate change. Mosquito-borne disease transmission is climate sensitive for several reasons mosquitoes require standing water to breed, and a warm ambient temperature is critical to adult feeding behavior and mortality the rate of larval development and speed of virus replication (Focks *et al*., 1993; Patz *et al*., 1998). With the change in temperature rate of flavivirus replication increases within the mosquito vector, depending on the serotype, amount of virus ingested, and possibly the species of mosquito involved. Viral replication is limited to cells of the posterior midget after the mosquito completes feeding on a viraemic human host. The increase in surrounding temperature may shorten the extrinsic incubation period in two ways. Firstly, the period of viral replication is reduced. Secondly, the time taken for the virus to travel to the mosquito's salivary glands and be present when the mosquito takes its next blood meal is also shor4tened (Gubler and Kuno, 1997). Higher ambient temperatures reduce the size of larvae which leads to

smaller adult mosquitoes. These in turn must feed on humans or other blood sources more frequently to obtain proteins needed for egg production. Temperature affect flight performance of vector and it was found that sustained tethered flight occurred from 15-32^{0}c, with the optional temperature being 21^{0}C (Rowley and Graham, 1968).

3.4 Clinical Diagnosis of Dengue

Dengue virus infections may be asymptomatic or may lead to undifferentiated fever, DF or DHF with plasma leakage that may lead to hypovolaemic shock (DSS) (WHO, 1997). 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 hemorrhage (WHO, 2009). Symptomatic dengue virus infections were grouped into three categories: undifferentiated fever, DF and DHF. DHF was further classified into four severity grades with grades III and IV being defined as DSS. Currently the classification into DF/DHF/DSS as shown in appendix-II continues to be widely used (WHO, 1999).

3.4.1 Clinical Symptoms

Dengue infection is a systemic and dynamic disease. It has a wide clinical spectrum that includes both severe and non-severe clinical manifestations. After the incubation period (3 to 14 days with an average, 4-7 days), the illness begins abruptly and is followed by the three phases-febrile, critical and recovery (Gubler, 1998; WHO, 2009).

DF is characterized by sudden onset of fever and a variety of nonspecific signs and symptoms, including frontal headache, retro-orbital pain, body aches, nausea and vomiting, joints pain, weakness, and rash. Patients may be anorexic, have altered sensation, and have a mild sore throat. Constipation is occasionally reported; diarrhea and respiratory symptoms are infrequently reported and may be due to concurrent infections. The fever may last for 2 to 7 days. The fever may drop after a few days, only to rebound 12 to 24 h later (saddle back). Rash is variable but occurs upto 50% of patients as either early or late eruptions. Clinical laboratory findings associated with DF include a

neutropenia followed by a lymphocytosis. Liver enzyme levels in the serum may be elevated (Gubler, 1998).

DHF is primarily a disease of children under the age of 15 years, although it may also occur in adults. It is characterized by sudden onset of fever, which usually lasts for 2 to 7 days and a variety of non specific signs and symptoms. Fever is occasionally biphasic. Typical DHF symptoms include hemorrhagic tendencies evidenced by at least a positive tourniquet test; petechiae, ecchymoses or purpura; bleeding from mucosa, gastrointestinal tract, injection sites or other locations; hemataemesis or malaena; thrombocytopenia (100,000/mm^3 or less), Plasma leakage evident by rise in the hematocrit (Gubler, 1998; WHO, 2009). Shock occurs when a critical volume of plasma is lost though leakage. The patient is considered to have shock if the pulse pressures (i.e. the difference between the systolic and diastolic pressures) is 20 mm Hg in children or he/she has signs of poor capillary perfusion (cold extremities delayed capillary refill, or rapid pulse rate). In adults, the pulse pressure of 20 mm Hg may indicate a more severe shock (WHO, 2009).

3.5 Pathogenesis of DHF/DSS

Pathogenesis of DHF is a controversial matter. Several hypotheses for the pathogenesis of DHF have been proposed. Among them, Antibody-Dependent Enhancement (ADE) of infection has long been thought to play a central role. Virus virulence, the capacity of a virus to produce disease in a host, is an alternative hypothesis for the pathogenesis of DHF/DSS (Guzman and Kouri, 2001; Lei *et al*., 2008). Cross reactive antibodies that lack neutralizing activity are induced in the primary infection. Serotype cross-reactive antibodies from the previous infection bind to virions without neutralizing and enhance the entry of virus into monocytes. The number of virus infected monocytes increases. As a result, the level of dengue virus-specific T cell activation is markedly enhanced. The T cells, especially the cross-reactive T cells, produce cytokines such as IFN-γ, IL-2 and TNF- and lyse dengue virus infected monocytes. TNF- is also produced by activated monocytes. The complement cascade is activated by a virus-antibody complex as well as by several cytokines to release C3a and C5a which also have direct effects on vascular

permeability. The synergistic effects of IFN- , TNF- and activated complement proteins trigger plasma leakage of endothelial cells in secondary dengue virus infection (Kurane, 2007; Lei *et al*., 2008).

Besides secondary infection, chronic diseases such as bronchial asthma and diabetes have been suggested as risk factors for DHF. Finally, whites have higher risk of developing DHF than blacks. DEN-2 is known to replicate in higher concentration in the peripheral blood cells of whites compared with those of blacks (Guzman, *et al*., 1999).

Cytokines that may induce plasma leakage such as IFN-γ, IL-2 and TNF- are increased in DHF cases. Also, IFN-γ enhances uptake of dengue particles by target cells through increasing Fc cell receptors. Others cytokines such as IL-6, IL-8, and IL-10 are also increased. A protein of 22-25-kDa has been associated with the pathogenesis of DHF. Dengue virus through an indirect more than a direct mechanism could mediate endothelial cell activation. It has been demonstrated that dengue infected peripheral blood monocytes in ADE conditions generate soluble mediators that activate endothelial cells through the enhanced expression of adhesion molecules such as VCAM-1 and ICAM-1. At the same time high levels of TNF- cold be responsible in part for transient vascular damage. The role of TNF- in the pathogenesis of the disease is critical and it probably initiates several processes relating to plasma leakage and Hemorrhage (Guzman and Kouri, 2001).

While many, perhaps even most cases of DHF/DSS occur in patients experiencing a second dengue virus infection, or in very young children with remaining maternal antibodies to dengue virus, DHF is also seen in primary dengue infections (Barnes et al., 1974). Viral virulence factors may play an essential role in the pathogenesis of DHF/SSS. Viral virulence factors may amongst other encompass the ability to (i) infect more cells, (ii) generate more progeny virus, (iii) cause a more severe inflammation, and (iv) evade immune response effectors mechanisms. Different strains of DENV-2 behave differently in assays for ADE (McBride *et al*., 2000). It has been assumed that virulent DENV strains cause DHF, while avirulent DENV strains cause DF. It was reported that the determinants for virulence resided at the amino acid 390 of the E protein, in the 5 non-

translated region and in the upstream 300 nucleotides of the 3 non translated region (Leitmeyer *et al*., 1999). The other group demonstrated non-synonymous amino acid replacement in the preM, NS1, NS2A, NS3 and NS5 by analyzing multiple strains of DENV-2 (Pandey and Igarashi, 2000).

3.6 Immune Response

The mechanisms of dengue-induced disease and immunity are poorly defined, and the protective versus the pathogenic nature of the immune response to DVI is yet unclear. Clinical, epidemiological, and laboratory studies suggest that the protective components of the immune system against DEN infection include interferons (IFNS), antibodies and T cells, whereas the immune pathogenic mechanisms may involve sub neutralizing concentrations of dengue-specific, antibodies, antibodies cross-reactive to host antigens, and T cells (Shresta *et al*., 2004).

Infection with one serotype confers lifelong homotypic immunity, but only short term (approximately three to six months) cross protection against heterotypic serotypes (Schieffelin *et al*., 2010). Immunity against DENV is determined by production of neutralizing antibodies. The immune response is monotypic; it does not protect against an infection by another serotype. The immune response to secondary infections, which does not neutralize the virus, may even increase the risk of complications (Halstead, 2004). Dengue virus-specific memory $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ lymphocytes are detectable in humans after natural dengue infections. Infection with a single dengue serotype induces both serotype-specific and serotype-cross reactive $CD4^+$ memory T cells, while $CD8^+$ T lymphocytes have virus-specific cytotoxic activity (Gubler, 1998). Primary, secondary, and even tertiary dengue infections can be observed taking into account the existence of four serotypes. During a primary infection, individual develops IgM after 5-6 days and IgG antibodies after 7-10 days. During a secondary infection high levels of IgG are detectable even during the acute phase and they rise considerably over the next 2 weeks. IgM levels are lower and in some cases absent during secondary infection. IgM antibodies suggest a recent infection although they are still present after 2-3 months. High

titres of IgG are criterion of secondary infection (Guzman and Kouri 2004; PAHO, 1994; WHO, 1997; Kuno, *et al*., 1991).

Total and dengue specific IgE antibody levels are higher in patients with DHF and DSS compared with those with DF (Koraka *et al*., 2003). Moreover, total IgE levels are significantly higher in those previously exposed to dengue infection (Miguez-Burbano *et al*., 1999). During severe dengue infection some studies suggest there are suppressed TH1 responses whereas others report predominant TH2 responses (Guzman and Kouri, 2002).

Innate immunity during early DVI remains poorly understood. Type I and Type II interferons can contribute to control of viral replication. Natural killer cells are activated in DVI and may contribute to killing of infected cells by cytokine release or Antibody Dependent Cellular Cytotoxicity (ADCC). Complement is also activated in acute DVI and soluble NS1 may be important in this process (Cameron *et al*., 2006).

3.7 Laboratory Diagnosis of Dengue

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 disease), 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. 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. A range of laboratory diagnostic methods has been developed to support patient management and disease control. The choice of diagnostic method depends on the purpose for which the testing is done (e.g. clinical diagnosis, epidemiological survey, and vaccine development), the type of laboratory facilities and technical expertise available, costs and the time of sample collection (WHO, 2009).

Three factors have been fundamental in dengue diagnosis: development of ELISA for dengue-specific IgM detection; mosquito cell lines and monoclonal antibody development for viral isolation and identification; and most recently the introduction of RT-PCR for molecular diagnosis and strain characterization. These three methods cover the serological, virological, and molecular diagnosis of dengue (Guzman and Kouri, 1996).

3.7.1 Culture Method

In the early stage of infection, isolation and identification of dengue virus is traditionally the only way to diagnose a current dengue infection (Buchy *et al*., 2006). Cell culture is the most widely used method for dengue virus isolation and remains the 'gold standard' although it has gradually been replaced by the RT.PCR method for rapid diagnosis. This is mainly due to its lower sensitivity and the fact that a longer time for detection is required if indirect immune fluorescence is performed to identity the isolated virus with dengue-or serotype-specific monoclonal antibodies (Gentry *et al*., 1982; Henchal *et al*., 1983; WHO, 2009). However, the molecular method based on RT-PCR has been combined with the cell culture method to improve the sensitivity and reduce the time needed to identify the cultured viruses (Oliveira *et al*., 2003).

Specimens for virus isolation should be collected early in the course of the infection, during the period of viraemia (usually before day 5). Virus may be recovered from serum, plasma and peripheral blood mononuclear cells and attempts may be made from tissues collected at autopsy (e.g. liver, lung, lymph nodes, thymus and bone marrow). Because dengue virus is heat-labile, specimens awaiting transport to the laboratory should be kept in a refrigerator or packed in wet ice. For storage up to 24 hours specimens should be frozen at -70^{0}c in a deep-freezer or stored in a liquid nitrogen container. Storage even for short periods at -20^{0}c is not recommended. Formation of immune complexes due to the presence of large quantities of neutralizing antibodies in secondary dengue patients may interfere with virus isolation (WHO, 2009).

Identification of the dengue virus is generally accomplished using immunofluorescence techniques with serotype specific monoclonal anti-dengue antibodies on mosquito head

squashes or infected cells. Some strains are not easily identified because of a low concentration of virus. Plaque assay is the gold standard methodology for the quantification of dengue virus (Buchy *et at*., 2006). The method of choice for dengue virus identification is indirect immunofluorescence assay (IFA) with serotype-specific monoclonal antibodies produced in tissue culture or mouse ascitic fluids and an anti-mouse IgG-fluorescein isothiocyanate conjugate (Gubler and sather, 1988; Gubler *et al*., 1984; Guzman and Kouri, 1996; Henchal *et al*., 1983). In those laboratories that do not have immune-fluorescence capability, DENVs can also be identified using monoclonal antibodies in an antigen capture ELISA (Henchal *et al*., 1982).

The isolation of viruses from clinical samples can be conveniently carried out with cultured mosquito cells, such as the AP-61, Tra-284, C6/36, AP64 and CLA-1 cell lines or mammalian cells, such as the LLCMK2, Vero, and BHK21 cell lines (Guzman and Kouri, 1996). The intrathoracic inoculation of mosquitoes *(Ae. aegypti, Ae. albopictus, Toxorrhynchites splendens, Tx. amboinensis)* is the most sensitive system for the isolation of dengue virus, but because of the particular technical skill and special containment facilities required for direct inoculation of mosquitoes, cell culture is preferable for routine diagnosis. At present, virus isolation with the C6/36 cell line with acute-phase serum or plasma from patients is the method of choice for routine dengue virus isolation (Shu and Hung 2004; Bucchy *et al*., 2006).

The oldest and least sensitive method for isolating the virus is through the intracerebral inoculation of suckling nice. This technique is used only when no other methods are available. Although many animals develop symptoms or signs indicating encephalitis, a large number of animals exhibit no signs of illness (Guzman and Kouri, 2004). A wide variety of cell types are susceptible to infection by DENV. In the past, BS-C-1 cells (African green monkey kidney cells) and suckling mice were used for virus isolation. Using these cells, virus isolation is time consuming, slow and expensive. Moreover, because of low sensitivity and requirement of numerous passages, these methods are no longer recommended for isolation of DENVs (Gubler *et al*., 1984).

3.7.2 Serological Methods

Diagnosis of dengue by the recovery of virus or the detection of antigens is preferable to serological diagnosis; however, the latter is used to confirm most dengue infections. Although serological assays can, in many instances provide a presumptive diagnosis of recent infection from a single serum specimen, a conclusive diagnosis of acute infection can be made only when rising levels of anti-dengue immunoglobulin are detected in paired sera. The diagnosis of acute dengue infection is possible on this basis because antibody levels are known to rise only for 2-4 weeks following infection. The subsequent decline to baseline levels requires another 6-24weeks, during which time single serum assays may still reveal elevated anti-dengue IgM or IgG antibody. The most commonly used serological techniques for the diagnosis of dengue infection are MAC-ELISA and the HI test (WHO, 1997).

Five basic serologic tests have been routinely used for diagnosis of dengue infection, hemagglutination-inhibition (HI), complement fixation (CF), neutralization test (NT), IgM-Capture ELISA (MAC-ELISA), and indirect IgG ELISA (Gubler, 1998). Routinely, detection of rising antibody by the hemagglutination inhibition test (HI) is recommended as the gold standard method (WHO, 1997). At present, IgM detection has been developed and claimed to be useful for early diagnosis (Innis *et al*., 1989).

The serological diagnosis of dengue virus infection is rather complicated for the following reasons: (i) Patients may have multiple and sequential infections with the four dengue virus serotypes due to a lack of cross protective neutralization antibodies, (ii) Multiple and sequential flavivirus infection make differential diagnosis difficult due to the presence of preexisting antibodies and original antigenic sin in regions where two or more flaviviruses are co-circulating, (iii) IgG antibodies have high degrees of cross-reactivity to homologous and heterologous flavivirus antigens, and (iv) The sero-diagnosis of past, recent and present dengue virus infections is difficult due to the long persistence of IgG antibodies (10 month, as measured by E/M-specific capture IgG ELISA, or lifelong, as measured by E/M antigen-coated indirect IgG-ELISA in many dengue patients with secondary infections (Innis *et al*., 1989; Gubler 1996).

3.7.2.1 Detection of Antigens

Flavivirus antigens may be detected in peripheral blood leukocytes from patients with dengue especially during the febrile phase of illness. Dengue antigens also may be found in the liver and lung at autopsy, and less often in the thymus, lymph nodes, skin, spleen, bone marrow and serosa. Fluorescent antibody, immunoperoxidase and avidin-biotin enzyme assays have been standardized in a number of research laboratories, permitting the visualization of viral antigen in acetone-fixed leukocytes, snap-frozen tissue and even formalin-fixed tissue after limited protease-digestion (to reveal antigens cross-linked by formalin). Tissues should be collected as soon after death as possible since a delay of even 24 hours compromises antigen staining (WHO, 1997).

Progress toward the detection of antigen in acute-phase serum, samples by serology has been slow due to the low sensitivity of the assay for patients with secondary infections, as such patients have preexisting virus-IgG antibody immune complexes (Shu and Huang, 2004). New developments in ELISA and dot blot assays directed to the envelope/membrane (E/M) antigen and the non-structural protein1 (NS1) demonstrated that high concentrations of these antigens in the form of immune complexes could be detected in patients with both primary and secondary dengue infections up to nine days after the onset of illness (WHO, 2009).

Detection of virus antigen is a promising tool for surveillance. Distinct advantage is that it can be performed under field conditions with the availability of a fluorescence microscope objective attachment to a standard laboratory microscope for field use (Polsuwan *et al*., 1992). It should now be possible to perform the virus antigen detection test even in peripheral laboratories.

3.7.2.2 Detection of Anti Dengue Antibodies

Several methods have been described for the serological detection of dengue virus-specific antibodies, including the hemagglutination inhibition (HI) test, the neutralization test, the indirect immune fluorescent-antibody test, ELISA, CF, dot blotting, western blotting and the rapid immunochromatographic test (for which many commercial kits are

available). Among these, capture IgM and/or IgG ELISA, antigen-coated indirect IgM and/or IgG ELISA, and the HI test are the most commonly used serological techniques for the routine diagnosis of dengue virus infections (Shu and Huang, 2004).

MAC-ELISA

Anti-dengue IgM detection using enzyme-linked immunosorbent assay (ELISA) represents one of the most important advances and has become an invaluable tool for routine dengue diagnosis. Specifically, MAC-ELISA (IgM antibody capture ELISA) diagnosis is based on detecting dengue-specific IgM antibodies in the test serum by capturing them using anti-human IgM antibody previously bound on a solid phase. In general, 10% false negative and 1.7% false positive reactions have been observed (Guzman and Kouri, 2004). A primary infection with dengue is characterized by a slow and low titre antibody response. IgM antibody is the first immunoglobulin isotype to appear. Anti-dengue IgG at low titre is detectable at the end of the first week of illness, increasing slowly thereafter. In contrast, during a secondary infection antibody titres rise extremely rapidly and antibody reacts broadly with many flaviviruses. High levels of IgG are detectable even in the acute phase and they rise dramatically over the following two weeks. IgM antibody is detectable by day 5 of illness in 80% of all dengue cases and by day 6-10 of illness in 93-99% of cases, and may then remain detectable for more than 90 days. IgM antibody capture enzyme-linked immune sorbent assay (MAC- ELISA) has become an important tool in the routine diagnosis of dengue; this technique has a sensitivity and specificity of approximately 90% and 98% respectively, but only when used 5 or more days after the onset of fever (Buchy, *et al*., 2006; PAHO, 1994). For the MAC- ELISA total IgM in patients' sera is captured by anti-μ chain specific antibodies (Specific to human IgM) coated onto a micro plate. Dengue-specific antigens, from one to four serotypes are bound to the captured anti-dengue IgM antibodies and are detected by monoclonal or polyclonal dengue antibodies directly or indirectly conjugated with an enzyme that will transform a non-coloured substrate into coloured products. The optical density is measured by spectrophotometer. Serum, blood on filter paper and saliva, but not urine can be used for detection of IgM if samples are taken within the appropriate

time frame. Serum specimens may be tested at a single dilution or at multiple dilutions (WHO, 2009).

MAC- ELISA has good sensitivity and specificity but only when used five or more days after the onset of fever. Cross-reactivity with other circulating flaviviruses such as Japanese encephalitis, St Louis encephalitis and yellow fever, does not seem to be a problem but some false positives were obtained in sera from patients with malaria, leptospirosis and past dengue infection. MAC- ELISA has the advantage that, it may be used without modification to detect anti-flaviviral IgM in cerebrospinal fluid. MAC-ELISA provides more information, is more efficient than other serological tests and is especially valuable for laboratories that perform a high volume of testing. A barrier to its wider use is the lack of standardized reagents (WHO, 1997; WHO 2009).

IgG-ELISA

The classic IgG-ELISA is used for the detection of past infection with the dengue. It was the same antigens as the MAC-ELISA. The assay is usually performed with multiple dilutions of the sera tested to determine an end point dilution. The higher the end-point dilution, the more robust the response obtained after the infection (Buchy *et al*., 2006). The use of E/M-specific capture IgG-ELISA (GAC) allows detection of IgG anti-bodies over a period of 10 months after the infection. IgG antibodies are lifelong as measured by E/M antigen-coated indirect IgG-ELISA, but a fourfold or greater increase in IgG antibodies in acute and convalescent paired sera can be used to document recent infections. Test results correlate well with HI (WHO, 2009).

This method can be used to detect IgG antibodies in serum or plasma and filter-paper stored blood samples and permits identification of a case as a primary or secondary dengue infection. Although the detection of specific IgG has been superseded in the diagnosis of acute infection, sero-epidemiological studies are best carried out using ELISA to detect specific IgG (Buchy *et al*., 2006; Fernandez and Vazquez, 1990; Vazquez *et al*., 1991; Vazquez *et al*., 2007). In general, IgG ELISA lacks specificity within the flavivirus serocomplex groups. Following viral infections, newly produced

antibodies are less avid than antibodies produced months or year after infection (WHO, 2009).

A dengue virus E/M protein specific IgM/IgG ratio can be used to distinguish primary from secondary dengue virus infections. IgM/IgG-Capture ELISAs are the most common assays for this purpose. In some laboratories, dengue infection is defined as primary if IgM/IgG OD ratio is greater than 1.2 (Using patient's sera at 1/100 dilution) or 1.4 (Using patient's sera at 1/20 dilutions). The infection is secondary if the ratio is less than 1.2 or 1.4 (WHO, 2009).

Haemagglutination Inhibition (HI)

The HI test is simple, sensitive and reproducible and has the advantage of using reagents that may be prepared locally. It requires only minimal equipment, and is very reliable if properly done (WHO, 1997; Clarke and Casals, 1958). Because HI antibodies persist for longer periods up to 48 years and probably longer, the test is ideal for sero-epidemiologic studies (Halstead, 1974). HI test is based on the ability of dengue antigens to agglutinate red blood cells (RBC) of ganders or trypsinized human O RBC. Anti-dengue antibodies in sera can inhibit this agglutination and the potency of this inhibition is measured in an HI test. Serum samples are treated with acetone or Kaolin to remove non-specific inhibitors of haemagglutination, and then adsorbed with gander or typsinized type O human RBC to remove non-specific agglutinins. Each batch of antigens and RBC is optimized. Optimal pH of each dengue haemagglutinin requires the use of multiple pH buffers for each serotype (WHO, 2009). HI antibody usually begins to appear at detectable levels (titer of 10) by day 5 or 6 of illness, and antibody titers in the convalescent phase serum specimens are generally at or below 640 in primary infections. A titre of 1,280 in an acute-phase or early convalescent phase serum sample is considered presumptive evidence of a current dengue infection. Such high levels of HI antibody persist for 2-3 months in some patients, but antibody titres generally begin to wane by 30 to 40 days and fall below 1,280 in most patients (Gubler, 1998).

Optimally the HI test requires paired sera obtained upon hospital admission (acute) and discharged (convalescent) or paired sera with an interval of more than seven days. The

assay does not discriminate between infections by closely related flaviviruses or between immunoglobulin isotypes. The response to a primary infection is characterized by the low level of antibodies in the acute phase serum drawn before day 5 and a slow elevation of HI antibody titres thereafter. During secondary dengue infections HI antibody times rise rapidly, usually exceeding 1:1280. Values below this are generally observed in convalescent sera from patients with primary response (WHO, 2009). The major disadvantage of the HI test is its lack of specificity, which generally makes it unreliable for identifying the infecting virus serotype (Gubler, 1998).

Complement Fixation (CF)

The complement fixation test may also be used in serological diagnosis, although it is the least sensitive serological assay, and other assays have generally replaced this method (WHO, 1997). It is more difficult to perform, requires highly trained personnel and therefore is not used in most dengue laboratories. It is based on the principle that complement is consumed during antigen-antibody reactions (Casey, 1965). CF antibodies generally appear later than HI antibodies are more specific in primary infections, and usually persist for short periods, although low levels of antibodies persist in some persons. It is a valuable test to have in a diagnostic laboratory because of the late appearance of CF antibodies; some patients thus show a diagnostic rise in antibody titres by HI or ELISA. The greater specificity of the CF test in primary infections is demonstrated by the monotypic CF responses when HI responses are broadly heterotypic; it is not specific in secondary infections. The CF test is useful for patients with current infections but is of limited value for sero-epidemiologic studies. Because complement fixing antibody typically appears later than IgM or HI antibody, CF can be useful in confirming dengue infection in patients with paired serum samples taken late in the infection (Gubler and sather 1988, WHO, 1997).

Particle Agglutination (PA)

PA is used to detect DENV specific IgG and IgM in human serum or plasma samples. The test kit consists of two parts, anti-human IgG or IgM antibody-coated microplate and purified DENV antigen coated Ha-Ny (hydroxyapatite-coated nylon) beads. The

microplate captures human IgG or IgM antibodies from the serum sample. The surface of Ha-Ny beads is coated with four serological types of DENV antigens. The beads can bind to anti-dengue virus specific IgG molecules, which are captured on the microplate, adhere to face of the wells. DENV antigen-coated Ha-Ny beads adhere to the face of the wells, when the test sample is anti-DENV IgG or IgM positive. On the other hand, the Ha-Ny beads sediment at bottom of the well if the serum or plasma is not contained with the anti-virus specific antibody (Yamamoto *et al*., 2000).

Neutralization Test (NT)

The NT is the most specific and sensitive serologic test for dengue viruses. Although several neutralization tests have been described for dengue virus, the most sensitive and specific method is the serum dilution virus-constant, plaque reduction test. In general, neutralizing-antibody titres rise at about the same time or slightly more slowly than HI and ELISA antibody titres but more quickly than CF antibody titres and persist for at least 48 years. Because the NT is more sensitive, neutralizing antibodies are present in the absence of detectable HI antibodies in some persons with past dengue infections (WHO, 1997; Gubler; 1998). The NT can be used to identify the infecting virus in primary dengue infections. Because of the long persistence of neutralizing antibodies, the test may also be used for sero-pidemiological studies. The major disadvantages are the expense, time required to perform the test and technical difficulty. It is therefore not used routinely by most laboratories (Gubler, 1998).

Plaque reduction and neutralization test (PRNT) is the most specific serological tool for the determination of dengue antibodies and issued to determine the infecting serotype in convalescent sera. This assay measures the titre of neutralizing antibodies in the serum of the infected individual and determines the level of protection the individual had against the infecting virus. The assay is based on the principle of interaction of virus and antibody, resulting in inactivation of virus such that it is no longer able to infect and replicate in cell culture. Some of the variability found in this assay is attributable to differences in interpretation of the results. The cell lines and virus seeds used as well as the dilution of the sera accounts for these differences (Buchy, *et al*., 2006).

Microneutralization Assay (MA)

The microneutralization assay is based on the same principle as neutralization test however instead of counting the number of plaques per well the assay uses a colorimetric measurement of the virus induced cell lysis to determine the end-point dilution. This assay was developed to utilize less reagents and for high-throughput purposes for larger number of samples for testing. Some of the limitations of the assay include the quantitative aspects of the classic PRNT (Buchy *et al*., 2006).

Rapid Immunochromatographic Test

The rapid immunochromatographic test is used for the detection of anti-dengue virus IgM and IgG antibodies. The test is available as a commercial product (for example, PanBio test, Standard Diagnostic test and Combo Q check test). In this assay, antibody to DENV is determined by a rapid colloidal gold-based immunochromatographic test for the separate determination of IgM and IgG antibodies in a Capture assay.

Specific human IgM and IgG binding proteins are immobilized on the nitrocellulose membrane respectively, as two individual test lines (IgM and IgG line) in the test zone (T) of the test device. Highly purified recombinant dengue viral proteins are conjugated to colloidal gold particles in the sample path. Patient serum is added to the sample wells of the device. Specific antibodies (IgM or IgG) of DENV if present, form gold conjugated antigen antibody complex on the test zones, resulting in a colored IgM test line or IgG test line. A band in control line in the control region (C) appears when the test has been performed properly, regardless of the presence or absence of anti-dengue virus antibodies in the specimen (Palmer *et al*., 1999; Vaughn *et al*., 1999).

3.7.3 Molecular Techniques

In recent years, PCR has become an important tool for the diagnosis of dengue for laboratory screening including entomological surveillance and for molecular epidemiological studies (Rosario *et al*., 2001). It has also proven useful as a research tool in pathogeneses, antiviral during and vaccine studies.

Reverse Transcriptase PCR (RT-PCR)

Reverse transcriptase-PCR has been developed for a number of RNA viruses in recent years and has the potential to revolutionize laboratory diagnosis; for dengue, RT-PCR provides a rapid serotype specific diagnosis. The method is rapid sensitive, simple, and reproducible if properly controlled and can be used to detect viral RNA in human clinical samples, autopsy tissues, or mosquitoes (Deubel, 1997; Guzman and Kouri, 1996; Lanciotti *et al*., 1992; Vorndam and Kuno, 1997). Since the 1990s, several RT-PCR assays have been developed. They offer better sensitivity compared to virus isolation with a much more rapid turnaround time (WHO, 2009). The nested RI-PCR assay developed by Lanciotti *et al*., (Lanciotti *et al*., 1992) is the PCR assay routinely used by some laboratories for the identification of dengue virus. This comprises a two-step PCR reaction involving an initial reverses transcription and amplification step using universal dengue primers targeting a region of the virus genome (C-prM) followed by a second 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 different sized bands observed are compared with a standard marker for the relative molecular mass of nucleic acids. Dengue serotypes are identified by the size of their bands (Harris *et al*., 1998. Buchy *et al*., 2006)

Compared to virus isolation, the sensitivity of the RT-PCR methods varies from 80% to 100% and depends on the region of the genome targeted by the primers, the approach used to amplify or detect the PCR products (e.g. one-step RT-PCR versus two-step RT-PCR), and the method employed for sub-typing (e.g. nested PCR, blot hybridization with specific DNA probes, restriction site-specific PCR, sequence analysis, etc). To avoid false positive results due to non-specific amplification, it is important to target regions of the genome that are specific to dengue and not conserved among flavi- or other related viruses. False-positive results may also occur as a result of contamination by amplicons from previous amplification. This can be prevented by physical separation of different

steps of the procedure and by adhering to stringent protocols for decontaminations (WHO, 2009)

Real-Time RT-PCR

The real time RT-PCR assay is a one-step assay system using primer pairs and probes that are specific to each dengue serotype. The use of fluorescent probe enables the detection of the reaction products in real time without need for electrophoresis (Buchy *et al*., 2006). Many real-time RT-PCR assays have been developed employing TaqMan or SYBR Green technologies. The TaqMan real-time PCR is highly specific due to the sequence-specific hybridization of the probe. Nevertheless, primers and probes reported in publications may not be able to detect all dengue virus strains; the sensitivity of the primers and probes depends on their homology with the targeted gene sequence of the particular virus analyzed. The SYBR green real-time RT-PCR has the advantage of simplicity in primer design and uses universal RT-PCR protocols but is theoretically less specific (WHO, 2009).

Real-time RT-PCR assays are either 'singleplex' (i.e. detecting only one serotype at a time) or 'multiplex' (i.e. able to identify all four serotype from a single sample). The multiplex assays have the advantage that a single reaction can determine all four serotypes with the potential for introduction of contamination during manipulation of the sample. However the multiplex real-time RT-PCR assays, although faster are currently less sensitive than nested RT-PCR assays. An advantage of this method is the ability to determine viral titre in a clinical sample, which may be used to study the pathogenesis of dengue disease (Vaughn *et al*., 2000).

Isothermal Amplification

The NASBA assay, an isothermal RNA-specific amplification assay, has been developed for the detection of viral and bacterial RNA in clinical samples. Wu and Coworkers (Wu *et al*., 2001) reported on the detection of dengue viruses by the NASBA assay, which had high degrees of sensitivity and specificity. Since the amplification procedure used with the NASBA assay is entirely isothermal and is conducted at 41^0C, it would be

suitable for epidemiological studies in the field (Shu and Huang, 2004). The NASBA assay does not require thermal cycling instrumentation. The initial stage is a reverse transcription in which the single stranded RNA target is copied into a double-stranded DNA molecule that serves as a template for RNA transcription. Detection of the amplified RNA is accomplished either by electrochemiluminescence or in real-time with fluorescent-labeled molecular beacon probes. Loop mediated amplification methods have also been described but their performance compared to other nucleic acid amplification methods are not known (WHO, 2009).

The development and evaluation of a simple, rapid, and cost effective one step, real time, and quantitative reverse transcriptase loop mediated isothermal amplification (RT-LAMP) assay is used for rapid detection and differentiation of dengue virus serotypes. The RT-LAMP assay is a novel approach to nucleic acid amplification and is based on the principle of a strand displacement reaction and stem loop structure that amplifies the target with high degree of specificity and selectivity and with rapidity under isothermal conditions, thereby obviating the need for the use of a thermal cycler. The amplification efficiency of the RT-LAMP method is extremely high due to continuous amplification under isothermal conditions, which results in the production of a large amount of target DNA as well as a large amount of the byproduct magnesium pyrophosphate which leads to turbidity. Therefore, quantitative detection of gene amplification is possible by real time monitoring of the turbidity in an inexpensive photometer. In addition, the higher amplification efficiency of the RT-LAMP method enables simple visual observation of amplification with the naked eye under a UV lamp in the presence of an intercalating dye, such as SYBR Green I or ethidium bromide. Thus, the RT-LAMP assay has emerged as a powerful gene amplification technique for rapid identification of microbial infections and has been applied to the identification of West Nile (WN) virus and sudden acute respiratory syndrome associated Corona virus (Manmohan *et al*., 2005).

Hybridization Probes

The hybridization probe method detects viral nucleic acids with cloned hybridization probes (Deubel, 1997, Vorndam and Kuno, 1997). Probes with variable specificity

ranging from dengue complex to serotype specific can be constructed depending on genome sequence used. The method is rapid and relatively simple and can be used on human clinical samples as well as fixed autopsy tissues. Unfortunately, hybridization probes have not been widely used or evaluated in the diagnostic laboratory. Preliminary data suggest that this method is less sensitive than RT-PCR, but like PCR, the outcome of the test is not influenced by the presence of neutralizing antibodies or other inhibitory substances (Gubler, 1998).

Immunohistochemistry Method

A major problem in dengue laboratory diagnosis has been confirmation of fatal cases. In most instances, only a single serum sample is obtained and serologic testing is therefore of limited value. Also, most patients die at the time of or slightly after defervescence, when virus isolation is difficult. With the help of **i**mmunohistochemistry, it is now possible to detect DENV antigen in a variety of tissues (Hall *et al*., 1991). Although immunofluorescence tests were used in the past, methods involving enzyme conjugates such as peroxidase and phosphatase in conjunction with either polyclonal or monoclonal antibodies are greatly improved (Zaki and Peters, 1997). Because tissues can be fresh or fixed, autopsies should be performed in all cases of suspected DHF with a fatal outcome (Gubler *et al*., 1979).

3.8 Treatment

There is no specific medicine for the treatment of the disease. However proper and early treatment can relieve the symptoms and prevent complications and death. Aspirin and Brufen should be avoided in DF, as it is known to increase the bleeding tendency and also it increases the stomach pain. Paracetamol can be given on medical advice. If one or more signs of DHF are seen, the patient should be taken to the hospital immediately. Fluid is given to drink while transferring the patient to the hospital (Thapa *et al*., 2007).

Early and effective replacement of plasma losses with plasma expander or fluid and electrolyte solution results in a favourable outcome in most cases. With adequate and appropriate fluid administration, DSS is rapidly reversible. Early and rapid resuscitation

from shock and the correction of metabolic and electrolytic disturbances will prevent disseminated intravascular coagulation. In case of DHF, thirst and dehydration results from high fever, anorexia and vomiting; thus fluid intake by mouth should be ample. An electrolyte replacement solution or fruit juice is preferable to plain water oral rehydration solution, as for the treatment of diarrheal disease, is recommended. During the acute febrile phase there is some risk of convulsions. Antipyretics may be indicated in patients with hyperpyrexia, particularly those with a history of febrile convulsions. Salicylates should be avoided since they may cause bleeding and acidosis. Paracetamol is preferable to reduce fever but should be used with caution parental fluid therapy can be given in an outpatient rehydration unit for patients whom fever, vomiting or anorexia produce dehydration (WHO, 1997).

Shock is medical emergency. The immediate administration of intravenous fluid to expand plasma volume is essential. Ringer's lactate, Ringer's acetate or 5% glucose diluted in physiological saline should be administered as a rapid (< 20 minutes) intravenous bolus (10-20 ml/kg). Another bolus bringing the fluid dose to 20-30 ml/kg can be administered if necessary. If shock persists, oxygen should be given and the haematocrit should be checked. A drop in haematocrit, e.g. from 50% to 40%, with no clinical improvement despite adequate fluid administration, indicates a significant internal hemorrhage. Transfusion with fresh whole blood is preferable, and the amount given should be such that the normal red blood cell concentration is not exceeded (WHO, 1997).

3.9 Prevention and Control

Prevention and control of dengue and DHF has become more urgent with the expanding geographic distribution and increased disease incidence. Unfortunately, tools available to prevent dengue infection are very limited (Gubler, 1998).

Dengue prevention and control will be implemented through the bi-regional dengue strategy (2008-2015) of the South-East Asia and western pacific regions. This consists of six elements: (i) Dengue surveillance, (ii) Case management, (iii) Outbreak response, (iv) Integrated vector management, (v) Social mobilization and communication for

dengue and (vi) Dengue research (a combination of both formative and operational research). Lack of reporting remains one of the most important challenges in dengue prevention and control (WHO, 2009).

Effective disease prevention programs must have several integrated components, including acute laboratory-based surveillance, emergency response, and education of the medical community to ensure effective case management, community-based integrated mosquito control, and effective use of vaccines when they become available (Gubler, 1988; Gubler and Casta-Velez, 1991).

Active disease surveillance is an important component of a dengue prevention program. In addition to monitoring secular trends, the goal of surveillance should be to provide an early-warning or predictive capability for epidemic transmission. If epidemics can be predicted, they can be prevented by initiating emergency mosquito control. For epidemic prediction, health authorities must be able to accurately monitor dengue virus transmission in a community and be able to tell at any point in time where transmission is occurring, which virus serotypes are circulating, and what kind of illness is associated with dengue infection (Gubler, 1998).

Prevention and control of dengue and DHF currently depends on controlling the mosquito vector *Aedes* in and around home, where most transmission occurs. *Ae. aegypti* should be the main target of surveillance and control activities. Other species should be considered for vector control only where there is reliable evidence that they play an epidemiologically significant role in the transmission of dengue infections. Space sprays with insecticides to kill adult mosquitoes are not usually effectives unless they are used indoors. The most effective way to control the mosquitoes that transmit dengue is larval source reduction i.e. elimination or cleaning of water-holding containers that serve as the larval habitats for *Ae. aegypti* in the domestic environment. The most effective means of vector control is environmental management, which includes planning, organization carrying out and monitoring activities for the modification or manipulation of environmental factors with a view to preventing or reducing vector propagation and human-vector-pathogen contact. Environmental management should focus on the

destruction, alteration, disposal or recycling of containers and natural larval habitats that produce the greatest number of adult mosquitoes in each community (WHO, 1997; Gubler 1998).

3.9.1 Vaccine Status

There is an urgent need for a safe and effective vaccine for dengue. The association of increased disease severity in the people with heterotypic immunity necessitates a vaccine that will confer long-term protection against all four serotypes (Halstead, 1988). Dengue poses some particular challenges for vaccine developments. There are four serologically distinct viruses and long-term immunity is specific for the infecting serotype. Sequential infections with different serotypes may result in severe disease. Since the vaccine is to be used in endemic areas, there must be no risk that preexisting anti-dengue virus antibody in a vaccine will enhance the vaccine infection and cause severe disease, and the vaccine must induce simultaneous, life-long immunity to all four virus serotypes if it is not to sensitize vaccines to severe disease following a natural dengue virus infection. Added to these difficulties is the absence of an animal model of dengue hemorrhagic fever in which to test a vaccine and the lack of definitive markers of virus attenuation (Aaskov, 2003).

Despite formidable challenges to developing tetravalent dengue vaccines, significant progress has been made in recent years and the pace towards clinical efficacy trials has accelerated substantially. A primary immunological mechanism that confers protection from dengue illness is virus neutralization through antibodies and all current dengue vaccine candidates aim to elicit high levels of neutralizing antibody. Live attenuated vaccines (LAVs) can induce durable humoral and cellular immune responses since they most closely mimic a natural infection. Several live attenuated vaccines are in advanced stages of development. One is a chimeric tetravalent vaccine in which the structural genes (prM and E) of each of the four dengue viruses were inserted individually to replace those of yellow fever virus in the backbone of the yellow fever 17D vaccine. Dengue vaccines in advanced preclinical development include DENV-DENV chimeras. In this vaccine, the prM and E protein genes of DENV-1, DENV-3 and DENV-4 were each inserted into the infectious clone of PDK-Passaged, attenuated DENV-2 (DK 53). There

is also work in progress on submit vaccines based on domain III of E protein, which is considered to be the principal neutralizing, epitope region of the virus, employing different strategies to increase immunogenicity (WHO, 2009).

CHAPTER IV

4. MATERIALS AND METHODS

4.1 Materials

A complete list of equipments, chemicals and other supplies used during the entire study period was given in appendix-I.

4.2 Methods

The study was designed as a descriptive cross-sectional. The study was carried from June 2010 to November 2010. The total number of 271 serum samples was collected from Narayani Sub-regional Hospital (NSH), Birgunj (173); Sukraraj Tropical and Infectious Disease Hospital (STIDH), Kathmandu (33); Koshi Zonal Hospital (KZH), Biratnagar (32) and Dhading District Hospital (DDH), Dhadingbesi (33). Serum samples were collected from individuals experiencing a febrile illness clinically consistent with dengue infection, selected according to the inclusion and exclusion criteria (WHO, 2009). Verbal consent was obtained from all the responding individuals. Personal details of patient about the symptoms, age, sex etc. were obtained through a questionnaire method by direct interview. Laboratory setting was done at Everest International Clinic and Research Center (EICRC), Kalanki, Kathmandu.

4.2.1 Sample Selection Criteria:

Case Inclusion Criteria

A case was included if there was high fever with clinical symptoms suggestive of dengue infection (WHO, 2009).

Case Exclusion Criteria

A case was excluded, if the case was provisionally diagnosed as infection other than dengue and being treated for bacterial and parasitic infections (WHO, 2009).

4.2.2 Sample Collection, Storage and Transport

The blood samples (5 ml from adult and 3 ml from children) were collected from each suspected cases in sterile, clean, dry and labeled test tube. The collected blood in test tube was allowed to clot at room temperature. Then the blood in test tube was centrifuged at 3000 rpm for 5 minutes and the serum was separated. The serum was then collected in vial and stored at 4^0C for a week. Then, the samples were transported to EICRC maintaining reverse cold chain. Aliquots for ELISA and RT-PCR were made and stored at 2-8^0C until tested.

Figure 2: Site of sample collection.

4.2.3 Laboratory Tests

4.2.3.1 IgM-Capture ELISA

The IgM-capture ELISA was performed according to standard protocol of manufacturer (SD Korea). During the testing procedure, the protocol was strictly followed to achieve high level of accuracy (Appendix-VI).

Procedure:

All reagents were equilibrated to room temperature (20-25°C) before commencing assay.

Serum Predilution

Positive control, negative control and patients' serum samples were diluted. For this, 10 µl of serum sample/positive/negative control was diluted to 990 µl of serum diluents (1:100).

Preparation of Antigen

One bottle of dengue antigen provided in the kit was diluted using 1.5 ml of the conjugate diluents. The anti-dengue HRP conjugate was diluted with diluted dengue antigen in 1:1 ratio. The mixture solution was gently mixed and left at room temperature (20-25°C) for 60 minutes.

Assay Plate

The required numbers of micro wells were removed from the foil sachet and were inserted into the strip holder. Five micro wells were required for controls; positive control (P) in duplicate and negative control (N) in triplicate. Within 10 minutes after mixing the monoclonal antibody (Mab) tracer and diluted antigen, 100 µl diluted serum sample and controls were pipetted into their respective microwells of the assay plate. The plate was covered and incubated for 1 hour at 37°C. After incubation, wells were washed five times with diluted wash buffer. The diluted anti-dengue HRP conjugate solution was mixed before transfer. Hundred micro litre of diluted anti-dengue HRP conjugate solution was pipetted into the wells. The plate was covered and incubated for 1 hour at 37°C. The

wells were washed five times with diluted wash buffer and 100 µl of mixed TMB solution was pipetted into each well. Timing from the first addition, the plate was incubated at room temperature (15-30°C) for 10 minutes. A blue colour was developed. Then 100 µl of stop solution was pipetted into all wells in the same sequence and timing as the TMB addition. It was mixed well. The blue colour was changed to yellow. The absorbance of each well was read within 30 minutes at a wave length of 450 nm with a reference filter of 620 nm by using Multi-ELISA Reader model 2010 (Anthos, Austria).

4.2.3.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA Extraction (The QIAamp Viral RNA Mini kit)

ELISA negative acute febrile serum samples were taken for RNA extraction. Five hundred sixty micro liter of prepared buffer AVL containing carrier RNA was added into a 1.5 ml micro centrifuge tube. Hundred forty micro litre of serum was added to the micro centrifuge tube and was mixed by pulse-vortexing for 15 S. After mixing, it was incubated at room temperature (15-25ºC) for 10 min. The micro centrifuge tube was briefly centrifuged to remove drops from inside of the lid. Five hundred sixty micro litre of ethanol (96-100%) was added to the sample and mixed by pulse-vortexing for 15 seconds. After mixing, it was briefly centrifuged to remove drops from the inside of the lid. In a 2 ml collection tube, 630µl of the solution was carefully applied to the QIAamp mini spin column without wetting the rim. The cap was closed and centrifuged at 8000 rpm for 1 min. The QIAamp spin column was placed into a clean collection tube and the tube containing the filtrate was discarded.

The QIAamp spin column was carefully opened and the previous step was repeated. The QIAamp spin column was opened and 500 µl of buffer AW1 was added. Cap was closed and centrifuged at 8000 rpm for 1 min. The QIAamp spin column was placed into a clean collection tube and the tube containing the filtrate was discarded. The QIAamp spin column was opened and 500 µl of buffer AW2 was added. The cap was closed and centrifuged at full speed (14,000 rpm) for 3 min. The QIAamp spin column was placed in a clean collection tube. The old collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 600 µl of buffer AVE was added. The

cap was closed and incubated at room temperature for 1 min. Finally, it was centrifuged at 8000 rpm for 1 min. Viral RNA was stored at $-20^{0}C$ until use.

cDNA Preparation

A volume of 10 µl of RNA sample was taken in a PCR tube. To that 1 µl of antisense primer (50 pMol/ml) and 4 µl of 2.5 mM dNTP mixture was added. The reaction mixture was transferred to pre-warmed thermal cycler. Thermal cycler was calibrated at 65°C for 15 min. To the tube, 4 µl of first strand buffer, 2 µl of 0.1 MDTT and 1 µl of Prime RNase inhibitor was added and mixed. It was incubated in thermal cycler at 42°C for 2 min. Superscript RNase inhibitor (1 µl) was added to the mixture and incubated at 42°C for 50minutes in thermal cycler. Finally the reaction was inactivated by heating at 70°C for 15 min. and template or cDNA obtained was kept at 4°C until use.

Polymerase Chain Reaction

A volume of 4.1 µl mixture solution [10X Taq buffer-2 µl + dNTPs-1.6 µl + Takara ExTaq-0.1 µl + primer F(DC-2C)-0.2 µl + primer R(DC-1S)-0.2 µl], 3 µl of cDNA and 12.9 µl of distilled water were taken in a PCR tube and mixed by gentle tabbing.

The mixture was then incubated in pre-warmed thermal cycler and the condition was:

Temperature (°C)	Time
94	2 min. (1 Cycle)
94	30 sec.
54	30 sec.
72	1 min.
72	5 min.

No. of cycles: 32 Holding temperature: 10°C

Finally, agarose gel electrophoresis was performed for detection of PCR products.

Agarose Gel Electrophoresis

Agarose powder of 0.5 gm in 23ml TAE buffer was added and dissolved by boiling in the microwave oven. The gel was left for cooling upto 50-60°C and poured in mould of 8 well and left for solidification. The gel was kept in electrophoresis tank and TAE buffer (1X) was poured. Amplified PCR products (10 µl) was mixed with 2 µl of loading dye on a paraffin film plastic sheet and then loaded in the respective well. Voltage was set at 100V for 40min. and electrophoresis was run. After electrophoresis, gel was kept for staining in the solution of ethidium bromide in TAE buffer. Finally, the gel was taken for photo documentation in UV trans-illuminator.

4.2.4 Interpretation of the Result

4.2.4.1 ELISA Result Analysis

The test was interpreted either positive or negative on the basis of absorbance with respect to cut-off value. If absorbance of the sample was greater than cut-off value, the sample was considered positive and if the absorbance of sample is less than cut-off value, the sample is negative.

Cut-off value = Mean absorbance of negative controls + 0.300

4.2.4.2 Electrophoresis Gel Analysis

For positive result, the band of the test sample should be at 400-500 bp for dengue consensus when compared with the 1 kb DNA ladder. The band obtained in our test met above condition so that was positive for dengue consensus.

4.2.5 Statistical Analysis

The collected data was analyzed to find out the age, sex-wise, hospital wise and occupation wise distribution of the cases. Chi-square value (P value) was determined to find out whether the findings were statistically significant or not. The collected data were analyzed using statistical package for social science (SPSS) software (version 16.0).

CHAPTER V

5. RESULTS

A total of 271 serum samples were collected, transported and tested by using IgM-Capture ELISA and RT-PCR for dengue during the study period. The suspected cases were grouped according to socio-demographic characteristics of patients. The positivity rate was also described according to socio-demographic characteristics.

5.1 Socio-demographic Status of the Suspected Dengue Cases.

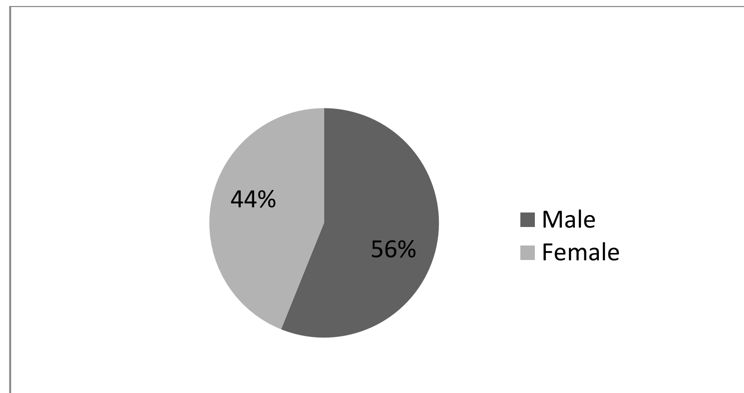


Figure 3: Sex wise distribution of suspected dengue cases.

Out of 271 suspected dengue cases investigated during the study period, 152 (56%) were males and 119 (44%) were female (fig.3).

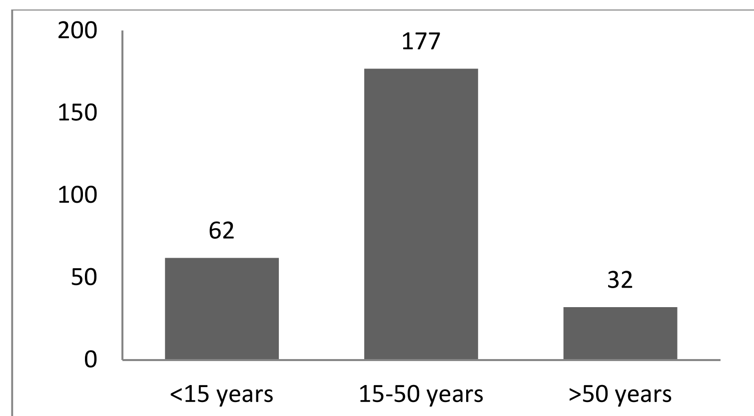


Figure 4: Age wise distribution of suspected dengue cases.

The patients under investigation were in the age range of 10 months to 80 years. The highest numbers of cases 177 (22.9%) were from age group 15-50 years and least number of cases 32 (11.8%) were from over 50 years age group (fig.4).

Among all participating hospitals, the highest numbers of cases 173 (63.8%) were from NSH and least number of cases 32 (11.8%) were from KZH (Table 1).

Table 1: Hospital Wise Distribution of Suspected Dengue Cases

Hospital	No. of dengue cases (%)
NSH	173 (63.8)
KZH	32 (11.8)
STIDH	33 (12.2)
DDH	33 (12.2)
Total	271 (100)

NSH: Narayani Sub-regional Hospital, KZH: Koshi Zonal Hospital

STIDH: Sukraraj Tropical and Infections Disease Hospital,

DDH: Dhading District Hospital

Table 2: Occupation wise Distribution of Suspected Dengue Cases

Occupation	No. of dengue suspected cases (%)
Agriculture	52 (19.2)
Labour	13 (4.8)
Job holder	12 (4.4)
Business	17 (6.3)
Students	93 (34.3)
Housewife	64 (23.6)
Others	20 (7.4)
Total	271 (100)

Occupation wise distribution of suspected dengue cases (Table 2) showed that out of 271 cases, the highest number of cases 93 (34.3%) were recorded among students. The least numbers of cases 12 (4.4%) were recorded among the labour group (Table 2).

5.2 Sociodemographic Characteristics of Positive DENV Cases

Sex Wise Distribution of Positive DENV Cases

Among the 152 male suspected DENV cases tested, 29 (19.0%) showed positive result for anti-dengue IgM antibody and out of 119 female cases 10(8.4%) showed positive result for anti-Dengue IgM antibody. Statistically there is significant relationship (p = 0.013) between male and female for the occurrence of disease (Table 3).

Table 3: Sex Wise Distribution of Positive DENV Cases

Sex	Total no. of sample	No. of positive samples (%)	% of positive cases in total	Statistics
Male	152	29 (19.0)	10.7	^{2}=6.174
Female	119	10 (8.4)	3.7	p=0.013
Total	271	39 (14.4)		

Age Wise Distribution of DENV Cases

Age wise result for anti-dengue IgM showed higher positive result among the age group 15 to 50 years (18.1%) which constituted 11.8% of total cases and least in age group above 50 years (3.1%) which comprised 0.4% of total cases. Statistically, there is significant relationship (p=0.041) between age group and occurrence of disease (Table 4).

Table 4: Age Wise Distribution of IgM ELISA Positive DENV Cases

Age (years)	No. of cases	No. of positive cases (%)	% of positive cases in total	Statistics
< 15	62	6 (9.7)	2.2	^{2}=6.369
15-50	177	32 (18.1%)	11.8	p=0.041
> 50	32	1 (3.1)	0.4	

Occupation Wise Distribution of DENV Cases

The dengue IgM positive cases were compared according to their occupation. IgM positive cases was observes highest among business group (29%) which constituted 1.8% of total cases analyzed and least observation was found in other group (5%). Statically there is no significant relationship (p=0.173) between occupation groups and the occurrence of the disease (Table 5).

Table 5: Occupation Wise Distribution of IgM ELISA Positive DENV Cases

Occupation	No. of sample	No. of positive samples (%)	% of positive cases in total
Agriculture	52	8 (15.4)	2.9
Labour	13	1 (7.7)	0.4
Jobholder	12	3 (25)	1.1
Business	17	5 (29)	1.8
Student	93	16 (17.2)	5.9
Housewife	64	5 (7.8)	1.8
Others	20	1 (5)	0.4
Total	271	39 (14.4)	

Statistics (^{2}=9.002; p=0.173)

Hospital Wise Distribution of DENV Cases

Hospital wise distribution of IgM ELISA positive DENV cases showed highest in STIDH (75.7%) which constitutes 9.2% of total. Lowest number of cases was found from DDH (3.1%) comprising 0.4% of total samples tested (Table 6)

Table 6: Hospital Wise Distribution of IgM ELISA Positive DENV Cases

Hospital	No. of sample	No. of positive cases (%)	% of positive cases in total
NSH	173	9 (5.2)	3.3
KZH	32	4 (12.5)	1.5
STIDH	33	25 (75.7)	9.2
DDH	33	1 (3.1)	0.4
Total	271	39 (14.4)	

NSH: Narayani Sub-regional Hospital,KZH: Koshi Zonal Hospital

STIDH: Sukraraj Tropical and Infections Disease Hospital,

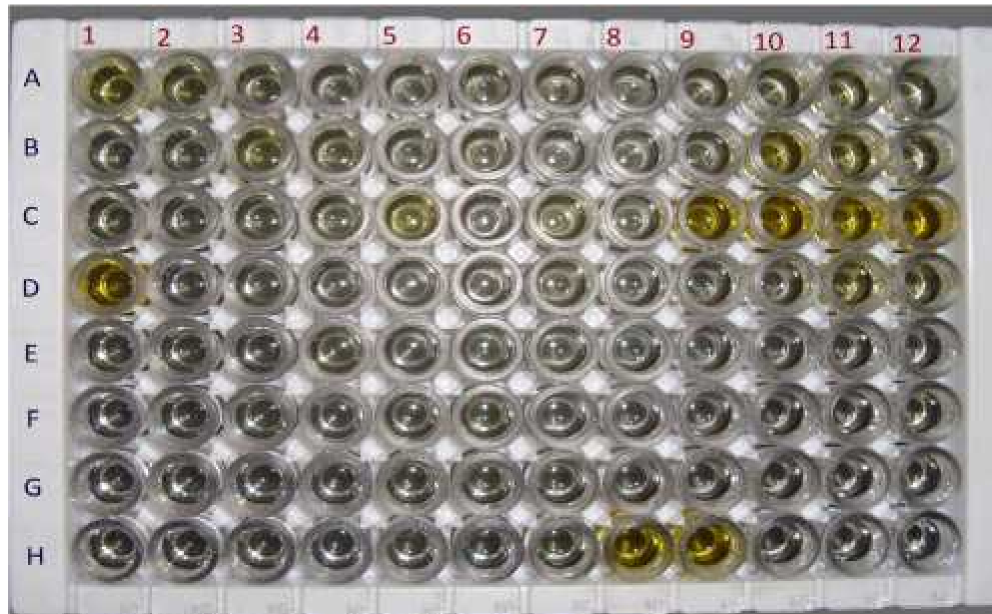
DDH: Dhading District Hospital

Diagnostic Test Wise Distribution of DENV Cases

Out of 271 IgM ELISA performed serum samples of dengue cases 39 (14.4%) were found to be positive. RT-PCR was performed from 21 acute, febrile seronegative serum samples and 1 (4.7%) was found to be RT-PCR positive for dengue consensus (Table 7).

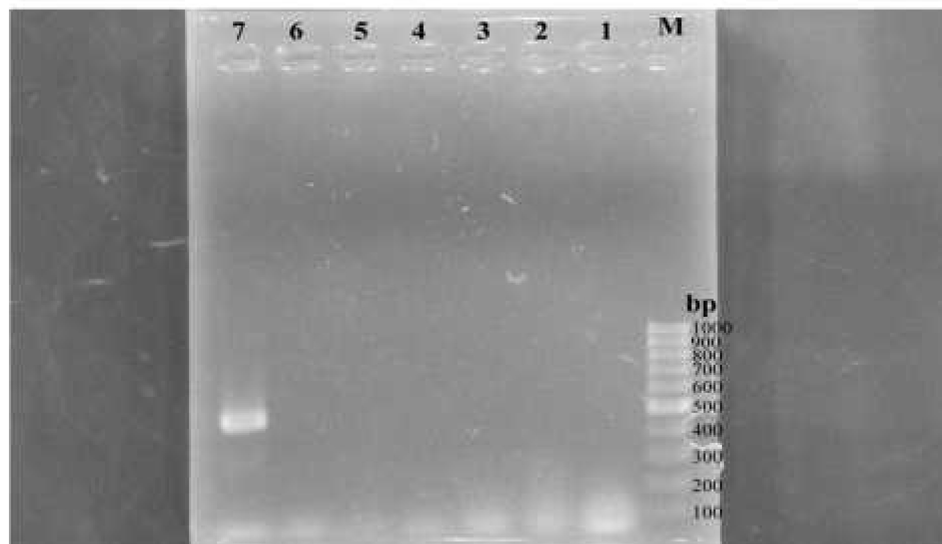
Table 7: Diagnostic Test Wise Distribution of DENV Cases

Diagnostic test	No. of tested sample	No. of positive sample	% of Positive
IgM-ELISA	271	39	14.4
RT-PCR	21	1	4.7



Photograph 1: Microtiter wells after addition of stop solution

(A1-A12, B1-B12, C1-C12, D1-D12, E1-E12, F1-F12, G1-G12 and H1-H7=Serum samples; H8-9= Positive control; H10-12= Negative control)



Photograph 2: Agarose gel electrophoresis of RT-PCR products

(Lane 1-7: samples, Lane M: Marker)

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Dengue has been a concern of its panic merely in developing countries because the management of case is poor and active surveillance has not been commenced yet. Many factors are associated with dengue, including those related to the increased likelihood of contact between the vector and the host such as household or population density. The factors that promote vector proliferation, including environmental conditions (temperature, humidity and altitude), poor sanitation or availability of potential breeding sites are important constituents of contracting dengue. The global prevalence of dengue has increased substantially recently. Dengue is endemic in 100 countries globally and it is serious problem in South-East Asia, Africa, the Western Pacific, the Americas, Africa and the eastern Mediterranean area with imported cases everywhere (WHO, 2009).

In Nepal, the epidemics of DENV infections appear in rainy and post rainy seasons. It is experienced that population growth in the country has led to unplanned and uncontrolled urbanization, which in turn has led to deterioration of housing and of water, sewage and waste management system in many parts of Nepal. The crowded human population living in intimate contact with increasingly higher densities of mosquito populations creates ideal conditions for increased mosquito-borne diseases such as DVI. In addition, human are frequently exposed to mosquitoes bites due to use of short sleeve cloths during hot and humid conditions. Possibility of global warming increases mosquito population and frequently travelling of infected cases might lead to expansion of this disease to hilly region (WHO/SEARO, 2006).

Present study was a descriptive cross-sectional epidemiological study covering four hospitals of the country. The study was carried out during post monsoon period from June 2010 to November 2010. The cases coincide mainly with the post monsoon period of subnormal rainfall because the relative prevalence and distribution of *Ae. aegypti* larval indices is highest during the post monsoon period. Heavy rainfall subsequently

leads to decrease in temperature during the later part of monsoon period. The result is increase in relative humidity and abundant stocks of fresh water reservoir generated due to rain, develop optimum conditions for mass breeding and propagation of vector and transmission of the virus (Chakravorti and Kumaria, 2005).

There are various techniques available to diagnose the dengue infection. The present study was conducted based on ELISA technique that was targeted to detect the anti-dengue IgM antibodies which appear in blood from day 5 to a month after the onset of fever. This technique has been proved to be reliable and cost effective serological method for dengue diagnosis and sero-epidemiology of dengue (PAHO, 1994; Gubler and Sather, 1988). IgM-Capture ELISA can be applied to the sero-diagnosis of DVI because ELISA was highly specific as only 10.7 % DVI cross reacted with JEV antigen (Sherchand *et al*., 2001).

During the study period, a total of 271 serum samples from dengue suspected cases were collected from four hospitals for anti-dengue IgM antibody ELISA assay and RT-PCR test. After processing of the collected specimens, the study results showed the significant number of positive cases of DVI. The positive cases include all the cases which were positive to either IgM ELISA or RT-PCR.

Out of 271 serum specimens tested by ELISA, 39 were found to be positive for anti-dengue IgM antibody which comprises 14.4% of the total tested specimens. Out of 21 seronegative, acute febrile cases tested for RT-PCR, 1 was found to be positive comprising 4.7% of samples tested for RT-PCR. The sero-positivity of the study was in accordance with some of the previous findings from Nepalese studies carried out by Pun (29.3%) in 2009, Sah (27.3%) in 2008 and Sherchand (10.4%) in 2007. Higher positivity rate than one could be due to variation in geographical distribution. The growth of population and urbanization, increased rate of deforestation, change in environmental conditions may contribute to the increase in prevalence of the disease. The increased rate of migration due to open border might also be the predisposing factor as Terai belt of Nepal is bordered with India. Low prevalence of DVI may be due to increase in

awareness programmes, change in personal as well as community behavior, increased preventive measures.

Sex wise distribution of suspected dengue cases was high in male 152 (56.1%) and low in female 119 (43.9%). The ratio of male to female suspected cases was 1.27:1. As the number of suspected male cases was higher the number of positive cases was also higher in males than in female. Out of 39 positive IgM ELISA positive DENV cases, 29 were male patients who comprise 19% of the total male and 10 were female patients which comprise 8.4% of the total female. The ratio of dengue positive cases in male to female was found to be 2.9:1. This ratio was in accordance with ratio of male to female suspected cases. Statistically there is significant relationship ($p=0.013$) between sex and the occurrence of disease. The numbers of cases were generally more in males because males are more likely to be exposed to mosquitoes during their outdoor activity. The higher numbers of cases of males may be due to the reason that females are less likely to be taken for care at a hospital when ill. This is a social taboo in developing countries like Nepal (WHO, 2009; Mohammed *et al*., 2005).

The age wise distribution of suspected dengue cases revealed that the highest number of cases 177 (65.3%) was from age group 15-50 years. Sixty two cases (22.9%) were from age group below 15 years. The least numbers of cases 32 (11.8%) were from age group above 50 years. The positive cases according to the age of the suspected were highest (32) in the age group 15-50 years which comprises 11.8% of the total suspected cases followed by age group below 15 years which accounted 2.2% of the total cases tested. The result is in harmony with the data obtained in outbreak of dengue in Nepal in the year 2006 in which dengue positive cases were recorded in age group greater than 15 years (WHO/SEARO, 2006). The age group below 15 years is pediatric age group, 15-50 years is middle or active age group and over 50 years is old age group. The middle age group is more active in outdoor activity so there is increased risk of vector contact with this age group. This age group is economically more significant group. Hence the possibility of attending to hospital is high in this age group than other. In this study the age is found to be statistically significant to the occurrence of the infection ($p = 0.041$).

The reason for the lower positivity in the younger age group could be due to improper clinical selection of cases; DF in younger age group manifests as rather undifferentiated illness, such as upper respiratory like infection accompanied by headache and mild gastrointestinal complaints. Pre-adolescent children exhibit a DF like illness but are not as severely incapacitated as adults. The disease in adults is severe enough that patients feel sick and demand medical attention. This seems to be the reason why adult patients are particularly apparent during dengue epidemics.

Out of 39 positive cases, anti-dengue IgM positive were highest in Sukraraj Tropical and Infection Disease Hospital, that constituted 11.8% of total suspected. The least number of cases was found in samples obtained from Dhading District Hospital which comprises 0.4% of total samples tested. Sukraraj Tropical and Infections Disease Hospital (STIDH) is the referral central hospital of Nepal. Many patients are referred from different places of Nepal to STIDH. Hence highest numbers of positive cases attend to this hospital. In 2010, patients from more than 20 districts were attended to STIDH. Among the patient admitted to STIDH, many patients were from Chitwan, Butwal, Nawalparasi, Rupandehi. It may be due to the warmer temperature and being the Terai region bordered by India and the presence of the vectors in the region as well.

The occupation wise distribution of suspected. Dengue cases revealed that the highest number of cases (93) were from occupation group student which accounted 34.3% of the total followed by house wife (64 cases, 23.6%) and the least number of cases (12 cases, 4.4%) were from occupation group job holder. Others group constitute 20 cases (7.4%). Other group comprises children (< 5 years), Driver, Painter, etc.

The occupation group, business (29%) was found most affected followed by job holder (25%), and least in others and labour groups (0.4%) statistically, there is no significant relationship between occurrence of the disease and occupation groups (p=0.173). The higher positivity in occupation group business might be due to businessman frequently involved in travel from one place to other and in outdoor activities and there may be chance of being bitten by vector mosquitoes. The finding were not in accordance with

other findings in Nepalese studies have reported agriculture group as the most affected occupation group Sah OP (2008) and Sah Y (2010).

In addition to the serological diagnosis, molecular typing of dengue from serum sample was done by using one step RT-PCR reaction involving a complete set of reverse transcription and amplification step using consensus dengue primers targeting a region of the virus genome for identifying the presence of dengue in the suspected serum. The products of these reactions were separated by electrophoresis on an agarose gel.

In this study, RT-PCR was performed in 21 serum samples having fever less than 5 days that were negative for IgM ELISA test. In positive ELISA sample, the antibody produced by the patients neutralize virus and there is very less chance of extracting RNA. The RT-PCR method relies on a combination of two steps: generation of a cDNA copy of the RNA genome by reverse transcription and subsequent Taq polymerase mediated amplification of this cDNA. The use of primers homologous to conserved dengue virus was amplified in the amplification reaction (Morita *et al*., 1991).

In our study, out of 21 samples one was positive in RT-PCR reaction for dengue consensus. The reason for low positivity might be due to inaccurate information about onset of fever of the patients during collection of samples. This could lead to neutralization of virus by the antibody produced during late collection. It also might be due to degradation or deterioration of virus because of thawing of sample during transportation and storage. Other explanation might be lack of recent DVI in the febrile patients suspected of dengue; the fever might be due to other viral agents which should be studied in details.

6.2 Conclusion

The sero-prevalence found from this study was 14.4% with higher prevalence among males and productive age group (15-50 years). RT-PCR test of 21 seronegative serum samples showed 4.7% positivity.

CHAPTER- VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

The sero-epidemiological and molecular study of DVI was conducted in four different hospitals of Nepal from June to November 2010. ELISA Assay was performed to detect the anti-dengue IgM antibody and the sero-prevalence of dengue was found to be 14.4% (39 out of 271 samples). RT-PCR was performed to detect genetic material of DENV and out of 21 serum samples tested one was found to be positive for dengue consensus. Of the total 271 suspected cases, 44% were male patients and 56% were female patients with confirmed positive cases 10.7% and 3.7% respectively. The highest positive cases were from age group 15-50 years which constitutes 11.8% of total. The highest positive cases were from Sukraraj Tropical and Infections Disease Hospital, Kathmandu (9.2%) followed by Koshi Zonal Hospital, Biratnagar (0.4%). The highest positive cases were found in occupation group student (5.9%) followed by agriculture (2.9%). Statistically there was no significant difference between occupation group and DVI while other variables were significant.

7.2 Recommendations

1. Presence of DVI in hilly region suggests that the prevention and control measures should cover all regions of country.
2. Prevention and control strategy of government should focus on productive age group.
3. Optimization of RT-PCR should be done in context of Nepal.

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APPENDIX-I: Materials

A) Reagents/chemicals

Primer	Gnet
Carrier RNA to buffer AVL	Qiagen
Buffer AW1	Qiagen
Buffer AW2	Qiagen
Stable 100bp DNA ladder	Sigma Genosys
Loading dye	Sigma Genosys
Tris acetate EDTA	Wako Company, Japan
Absolute ethanol	Hong Yong chemical, China
Distilled water	Utsav Laboratories

B) Glasswares

Beaker	Borosil
Pipettes	Borosil
Conical flask	Borosil
Measuring cylinder	Borosil

C) Equipments

Microcentrifuge	Eppendroff
Refrigerator	Sanyo
Multi ELISA reader model 2010	Anthos, Austria
Thermocycler	Takara Dice Mini, Japan
Vortex shaker	Genie
Electrophoretic tank	Mupid-exu, Japan
Electronic scale	QHAS Corporation, USA
Illuminator	Bio-pyramid
Oven	CG
Digital camera	Canon
Cold chamber	Diversified Biotech

Ice box	Rush
Autoclave	Life

D) Pipettes and tubes

Micropipette	Eppendroff
Micropipette	Eppendroff
PCR tubes	ABgene
Filter tips	Eppendroff

E) ELISA Kit (Standard Diagnostic INC.,Korea)

Anti-human IgM Coated Micro wells- (Assay plate)

Dengue antigen powder (Dengue 1, 2, 3 & 4 antigens pool)

HRP (Horseradish peroxidase) Conjugated Monoclonal Antibody Tracer

Conjugate diluents (Phosphate Buffer saline containing preservatives)

Sample diluents (Tris buffer saline with preservatives)

Negative Control Serum (Inactive anti-dengue IgG/IgM antibody positive human serum)

Positive Control Serum (anti-dengue IgG/IgM antibody negative human serum)

TMB substrate A (Hydrogen peroxidase with preservatives)

TMB substrate B (Tetramethylbenzidine with HCl and Penicillin)

Wash buffer (concentrate of phosphate buffered saline with Tween 20)

Stop Solution (1.6N Sulfuric acid)

APPENDIX-II

Table no. 1 Grading the Severity of Dengue Infection (WHO, 1999)

DF/DHF	Grade*	Symptoms	Laboratory
DF		Fever with two or more of the following signs: headache, retro -orbital pain, myalgia, arthralgia	Leukopenia occasionally. Thrombocytopenia, may be present, no evidence of plasma loss
DHF	I	Above signs plus positive tourniquet test	Thrombocytopenia <100,000, Hct rise >20%
DHF	II	Above signs plus Spontaneous bleeding	Thrombocytopenia <100,000, Hct rise >20%
DHF	III	Above signs plus circulatory failure (weak pulse, hypotension, restlessness)	Thrombocytopenia <100,000, Hct rise >20%
DHF	IV	Profound shock with undetectable blood pressure and pulse	Thrombocytopenia <100,000, Hct rise >20%

* DHF Grade III and IV are also called as Dengue Shock Syndrome (DSS)

APPENDIX-III: Reagents Preparation

Preparation of TAE buffer

25gm of TAE powder is added to 100ml of distilled water to make 25x TAE buffer.

Preparation of Buffer

Addition of carrier RNA to buffer AVL

Check buffer AVL for precipitate, and if necessary incubate at 80^{0}C until the precipitate is dissolved.

Add 1ml of buffer AVL to one tube of lyophilized carrier RNA. Dissolve carrier RNA thoroughly .Transfer to the Buffer AVL bottle, and mix thoroughly before using buffer AVL for first time.

Carrier RNA is stable for up to 1year when stored at room temperature (15-25^{0}C).Carrier RNA dissolved in buffer AVL, however, should be stored at 2-8^{0}C and will be stable for up to 6 months.

Buffer AW1

Buffer AW1 is supplied as a concentrate contains guanidine hydrochloride. Before using for the first time, add 125ml of ethanol (96-100%) as indicated in bottle to obtain 220ml.

Buffer AW2

Buffer AW2 is supplied as a concentrate contains chaotropic salt and sodium azide. Before using for the first time, add 160ml of ethanol (96-100%) as indicated in bottle to obtain 226ml.

APPENDIX-IV

Table no.2 Summary of Characteristics of Dengue Diagnostic Methods (WHO, 2009)

Diagnostic Methods	Diagnosis of acute infection	Time to result	Specimen	Time of collection after onset of symptoms	Facilities
Viral isolation and Serotype identification	Confirmed	1-2 weeks	Whole blood, serum, tissues	1-5 days	Mosquito or cell culture facilities, BSL-2/BSL-3 laboratory, fluorescence microscope or molecular biology equipment
Nucleic acid detection	confirmed	1 or 2days	Tissues, whole blood, serum, plasma	1-5days	BSL-2 Laboratory equipment for molecular biology
Antigen detection	Not yet determined	1day	Serum	1-6days	ELISA facilities
	Confirmed	>1day	Tissue for immunochemistry	NA	Facilities for histology
IgM ELISA	Probable	1-2 days	Serum, plasma, whole blood	After 5 days	ELISA facilities
IgM rapid test		30mins			No additional supplies
IgG (paired sera by ELISA, HI or neutralization test)	Confirmed	7days or more	Serum, Plasma, whole blood	Acute sera, 1-5days; convalescent after 15 days	ELISA facilities BSL-2 laboratory for neutralization assay

APPENDIX-V: PCR Primers and Mixture

Target (Purpose)	Primer Code	Direction	Nucleotide sequence(5' to 3')	Position in Viral Genome	Product (bp)
Dengue consensus	DC-1S	Sense	TCA-ATA-TGC-TGA-AAC-GCG-CGA-GAA-ACC-G	D1:132-159, D2:134-161, D3:132-159, D4:136-163	511
	DC-2C	Antisense	TTG-CAC-CAA-CAG-TCA-ATG-TCT-TCA-GGT-TC	D1:642-614, D2:644-616, D3:642-614, D4:646-618	
DENV-1 specific	D1-S	Sense	GGA-CTG-CGT-ATG-GAG-TTT-TG	2256-2275	490
	D1-C	Antisense	ATG-GGT-TGT-GGC-CTA-ATC-AT	2745-2726	
DENV-2 specific	D2-S	Sense	GTT-CGT-CTG-CAA-ACA-CTC-CA	1203-1222	230
	D2-C	Anti sense	GTG-TTA-TTT-TGA-TTT-CCT-TG	1432-1413	
DENV-3 specific	D3-S	Sense	GTG-CTT-ACA-CAG-CCC-TAT-TT	2253-2272	320
	D3-C	Anti sense	TCC-ATT-CTC-CCA-AGC-GCC-TG	2572-2553	
DENV-4 specific	D4-S	Sense	CCA-TTA-TGG-CTG-TGT-TGT-TT	3973-3992	399
	D4-C	Antisense	CTT-CAT-CCT-GCT-TCA-CTT-CT	4371-4352	

APPENDIX -VI: SD Dengue IgM-Capture ELISA

Explanation of the Test

Dengue viruses, transimitted by the mosquito, *Aedes aegypti* and *Aedes albopictus* mosquitoes, are widely distributed throughout the tropical and subtropical areas of the world. There are four known distinct serotypes (dengue 1, 2, 3 and 4). Dengue is considered to be the most important arthropod borne viral disease due to the human morbidity and mortality it causes. Primary Dengue infection is associated with mild to high fever, headache, muscle pain and skin rash. Immune response includes IgM antibodies produced by 5th day of symptoms and persist for 30-60 days. IgGs appear the 14th day and persist for life. Secondary infections often often result in high fever and in many cases with hemorrhagic events and circulatory failure. Secondary infections show that IgGs rise within 1-2 days after the onset of symptoms and induce IgM response after 20 days of infection.

SD Dengue IgM Capture ELISA is an enzyme-linked immunosorbent assay for the qualitative detection of IgM antibodiesagainst dengue virus antigen in human serum. SD Dengue IgM Capture ELISA contains a mocroplate, which is pre-coated with mouse monoclonal anti-human IgM antibodies on well. During first incubation with microplate, anti-dengue IgM antibody in patient serum is bound to mouse monoclonal anti-human IgM antibodies on well, and then is bound to mixture of dengue antigen and mouse monoclonal anti-dengue HRP Conjugate. Following this incubation, all unbound materials are removed by aspiration and washing. The residual enzyme activity found in the wells will thus be directly proportional to the dengue IgM antibody concentration in patient serum and evidenced by incubating the solid-phase with a substrate solution (TMB) in a substrate buffer. Colorimetric reading will be performed by using a spectrometer at 450 nm.

SD Dengue IgM Capture ELISA is for the qualitative detection of anti-dengue IgM antibody with high degree of sensitivity and specificity. This test is intended for professional use as an aid on the diagnosis of dengue virus infection.

Intended Use Purpose

The SD Dengue IgM Capture ELISA kit is an enzyme-linked immunosorbent assay for the qualitative detection of IgM antibodies specific to Dengue virus in human serum. The SD Dengue IgM Capture ELISA kit is intended for professional use as an aid on the diagnosis of dengue virus infection and reactive samples should be confirmed by a supplemental assay such as RT-PCR and HI (Hemagglutination inhibition) test as gold standard.

Materials Required But Not Provided

1. Precision micropipette and disposable tips
2. Waste discard container with suitable fresh disinfectant
3. Automated plate washer (Optional) or suitable equipment for washing 8 microwell strips
4. A spectrometer or ELISA plate reader able to read a 96 microwell plate of 8 microwell strips at an absorbance or 450 nm with a reference at 620 nm.
5. Incubator capable of maintaining temperature at 37±1°C

Preparation, Storage and Re-use of Kit Components

In order to ensure the optimal kit performance it is important that unused kit components are stored according to the following instructions;

1. Coated microplate: open the plate by cutting along the seal. Break off the required number of microwells and relocate them into the frame. Return all unused microwells and strips to the resealable plastic pouch with the desiccant. Carefully reseal the pouch and store at 2-8 °C. Microwells may be used for up to 1 month after initial opening, provided they are stored in this manner.

2. Enzyme conjugate: provided as X2 concentrate.
   (Preparation of working enzyme conjugate)
   Mix 1:1 dilution of enzyme conjugate (X2 concentrated) with diluted dengue antigen in a tube (X2 concentrated enzyme conjugate 10 ml + diluted dengue

antigen 10 ml). Prepare the volume of working enzyme conjugate as required on the time of use.

3. Washing solution: provided as X20 concentrate.
   (Preparation of working washing solution)
   Prior to use, take 50 ml of washing solution (X20 concentrated), and then fill-up to 1000 ml with distilled water. If undissolved crystals are present in washing solution, re-suspend the solution by placing the vial at 37 °C for few minutes.

4. TMB substrate A, B
   (Preparation of working substrate solution)
   Prior to use, Mix 1:1 dilution of TMB substrate A with TMB substrate B in a tube. (TMB substrate A 10 ml + TMB substrate B 10 ml)

Precaution and Warnings

In order to obtain reproducible results, the following rules must be observed.

1. For in vitro diagnostic use only.
2. This test should be performed on serum only. The use of whole blood, plasma or other specimens has not been established.
3. All reagents must be placed to room temperature before testing (15-30 °C).
4. As TMB is susceptible to contamination from metal ions, do not allow the TMB substrate A and B to come into contact with metal surfaces.
5. Avoid prolonged exposure to direct light.
6. Do not mix reagent of different lots.
7. Use thoroughly clean glassware, free from contamination of metal ions or oxidating substances.
8. Use disposable gloves while handling potentially infectious material and performing the assay.

9. TMB substrate A/B and stopping solution should be handled with care. Avoid contact with skin, eyes and mucous membrane. In case of accident rinse thoroughly with running water.
10. Sodium azide inhibits conjugate activity. Clean pipette tips must be used for the conjugate addition so that sodium azide is not carried over from other reagents.

Specimen Collection/Storage and Preparation

1. Collect the whole blood by venipuncture.
2. Centrifuge whole blood to get serum specimen.
3. If specimens are not immediately tested they should be refrigerated at 2-8 °C. for storage periods greater than three days , freezing is recommended. They should be brought to room temperature prior to use.
4. Specimens containing precipitate may yield inconsistent test results. Such specimens must be clarified prior to assaying.

Test Procedure

Sample Predilution

1. Prepare the microplate wells and other reagents, and place them at room temperature (15-30 °C).
2. Take the required number of microwells from the foil pouch and the remaining unused microwells have to be sealed tightly in the foil pouch. Five microwells are required for Negative control (N) in triplicate, Positive control (P) in duplicate.
3. Using suitable test tubes or a microplate, dilute the Negative Control (N), Positive control (P) and patient's samples (S) 1/100 with sample diluents, respectively; (Example) 990 µl of sample diluent + 10µl of Negative/Positive controls and patient's samples and mix well.

ELISA Procedure

a. Dengue antigen

1. Dilute a bottle of dengue antigen powder using 1.5 ml of the conjugate diluent.
2. Dilute the anti-dengue HRP conjugate 1:1 with diluted dengue antigen of above (example) 1.5 ml of anti-dengue HRP conjugate + 1.5 ml of diluted dengue antigen. This is sufficient for 3 strips (24 wells).
3. Gently mix and leave at room temperature (15-30 °C) for 60 minutes. Discard the unused diluted dengue antigen.

b. Assay plate

1. After mixing the diluted anti-dengue HRP conjugate, pipette 100 µl of diluted controls and patient's samples into their respective wells of microplate. Five microwells are required for negative control (N) in triplicate, positive control (P) in duplicate.
2. Cover the microplate with adhesive plate sealer. Incubate the wells at 37±1 °C for 60 minutes.
3. Wash the wells 5 times with 350 µl of diluted washing solution, giving at least 10 seconds soak time for each wash and aspirate all liquid from the wells.

   Washing procedure

   a. Automated washer

   1. Completely aspirate all wells.
   2. Fill all wells (350 µl of diluted washing solution) during wash cycle.
   3. On completion of 5 washes, invert plate and tap firmly on absorbent paper tower to ensure all washing solution is removed.

   b. Manual washing

   1. Discard contents of plate in appropriate waste container.
   2. Fill wells with diluted washing solution. Avoid bubbling of washing solution as this may reduce wash efficiency. Discard washing solution from well immediately.
   3. Repeat step 2 another four times. This will make a total of five washes with washing solution.

    4. After the final wash, discard contents of wells and tap the plate on absorbent paper towel to ensure all washing solution is removed

4. Mix the diluted Anti-dengue HRP conjugate solution before transfer. Pipette 100 µl of the diluted Anti-dengue HRP conjugate solution into the appropriate wells of microplate.
5. Cover the microplate with adhesive plate sealer. Incubate the wells at 37±1 °C for 60 minutes.
6. Wash the wells 5 times with 350 µl of diluted washing solution, giving at least 10 seconds soak time for each wash and aspirate all liquid from the wells.
7. Mix the TMB substrate A and TMB substrate B. (1:1); (Example) 5 ml of TMB substrate A + 5 ml of TMB substrate B. this is sufficient for 12 strips (96 wells).
   Caution: when mixing TMB A and TMB B solution, some crystals may be generated. In this case, make the substrate solution again with gentle mixing.
8. Pipette 100 µl mixed TMB solution into each well.
9. Incubate for 10 minutes at room temperature (15-30 °C). A blue color will develop.
10. Pipette 100 µl of stopping solution into each wells in the same sequence and timing as the TMB addition. Mix well. The blue color will change to yellow.
11. Within 30 minutes, read the absorbance of each well at a wavelength of 450 nm with a reference filter of 620 nm.

Internal Quality Control

The individual values of the absorbance for the control sera are used to calculate the mean value if

0.000 A (neg.) 0.300

A (pos.) 1.000

If one of the absorbance values of negative controls is outside the specification, this value can be neglected. Both absorbance values of the positive control must comply with the specification. If these specifications are not met, the test is to be repeated.

Interpretation of the Test

1. Evaluation

   Calculate the mean absorbance of the negative controls, then calculate the cut-off value by adding 0.300.

   A (neg.) + 0.300 = Cut-off value

   Based on the criteria of the test, the samples are classed as follows:

   Test results:

   i. A (sample)< Cut-off value = Anti-dengue IgM negative
   ii. A (sample)   Cut-off value = Anti-dengue IgM positive

Samples with a test result, which is equal to or greater than cut-off value, should first be retested in duplicate. If, in the retest, the mean absorbance is again equal to or greater than the cut-off, such samples should always be verified using a confirmatory test.

2. Result interpretation
   - Negative result: No detectable IgM antibody. An additional sample should be tested in 17-14 days if early infection is suspected.
   - Positive result: Presence of detectable anti-dengue IgM antibodies indicates primary dengue virus infection.

Limitation and Interferences

1) The test procedure, precautions and interpretation of results sections for this test kit must be followed closely when testing.
2) Testing should only be performed on patients with clinical symptoms or when exposure is suspected.
3) Samples
   - Pasteurized samples (no less than 10 hours at 60°C) may lead to diminished reactivity and therefore should not be used.
   - Haemolytic samples should be centrifuged before use to avoid interference by cellular constituents.

- Lipaemic and icteric samples may impair the test results.
- Rheumatoid factors can lead to elevated reactivity if contained in the samples.

4) Serological cross-reactivity across the flavivirus group (between dengue 1-4, Murray valley encephalitis, Japanese encephalitis, Yellow fever and West Nile viruses) is common. These diseases must be excluded before confirmation of diagnosis.
5) Heterophilic antibodies are a well-recognized cause of interference in immunoassays. These antibodies to animal IgM may cross-react with reagent antibodies and generate a false positive signal.
6) Failure to add specimen in the procedure could result in a falsely negative test. Repeat testing should be considered where there is clinical suspicion of infection.

Limitation of the Test

A negative result does not preclude the possibility of infection with dengue virus infection. Other clinically available tests are required if questionable results are obtained. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.

Expected Values

SD Dengue IgM-Capture ELISA has been compared with HAI. The overall accuracy is greater or equal to 90%.

APPENDIX -VII: Questionnaire

Dengue Case Details Form

Bed No: ……

Name of the hospital: …………………………………………………………………..

Full name of the patient: ………………………………………………………………….

Age /Sex: ……………………………. Occupation: ……………………………………

Address: District ……………………………VDC/Municipality: ……………………

Ward No: ……………………   Tole: …………………………….

Travel history of patient's within 14 days before of fever onset: ☐ Yes ☐ No

If yes place: ……………………………………………..

Date of hospitalization: …………………………………

Date of fever onset: …………………………………..

Clinical findings (if present, check the box): ☐ Yes ☐ No

Fever:	☐	Arthralgia:	☐
Headache:	☐	Rash:	☐
Retro-orbital pain:	☐	Hemorrhagic manifestation:	☐
Myalgia:	☐		

Provisional Diagnosis (From Physician) ……………………………………………….