CHAPTER I

1. INTRODUCTION

Dengue is an acute febrile illness caused by a virus that is transmitted from human to human via species of *Aedes aegypti* and *Ae. albopictus* mosquitoes. (Gubler,1998).The incidence of disease has been estimated to be 50-100 million cases per year which Is likely to increase due to expanding geographic distribution of both virus and vectors (Gibbons and Vaughn,2002).

Four dengue virus (DV) serotypes viz DEN-1, DEN-2, DEN-3 and DEN-4 are the responsible for the disease. The viral infection in human causes a spectrum of illness ranging from asymptomatic or mild febrile illness i.e. Dengue fever, which may evolve to severe disease forms i.e. Dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS). Secondary dengue virus infection (DVI) has been mainly associated with the severe form of the disease (Rothman, 2004).

Majority of the infection are asymptomatic or mildly symptomatic but other causes involves severe clinical presentation. It may lead to fever, headache, pain in various body parts prostration, rash, lymphadenopathy and leukemia. The severe form are DHF, DSS, which manifest with bleeding and shock (Yi-chung and kuie-Hsing, 2008). The major pathological hallmark that distinguish DHF from DF is plasma leakage due to increase vascular permeability which could be related to the release of immune mediators such as cytokines.

In recent years, the global prevalence of DV has grown dramatically. It has been recognized in over 100 countries and 2.5 billion people live in area where dengue is endemic (Gibbons and Vaughn, 2002). The emergence and reemergence of DF and DHF is directly related to the increase in density and geographic distribution of the vectors. In 2008, for the South East Asia region whole there is about 18% increase in the number of reported cases and about 15% increase in the number of reported dengue deaths as compared to the same period in the previous year. There was substantial increase in the reported case of dengue in Thailand, Indonesia and Myanmar. The peak months in 2008

of dengue transmission was February in Indonesia, June in Thailand and July in Myanmar. The case fatality rate in Thailand is above 0.2% in Indonesia and Myanmar it is around 1%. However there are some out breaks away the urban areas that have case fatalities even up to 3 to 5 % in India, Indonesia and Myanmar (WHO, 2008).

Nepal being bordered by India in the eastern, western and southern belts, DF/DHF has been considered to be a possible public health threat. DF is the emerging disease affecting Nepal since 2004 although DF has been already observed in foreign visitor earlier (Pandey *et al.,* 2008; Takasaki *et al.,* 2008). Nepal reported indigenous transmission of dengue cases in November 2006 (WHO/ SEARS, 2006). The outbreak occurred in Nepal following the Indian epidemic of DF/DHF in September-October 2006.

The basic methods routinely practiced by most laboratories are enzyme linked imunosorbent assay (ELISA) and hemagglutination inhibition (HI) for the detection of DV specific antibody (Guzman *et al*, 1996; Innis *et al.,* 1989 and WHO, 1997). Virus isolation through a mosquito cell line (C6/36) from acute phase serum sample is method of choice and remains the gold standard, although it takes weeks to complete the process. The molecular techniques such as RT-PCR has been used to detect dengue virus RNA in the plasma or serum of patients in order to obtain accurate result in time in acute cases and to avoid the cross reactivity among flaviviruses (WHO, 2009; Sadon *et al.,* 2008). The collection, storage and transportation of the blood for the viral identification is difficult due to inadequate facilities in tropics region for maintaining the viral integrity, so recently the use of dried blood filter papers has been used which is believed to overcome these difficulties. Moreover the capillary blood samples has been used to detect dengue virus as the viral particles persist longer in capillary blood than in peripheral blood (Prado *et al.,* 2005; Michaud *et al.,* 2007; Matheus *et al.,* 2007).

At present, diagnosis and management of dengue & Japanese encephalitis (JE) in Nepal is based on patient's clinical symptoms due to lack of diagnostic facility (Pandey *et al.,* 2003). The threat of the DVI in Nepal is emerging as the disease caused significant morbidity and mortality in the neighboring country. Though there is high risk of dengue in Nepal, there are only few studies on the sero-prevalence of the disease. This study

would initiate in establishing sero-epidemiological studies and its implication for crafting appropriate future intervention of dengue in Nepal. The information generated through this study would be significant 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 Objective

Sero-Diagnosis of Dengue Virus in different Hospitals of Nepal.

2.2 Specific Objectives

1. To detect the anti- dengue IgM In the serum samples of the suspected dengue patients by ELISA.
2. To describe -demographic distribution of clinically suspected cases .

CHAPTER III

3. LITERATURE REVIEW

3.1 Epidemiology of Dengue

Dengue is the most widespread vector-borne viral disease in the world (WHO, 1997). Dengue infection has spread progressively to most tropical and subtropical countries during the past 60 years, particularly to countries in Southeast Asia, the western Pacific, and Latin America. Over half of the world's population lives in these areas of risk of infection, and these are popular tourist destinations too. It is estimated that 50-500 million cases of DF occur annually, corresponding to an incidence rate of 2.5-5 %. These cases result in hundreds of thousands of hospitalization and about 20,000 deaths each year (Beatly, 2006). A severe form of the disease, DHF was first recognized in the Philippines in 1953 (WHO, 1997).

Dengue is an old disease and has had a wide global distribution in the tropics and subtropics for over 200 years (Monath, 1994). The epidemics of an illness compatible with DF were first reported in the medical literature in 1779 and 1780, and until the 1939–45 war pandemics of DF occurred every 10–30 years.

The global resurgence and emergence of DF and DHF are rather complex and not well understood. It is believed that activities related to World War II contributed to the emergence of DHF in South East Asia in 1950s. Ecological disturbance and demographic changes resulted in dramatic increase in *Ae. aegypti* mosquito population and dengue transmission. In addition to movement of indigenous population, the movement of foreign soldiers and expatriates, most of them were susceptible to dengue, aided in the spread of DV. The high rate of transmission associated with epidemic activity and transmission by vector and to vertebrate host in new geographic areas can result in selective pressure that lead to genetic changes in the pathogen. These new strains of virus may have greater epidemic potential and virulence. Finally, the lack of effective vector control and deterioration in the ability of the public health infrastructure to deal with

vector borne diseases have contributed to the widespread and increased epidemic activity (Gubler *et al.*, 1997).

3.1.1 Molecular Epidemiology

The techniques of RNA fingerprinting, RNA-DNA hybridization, antigenic analysis, short RNA oligonucleotide fingerprinting for determining the origin and spread of dengue epidemics has been replaced by nucleotide sequencing of the entire E gene or the E-NS1 in studying molecular epidemiology. Within 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. PCR combined with the nucleotide sequencing and restriction enzyme analysis has become a powerful tool for dengue strain characterization. Nucleotide sequencing studies have allowed classification of dengue viruses into different genotypes according to their nucleotide sequences (Rico-Hesse, 1990; Deubel *et al.*, 1993). Phylogenetic analysis of sylvatic and endemic/epidemic strains suggests that each serotype emerged separately from a sylvatic ancestor (Wang *et al.*, 2000) and this emergence is estimated to have occurred about 125–320 years ago, varying by serotype (Twiddy *et al.*, 2003).

Based on sequences of the complete envelope (E) gene or the E-NS1, DENV1 is currently divided into four to five genotypes, including a sylvatic clade (Zhang *et al.*, 2005; Holmes and Twiddy, 2003). DENV2 is divided into six subtypes, designated as Sylvatic, American, Cosmopolitan, Asian 1, Asian 2, and Asian-American, although the two Asian subtypes have on occasion been collapsed into a single Asian genotype (Salda *et al.*, 2005; Holmes and Twiddy, 2003; Rico-Hesse *et al.*, 1998). DENV3 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). Finally, DENV4 is divided into two 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). Overall, as further sequences become available, these genotypic structures are likely to be revised, possibly with the appearance of a new genotype or the collapse of two or more genotypes into one.

3.1.2 Sero -Epidemiology

A global pandemic of dengue begun in Southeast Asia after World War II and has intensified during the last 15 years. Epidemics caused by multiple serotypes are more frequent, the geographic distribution of dengue viruses has expanded, and DHF has emerged in the Pacific region and the Americas **(**Gubler and Clark, 1995).

Serological study performed in Thailand from 1973 to 1999 showed that 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).

Serological surveys conducted in Indonesia showed that DEN-1 and DEN-2 were the prevalent serotypes until the late 1980s; however the DEN-3 serotype has been the predominant serotype in the recent outbreaks. DEN–4 has been isolated in almost all epidemics; it is primarily detected in secondary dengue infections (Sukri *et al.,* 2003).

A total of 26 laboratory confirmed dengue patients were analyzed retrospectively who returned to German during the period 1993-2001. Of the total, 25 cases were determined to be primary infection and one was determined to be secondary infection, based on antibody response. All 26 serum samples obtained at first presentation were used for virus isolation. DV was isolated from 11 of 26 (42.3 %) acute serum samples from C6/36 cell line. The DVs detected were DEN-1 and DEN-2 by RT-PCR. Attempts to isolate the virus seem promising when patients are febrile. Once fever has subsided, virus isolation attempts usually result negative (Teichmann *et al*., 2003).

In Bangladesh, dengue outbreak occurred in 2002 where 6,132 clinical cases of dengue were reported. Of the total 200 clinically diagnosed dengue cases virus isolation and IgM-capture ELISA confirmed that 100 (50 %) cases were dengue. Of these 100 dengue positives, 192 (92%) cases were positive only. For IgM and 8 (8%) cases were positive only for the diction of virus. DEN-2 is found to be responsible for the outbreak (Islam *et al.,* 2006).

In the first outbreak of dengue in makkah, Saudi Arabia from April to July 2004. DV infection was confirmed by a positive IgM caputure ELISA or RT-PCR. Total 160

clinically suspected patients, 91 (64 by IgM capture ELISA, 14 by RT-PCR & 13 by both) to have DVI. Dengue serotypes 2 & 3 were identified in 19 and 4 patients respectively. Most patients were young adults with median age of 26 (range=6.94) years and male:female ratio of 1:5:1. The common symptoms were fever (100%), malaise (83%), musculoskeletal pain (81%), headache (75%), nausea (69%), vomiting (65%) and abdominal pain (48%) (Khan *et al*., 2008).

DV outbreak occurred in 2005 in Brunei. A total of 271 samples from the patients suspected of having DVI were selected and analyzed. The result showed that 45 people positive for dengue-specific IgM (27 males and 18 Females), while (RT-PCR) detected dengue viral RNA in 12 patients, 3 identified as DEN-1 and 9 as DEN-2 (Osman *et al.*, 2007).

In Japan, DV serotype 2 was detected by TaqMan RT-PCR from an imported dengue patient from Nepal. Anti-dengue immunoglobulin M (IgM) antibodies and anti-dengue immunoglobulin G (IgG) antibodies were assayed with IgM capture ELISA kit and dengue IgG indirect ELISA kit respectively. Serum samples were negative on day one and positive for anti-dengue IgM antibodies on days 3, 5, and 10 after the onset of illness. Those were positive for anti-dengue IgG antibodies on day 1, 3, 5 and 10 (Takasaki *et al.*, 2008).

A major DHF outbreak occurred in Delhi in 1996. Following this, another outbreak was reported in the year 2003. Out of 1820 serum samples received from suspected cases in three years (2003, 2004 & 2005), 811 (44.56%) were confirmed as dengue infection serologically. All four dengue serotypes were seen co-circulating in the year 2003, followed by complete predominance of dengue serotype 3 in 2005 (Gupta *et al.*, 2006).

In 2005 outbreak in Pakistan, dengue virus infection was confirmed for 42 of the 106 patients. Serum samples from 39 patients contained anti–dengue virus immunoglobulin M (IgM) antibody. DEN-3 was found to be responsible for the outbreak that showed significant similarity with a dengue serotype 3 isolate from India in 2004 (Jamil *et al.*, 2007).

In 2008, DEN-4 emerged in Northeastern Peru, causing a large outbreak and displacing DEN-3 which had predominated for the previous 6 years. Phylogenetic analysis of 2008 and 2009 isolates support their inclusion into DEN-4 genotype II, forming a lineage distinct from strains that had previously circulated in the region (Brett *et al.*, 2008).

The accuracy and speed of the RT-PCR assay make it an appealing test for the diagnosis of dengue and for epidemiologic surveillance. In diagnostic laboratories currently using traditional isolation or serological methods, this assay could be used to complement existing techniques or in some cases to replace them. In addition, the basic methodology of directly amplifying larger regions of the genome for rapid sequence analysis, which is potentially useful for both epidemiologic analysis and evolutionary studies (Lindsay et al., 1997).

3.1.3 Transmission of Dengue Virus

Dengue Viruses are transmitted to humans through the bite of infected *Aedes* mosquitoes (*Ae. aegypti, Ae. albopictus*, and *Ae. polynesiensis*), principally *Ae. aegypti* (Gubler, 1998). Once infected, mosquito remains infected for life, transmitting the virus to susceptible individuals during probing after an incubation period of approximately 1 week, depending upon ambient temperature (Watts *et al*., 1987). Infected female mosquitoes may also pass the virus to progeny by transovarial transmission. Humans are the main amplifying host of the virus, although monkeys have also been reported. Thus a single infected mosquito may transmit the virus to several susceptible humans over its life time (Rosen and Shroyer, 1983).

There is an enzootic Dengue transmission cycle in the forest involving *Aedes* mosquitoes and lower primates in Africa and Asia but because there is rarely movement of the enzootic cycle into urban areas, the most important cycle is the urban transmission cycle. 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.

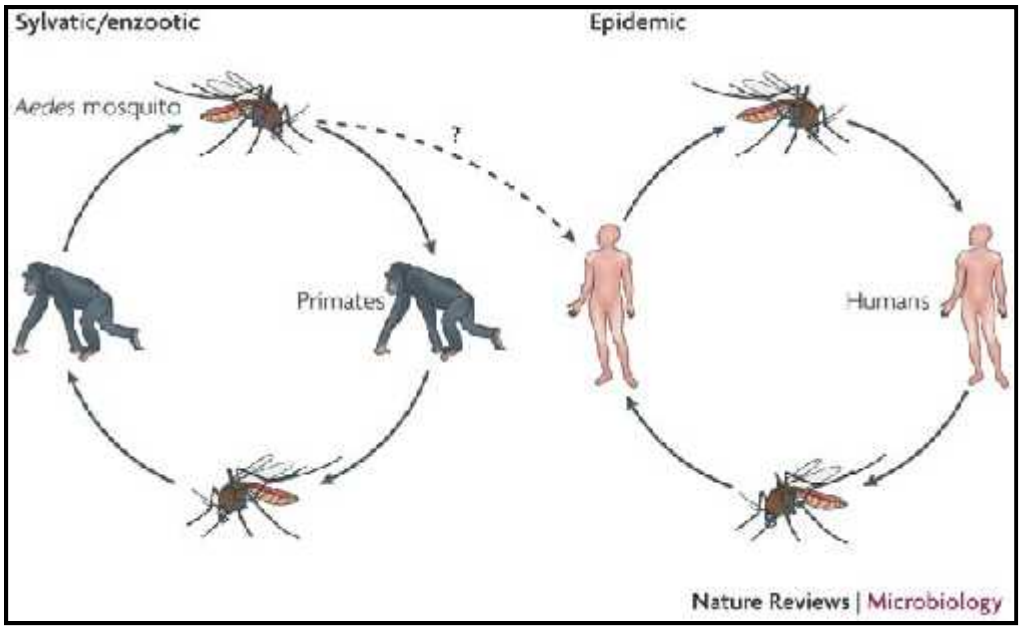


Figure 1**:** Sylvatic and Urban Dengue Transmissions Cycles (Whitehead *et al*., 2007)

Transmission rates are related to numerous environmental factors, population and human behaviors in intricate relationship with one another. These include the virus type and immunity, the virus EIP, prevalence of water holding vessels associated with *Ae. aegypti* breeding, weather and climate, densities of mosquitoes, infected humans and susceptible humans (Schreiber, 2001).

3.1.4 Dengue Virus

Morphology and Structure

DENV is a member of the Flaviviridae family and is grouped within the flavivirus genus together with other pathogenic viruses including West Nile virus (WNV), Japanese encephalitis virus (JEV), Tick borne encephalitis virus (TBEV) and Yellow fever virus (YFV) (Perera and Kuhn, 2008). The mature virion is icosahedral in shape and is about 50 nm in diameter. The viral genome consists of a positive-sense RNA of 10.7 Kb with a single long open reading frame that is translated as a polyprotein of about 3388 amino acids. This RNA encodes three structural proteins (C, prM and E) that form the components of the virion, and 7 non-structural proteins (NS1, NS2A/B, NS3, NS4A/B,

NS5) involved in viral RNA replication. The genome of DV is arranged in the order 5'-C-PrM (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (Zhang *et al.*, 2003; Kuhn *et al.*, 2002; Leitmeyer *et al.*, 1999).

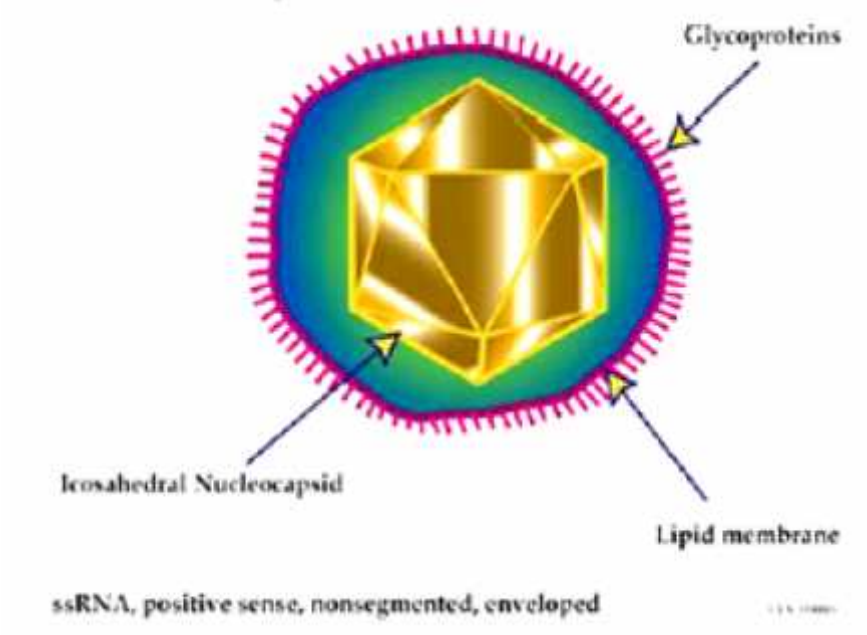


Fig. 1: Morphology of Dengue Virus

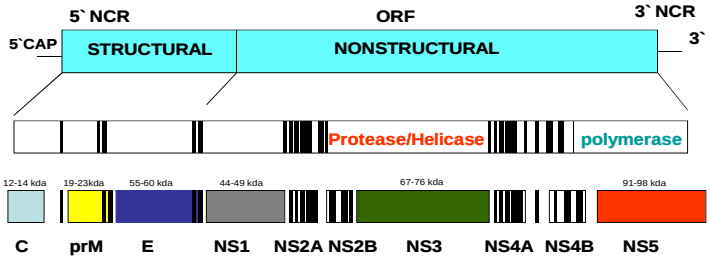


Fig. 2: Mature proteins **generated by proteolytic processing cascade**

(Source: Fields virology 2001)

The structural proteins are in the N-terminal region and are anchored in the endoplasmic reticulum by multiple membrane-spanning amino acid sequences. The polypeptide is

subsequently cleaved by cellular and virally encoded proteinases and glycosylated by cellular glycosyltransferases to yield three structural proteins: anchored capsid (anC) consisting of 113 residues, pre-membrane (prM) consisting of 166 residues and glycoprotein (E) consisting of 495 residues. Signal and stop-transfer sequences direct the translocation of the polyprotein back and forth across the membrane. The nucleocapsid core of the mature virion consists of the genomic RNA surrounded by multiple copies of the capsid protein C. This core is enveloped by a thick lipid bilayer derived from the endoplasmic reticulum of the host cell. Outside the membrane envelope is a layer of 180 copies of the E glycoprotein organized into a herringbone pattern plus 180 copies of the M protein. It has also been reported that both the E and M proteins are anchored in the membrane by their C-terminal domains. (Umareddy *et al.,* 2007; Modis *et al.,* 2005; Zhang *et al.,* 2003; Burke and Monath, 2001).

Replication

DV replicates in a wide variety of culture cells of both vertebrate and arthropod origin. Flaviviruses enter cells via clathrin-mediated endocytosis and fuse from within acidic endosomes, through which the viral genome gains access to the target cell cytoplasm (Rodenhuis-Zybert *et al.,* 2010). The mammalian cell generated virus enters cells mainly by receptor mediated endocytosis. Antibody dependant enhancement (ADE) can mediate virus attachment and uptake by binding the virus-antibody complex to cellular FC receptors. The virus-specific antibody and the Fc receptor together appear to act together as a co-receptor, enhancing the efficiency of virus binding and increasing the number of infected cells (Gollins and Porterfield, 1986). However, DEN-2 may enter human peripheral blood monocytes by direct fusion with the plasma membrane. Penetration and uncoating occur by endocytosis with the formation of coated vesicles. Once a virus is inside the cells, uncoating of the nucleo-capsid is accomplished by an acid dependent fusion of viral and endosomal membrane. Lysosomotropic amines increase the pH of the endosome and block the acid dependent fusion, inhibiting the early phase of viral replication. It is believed that once uncoating is completed, replicaton proceeds with the specific virus. Once inside cells, virus replication starts by translating uncoated

messenger sense viral genomic RNA and assembling replication machinery (Lindenbach and Rice, 2001).

Replication of the viral genome primarily occurs in the cytoplasm of infected cells. Initially, the incoming viral RNA is translated into a polyprotein, which is then directed to the endoplasmic reticulum (ER). Signal sequences within the polyprotein translocate NS1 and the ectodomains of prM and E into the lumen of the ER while the C, NS3 and NS5 proteins are localized to the cytoplasm. NS2A/B and NS4A/B remain predominantly as transmembrane proteins (Perera and Kuhn, 2008; Lindenbach and Rice, 1999). Processing of this polyprotein is a fundamental process that must occur before viral RNA replication can proceed. This task is carried out by host signalases that reside in the lumen of the ER and the viral NS3 protein and its co-factor, NS2B that reside in the cytoplasm (Falgout and Markoff, 1995; Falgout *et al.,* 1991). Structural insights into the viral replication proteins have been limited to NS2B/NS3 and NS5 (Wu *et al.,* 2003). However, a number of ultrastructural studies have also been done that demonstrate substantial re-arrangements of internal membranes permitting facile virus RNA synthesis and assembly (Mackenzie *et al.,* 1999). The NS4A protein has been implicated in this alteration of intracellular host membranes but the mechanism by which this occurs is unknown (Miller *et al.,* 2007).

3.1.5 Vector

Ae. aegypti is a tropical and subtropical species of mosquito found around the globe, usually between latitudes 35^0N and 35^0S. Although *Ae. aegypti* has been found as far north as 45^0N, such invasions have occurred during the warm season, and the mosquitoes have not survived the winters. Distribution of *Ae. aegypti* is also limited by altitude. It is usually not found above 1000m but has been reported at 2121m in India, at 2200m in Columbia, where the mean annual temperature is 17^0C. *Ae. aegypti* is one of the most efficient mosquito vectors for arboviruses, because it is highly anthrophilic and thrives in close proximity to humans and often lives indoors. 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, they are less efficient epidemic vectors than *Ae. aegypti* (WHO, 1997).

3.1.6 Host

Each of the four DV serotypes has been associated with DF and DHF in humans. The first infection produces lifelong immunity to the infecting serotype but not to the other three serotypes. Transmission of DV 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. This leads 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).

3.2 Dengue Burden: The Global Scenario

3.2.1 Dengue Situation in Nepal

It is not clear the precise year in which dengue was introduced in Nepal, but sporadic cases were reported in foreigners visiting Nepal in the late 1980s and 1990s. DVI had been reported in foreigners in Nepal in those visiting Nepal and the infected numbers were five in the year 1987, 1992, 1994, 1997 and 1998 (Kurane *et al*., 2000). Since then, the first case of DF was reported in Nepal in a Japanese volunteer in the year 2004 (Pandey *et al.*, 2004).

The outbreak of DF was documented in November 2006 in several locations of terai region of Nepal, bordering with Indian state of Bihar. During this outbreak, 23 confirmed DF cases were recorded. The outbreak has occurred in Banke, Bardiya, Dang, Kapilbastu, Parsa, Rupandehi and Jhapa districts. Ninety four percent patients were adults and male to female ratio was 4:1. DEN-1, DEN-3 and DEN-4 have been found in Nepal indicating the possibility of severe form of disease i.e DHF during outbreak. No DF related deaths have been recorded in Nepal (WHO/SEARO, 2006). However the isolation of DV type 2 was reported from a dengue patient returning to Japan from Nepal in October, 2004. The

isolated DEN-2 (GenBank accession number is AB194885) was 98 % homologous with DV type 2 isolate from India (Takasaki *et a*l., 2008).

3.2.2 Dengue Situation in SEARO

During the 1960s and 1970s, seasonal and cyclical epidemic patterns were observed with large outbreaks occurring at 2-3 years interval. 1070207 cases and 42808 deaths were reported, mostly in children during the period. In 1980s DHF/DSS spreaded to China, Indonesia, Malaysia, Myanmar, Philippines, Thailand and Vietnam affecting even rural villages (WHO, 1997). In South East Asia Region (SEAR), total cases reported were 188,684 in 2006. In the same year, the reported dengue cases have been increased in Indonesia, Thailand, India, Sri Lanka, Maldives, Bangladesh and Bhutan. All four dengue serotypes are present in this region. The disease is among the ten leading causes of hospitalization and deaths in at least eight tropical Asian countries (WHO/SEARO, 2006).

3.2.3 Dengue Situation in Americas

Epidemics of classic DF occurred in the Careabean and Northern South America in 1963-64, 1968-69, 1972-75 and 1977-78, but only sporadic suspected cases of DHF had been reported in the Americas. However, in 1981 an outbreak of DHF/DSS occurred in Cuba that marked the start of DHF in the region of Americas. During this epidemic 344203 cases of dengue were reported, including 10312 patients classified as severely ill according to WHO criteria. During the same epidemic, 158 deaths were reported. The second largest outbreak of DHF/DSS in the region occurred in Venezuela from October 1989 to April 1990. DV serotypes 1, 2 and 4 were isolated during these outbreaks. Cases of DHF have been reported in the Americas nearly every year since 1981. Dengue has been recorded in virtually all Latin American countries, with the possible exception of Argentina, Chile and Uruguay, and it appears that DHF/DSS is generally becoming endemic in several countries of the Americas (WHO, 1997).

3.2.4 Dengue Situation in Africa

Although the history of dengue in Africa is poorly documented, it is known that dengue has been on the continent since the last of the 20th century. All four serotypes of DV have caused outbreaks in this region. In the 1960s, DEN-1, 2 and 3 were isolated for the first time from samples taken from humans in Nigeria (Cary, 1971). Subsequently, dengue has been found in Senegal. After the first outbreak of dengue in South Africa in 1926-1927, cases of the disease imported from India have been detected in the 1980s. Similarly, DF was reported from Kenya, Mozambique and Sudan (Sang, 2006). The first reported epidemic of DF in Cape Verde (WHO, 2009)

3.3 Clinical Diagnosis of Dengue

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 (4-10 days), the illness begins abruptly and is followed by the three phases- febrile, critical and recovery (WHO, 2009).

3.3.1 Severe Dengue

Severe dengue is defined by one or more of the following: (i) plasma leakage that may lead to shock (dengue shock) and/or fluid accumulation, with or without respiratory distress, and/or (ii) severe bleeding, and/or (iii) severe organ impairment (WHO, 2009).

The patient is considered to have shock if the pulse pressure (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).

3.3.2 Grading the Severity of Dengue Infection

To decide about where to treat the patient, it is important to classify the severity of dengue infection. The severity of dengue infection is classified into the grades described in table below (WHO, 1999).

Table 1 Grading the Severity of Dengue Infection

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)

3.4 Pathogenesis of DHF/DSS

The dominant host cell receptor for virus entry has not been identified, although co-receptor such as DC-SIGN on dendritic cells has been reported. The virus multiplies

on skin dendritic cells, tissue macrophage, peripheral blood monocytes and hepatocytes, but not endothelial cells. The magnitude of viraemia and NS1 antigenaemia has been associated with disease severity, including complement activation as well as cytokines and chemokines activities (Cameron *et al.,* 2006).

Several hypotheses for the pathogenesis of dengue hemorrhagic fever have been proposed. Among them, Antibody-Dependent Enhancement (ADE) of infection has long been thought to play a central role (Halstead, 1970; Halstead, 2003). Virus virulence, the capacity of a virus to produce disease in a host, is an alternative hypothesis for the pathogenesis of DHF/DSS (Lei *et al.,* 2008). The different manifestations of DF, DHF, and DSS may be caused by variants of dengue virus with different degree of virulence. The risk of DHF/DSS is higher in secondary infections with dengue virus of serotype 2 compared with that of the other serotypes (Rico-Hesse *et al.,* 1997). Structural molecular differences have also been found among various isolates of DF and DHF patients (Leitmeyer *et al.,* 1999; Pandey *et al.,* 2000). Furthermore, it was reported that high dengue viremia titer was associated with increased disease severity (Vaughn *et al.,* 2000). Several factors such as cytokine storm phenomenon, individual's genetic background, virus strain differences, levels of virus circulating in individuals during the acute phase, the nutritional status of the infected individual have been associated to pathophysiological conditions. Moreover, hyper-thermal factors, physical status of virus in viremic individuals, conditioning of neutralizing antibody assay in dengue virus infection, concept of vector transmission, and innate immune system have been also found to be associated to pathophysiologiacl conditions (Noisakran and Perng, 2008; Pang *et al.,* 2007; Sierra *et al.,* 2007; Diamond *et al.,* 2000; Gubler *et al.,* 1981).

A series of studies have suggested that plasma leakage, which differentiates DHF from DF, is caused by malfunction of vascular endothelial cells induced by cytokines and chemokines rather than by destruction of the small vessels (Rothman and Innis, 1999). Plasma level of various cytokines is significantly higher in DHF than DF. The cytokines elevated in patients with DHF include TNF-α, IL-2, IL-6, IL-8, IL-10, IL-12 and IFN-γ. The levels of IL-8 and monocyte chemoattractant protein (MCP)-1 are also elevated in the pleural effusion from DHF patients (Aviruthan *et al*., 1998). However it is not clearly

understood how these cytokines cause malfunction of vascular endothelial cells and lead to plasma leakage.

Activation of complement is another important clinical manifestation in DHF. It was reported that the level of C3a and C5a complement activation products are correlated with the severity of DHF and the level of C3a and C5a reached the peak at the time of defervescence when plasma leakage becomes most apparent (Malasit, 1987). This is consistence with the assumption that complement activation is also responsible for the pathogenesis of DHF. The mechanism of complement activation in DHF is not completely understood. Circulatory immune complexes are present in the DHF patients, and it has been assumed that the complement is activated by the immune complexes (Aviruthan *et al*., 2006). Further it was reported that DV infected monocytes and endothelial cells activate complement via classical and alternate pathways.

Greater than 90% of DHF cases occur during secondary infections (Halstead *et al.*, 1970). A secondary dengue infection is caused by a different serotype of DV than that which caused a primary infection. Cross-reactive antibodies that lack neutralizing activity are induced in the primary infection. In secondary infection, dengue virus and non-neutralizing antibodies form virus–antibody complexes. Virus–antibody complexes bind to Fcγ receptors on target cells and result in enhancement of dengue virus infection. The non-neutralizing, cross-reactive antibodies thus markedly augment dengue virus infection of Fcγ receptor-positive cells and enhance the entry of virus into monocytes. The number of virus-infected monocytes increases. This phenomenon is called antibody-dependent immune enhancement (ADE) (Kurane *et al.*, 1991).

ADE increases the number of antigen presenting cells (APCs) that stimulate dengue cross reactive memory CD4 and CD8 T cells. 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 TNFα and lyse dengue virus-infected monocytes. TNFa 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. Positive feedback effects of

activated DV specific T lymphocytes on monocytes, through the action of IFN-γ, further contribute to the dysregulation of cytokine production. The synergistic effects of IFN-γ, TNFα and activated complement proteins trigger plasma leakage of endothelial cells in secondary dengue virus infection. However, the pathways leading to elevated levels of these vasoactive molecules are set in motion much earlier in the infection (Rothman 2004; Lei *et al.,* 2008; Rothman and Ennis, 1999; Green *et al.,* 1999).

Although DHF occurs more frequently in secondary infection, DHF also occurs in primary infection. This suggests that virulence of the virus contributes to the development of DHF. It has been assumed that virulent DV strain causes DF. It was reported that the determinants for virulence resided at the amino acid 310 of the E protein, in the 5' untranslated 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 DV type 2 (Pandey and Igarishi, 2000; Pandey *et al.,* 2000).

Most of the DHF/DSS in children are secondary infection; however the DHF/DSS in infants are primary infection. The levels of maternal dengue virus antibodies in the infants needed to decline to the levels that can enhance dengue virus infection and lead to DHF (Kliks *et al.,* 1988) which is consistent with the idea that enhancing antibodies increase the number of dengue virus-infected cells and the levels of viremia and lead to DHF.

3.4.1 Immune Response

After primary dengue infection, antibodies form against both structural and non-structural viral proteins. A primary infection with dengue is characterized by a slow and low titer antibody response. The precise roles of these different antibodies are not known, antibodies against viral NS1 have been shown to induce endothelial cell apoptosis in a caspase dependent manner (Lin *et al.,* 2002). After binding with antigen, different IgG subclasses vary in their capacity to activate the classical complement pathway; IgG1 being very effective whereas IgG2 being less so. Higher levels of dengue virus specific IgG1 and IgG4 and lower levels of IgG2 are seen in patients with DHF and dengue shock

syndrome compared with those with dengue fever (Koraka *et al.,* 2001; Thein *et al.,* 1993). Since complement activation could contribute to increased vascular permeability and abnormalities in coagulation, the predominating dengue specific IgG subclass may be important in the pathogenesis of severe disease (Koraka *et al.,* 2003).

Antibodies (IgM and IgG) are likely to be critical effectors in the resolution of DVI and long term immunity. Antibody may provide immune protection by blocking cellular attachment, viral fusion or by antibody dependent cellular cytotoxicity (ADCC). ADCC has been associated with severe dengue (Cameron *et al*., 2006). A primary infection with dengue is characterized by a slow and low titer antibody response. IgM antibody is the first immunoglobulin isotype to appear. Anti-dengue IgG at low titer is detectable at the end of the first week of illness, increasing slowly thereafter. In contrast, during a secondary infection antibody titers rise extremely rapidly (Innis *et al*., 1989).

Total and dengue specific IgE antibody levels are higher in patients with DHF and dengue shock syndrome compared with those with dengue fever (Koraka *et al.,* 2003). Moreover, total IgE levels are significantly higher in those previously exposed to dengue infections (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).

Serum concentrations of tumour necrosis factor- α (TNF- α), interleukin (IL)-2, IL-6, and IFN- γ are highest in the first three days of illness whereas IL-10, IL-5, and IL-4 tend to appear later (Chaturvedi *et al.,* 1999). Increased levels of IL-13 and IL-18 have also been reported during severe dengue infections, with highest levels seen in patients with grade IV DHF. Serum IL-12 levels are highest in patients with dengue fever. DHF patients have higher levels of TNF- α, IL-6, IL-13, IL- 18, and cytotoxic factor compared with DF patients. These cytokines have been implicated in causing increased vascular permeability and shock during dengue infections (*Mustafa et al.,* 2001; *King et al.,* 2000; *Vitarana et al.,* 1991). Moreover, cytotoxic factor, produced by CD4+ T-cells, induces macrophages to produce the proinflammatory cytokines IL- 1α, TNF- α, and IL-8. Serum

IL-6 and IL-8 concentrations are higher in patients with DHF and dengue shock syndrome (Juffrie *et al.*, 2001).

The similarity between DVs account for cross reactivity in the humoral and cellular immune response. With acute secondary dengue, there is massive activation, proliferation and programmed cell death of dengue specific T cell clones generated during previous infection. Finally, there is a correlation between the magnitude of the peripheral blood T cell response and disease severity, although in many cases this association is observed well after the acute symptoms have resolved. So, DV specific T cells are not usually detectable in the peripheral blood during the febrile phase of the illness (Cameron et al., 2006).

3.5 Treatment

No specific anti-viral treatment against the dengue virus are currently available, adequate supportive care and treatment could control its morbidity. Early and effective replacement of plasma losses with plasma expander or fluid and electrolytes result in a favorable outcome in most of the cases (WHO, 1997).

Although 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 a*l., 2007).

3.6 Prevention and Control

Prevention and control of DF and DHF has become more urgent with the expanding geographic distribution and increased disease incidence. Unfortunately, tools available to prevent DVI are very limited. There is no vaccine currently available and options for mosquito control are limited.

Environmental control methods include: reducing vector breeding sites, solid waste management, modification of manmade breeding sites, and improvements in house design as well as personal protection. Public education programmes play a vital role if they are effectively conducted. Biological control methods are targeted against the larval stages of the dengue vector. They include the use of larvivorous fish such as *Gambusia affinis* and *Poecilia reticulate*, endotoxin producing bacteria (*Bacillus thuringiensis* serotype H-14 and *B. sphaericus* are currently used), and copepod crustaceans. *B. thuringiensis* serotype H-14 is more effective against *Ae. aegypti* with very low levels of mammalian toxicity, and has therefore been accepted for use in household containers storing water. Chemical control includes the application of larvicidal insecticides or space spraying. Space spraying is more widely used as larvicidal insecticides cost more. It may be applied as thermal fogs or as ultra low volume sprays. Insecticides used for treating containers that hold water includes Temephos 1% sand granules and insect growth regulators. Regular monitoring of resistance patterns is essential as resistance to Temephos has been reported among some aedes mosquito species in the South East Asian Region. Although insecticides such as Malathion 4%, fenitrothion 1%, or pirimiphos-methyl have proved to be very effective in many control programmes, mosquito vectors develop different patterns of resistance to them (*Kay et al., 2002; Katyal et al., 2001; Soni et al., 2001; Dung et al., 1999;* Gratz *et al.,* 1999; WHO, 1999).

Attempts are being made to prevent infection by developing vaccine that would protect against all four serotypes of the DV. WHO has designated the DV as a high priority target for accelerated vaccine development. This work is conducted by a steering committee on dengue and JE vaccines, established in 1984. In the area of dengue vaccine, the main purpose of the steering committee is to promote and facilitate the development of candidate vaccines with a view to expediting their introduction in developing countries. This involves the evaluation of new biotechnological approaches, active participation in clinical trials of candidate vaccines, and the facilitation of vaccine introduction through the planning and assessment of low cost vaccination schedules. The steering committee has supported some research projects that have led to the development of candidate vaccines now undergoing clinical evaluation (Pervikov, 2000).

3.7 Clinical Diagnosis of Dengue

The clinical presentations of dengue virus infection range from asymptomatic, or a mild self-limited illness, dengue fever (DF) to a severe and potentially life-threatening disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Wang *et al*., 2002). The group progressing from non-severe to severe disease is difficult to define, but this is an important concern since appropriate treatment may prevent these patients from developing more severe clinical conditions. Triage, appropriate treatment, and the decision as to where this treatment should be given (in a health care facility or at home) are influenced by the case classification for dengue (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 dengue shock syndrome (DSS). Currently the classification into DF/DHF/DSS continues to be widely used (shown in appendix-III) (WHO, 1997).

The classification into levels of severity has a high potential for being of practical use in the clinicians' decision as to where and how intensively the patient should be observed and treated (i.e. triage, which is particularly useful in outbreaks), in more consistent reporting in the national and international surveillance system, and as an end-point measure in dengue vaccine and drug trials (WHO, 2009).

3.7.1 Clinical Symptoms

Symptoms of DF include fever, headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations and leucopenia. Symptoms of DHF include fever or history of acute fever lasting 2-7 days, occasionally biphasic, hemorrhagic tendencies evidenced by at least a positive tourniquet test; petechiae, ecchymoses or purpura; bleeding from mucosa, gastrointestinal tract, injection sites or other locations; hematemesis or malaena; thrombocytopenia (100,000/mm^3 or less), plasma leakage evident by rise in the hematocrit. Symptoms of DSS include all of the criteria for DHF must be present, plus evidence of circulatory failure manifested by rapid and weak pulse, narrow pulse

pressure (<20 mmHg), hypotension for age and cold, clammy skin and restlessness (WHO, 2009).

3.8 Laboratory Diagnosis of Dengue

Efficient and accurate diagnosis of DVI is of primary importance for clinical care (i.e. early detection of severe cases, case confirmation and differential diagnosis with other infectious diseases), surveillance activities, outbreak control, pathogenesis, academic research, vaccine development, and clinical trials. The four basic methods routinely practiced by most laboratories are DV isolation and characterization, detection of DV specific antibodies, detection of dengue antigen and detection of viral nucleic acid by nucleic acid amplification technique. The summary of characteristics of dengue diagnostic methods is shown in appendix-IV. After the onset of illness, DV can be detected in serum, plasma, circulating blood cells and other tissues for 4-5 days. During the early stages of the disease, virus isolation, nucleic acid detection 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 (WHO, 2009).

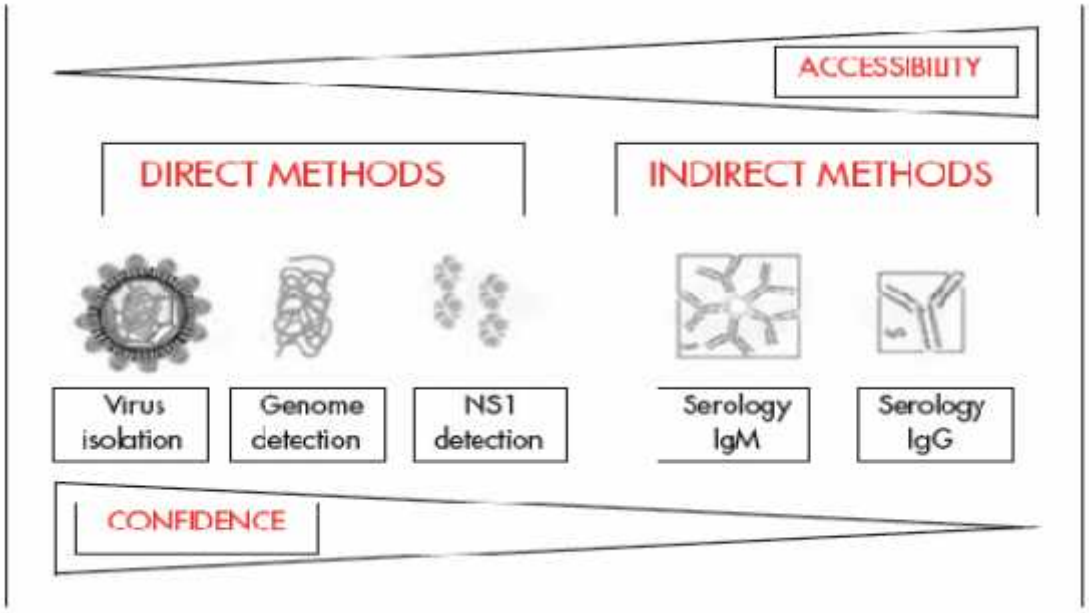


Figure 2: Comparision of diagnostic tests according to their accessibility and confidence (WHO,2009; Innis, 1995; Shope, 1990).

3.8.1 Virus Isolation

Virus culture is the best method for isolation of virus that depends upon the available laboratory facilities. No single isolation system is adequate for all arboviruses. Identification of the infecting DV serotype depends upon isolation of virus in a sensitive host system followed by serotype identification using reference monoclonal antibodies. A wide variety of cell types are susceptible to infection by DV. 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 DVs (Gubler *et al*., 1984).

Methods selected for virus isolation depend upon the laboratory facilities available. No single isolation system is adequate for all arboviruses. Identification of the infecting dengue virus serotype depends upon isolation of virus in a sensitive host system followed by serotype identification using reference monoclonal antibodies. A successful isolation of virus followed by sero-typing usually takes more than two weeks which procrastinates the inevitable vector control under epidemic situations. DVs do not grow well in vitro normally. However, at present some more sensitive isolation systems comprising certain insect species such as non blood-sucking Toxorhynchites mosquitoes are available where in the inoculation and isolation of DVs are successfully accomplished (Philip *et al*., 2006).

3.8.1.1 Suckling Mice

All the four DVs have been successfully isolated in BS-C-1 cells (African green monkey kidney cells) or 1-3 days old baby mice using a soup prepared from *Ae. aegypti* (Singhraj *et al*., 1966 and Carey *et al*., 1964). Baby mice are very insensitive before inoculating an evidence of infection. In spite of this, suckling mice are important as it is generally not possible to detect the virus in other animal host body (*e.g.*, mosquitoes, ticks) when in low quantity. Mice are inoculated intracranial with classified suspensions of clinical specimens or macerated arthropod pools or animal tissues. Since the suckling mice are readily available in all laboratories and have certain practical advantages over others, the

supernatant of the mosquito soup after centrifugation is inoculated intracerebrally into suckling mice for virus isolation. Dengue serotypes 1 and 4 were isolated from *Ae.aegypti* in 1961 from Vellore, in Tamil Nadu State, by inoculating infant mice (Carey *et al*., 1966).

3.8.1.2 Mosquito Inoculation

Mosquito inoculation is the most sensitive method for DV isolation (Gubler and Sather, 1988). Four mosquito species have been used for virus isolation; *Ae. aegypti, Ae. albopictus, Toxorhynchities amboinensis and T. splendens.* Male and female mosquitoes are equally susceptible; DV generally replicates to high titer as early as 4-5 days depending on the temperature of incubation. DV replicates in most mosquito tissues including the brain. This technique is not being used nowadays because it is labor intensive and require an insectary to produce large numbers of mosquitoes for inoculation (Rosen and Shroyer, 1983; Rosen and Gubler, 1974).

3.8.1.3 Mosquito Cell Cultures

Mosquito cell cultures are the most recent addition to DV isolation. The first cell line developed, and still the most widely used is the C6/36 clone of *Ae. albopictus*. The use of this cell line has provided a rapid, sensitive and economical method for DV isolation. The sensitivity of the mosquito cell line may vary with the strain of the virus. Several continuous mosquito cell lines have been shown to be highly susceptible to dengue virus infection. (Gubler ,1984; Igarashi 1978 and Tesh 1979).

Dengue virus isolation by mosquito cell cultures have proved to be more sensitive than mice or mammalian cell culture systems. Some prefer to use cytopathic effect to detect infection especially with AP-61 cells. This has the disadvantage of not producing cytopathic effects (rounding, refraction of light, detachment from the substrate) and requires secondary step for recognizing presence of virus in the culture. Intra-thoracic inoculation of *Toxorhynchites* mosquitoes (which do not take blood meals) or *Aedes* mosquitoes has also been used. In routine diagnosis, the C6/36 cell lines have become most widely used (Igarashi, 1978).

3.8.2 Detection of Anti-dengue Antibody

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 immunofluorescent- antibody test, ELISA, complement fixation, dot blotting, Western blotting, and the rapid immunochromatography 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).

3.8.2.1 Hemagglutination Inhibition (HI)

HI for dengue is simple and more sensitive than electrophoretic methods, being able to detect less than 1µg/ml of antibodies (Roitt, 2001). HI is sensitive, easy to perform, reliable and most frequently used method for diagnosis of dengue infection. Because HI antibodies persist for long periods up to 48 years and probably longer (Halstead, 1974), the test is ideal for sero-epidemiological studies. HI antibody usually begins to appear at detectable level by day 5 or 6 of illness and antibody titer in convalescent phase serum specimens are generally at or below 640 in primary infection. A titer of ≥ 1,280 in an acute phase or early convalescent phase serum is considered presumptive evidence of a current dengue infection. Such high level of HI antibody persists for 2-3 months in some patients, but antibody titer generally begins to wane by 30-40 days and fall below 1,280 in most patients (Gubler, 1988). The major disadvantage of the HI test is its lack of specificity, which generally makes it unreliable for identifying the virus serotype. However, some patients with primary infections show a relatively monotypic HI response that generally correlation with virus isolated.

3.8.2.2 Particle Agglutination (PA)

PA is used to detect DV 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 (strip) and purified DV 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 DV antigens. The beads can bind to anti-dengue virus specific IgG molecules, which are captured on the microplate, adhere to face of the wells. DV antigen-coated Ha-Ny beads adhere to the face of the wells, when the test sample is anti-DV 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).

3.8.2.3 Neutralization Test (NT)

The NT is the most specific and sensitive serologic test for DVs. The most common protocol used in dengue laboratories is the serum dilution plaque reduction NT. In general, neutralizing antibody titers rise at about the same time or slightly more slowly than HI and ELISA antibody titers but more quickly than CF antibody titers and persist for more at least 48 years. Neutralizing antibodies are present in the absence of detectable HI antibodies in some persons with past dengue infection. NT can be used to identify the infecting virus in primary dengue infections. The major disadvantages are the expense, time required to perform the test and technical difficulty. It is therefore not used routinely by most laboratories (Russell and Nislak, 1967).

Plaque reduction and neutralization test (PRNT) is the most specific serological tool for the determination of dengue antibodies and is used to determine the infecting serotype in convalescent sera. This assay measures the titer 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 principal 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).

3.8.2.4 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).

3.8.2.5 Complement Fixation Test (CFT)

The CF test is not widely used for routine dengue diagnostic serologic testing. 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. CF antibodies generally appear later than HI antibodies, are more specific in primary infections, and usually persist for short period, 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 titers by CF but have only stable antibody titers 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 (WHO, 1997; Casey, 1965).

3.8.2.6 Enzyme-linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) test measures the proportion of IgM and IgG anti-dengue antibodies, including IgA (Cuzzubbo *et al*., 1998; Figuerede *et al.*, 1987; Innis *et al*., 1989; Tio and Malasit, 1995). In primary or secondary dengue infections, IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) can measure a rising dengue specific IgM, even in sera samples collected at 1-day to 2-day intervals in the acute phase. Specimens collected over an interval of 2-3days spanning the day of defervescence are also usually diagnostic in MAC-ELISA. In cases where only a single specimen is available, detection of anti-dengue IgM permits the

diagnosis of recent dengue infection, even in primary infections where the level of HI antibody would not be diagnostic. By day 5 of illness, most patients (80%) develop detectable IgM antibody in the acute phase serum and by day 6-10 days of illness in 93-99% of cases. IgM persists for more than 90 days, but in most patients it has waned to an undetectable level by 60 days. A small percentage of patients with secondary infections have no detectable IgM antibody (WHO, 1997; PAHO, 1994).

One of the main advantages of the test is the ability to distinguish primary from secondary infections based on the IgM/IgG ratio. In primary infections, the IgM/IgG ratio in acute or convalescent sera usually exceeds 1.5, whereas in secondary infections, the quantity of IgG antibodies is much higher than that of IgM antibodies. This technique has a sensitivity and specificity of approximately 90 % and 98 % respectively, but when used 5 or more days after the onset of fever (Buchy *et al*., 2006; WHO, 1997; PAHO, 1994; Gubler, 1988).

Serum sample is added to wells of the assay plate. Serum antibodies of the IgM class, when present, combine with anti-human IgM antibodies attached to the polystyrene surface of the microwell test strips. Dengue 1-4 antigens are diluted separately to the correct working volume with antigen diluents. An equal volume of the HRP-conjugated monoclonal antibody (MAb) is added to each diluted antigen, which allows the formation of antigen-MAb complexes. Residual serum is removed from the assay plate by washing and complexed antigen-MAb is added to the assay plate. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) is added. The substrate is hydrolyzed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow; colour development is indicative of the presence of the respective anti-dengue IgM antibodies in the test sample (Protocol **,** Panbio Australia).

3.8.2.7 Dot-blot Immunoassay

Dot-blot immunoassay technology is relatively new and reagent and test procedures are developing. At least one dot-blot immunoassay for dengue antibodies is available

commercially, as greater interest is developing among commercial manufacturers. This test uses nitrocellulose membrane to capture the dengue virus antigen and drops of sera for antigen antibody reaction. The test kit, a modification of an EIA assay that has been exclusively evaluated, has high specificity and sensitivity (Palmer et al, 1999; Vaughn *et al*., 1999; Vaughn *et al*., 1998).

3.8.2.8 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 DV 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 human 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 DV if present, form gold conjugated antigen antibody complex on the test zones, resulting in a colored IgM test line or IgG test line. A built 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).

3.8.3 Detection of Antigens

The detection of antigen in acute-phase serum samples by serology has been slowly progressed due to the low sensitivity of the assay for patients with secondary infections, as such patients have preexisting virus-IgG antibody immunocomplexes. However, new developments in ELISA and dot blot assays directed to the envelop/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 (Young *et al.,* 2000; Alcon *et al.,* 2002; Koraka *et al.,* 2003).

The NS1 glycoprotein is produced by all flaviviruses and is secreted from mammalian cells. NS1 produces a very strong humoral response. Many studies have been directed at using the detection of NS1 to make an early diagnosis of dengue virus infection. Commercial kits for the detection of NS1 antigen are now available, though they do not differentiate between dengue serotypes. Fluorescent antibody, immunoperoxidase and avidin-biotin enzyme assays allow detection of dengue virus antigen in acetone-fixed leucocytes and in snap-frozen or formalin-fixed tissues collected at autopsy (WHO, 2009).

3.8.4 Molecular Techniques

3.8.4.1 Polymerase Chain Reaction (PCR)

PCR is an *in vitro* technique that allows the amplification of specific target DNA. They offer better sensitivity compared to virus isolation with a much more rapid turnaround time. All nucleic acid detection assays involve three basic steps: nucleic acid extraction and purification, amplification of the nucleic acid, and detection and characterization of the amplified product.

Extraction and purification of viral RNA from the specimen can be done by traditional liquid phase separation methods (e.g. phenol, chloroform) but has been gradually replaced by silica-based commercial kits (beads or columns) that are more reproducible and faster, especially since they can be automated using robotics systems. This technique potentially detects as little as a single copy of a specific target nucleic acid sequence by using oligonucleotide primers. These are short, single-stranded and complementary to the end of the template. The primers are extended on the single-stranded DNA template by DNA polymerase, in the presence of dNTPs under suitable reaction conditions. This result in the synthesis of new DNA strands that is complementary to the template strands. These strands exist at this stage as double-stranded DNA. Strand synthesis can be repeated by heat denaturation of double stranded DNA, annealing of primers by coding

the mixture and primer extension by DNA polymerase. Many laboratories utilize a nested RT-PCR assay, using universal dengue primers targeting the C/prM region of the genome for an initial reverse transcription and amplification step, followed by a nested PCR amplification that is serotype-specific (Lanciotti *et al*., 1992).

A combination of the four serotype-specific oligonucleotide primers in a single reaction tube (one-step multiplex RT-PCR) is an interesting alternative to the nested RT-PCR (Harris *et al*., 1998). The products of these reactions are separated by electrophoresis on an agarose gel, and the amplification products are visualized as bands of different molecular weights in the agarose gel using ethidium bromide dye, and compared with standard molecular weight markers. In this assay design, dengue serotypes are identified by the size of their bands (WHO, 2009).

PCR can be used to amplify target RNA if reverse transcription of the target RNA to cDNA is used in the initial step; this method is commonly used for the detection of viral RNA, such as HIV, CMV, dengue virus, etc. RT-PCR assay can detect a small amount of target RNA; unlike more limited biological amplification through culture, million-fold enzymatic amplification can be accomplished in a few hours. A number of methods involving primers from different locations in the genome and different approaches to detect the RT-PCR products have been developed over the past several years (Guzman and Kouri, 1996; Shope, 1990; WHO, 1997).

Several laboratories use RT-PCR for the detection of dengue viral RNA in various kinds of specimens, such as clinical samples, mosquitoes and cell cultivation (Chan et al., 1994; Chow *et al*., 1998). This method consists of two strategies for the identification of the four dengue virus serotypes: combination of the four serotypes specific oligonucleotide primer pairs in a single reaction tube, and the use of universal primers which require a subsequent step to classify a positive result (Lanciotti *et al*., 1992; Morita *et al*., 1991).

There are various applications of RT-PCR assay such as *in situ* PCR , which is used to detect dengue viral RNA in the tissue (Lucia and Kangluanpong, 1994) or quantitative RT-PCR for the detection of the number of dengue viral RNA (Laue *et al*., 1999; Wang

et al., 2000). This method is further used to detect the number of dengue viral RNA in clinical specimens so that it is useful for the treatment and study of clinical features.

3.8.4.2 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 a fluorescent probe enables the detection of the reaction products in real time without need for electrophoresis. Many real time RT-PCR assays have been developed either as 'singleplex' (only detecting one serotype at a time) or 'multipiex' (able to identify all four serotypes from a single sample). The multiplex assays have the advantage that a single reaction can be used to determine all four serotypes without the potential for introduction of contamination during manipulation of the sample. The fourplex real time RT-PCR assays are often less sensitive than nested RT-PCR assay but are faster. An advantage of this assay is the ability to determine viral load in a given sample, which is believed to be important in determining the severity of dengue disease (Buchy *et al*., 2006).

The primary advantage of the molecular tool lies in the speed at which specimens can be screened for the presence of DVs and also by its highly sensitive and specific detection. It is able to monitor the infection rate in mosquitoes, both adults and larvae, with a high degree of precision (Rohani *et al*., 1997).

3.8.4.3 Typing of Dengue Virus:

The detection of dv by pcr is not enough tools to determine which type of dvi actually occurred,hence two methods of detection of different type of dengue viz dengue 1,dengue 2,dengue 3 and dengue 4 by using their specific primers.nowadays,there are mainly 2 methods are available for typing of dengue virus.

1. Dengue Virus Typing by Second-round Amplification with Type-specific Primers (Nested PCR).

In this, A second amplification reaction was initiated with 10 µl of diluted material (1:100 in sterile distilled water) from the initial amplification reaction. The reaction

mixture contained all the components of initial amplification reaction with the following exceptions: second primer replaced with the dengue Virus type-specific primers and dithiothreitol and RT were eliminated. The samples were subjected to 20 cycles of denaturation (94°C,30second), primer annealing (55°C, 1 min), and primer extension (72°C, 2 min). A 15-µ1 portion of the reaction product was electrophoresed on a 4% composite agarose gel in 0.4 M Tris-0.05 M sodium acetate4.01M EDTA buffer. Because of the position of priming with each of the dengue virus type-specific primers, the size of the resulting DNA band was characteristic for each dengue virus type. (lanciotti *et al*,1991)

2. Detection and Typing of Dengue Viruses from Viremic Human Serum.

Human serum samples were obtained from patients with clinically characterized and virologically confirmed dengue infection and were tested by the RT-PCR assay. These samples had previously been shown to contain dengue viruses by isolation in C6/36 A. albopictus cell cultures or by intrathoracic inoculation of mosquitoes. Dengue virus serotypes were determined by an indirect immunofluorescence assay (IFA) with dengue virus type-specific monoclonal antibodies . (laciotti *et al*, 1991)

3.8.4.4 Hybridization Probes

The hybridization probe method detects viral nucleic acids with cloned hybridization probes. Probes with variable specificity ranging from dengue complex to serotype specific can be constructed depending on the genome sequences 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. 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 (Deubel and Pierre, 1994).

3.8.4.5 Loop Mediated Isothermal Amplification (LAMP)

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).

3.8.4.6 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 DV 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).

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 289 serum samples were collected from Narayani Sub-regional Hospital (NSH), Birgunj (173); Tanahu District Hospital (TDH), Damouli (42); Koshi Zonal Hospital (KZH), Biratnagar (32), Bharatpur hospital (BH) Chitwan (9), and Dhading District Hospital (DDH), Dhading Besi (33). Serum samples were collected from individuals experiencing a febrile illness clinically consistent with dengue infection, selected according to the inclusion and exclusion criteria. Patients' personal details about the symptoms, age, sex etc. were obtained through a questionnaire method by direct interview. The entire laboratory setting was done at Everest International Clinic and Research Center (EICRC), Kalanki, Kathmandu.

Selection Criteria:

4.2.1 Case Inclusion Criteria

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

4.2.2 Informed Consent

Written consent was obtained from all the responding patients.

4.2.3 Sample Collection, Storage and Transport

The serum samples from suspected cases were collected, stored and transported maintaining the reverse cold chain to EICRC.

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. After 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 and -20^0C until tested.

Figure 3: Site of sample collection in different district of Nepal.

4.2.4 Laboratory Tests

4.2.4.1 Detection of Anti-dengue IgM-Capture ELISA

The IgM-capture ELISA was performed according to standard protocol of manufacturer. During the testing procedure, the protocol provided by the Standard diagnostics was strictly followed to achieve high level of accuracy (Appendix-VIII for detail procedure).

SD Dengue IgM Capture ELISA Test

List of chemicals and reagents are given in Appendix-VIII.

Procedure:

All reagents are equilibrated to room temperature (20-25^{o}C) before commencing assay.

Serum Predilution

Positive control, negative control and patient 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

A bottle of Dengue antigen power was diluted using 1.5ml of the conjugate diluents. The anti-Dengue HRP conjugate was diluted with diluted Dengue antigen in 1:1 ratio (eg 1.5 ml of anti-dengue HRP +1.5 ml of diluted Dengue antigen). The mixture solution was gently mixed and left at room temperature (20-25^{o}C) for 60 minutes.

Preparation of TMB Substrate

In a tube, A TMB substrate A was mixed with TMB Substrate B in ratio 1:1 (eg 5 ml of TMB Substrate A +5 ml of TMB substrate B)

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 Mab tracer and diluted antigen, 100 µl diluted patient sample and controls were pipetted into their respective microwells of the assay plate. The plate was covered and incubated for 1 hour at 37^{o}C. After incubation, wells were washed five times with diluted wash buffer. The diluted anti-dengue HRP conjugate solution was mixed before transfer. Hundred microlitre of diluted anti-dengue HRP conjugate solution was pipetted into the wells. The plate was covered and incubated for 1 hour at 37 ^{o}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 ^{o}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).

4.2.5 Interpretation of the Result

4.2.5.1 ELISA Result Analysis

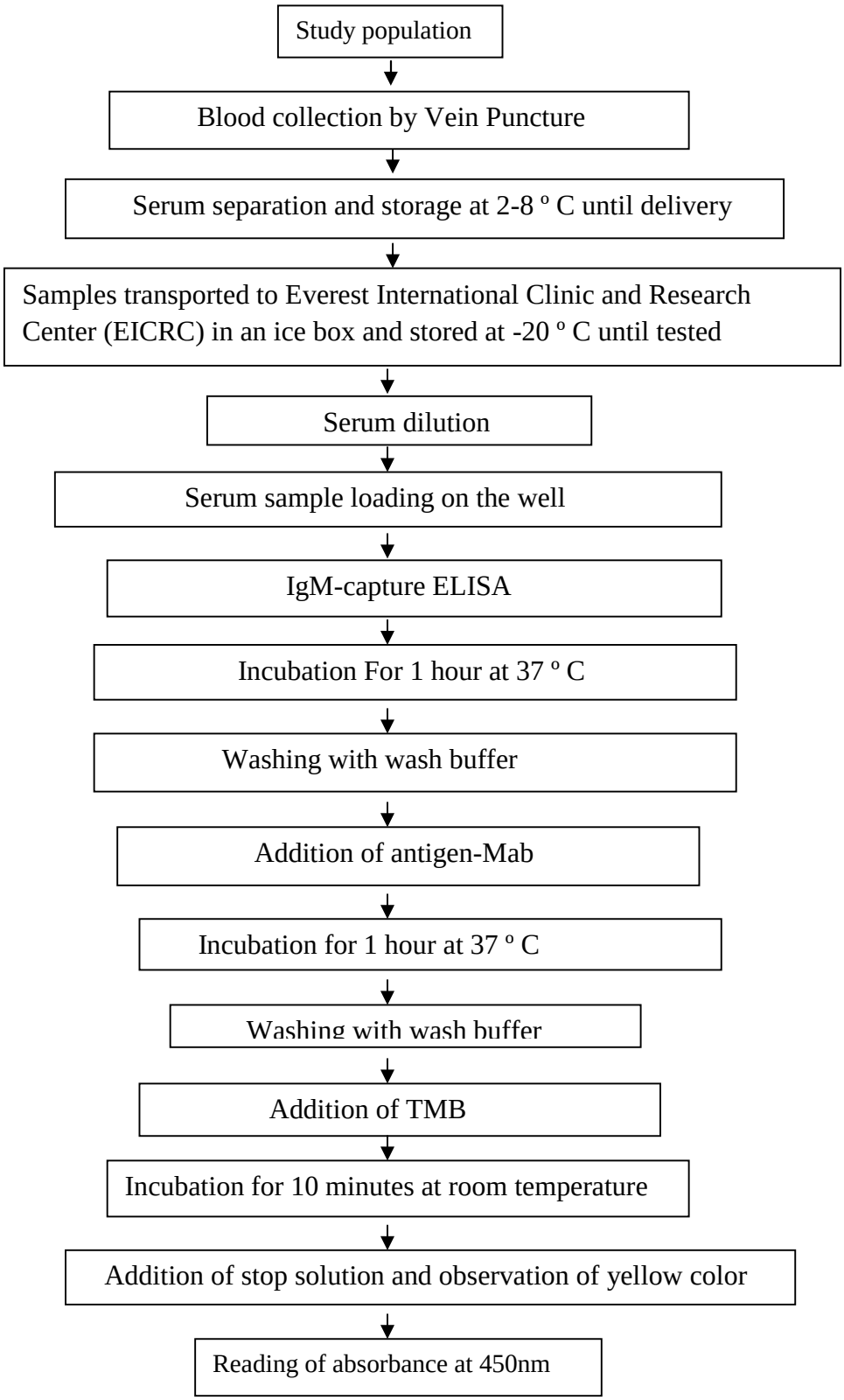
A negative result means that DV specific IgM cannot be detected. If a sample is assessed to be positive this means that virus specific IgM has been detected. The test is interpreted either positive or negative on the basis of absorbance with respect to Cut-off value. If absorbance of the sample are greater than cut-off value, the sample is considered positive and if the absorbance of sample are less than cut-off value, the sample is negative.

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

4.2.6 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 and 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 17.0).

Flow Chart Methodology for IgM Capture ELISA



CHAPTER V

5. RESULTS.

5.1 Socio Demographic Status of the Suspected Dengue Cases

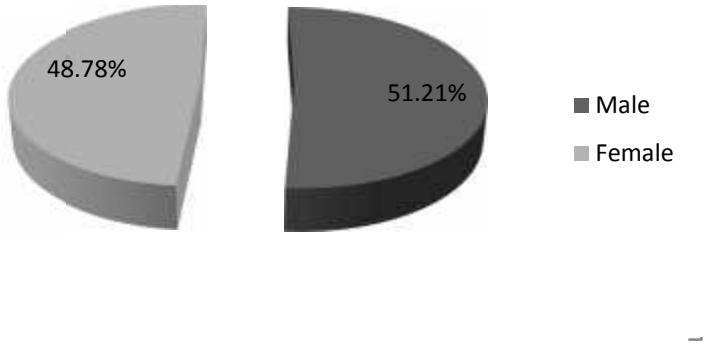


Figure 4: Sex Wise Distribution of Suspected Dengue Cases

Out of 289 suspected dengue cases investigated during the study period, 148 (51.21%) were males and 141 (48.78%) were females (fig-4)

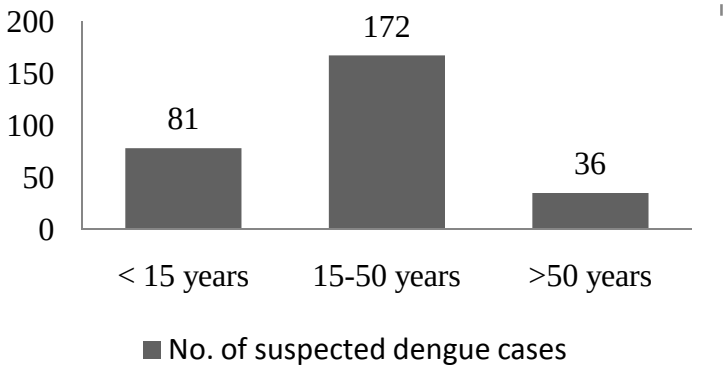


Figure 5: Age Wise Distribution of Suspected Dengue Cases

The patients under investigation were in the age group of 9 months to 80 years. The highest numbers of cases 167 (59.6%) were from age group 15-50 years and least number of cases 35 (12.5%) from over 50 years age group (Fig. 5).

Table 1: Occupation Wise Distribution of Suspected Dengue Cases

Occupation group	No. of suspected cases (%)
Agriculture	52 (18)
Labour	13 (4.6)
Job holder	10 (3.5)
Business	15 (5.3)
Student	101 (36.0)
House wife	68 (24.3)
Others	21 (7.5)
Total	289 (100)

5.2 Test Results by IgM-ELISA

Out of 289 IgM ELISA performed serum samples of dengue suspected cases, 26 (9%) were found to be positive.. (Table 2).

Table 2: Diagnostic Test Wise Distribution of DV Cases

Diagnostic Test	No. of Tested Sample Cases	No. of Positive Sample	% of Positive
IgM-ELISA	289	26	9

5.3 Sex Wise Distribution of DV Cases

Sex wise positive cases for dengue was observed high in male (10.8%) which constituted 5.5 % of total cases and low in female (7.1%) which comprised 3.5 % of total cases.

Table 3: Sex Wise Distribution of Positive DV Cases

Sex	Total no. of samples	Number of Positive samples (%)	% of positive cases in total	Statistics
Male	148	16 (10.8)	5.5	χ^2=3.573
Female	141	10 (7.1)	3.5	p=0.059
Total	289	26 (9)	9	

5.4 Age Wise Distribution of DV Cases

Age wise positive cases of dengue was observed highest in age group below 15 years (12.3 %) which constituted 3.5 % of total cases and least in age group 15-50 years (7.5%) which comprised 4.5 % of total cases (Table 4).

Table 4: Age Wise Distribution of Positive DV Cases

Age (years)	Total no. of samples	Number of Positive samples (%)	% of positive cases in total	Statistics
< 15	81	10 (12.3)	3.5	χ^2=2.82
15-50	172	13 (7.5)	4.5	p=0.244
> 50	36	3 (8.3)	1	
Total	289	26 (9)		

5.5 Hospital Wise Distribution of DV Cases

Hospital wise positive cases were observed highest in TDH (23.8%) and least in DDH (3.0 %) table-5.

Table 5: Hospital Wise Distribution of Positive DV Cases

Samples collection site	Total no. of samples	Number of Positive samples (%)	% of positive cases % in total
NSH	173	9 (5.2)	3.11
KZH	32	4 (12.5)	1.38
TDH	42	10 (23.8)	3.46
DDH	33	1 (3.0)	0.34
BH	9	2(22.2)	0.69
Total	289	26(66.7)	9

NSH: Narayani Sub-regional Hospital, KZH: Koshi Zonal Hospital,
TDH: Tanahu District Hospital, DDH: Dhading District Hospital, BH:Bharatput hospital

5.6 Occupation Wise Distribution of DV Cases

Occupation wise positive case was observed highest in business group (13.3%) which constituted 5.2 % of total cases and least in job holder and other groups.

Table 6: Occupation Wise Distribution of Positive DV Cases

Occupation group	Total number of samples	Number of Positive samples (%)	% of positive cases in total
Agriculture	55	7 (12.7)	19
Labour	13	1 (7.7)	4.5
Job holder	11	0 (0.0)	3.8
Business	15	2 (13.3)	5.2
Student	104	11 (10.6)	36
House wife	70	5 (7.1)	24.2
Others	21	0 (0.0)	7.3
Total	289	26(8.99)	100

Statistics: (χ^2=5.438; p=0.489)

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

The global prevalence of dengue has increased substantially recently. Dengue is endemic in ≥100 countries globally and it is serious problem in Southeast Asia, Africa, the Western Pacific, the Americas, Africa and the eastern Mediterranean area with imported cases everywhere. Dengue has been a concern because 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 include environmental conditions (temperature, humidity and altitude), poor sanitation or availability of potential breeding sites are important constituents of contracting dengue (WHO, 2009).

The study was a cross-sectional epidemiological study covering some hospitals of different region of dengue endemic areas in the country. The present study was carried out during post monsoon period from June to September 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 temperature remains almost constant and subsequent rainfall further leads to decrease in the temperature. 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 (Chakravarti and Kumaria, 2005).

Out of total 289 serum specimens collected from suspected dengue cases, 26 were found to be positive for anti-dengue IgM antibody which comprises 8.99 % of the total tested specimens. The sero-positivity of the study was is in accordance with some of the previous findings from Nepal studies carried out by Pun (29.3%) in 2009, Sah (27.3%) in

2008,Poudel (7.20) in 2009 and Sherchand (10.4%) in 2001 .The present study result shows less positivity rate than one of the above reports which 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.

IgM capture ELISA can be applied to the sero-diagnosis of DVI when both JEV and DV co-exist because ELISA was highly specific as only 10.7 % DVI cross reacted with JEV antigen (Sherchand *et al.*, 2001). Present study was based on ELISA test, which also detects cross reacting antibodies to other flavivirus such as JE virus. However, subjects with previous JE immunizations were excluded from the study. Therefore, chances of false positive results due to cross reactivity should be minimal.

Out of 26 positive cases observed in this study, 16 were male patients who comprises 10.8 % of the total male enrolled cases and 10 were female patients which comprise 7.1 % of the total female enrolled cases. The ratio of dengue positive cases in male to female was found to be 1.5:1. However, in this study the numbers of male cases (51.21%) was slightly higher than the female (48.78%).. Statistically there is no significant relationship ($p=0.139$) 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 172 (59.51%) was from age group 15-50 years. Eighty one cases (28.02%) were from age group below 15 years. The least numbers of cases 36 (12.45%) were from age group above 50 years. The positive cases according to the age of the suspected were highest (13) in the age group 15-50 years which comprises 7.5% of the total suspected cases followed by age group below 15 years which accounted 3.5% 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 no significant to the occurrence of the infection ($p = 0.244$).

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.

A similar study was done during epidemic in Bangladesh in 2002. Two hundred clinically suspected dengue patients were among 10 to 70 years old age group in which 100 (50%) were confirmed as dengue cases by virus isolation and dengue IgM capture ELISA. Of the 100 dengue-confirmed cases, the mean age was 29.0 (+/-12.4) (Islam *et al.,* 2005). In another study, serum samples were screened by IgM capture ELISA (PanBio, Australia) in which 664 (59.8 %) out of 1,110 samples tested were positive for dengue IgM antibodies. The predominant age group involved was 21-30 years (35.5%) followed by 11-20 years (25.6%) (Gupta *et al.,* 2006). A cross-sectional sero-epidemiological study was done in Singapore to determine the extent at which the Singapore population has been exposed to dengue infections. Dengue antibodies were measured with Panbio Dengue. Of 298 enrolled subjects (age 18-45 years), 135 (45 %) had a positive dengue serology. Similarly, 74 % (73/98) in the group 36-45 years old, 44 % (42/96) in 26-35 years old and 17 % (18/104) in 18-25 years old showed seropositivity. Age was found to be significant independent predictors (Wilder-Smith *et al.,* 2004).

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 26 positive cases, anti-dengue IgM positive were highest in Tanahu district Hospital (TDH), that constituted 23.8% of total suspected. The least number of cases was found in samples obtained from Dhading District Hospital which comprises 0.34% of total samples tested. The comparatively higher positive cases in Tanahu district Hospital might be due to travel to endemic region as one of the positive case from Tanahu had travel history to Chitwan or wide viral circulation. Besides, Tanahu is bordered with Chitwan, one of the dengue outbreak district of 2010 epidemic in Nepal. Tanahu also consists of a lots of marshy places which provide excellent mosquito breeding places. Further, this may be partially attributed by poor sanitation facilities contributing fertile breeding grounds for mosquitoes like *Aedes* species. The prevalence among patients from Narayani Sub-regional Hospital in this study was not in accordance with other findings at that hospital by Sah OP, 2008 who reported dengue sero-prevalence of 25 %. This might be due to decrease in vector population or awareness among population in that region.

The EDCD report suggests that dengue virus has been circulating in Nepal for several years. Thus, DF/DHF has likely been misdiagnosed, illness caused by dengue virus underestimated and no proper diagnosis or due to lack of enough knowledge about the disease in Nepal. Nepal has no dengue surveillance programs, and health professionals do not usually consider dengue as a differential diagnosis (EDCD/MHN, 2005/2006).

A similar result was obtained in the year 2003 in India when the study was designed to find out a relationship of dengue infection after post monsoon period. Only 3 cases (0.3 %) were confirmed serologically positive in the month of August and 68 cases (7.6 %) in the September. The maximum number of dengue specific antibody positive cases 583 (65.3 %) were reported during the month of October followed by 230 (25.8 %) cases in the November (Chakravarti and Kumaria, 2005).

The occupation wise distribution of suspected Dengue cases revealed that the highest number of cases (104) were from occupation group student which accounted 35.98 % of the total followed by house wife (70 cases, 24.22%) and the least number of cases (11 cases, 3.80%) were from occupation group job holder. Others group constitute 21 cases (7.26%). Other group comprises children (< 5 years), Driver, Painter, etc.

The occupation group, business (13.3%) was found most affected followed by Agriculture (12.7%), and least in others and job holder (0.0%) statistically, there is no significant relationship between occurrence of the disease and occupation groups (p=0.489). 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).

6.2 Conclusion

In 2010, total 289 samples collected and tested from five different hospitals of Nepal and 26 were found to be positive for DVI. Dengue was detected in Damouli, Biratnagar, Birgunj , Dhading Besi and Chitwan. The samples tested for anti-dengue IgM antibody by ELISA & the IgM positivity was 8.9%. The sero-prevalence of dengue has marginally increased so the concerned authority should initiate extensive surveillance of dengue virus infection and commence an integrated vector control programme in order to abate from a panic viral disease.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

The sero-epidemiological study of DVI was conducted in five hospitals of Nepal from June 2010 to November 2010. ELISA Assay was performed to detect the anti-dengue IgM antibody and the sero-prevalence of dengue was 8.99% (26). Of the total 289 suspected cases, 51.21% were male patients and 48.78% were female patients with the confirmed positive cases 5.5 % and 3.5 % respectively. The ratio of dengue positive cases in male to female was observed as 1.5:1. The highest positive cases were from age group below 15 years (12.3%) followed by age group above 50 years (8.3%). The highest positive cases were from Tanahu District Hospital, Damauli (23.8%) followed by Bharatpur hospital (22.2%). The highest positive cases were found in occupation group Business (13.3 %) followed by Agriculture (12.7%). Statistically there was no significant difference between occupation group, Sex, age with Dengue virus infection.

7.2 Recommendations

1. Sero-prevalence of dengue has marginally increased in Nepal therefore surveillance of dengue disease should be initiated
2. Presence of DVI in hilly region suggests that the prevention and control measures should cover all regions of country.
3. Prevention and control strategy of government should focus on productive age group.
4. Facilities to the Sero-Typing to all main hospital should be provided soon.

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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: 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 80^0C 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 (15-25^0C).Carrier RNA dissolved in buffer AVL, however, should be stored at 2-8^0C 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-III

Table no. 1 Grading the severity of dengue infection (WHO, 1997)

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-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

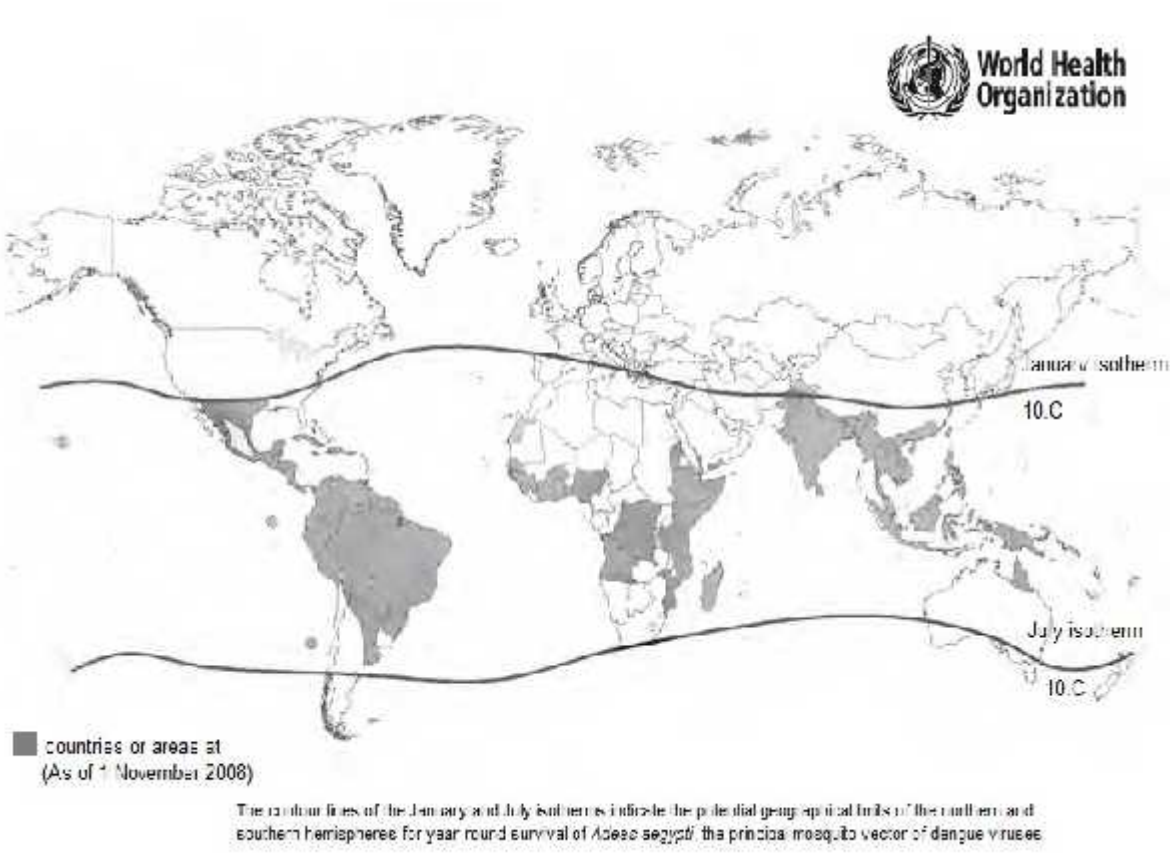

Figure 1 Countries/areas at risk of dengue transmission, 2008 (WHO, 2009).

APPENDIX -VI: DENGUE CASE DETAILS AND LAB FORM

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♑♂♎-''''''''''''''''''''॥

▭☒☹-''''''''''''''''''''

S. No. ……. Date of admission: | | | |

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

Address: District: ……VDC/Municipality: ……. Ward no.: ……

Occupation: Agriculture☐ Labour☐ Service ☐ Business☐Student ☐ if other……

Do you know Dengue? Yes ☐ No ☐

How does it transmitted? Mosquito ☐ Animal ☐ Others ☐

Do you use ? mosquito net ☐ Mosquito repellent ☐ Insecticidal spray ☐

How often do you use such? Regularly ☐ Occasionally ☐

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

Fever ☐ Headache ☐

Muscles or joint pain ☐ Retro orbital pain ☐

Hemorrhage ☐ Skin rash (Red Rashes) ☐

Lymphadenopathy ☐ If other then specify…….

Provisional Diagnosis (From Clinician): ……..

Travel history of patients within 14 days: before onset of fever: yes ☐ No ☐

Laboratory Diagnosis (filled by lab):

Specimen: Serum Date of collection: ……

ELISA test: For: IgM ☐ IgG ☐ **:** Positive ☐ Negative ☐

RT-PCR: Positive ☐ Negative ☐

APPENDIX -VII: 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 5^{th} day of symptoms and persist for 30-60 days. IgGs appear the 14^{th} day and persist for life. Secondary infections often often result in high fever and in many cases with haemorrhagic 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 solution 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 veinpuncture.
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

$0.000 \leq A(neg.) \leq 0.300$

$A(pos.) \leq 1.000$

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 specification 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.

   $$A(neg.) + 0.300 = \text{cut-off value}$$

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

i. A (sample)< cut-off ≡ anti-Dengue IgM negative

ii. A (sample)≥ cut-off ≡ 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%.