CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1Background

Dengue is a mosquito-borne viral illness caused by one of the four serotypes of the dengue virus (DENV; (DENV-1 to DENV-4) belonging to the family Flaviviridae. The virus serotypes are closely related but antigenically distinct. Dengue infections can result in a wide spectrum of disease severity ranging from an influenza-like illness (dengue fever; DF) to the life-threatening dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS)(WHO 2008).DF is characterized by high grade fever, sometimes biphasic, with headache, retro-orbital pain, myalgia or arthralgia, nausea or vomiting, skin rashes etc. DHF is a potentially life threatening complication of dengue characterized by high fever lasting 2 to 7 days, hemorrhagic phenomena (including vascular leakage of plasma), low numbers of platelets and sometimes circulatory failure. The condition of some patients progresses to shock. This is known as DSS (WHO 2009; WHO 1997). The risk of severe disease is much higher in secondary (with another serotype) rather than primary dengue virus infection (Deen et al 2006).

Dengue viruses (DVs) are transmitted to humans by mosquitoes from the *Aedes* genus; *Aedes aegypti* being the most competent epidemic vector. It lives in close association with humans because of its preference to lay eggs in artifici3al water holding containers in the domestic environment, and to rest inside houses and feed on humans rather than other vertebrates. Blood feeding and oviposition occur mostly in the morning and in the late afternoon. If a competent mosquito vector takes a blood meal from a person during the viremic phase (2 to 12 days), virus is ingested with the blood meal and infects the mosquito. After 8 to 12 days, depending on ambient temperature, the virus will disseminate and infect other tissues, including the mosquito salivary glands. When the mosquito takes a subsequent blood meal, virus is injected into the person along with the salivary fluids. Dengue virus infection has no apparent effect on the mosquito, which is infected for life (Gubler 2002).

Dengue is a climate sensitive vector borne disease, which in recent years has become a public health concern. Dengue is transmitting in tropical and sub-tropical regions around the world, predominantly in urban and suburban areas. Domestic Dengue Virus Infection (DVI) occurs in more than 100 countries and over 2.5 billion people live in the areas with a risk of dengue virus infection (Gibbons and Vaughn, 2002). Up to 100 million cases of DF and 500,000 cases of DHF and several thousand deaths are estimated to occur annually worldwide. 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 was 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 case fatality rate in Thailand is above 0.2% and around 1% in Myanmar (WHO 2009). The global prevalence of dengue has grown dramatically in the recent decades.

The four basic methods routinely practiced by most laboratories for dengue virus diagnosis are virus isolation and characterization, detection of DV specific antibodies, detection of dengue antigen and detection of viral nucleic acid by nucleic acid amplification technique (WHO 2009). Virus isolation through a mosquito cell line (C6/36) from acute phase serum or plasma sample is a method of choice and remains the gold standard. However, the confirmation of the infection with the virus and typing of the virus is based on molecular techniques such as Reverse transcription polymerase chain reaction (RT-PCR), Real time RT-PCR, Nested PCR and nucleic acid sequence based amplification (NASBA) and are gradually being accepted as new standards over virus isolation for detection of DV in acute phase serum samples (WHO 2009; Cardosa et al 1998). Serologically, DVI can be inferred by immunoglobulin M (IgM) and IgG capture enzyme linked immune-sorbent assay (ELISA), Particle agglutination test, Hemagglutination inhibition test, Complement fixation assay, Neutralization test and Rapid Immunochromatographic test (WHO 2009).

Nepal is bordered by India in the eastern, western and southern belts that is one of the countries with higher risk and so is more vulnerable to worse consequences of DVI. As with other vector borne diseases, outbreak of DF is related with increasing temperature, travel and frequent movement of people which is common due to open border between Nepal and India. DF was first reported in foreign visitor in Chitwan in 2004 (Pandey et al 2004). Nepal reported larger outbreak in 9 districts in 2006 (WHO 2009; EDCD 2008). The outbreak occurred in Nepal following the Indian, Pakistan and Bhutan epidemic of DF/DHF in September-October 2006 (EDCD 2008). The occurrence of DEN-1, DEN-2, DEN-3 and DEN-4 serotypes in the territory of Nepal augment the chances for the epidemic DF/DHF to be flourished in the country (WHO/SEARO 2006, Takasaki et al and Pandey et al 2008).

Several studies have reported the prevalence of DF in Nepal. The seroprevalence varies with time; 10% in 2007 (Pun et al 2011), 29.3% in 2008 (Pun et al 2011), 38.17% in 2010 (Pun et al 2012). Nepal experienced major outbreaks of DF in several districts in 2010. During the 2010 outbreaks, DF was reported from 24 districts at Sukraraj Tropical and Infectious Disease Hospital alone (Pun 2011). This indicates rapid geographical expansion is occurring within the country. Proper management of disease is required to prevent the increased threat of DVI in Nepal.

In Nepal diagnosis and management of dengue and other infectious diseases is based on clinical symptoms and many cases go undiagnosed due to lack of diagnostic facility. Thus, DF/DHF has likely been misdiagnosed and illness caused by dengue virus underestimated in Nepal. Nepal has no dengue surveillance programs, and health professionals do not usually consider dengue as a differential diagnosis (Pandey et al 2008). The 2010 epidemic of dengue indicates that dengue is increasing public health problem in Nepal. Fewer studies have been carried out for sero-prevalence of the disease in Nepal, though there is high risk of infection. This study aims to shed light into the clinical, epidemiological and serological aspects associated with DVI andits implications in future diagnosis, management, prevention and control of the disease.

1.2 OBJECTIVES

1.2.1 General objective:

To study the clinical, epidemiological and serological aspect of dengue virus infection in suspected hospital visiting patient.

1.2.2 Specific objectives:

1) To determine the proportion of dengue virus infection in relation to different socio-demographic status of the patients.
2) To determine the sensitivity and specificity of RDT and IgM Capture ELISA for the diagnosis of dengue virus infection.
3) To describe clinical features of serologically confirmed dengue cases.
4) To describe the risk factors and its impacts on dengue.

CHAPTERII

LITERATURE REVIEW

2.1 Historical review

The origin of dengue virus has been the subject of various discussions. Some early researchers speculated an African origin and distribution around the world with the slave trade (Smith 1956). More recently, it has been proposed that the viruses may have originated in a forest cycle involving lower primates and canopy-dwelling mosquitoes (Halstead 1992; Rudnick and Lim 1986; Smith 1956). All four serotypes have been documented in the forest cycle of Asia, while only one (DEN-2) has been documented in Africa (Cornet 1993; Rudnick and Lim 1986).

The mosquito, *A. aegypti*, is the principal vector of DVs and has an African origin and adapted to the peridomestic environment, breeding in water storage containers in African villages, prior to the slave trade. In the past, probably with the clearing of the forest and development of human settlements, DVs moved out of the Jungle and into a rural environment, where they were and still are transmitted to humans by peridomestic mosquitoes such as *Aedes albopictus*. Migration of people and commerce ultimately moved the viruses into the villages, towns and cities of tropical Asia, where the viruses were most likely transmitted sporadically by *A.albopictus* and other closely related peridomestic Stegomyia species. By the last decade of the twentieth century the four dengue serotypes and their major vector, *A. aegypti* have spread to nearly all tropical countries of the world. Molecular studies have been useful in determining evolutionary trends in dengue epidemics (Gubler 1998; Smith 1956). Dengue is also known by any of the pseudonyms: break-bone fever, dandy fever, denguero, bouquet fever, giraffe fever, polka fever, or the 5-day or 7-day fever (Sabin et al 1945).

2.2 Dengue the disease

Dengue is the most common arthropod-transmitted disease and it ranks as the most important mosquito-borne viral disease in the world. Some 2.5 billion

people living in tropical and sub-tropical regions are at risk of dengue infection, which equates to about two-fifths of humanity (Gubler & Clark 1995; WHO 2009). There is an estimated 50-100 million infections occurring globally every year, with 500,000 cases requiring hospitalization and causing 24,000 deaths (Halstead 1988; WHO 1997). Furthermore, the number of people living in tropical and sub-tropical regions is set to double by the end of the century (UNEP 2009; Holden 2009), thus making dengue an unqualified global threat to public health.

2.3 Dengue virus

2.3.1 Morphology and genome structure

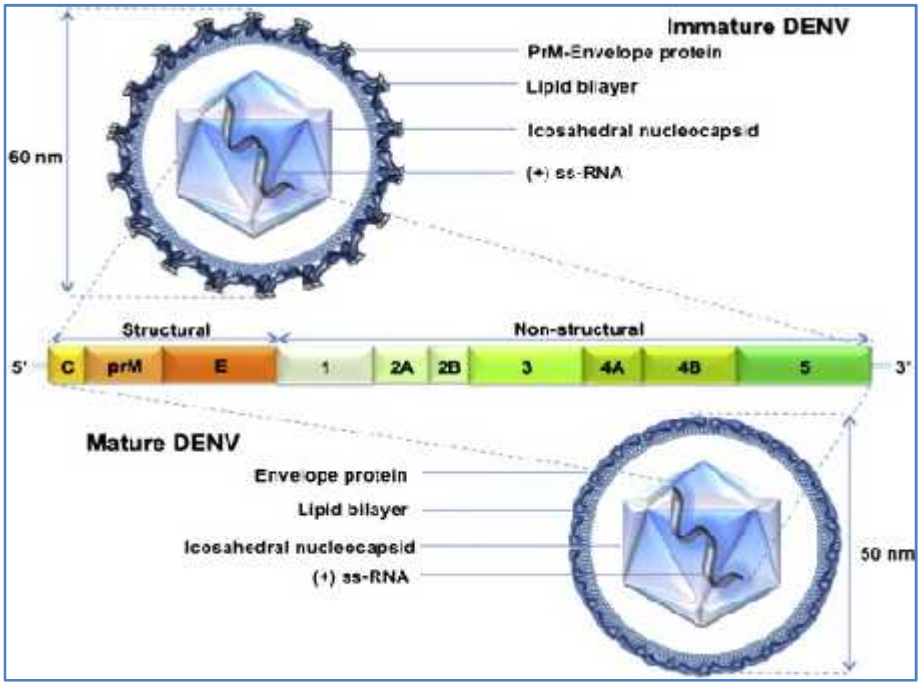

Figure 1: The Structural and Genomic Features of Dengue Virus

Source: Herrero et al 2013

The mature particles of dengue viruses are spherical with a diameter of 50 nm, containing multiple copies of the three structural virus proteins, a host derived membrane bilayer, and a single copy of a positive sense, single stranded RNA genome. The genome of the four viruses contains a single open reading frame of 10233 nucleotides encoding for a polyprotein ordered as 50-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5–30. The full length polypeptide is

processed by viral and host proteases into the capsid (C), membrane (M), and envelope (E) structural proteins that make up the virus and seven nonstructural proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. The C protein binds strongly with RNA to form the nucleocapsid. The prM glycoprotein represents the precursor of the mature M protein. It has a crucial role, as it prevents the E glycoprotein from undergoing an irreversible acid catalyzed conformational change during its transportation through host cell acidic compartments. The major structural protein (E) represents the target for neutralizing antibodies, protective immunity, and the antibody dependent enhancement phenomenon (ADE). Besides having hemagglutination activity, E glycoprotein mediates receptor binding and pH dependent membrane fusion activity. NS1 glycoprotein can be found on the cell surface and as a secreted form. It may have a role in RNA replication and in the pathogenesis of clinical disease. The NS3 protein has three enzymatic activities, a trypsin like serine protease, a RNA helicase, and a RNA triphosphatase activity. Together with NS2B, it forms the protease domain of the virus. Functions of NS2A, NS4A, and NS4B are not well known but are believed to play a role in RNA replication. NS5 is the largest and most conserved viral protein, and it is considered the putative RNA-dependent RNA polymerase responsible for viral RNA replication. The methyltransferase activity of this protein is probably involved in the methylation of the 50 cap structure (Kuhn et al 2002).

2.3.2 Replication

Dengue virus replicates in a wide variety of culture cells of both vertebrate and arthropod origin. The mammalian cell generated virus enters cells mainly by receptor mediated endocytosis. Antibody dependent enhancement (ADE) can mediate virus attachment and uptake by binding the virus-antibody complex to cellular Fc receptors (Gollins and Porterfield 1986, 1994). However, DEN-2 may enter human peripheral blood monocytes by direct fusion with the plasma membrane (Hase et al 1989). 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 P^H 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. Unfortunately very little is known about cell receptors and the early events of penetration and uncoating (Ishak et al 1988).The liver is an important site of DV replication & the source of some of the pathophysiologic aberration (Rosen et al 1989, Chung et al 1992).

2.4 The vector

The various serotypes of the dengue virus are transmitted to humans through the bites of infected *Aedes* mosquitoes, principally *A. aegypti*. This mosquito is a tropical and subtropical species widely distributed around the world, mostly between latitudes 35 ^{0}N and 35 ^{0}S (WHO 2009).

Females feed on any vertebrate host, but prefer humans. They fly upwind, following chemoattractant odors. The first step can be to enter a house. Blood feeding and oviposition occur mostly in the morning and in the late afternoon. Only the female bites for blood, which she needs to, mature her eggs. She takes a complete blood meal of 2–3 µl of blood, and will produce a batch of `about 100 eggs in approximately 3 days. Stomach distention triggers ovarian development. Thus, smaller blood meals produce fewer eggs, and refeeding with repeated biting by the same female occurs when the volume of ingested blood is too small for efficient egg production. Older populations, having taken many blood meals, have a greater potential for virus transmission. Females usually fly no more than 50 m. *A.aegypti* mosquitoes are peridomestic, that is, they prefer to rest inside the house, rather than in the garden. Most resting is on walls. This means that people, rather than mosquitoes, rapidly move the virus within and between communities. They can be transported by cars, trucks, aircraft, and even hurricanes for still longer distances. The mosquito's preferred breeding sites are in areas of stagnant water, such as flower vases, uncovered barrels, buckets, and discarded tires. The most dangerous areas are wet shower floors and toilet bowls, as they allow the mosquitoes to breed right in the residence. These mosquitoes can

live for months, yet most usually survive only a few weeks. Half of them die in the first week and 95% in the first month of life (Christopher 1960).

Dengue outbreaks have also been attributed to *A. albopictus*, *Aedes polynesiensis* and several species of the *Aedes scutellaris* complex. Generally, *A. aegypti* is the main vector in urban transmission, but *A. albopictus* is shown to be more competent and more susceptible to experimental infection. It is suggested that *A. albopictus* is a less selective feeder so it is of less epidemiological significance, as it may bite nonhuman hosts as well as humans, diluting its capacity to acquire and transmit dengue (WHO 2009).

2.5 Host

In humans, each of the four dengue virus serotypes has been associated with DF and DHF. DHF/DSS is associated with secondary-type dengue infections in individuals of one or more years of age and with primary dengue infections in infants born to dengue-immune mothers (Halstead et al1967; Halstead et al 1970). The acute phase of infection, following 3-14 days of incubation, lasts about 5-7 days and is followed by an immune response. The first infection produces life-long immunity to the infecting serotype but only temporary and partial protection against the other three serotypes and secondary or sequential infections are possible after a short time. Classic DHF/DSS is almost totally confined to children. From an admittedly small sample, the modal age of greatest susceptibility to shock is 8 to 10 years (Kouri et al 1989). Shock cases and deaths occur more frequently in female than male children (Halstead et al 1970), and black people are less susceptible to shock syndrome than are white and Asian people (Guzman et al 1990). Moderate to severe protein-calorie malnutrition reduces the risk of DHF/DSS in dengue-infected children (Thisyakorn and Nimmannitya 1993). Peptic ulcer and menstrual periods may be risk factors for severe bleeding in DHF/DSS patients (Rice 1923; Tsai et al 1991).

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 2009).

2.6 Transmission of Dengue Virus

The transmission cycle of the dengue virus by the *A. aegypti* mosquito begins with a dengue-infected person. The person will have virus circulating in the blood and viremia will last about 5 days. During the viremic period, an uninfected female mosquito bites the person and ingests the blood that contains the dengue virus. 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). Although, there is some evidence of transovarial transmission of dengue virus in *A. aegypti*, usually mosquitoes are only infected by biting a viremic person (CDC 2002). 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).

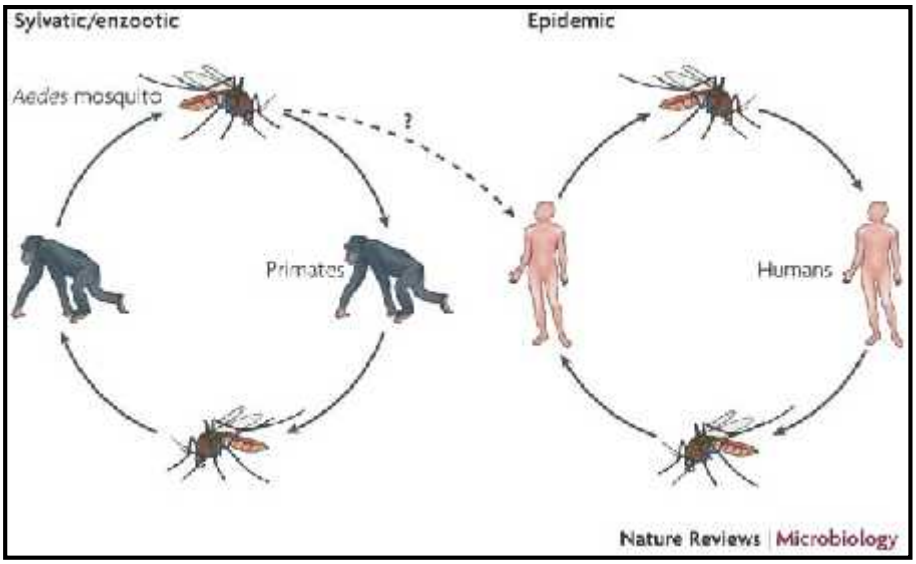

Figure 2: Sylvatic and Urban Dengue Transmissions Cycles (Whitehead et al 2007)

Within the mosquito, the virus replicates during an extrinsic incubation period of 8 to 12 days. The mosquito then bites a susceptible person and transmits the

virus to that person, as well as to every other susceptible person the mosquito bites for the rest of its lifetime. The virus then replicates in the second person and produces symptoms. The symptoms begin to appear on average 4 to 7 days after the mosquito bite. This is the intrinsic incubation period and it ranges from 3 to 14 days (CDC 2002).

Viremia begins slightly before the onset of symptoms. Symptoms caused by dengue infection may last 3 to 10 days, with an average of 5 days, after the onset of symptoms, so the illness persists several days after viremia has ended (CDC 2002).

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 viremia resulting from dengue infection of humans, the viruses are efficiently transmitted between mosquitoes and humans without the need for an enzootic amplification host. 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 *A.aegypti* breeding, weather and climate, densities of mosquitoes, infected humans and susceptible humans (Schreiber 2001).

2.7 Immune response

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).

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).

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

2.8 Dengue burden

2.8.1 Global scenario

The first outbreaks of an illness compatible with classical dengue fever took place in the Caribbean in 1635 and 1699, long before the reported simultaneous epidemics of 1779 and 1780 that came about in Asia, Africa and North America (Vasilakis et al 2008). In 1789 Benjamin Rush reported the first definitive case of the disease and coined the term ‘break-bone fever. Since then, major outbreaks have been recognized worldwide every 20 to 40 years (Gubler 1998).

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. In the last 50 years, incidence has increased 30 fold with increasing geographic expansion to new countries and, in the present decade, from urban to rural settings. An estimated 50 million dengue infections occur annually and approximately 2.5 billion people live in dengue endemic countries(WHO 2009).Some 1.8 billion (more than 70%) of the population at risk for dengue worldwide live in member states of the WHO South East Asia Region and Western Pacific Region, which bear nearly 75% of the current global disease

burden of dengue. In the South East Asia Region, epidemic dengue is a major public health problem in Indonesia, Myanmar, Thailand, Sri Lanka and Timor-Leste. More than 200,000 cases were reported in Indonesia, Myanmar, and Thailand together only in 2007. Frequency of cyclic epidemics and geographic expansion within the country are increasing in Bangladesh, India and Maldives. Dengue has spread in Bhutan and Nepal over the past 7 to 8 years. In the Western Pacific Region, the highest numbers of cases were reported in Cambodia, Malaysia, Philippines, and Viet Nam. Between 2001 and 2008, the number of cases and deaths reported were 1, 020, 333 and 4798 respectively in these countries. Dengue has also spread throughout the Pacific Island countries and areas. Between 2001 and 2008, the six most affected Pacific island countries and areas were French Polynesia, New Caledonia, Cook Islands, American Samoa, Palau and the Federal States of Micronesia. The total number of deaths for the six island countries was 34 (WHO 2009).

Interruption of dengue transmission in much of the WHO Region of the Americas resulted from the *A. aegypti* eradication campaign in the Americas, mainly during the 1960s and early 1970s. However, vector surveillance and control measures were not sustained and there were subsequent reinfestations of the mosquito, followed by outbreaks in the Caribbean, and in Central and South America (PAHO 1997). Dengue fever has since spread with cyclical outbreaks occurring every 3-5 years. The biggest outbreak occurred in 2002 with more than 1 million reported cases. From 2001 to 2007, more than 30 countries of the Americas notified a total of 4332731 cases of dengue. The number of cases of dengue hemorrhagic fever (DHF) in the same period was 106, 037. The total number of dengue deaths from 2001 to 2007 was 1299, with a DHF case fatality rate of 1.2%. The four serotypes of the dengue virus (DEN-1, DEN-2, DEN-3 and DEN-4) circulate in the region. The countries most affected by dengue in this region are Brazil, Columbia, Venezuela, Costa Rica, Honduras, Mexico, Cuba, Puerto Rico, Dominican Republic, Martinique and Trinidad and Tobago (PAHO 2008).

Countries with experience and future challenges of DVIs which are situated outside the WHO region of South East Asia, Western Pacific and the America

include Pakistan, Saudi Arabia, Yemen, Egypt, Sudan, Djibouti, Nigeria, Cote d'Ivore, Senegal, Mozambique and Burkina Faso (WHO 2009).

2.8.2 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).

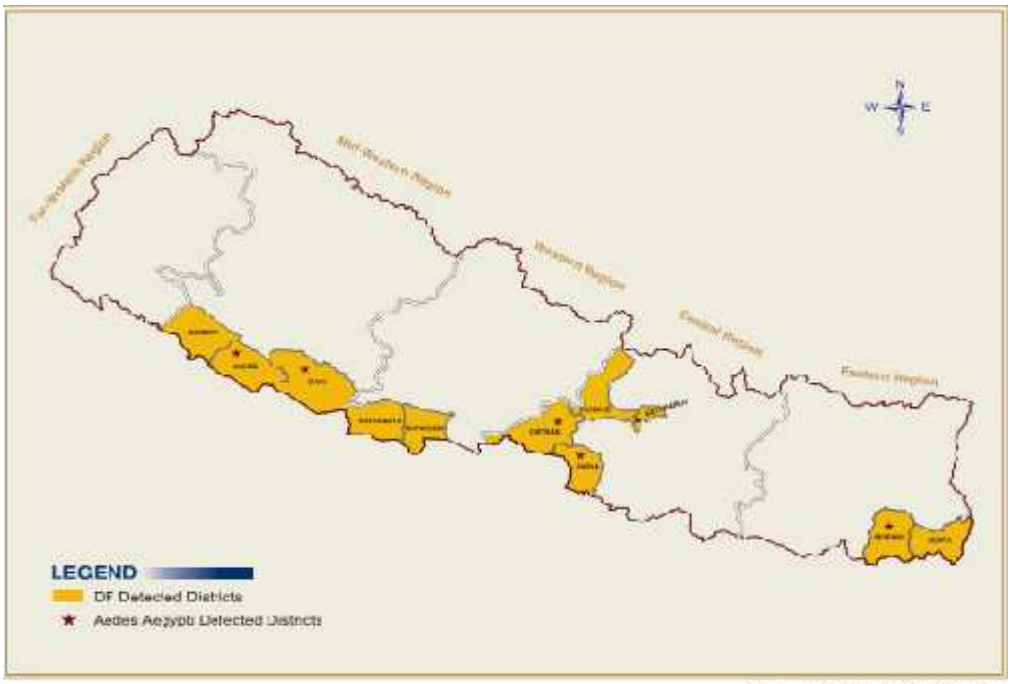

Figure 3: Map of Nepal showing the Dengue detected districts

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 al 2008). Nepal experienced major outbreaks of DF in several districts in 2010. During the 2010 outbreaks, DF was reported from 24 districts at Sukraraj Tropical and Infectious Disease Hospital alone (Pun, 2011). The 2010 dengue outbreak in Nepal highlights the expansion of DF/DHF to the hilly regions of Nepal for the first time (EDCD 2011). In 2010 outbreak, the virus serotype was DENV-1 (Pandey et al2013).

A.aegypti has been recorded and identified deploying entomological survey techniques in five major urban areas of terai region; Biratnagar and Bhadrapur (Eastern region), Birgunj (Central region), Tulsipur and Banke (Midwestern region), which is suggestive of possibility of local transmission of the disease (WHO/NHRC 2009). As in the Terai plains, the *A. aegypti* mosquito has been reported as the vector for the transmission of the virus in the highlands such as Kathmandu (Pandey et al2013).

2.9 Clinical Diagnosis

2.9.1 Clinical symptoms

Early clinical features of DVI are variable among patients, and initial symptoms are non-specific. Most dengue infections are symptomless or very mild characterized by undifferentiated fever with or without rash mainly in infants and young children. Older children and adults may develop a mild febrile syndrome or typical DF consisting of high fever, severe headache, myalgia, arthralgia, retro-orbital pain, and maculopapular rash. Those initial symptoms of DF are closely resembled with chikungunya fever, influenza like illness, leptospirosis, typhoid fever, acute tonsillitis, and malarial symptoms. The mildest form of clinical dengue infection is dengue fever, but because of the broad spectrum of signs and symptoms, the World Health Organization (WHO) has suggested there should not be a rigid clinical definition for DF. In some epidemics, DF may be accompanied by bleeding complications such as

epistaxis, gingival bleeding, gastrointestinal bleeding, hematuria and menorrhagia (WHO 1997).

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, increased vascular permeability (plasma leakage syndrome) evidenced by hemoconcentration (20% or greater rise in hematocrit above baseline value), leucopenia, thrombocytopenia, neutropenia and internal bleeding (bleeding from mucosa, gastrointestinal tract, injection sites or other locations) are the important laboratory findings associated with DHF.

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 (Halstead2008; WHO 2009b).

2.9.2 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 classification ofWHO2009b is given in (Apendix-B).

2.10 Laboratory Diagnosis of Dengue

Rapid and accurate diagnosis is of principal importance for clinical care, surveillance activities, outbreak control, academic research, vaccine development and clinical trials. No single method works as a method of choice at different stages of dengue progression. Additionally early clinical features of dengue infection are variable among patients, and initial symptoms are non-specific and similar to many other infectious disease. The diagnosis of dengue virus infection, therefore, requires specific laboratory confirmation. 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 Serum is the sample of choice for routine diagnosis; however, virus can also be detected in plasma, leukocytes and in autopsy tissues such as liver, spleen, lymph nodes, lungs and thymus (WHO 2009b).

Laboratory diagnosis of DVI can be made by the detection of specific virus (culture method), genomic sequence (molecular method), viral antigen and/or antibodies (serological method). Suckling mice inoculation, mosquito inoculation and mosquito cell culture are culture method for the identification of dengue virus. Polymerase chain reaction (PCR), real time RT-PCR, typing of DV, hybridization probes, loop mediated isothermal amplification (LAMP) and immuno-histochemistry method are different molecular techniques that can be used for the detection of the genomic sequence of the virus. There are 16 different serological tests like ELISA, microneutalization assay (dot-blot immunoassay and rapid immuno-chromatographic test), hemagglutination inhibition test, particle agglutination test, complement fixation test and neutralization test which are used for the detection of anti-dengue antibody in patient's serum. Of these serological tests, RDT and ELISA are widely used. The summary of characteristics of dengue diagnostics methods are given in (Appendix-C).

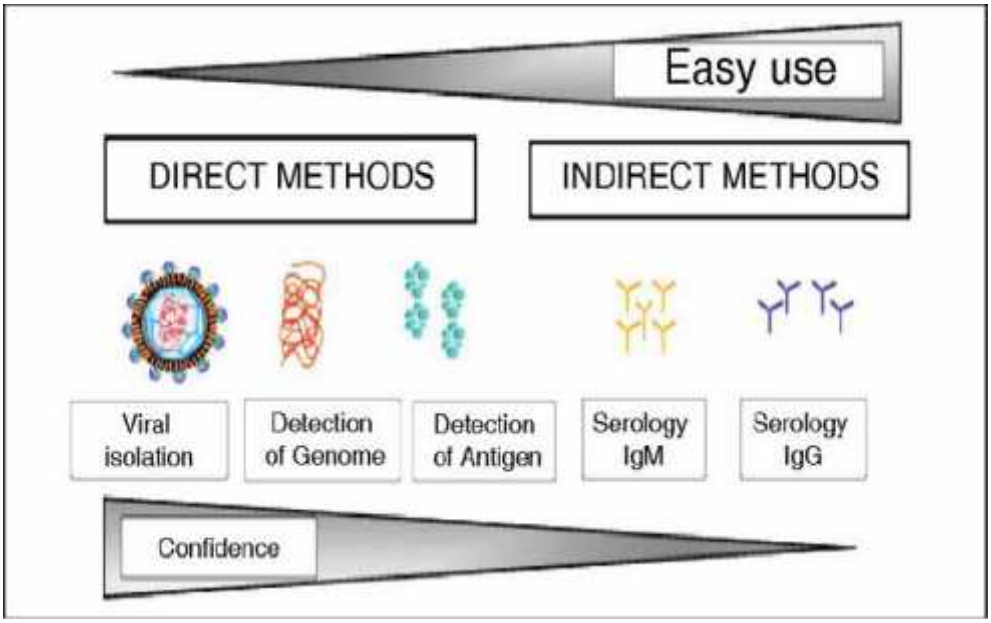

Figure 4: Diagnostic methods for dengue (WHO 2009b).

2.10.1 Serological Detection

During a primary dengue infection, anti-dengue IgM is detected 5 to 6 days after onset of fever persisting for 30 to 60 days. IgG levels slowly elevate and by the ninth to tenth day after onset are detectable, and persist for life.

According to Pan American Health Organization (PAHO1997) guidelines, by day five of illness, 80% of cases have detectable IgM antibody, and by day six to ten, 93 to 99% of cases have detectable IgM that may persist for over 90 days. Unlike a primary infection, a secondary infection with a heterologous serotype is characterized by a rapid (1 or 2 days after fever onset), high, and cross reactive IgG response. In some cases, an IgM response is not detectable. Specific IgA and IgE antibodies are also developed during both a primary and a secondary infection. The IgA is broadly cross-reactive to the four serotypes, and the level of IgE has been related to disease severity. To date, the detection of anti-dengue IgM is the best indicator of an active or recent infection (Guzman and Kouri 2008).

Several methods have been described for the serological detection of dengue virus-specific antibodies, including the Enzyme Linked Immunosorbent Assay (ELISA), hemagglutination inhibition (HI) test, the neutralization test, the indirect immunofluorescent antibody test, complement fixation, dot blotting, Western blotting, and the rapid immune chromatography 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).

2.10.2 Enzyme Linked Immuno sorbent Assay (ELISA)

Anti-dengue IgM detection using enzyme-linked immunosorbent assay (ELISA) represents one of the most important advances and has become an invaluable tool for routine dengue diagnosis and surveillance. Specifically, MAC-ELISA (IgM antibody capture ELISA) diagnosis is based on detecting dengue-specific IgM and/or IgG antibodies in the test serum by capturing them using anti human IgM antibody previously bound on a solid phase (Nawa et al 2001; Kuno et al 1991). In general, 10% false negative and 1.7% false positive reactions have been observed. Different formats such as capture ELISA, capture ultramicro ELISA, dot ELISA, AuBioDOT IgM capture and dipstick have been developed. Serum, blood on filter paper, and more recently saliva are useful for IgM detection if samples are taken within the appropriate

time frame (after five days of onset of fever). Different commercial kits for anti-dengue IgM and IgG detection are available, with variable figures of sensitivity and specificity (Guzman and Kouri 2004).

Although detection of IgM antibody to dengue virus by an envelope and membrane (E/M) specific capture IgM ELISA usually indicates an active or recent infection, the most reliable way to demonstrate active infection would be a significant (fourfold or greater) rise in IgM and/or IgG antibody titers between the acute and the convalescent phase sera. This could best be analyzed by an E/M specific capture IgM ELISA (for IgM antibodies) and an E/M antigen coated indirect IgG ELISA (for IgG antibodies) with serially diluted serum samples. For routine analysis, significant increases in IgM and/or IgG antibody levels, from negative or low optical density (OD) values in acute phase serum to positive and high OD values in convalescent phase serum, can be conveniently determined. Analysis of paired serum samples from both the acute and the convalescent phases by an E/M specific capture IgM and IgG ELISA to avoid false positive results in areas where dengue is highly endemic because of the long persistence of dengue virus specific IgG antibodies in many patients with secondary infections is recommended (Shu and Huang 2004).

Innis et al(1989) first proposed classification of primary and secondary infections by determining the ratio of the units of dengue virus IgM antibodies to the units of dengue virus IgG antibodies. They showed that the acute-phase sera of patients with primary dengue virus infections had higher IgM/IgG ratios, whereas patients with secondary infections had lower IgM/IgG ratios. This method has made a great contribution to the analysis of the immune status of patients with dengue. This technique was modified and simplified so that differentiation of primary and secondary dengue virus infection can be made by using the ratio of IgM/IgG readings directly (≥ 1.2 or <1.2, respectively) without calculating the antibody units through the use of a standard control (Shu et al 2003).

Recently, an NS1 isotype- and serotype-specific ELISA has been developed that can be easily and reliably used to differentiate (i) JE virus and dengue

virus infections, (ii) JE vaccination and JE infection, and (iii) primary and secondary dengue virus infections and (iv) for serotyping of dengue virus in patients with primary dengue virus infections (Shu and Huang 2004).

2.10.3 Rapid Immunochromatographic Strip Test

Many rapid test kits that use the principle of immunochromatography are commercially available. Most of these kits can simultaneously detect IgM and IgG antibodies to dengue virus in human whole blood, serum, or plasma within 5 to 30 min. Several evaluations that offer conclusions in favour or against these commercial kits are available. In a study by Sang et al(1998), a rapid immunochromatographic test was compared to the hemagglutination inhibition assay for separate determinations of dengue virus-specific immunoglobulin M (IgM) and IgG levels in paired serum specimens. The rapid test showed 99% sensitivity in the diagnosis of dengue virus infection and specificity in non flavivirus infections was 96%. Although the rapid test has the advantages of easy performance and the rapid provision of results, it should best serve as a screening test for clinicians in hospitals. Furthermore, these kits should not be used for surveillance for dengue disease in public health settings or in sero-epidemiological studies due to the high sensitivity of this assay for the detection of IgG and the long persistence of cross- reactive flavivirus IgG antibodies in the general population in many areas where dengue is endemic (Shu and Huang 2004).

2.11 Risk factor and Breeding place of DVI

Aegypti virus has been incriminated as the principal vector which is primarily an urban mosquito but sometimes it is also found in the periphery of cities breeding in rain water accumulated in tree holes. This species is mainly urban and semi-urban, breeding in domestic and peridomestic water storage containers. (WHO/NHRC 2009). *A.aegypti*, the most important vector, exploits domestic artificial water-holding containers, built-in cement containers holding water for bathing or flushing toilets are the most productive breeding sites(WHO 2009). Usually, epidemics are more intense in a city,

which is attributed to favourable epidemiological characteristics of rapidly growing urban agglomerations such as crowding, poor housing, as well as the existence of many putative breeding sites such as disposable containers, bottles and used tyres (Kuno 1995; Rigau-Perez et al 1998).Possible risk factors for dengue fever spread include:

Unplanned urban overpopulation of areas leading to inadequate housing and public health systems (water, sewerage and waste management)

- Poor vector control, e.g., stagnant pools of water for mosquito breeding
- Climate change andviral evolution.
- Increased international travel (recreational or business) to endemic areas.

2.12 Prevention and control of DVI

In absence of a safe and effective tetravalent vaccine for dengue viruses, vector control is the only method to prevent the disease (Ooi et al 2006). The most effective means of the vector control is environmental management, which includes planning, organization, carrying out and monitoring activities for the modification or manipulation of environmental factors with the view of preventing or reducing vector propagation and human-vector-pathogen contact. Environmental management includes improvement of water supply and storage, solid waste management and the modification of man-made larval habitats (WHO 2002).

Dengue prevention and control can be implemented through the Bi-regional Dengue Strategy (2008--2015) of the WHO South-East Asia and Western Pacific regions. This consists of six elements:

- Dengue surveillance
- Case management
- Outbreak response
- Integrated vector management
- Social mobilization and communication for dengue
- Dengue research (a combination of both formative and operational research).

CHAPTERIII

MATERIALS AND METHODS

3.1Materials

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

3.2 Methods

The study was designed as a descriptive cross-sectional. The study was carried out from July 2013 to December 2013. The total number of 261 serum samples was collected from Narayani Sub Regional Hospital and Bhawani Hospital and Research Centre, Birgunj, Parsa. 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 and clinical symptoms were obtained through a questionnaire method by direct interview in 'Dengue case details and Laboratory investigation Form (shown in appendix-E). The entire test was done at Everest International Clinic and Research Center (EICRC), Kalanki, Kathmandu.

Selection criteria:

3.2.1 Case inclusion criteria

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

3.2.2 Case exclusion criteria

A case was excluded, if laboratory testing suggested bacterial or any viral infection other than dengue infection or any other disease (WHO 2009).

3.2.3 Ethical clearance

Written consent was obtained from all the responding patients

3.2.4 Sample collection, storage and transport

The blood 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. After then, the serum samples were transported to EICRC maintaining reverse cold chain. Aliquots for RDT and ELISA were made and stored at 2-8^{o}C until tested

3.2.5 Clinical profile

A standardized form was used, on the day of admission, to collect information from suspected patients with dengue fever about demographic details, knowledge of dengue, risk factors, breeding places, use of preventive measures and following symptoms: fever, headache, lethargy, muscular pain, rash, retro-orbital pain, joint pain, , nausea, vomiting, abdominal pain and mucosal bleeding.

3.2.6 Laboratory Tests

3.2.6.1 Detection of Anti-Dengue IgM by RDT

Panbio Dengue Duo Cassette (Panbio, Australia)

Explanation of the test is provided in Appendix-G

Procedure:

All kit components and specimen were equilibrated to room temperature (20-25^{o}C) before commencing the assay.

The test device was removed from foil pouch and placed on a flat, dry surface. Using the 10 micro litre (µl) capillary pipette provided, 10 µl serum specimen was drawn and added into the circular sample well. The sample was allowed to absorb entirely into the specimen pad within the circular well. Two drops of buffer was added to the square well holding the buffer bottle vertically and 1 cm above the square well. Test results were interpreted exactly 15 minutes after adding the buffer to the cassette.

3.2.6.2 Detection of Anti-Dengue IgM by 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-F for detail procedure).

SD Dengue IgM Capture ELISA Test(Standard dignostic inc, Korea)

List of chemicals and reagents are given in Appendix-A

Procedure:

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

Serum Pre-dilution

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

Preparation of Tetra Methyl Benzidine (TMB) Substrate

In a tube, 5 ml of TMB substrate A and 5 ml of TMB substrate B was mixed.

Assay Plate

The required numbers of micro wells were removed from the foil sachet and were inserted into the strip holder. Five micro wells were required for controls: positive control (P) in duplicate and negative control (N) in triplicate. Within 10 minutes after mixing the monoclonal antibody (MAb) tracer and diluted antigen, 100 µl diluted 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).

3.2.7 Interpretation of the Result

3.2.7.1 Rapid Test Results Interpretation

One pink line “C” in the result window suggests no dengue infection, hence the negative test. Two pink lines “C” and “M” in the result window suggests the sample is positive for IgM antibodies to dengue virus. This is indicative of a primary dengue infection. The result is positive even if the “M” line is weak.

The results of line “G” indicating IgG antibodies were not interpreted and not included in this study.

3.2.7.2 ELISA Result Interpretation

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 is greater than cut-off value, the sample is considered positive and if the absorbance of sample is less than cut-off value, the sample is negative.

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

3.2.8 Quality Control

Quality control was applied in various areas during study period that ensured accuracy, reliability and reproducibility of the information generated by study.

Laboratory equipments like centrifuge, refrigerator, ELISA reader etc. were regularly monitored for their performance. Reagents were checked for manufacture, expiry date and proper storage. The reagents were prepared strictly following the manufacturer’s instruction and stored in proper conditions.

Aseptic method was followed during sample collection using sterile syringe and needle, disinfecting the skin over vein of the patient and collecting the blood in sterile bottles in order to avoid contamination. The sample was also processed in aseptic condition.

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

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

$$A\ (pos.) \geq 1.000$$

If one of the absorbance values of negative controls was outside the specification, the value could be neglected. Both absorbance values of the positive control had to comply with the specification. If these specifications were not met, the test had to be repeated.

3.2.9 Statistical Analysis

The collected data were analyzed using Statistical package for social science (SPSS) software (version 16.0).The data was analyzed to find out the relation of dengue with age, sex, occupation, knowledge of dengue, risk factors, breeding place and use of preventive measures. Chi square testwas determined to find out whether the findings were statistically significant or not andodds ratio to measure the strength of association between risk factor and outcome.

CHAPTER IV

RESULTS

During the study period, a total of 261 serum samples were collected, transported and tested by IgM Capture ELISA and Rapid Immunochromatographic Strip test kits (later referred to as RDT).

4.1 Socio-Demographic Study of Suspected Dengue Cases

4.1.1 Sex wise distribution of suspected cases

Out of 261 suspected dengue cases investigated during the study, 134(51.4%) were males and 127(48.6%) were females. Male to female ratio was 1.05:1.

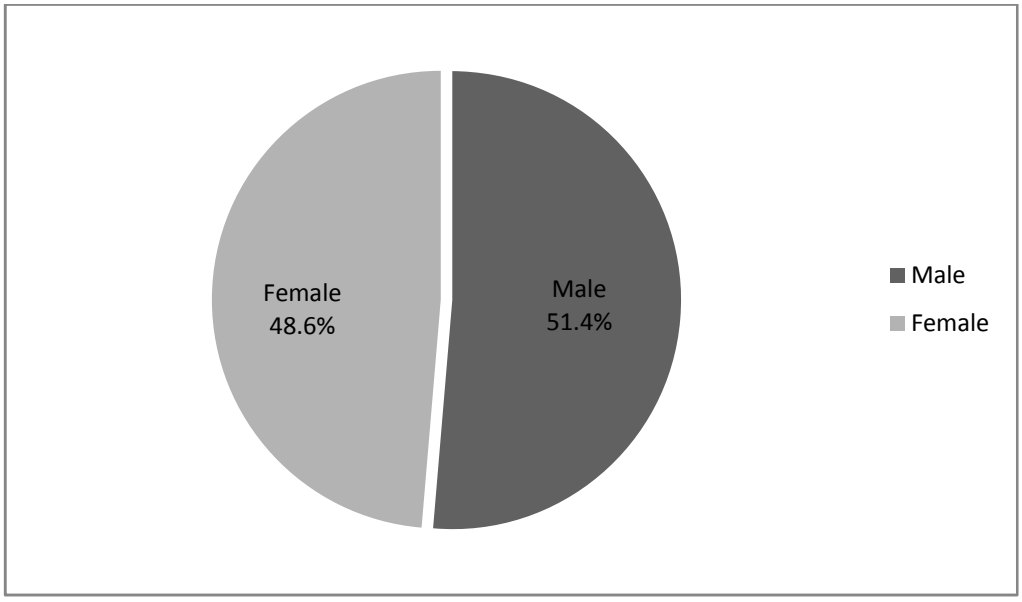

Figure 5Sex wise distribution of suspected cases

4.1.2 Age wise distribution of suspected cases

The cases under investigation were of the age 1 year to 80 years. The highest numbers of suspected cases 186(71.3%) were from the age group 15-50 years and least numbers of cases 28(10.7%) from the age group of 50 above. 47(18 %) cases belonged to the age group of below 15 years.

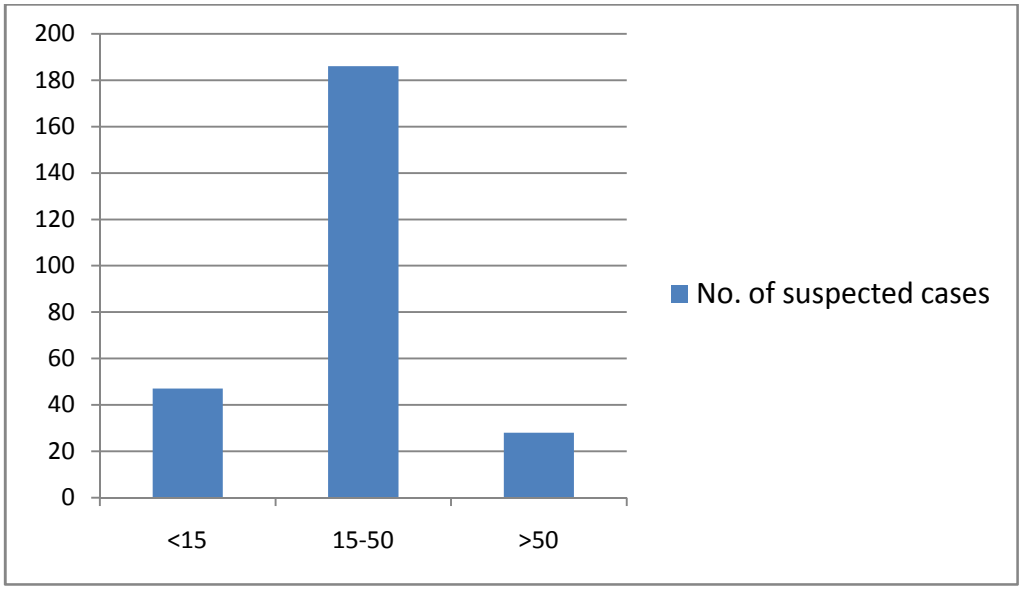

Figure 6**:** Age wise distribution of suspected cases

4.1.3 Profession wise distribution of suspected cases

Out of 261 serum samples of dengue suspected cases, the highest 90(34.5%) were students and the lowest 21(8%) were farmers.

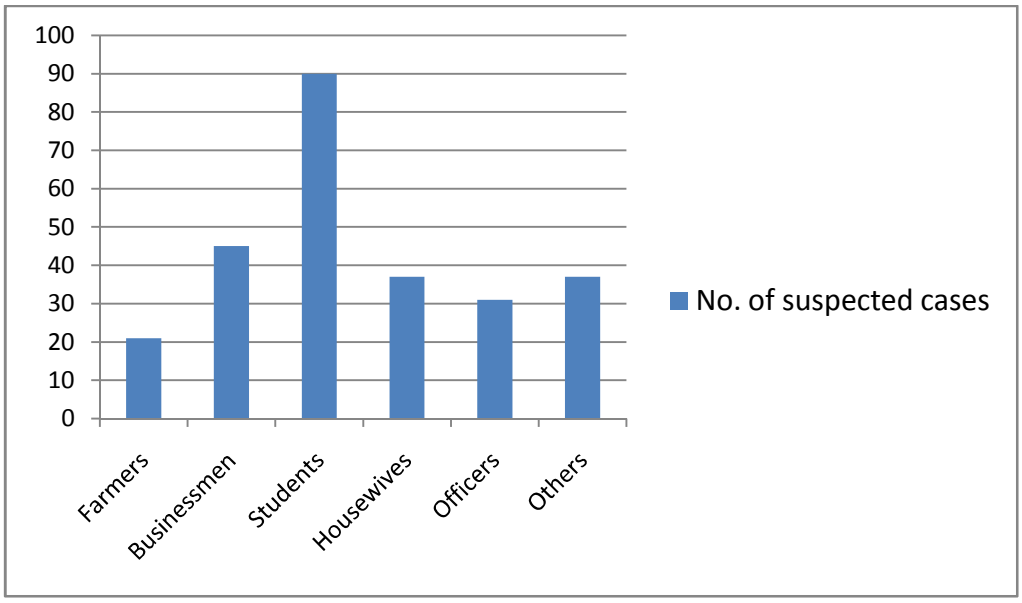

Figure 7 Profession wise distribution of suspected cases

4.2 Diagnostic Tests

Out of 261 serum samples of dengue suspected cases, 49(18.8%) were found to be positive for IgM antibody by IgM Capture ELISA and 40(15.3%) were found to be positive by RDT**.** (Table 1)

Table 1: Diagnostic Tests

Diagnostic Test	**Total no.of Sampletested**	**No.of positive cases(%)**
IgM Capture ELISA	261	49(18.8)
RDT	261	40(15.3)

4.3 Comparison between RDT and IgM-capture ELISA assay

Forty nine (18.8%) of 261 samples were IgM positive by IgM Capture ELISA and 36(73.5%) of the 49 IgM positives by IgM Capture ELISA were also positive by RDT. Four samples positive for IgM by RDT were negative by IgM Capture ELISA and 13 samples positive for IgM by IgM Capture ELISA were negative by RDT. Two hundred eight samples were Dengue IgM Negative by both IgM Capture ELISA and RDT (Table 2). Compared to IgM Capture ELISA, sensitivity and specificity of RDT is 73.46% and 98.1% respectively (Calculations in Appendix-D). The Kappa value of the test is 0.77. (Table 2)

Table 2: Comparison between RDT and IgM capture ELISA

		IgM capture ELISA		
		Positive	**Negative**	**Total**
RDT	Positive	36	4	40
	Negative	13	208	221
	Total	49	212	261

4.4 Age wise distribution of IgM positive cases

In 49 anti-dengue IgM positive cases, the highest number 40(81.6%) of patients were 15-50 years of age and the least; 4(8.2%) cases each were less than 15 and 5(10.2%) were from age group above 50. The mean age was 28.39 with standard deviation of 17.20.There is no significant association of the anti-dengue IgM positivity with age (p=0.124). (Table 3)

Table 3: Age wise distribution of IgM positive cases

Age	**Total no. of suspected case**	**No. of IgM positive case in suspected and %**	**%of IgM positive Case n(49)**	**p-value**
< 15	47	4(8.2)	1.6	
15 – 50	186	40(81.6)	15.3	0.124
>50	28	5(10.2)	1.9	
Total	261	49(100)	18.8	

4.5 Sex wise distribution of IgM positive cases

In 49 anti-dengue IgM positive cases, 33(67.4%) were males and 16(32.6%) were females (male to female ratio=2.06:1). There is significant association of the anti-dengue IgM positivity with sex group (p=0.013). (Table 4)

Table 4: Sex wise distribution of DV Cases

Sex	**Total no. of suspected case**	**No.of IgM positive case in suspected and %**	**% of IgM positive cases in total n(49)**	**p-value**
Male	134	33(67.4)	12.7	
Female	127	16(32.6)	6.1	0.013*
Total	261	49(100)	18.8	

⋆ Statistically significant

4.6 Profession wise distribution of IgM positive cases

Profession wise distribution of positive cases showed the highest number of anti-dengue IgM positive cases in students 16(6.2%), followed by Business 8(3%) housewife 8(3%) and officers 7(2.7%) out of total positive cases and lowest number of cases were found in others group 6(2.3%)and 4(1.6%) in farmers respectively. Profession was not found statistically significant among IgM positive cases. (Table 5)

Table 5: Profession wise distribution of positive case

Profession	**Total no.of suspected case**	**No. of Anti dengue IgM positive cases**	**% of IgM Positive cases**	**p-value**
Farmer	21	4	1.6	
Business	45	8	3	
Student	90	16	6.2	
Housewife	37	8	3	
Officer	31	7	2.7	0.899
Others	37	6	2.3	
Total	261	49	18.8	

4.7 Clinical features of anti-dengue IgM positive cases

Fever 49(100%), headache 32(65.3%), Joint pain 19(33.9, Retro-orbital pain 22(44.9%) and, Muscular pain 21(42.8%) were the major clinical manifestations in the anti-dengue IgM positive cases while Nausea 16(32.6%),Skin rash 11(22.4%), abdominal pain 11(22.4%), Lethargy15(30.6) and mucosal bleeding 6(12.2) were relatively low in the anti-dengue IgM positive cases. Retro orbital pain, skin rash, mucosal bleeding and joint pain was found statistically significant in relation to IgM detection. The highest

odds ratio 14.651 was found in mucosal bleeding and lowest odds ratio 1.118 in leathergy.(Table 6)

Table 6: Clinical manifestation in IgM positive cases

Clinical features	**No. of cases**	**% of IgM positive case**	**p-value**	**Odds ratio**
Fever	49	100	_	_
Headache	32	65.3	0.079	1.779
Nausea	16	32.6	0.104	1.750
Retro orbital pain	22	44.9	0.00*	7.037
Skin rash	11	22.4	0.02*	3.546
Joint pain	19	38.8	0.03*	2.723
Abdominal pain	11	22.4	0.284	1.515
Lethargy	15	30.6	0.747	1.118
Muscular pain	21	42.8	0.065	1.815
Mucosal bleeding	6	12.2	0.00*	14.651

⋆ Statistically significant

4.8 Relation between duration of febrile illness and IgM detection

In 261 suspected cases of DF, the duration of fever less than 5 days was found in 62(23.75%) cases while 199(76.25%) cases was found in 5 days and more. Anti-dengue IgM was detected in only 8(12.9%) cases out of 62 suspected cases with duration of fever less than 5 days and 41(20.6%) cases out of 199 suspected cases with duration of fever of 5 days and more. Out of 49, 16.3% of IgM positive cases were detected in duration of fever less than 5 days and 83.7% of IgM positive cases were detected induration of fever more than5 days. Detection rate of anti-dengue IgM positivity and duration of fever was not significantly associated (p=0.175). (Table 7)

Table7: Duration of Fever in relation to IgM detection

No. days of onset of fever	**Total no. of Suspected case and %**	**No. of IgM positive cases in suspected and%**	**% of IgM positive case n(49)**	**p-value**
< 5	62(23.75)	8(12.9)	16.3	
≥ 5	199(76.25)	41(20.6)	83.7	0.175
Total	261(100)	49	100	

4.9Relation between knowledge of dengue and IgM detection

In 261 suspected cases, knowledge of dengue was found among 172(65.9%) and IgM positive in 20(11.6%) which is 7.7% of total positive cases and knowledge of dengue was not found in 89(34.1%) and IgM positive in 29(32.6 %) which is 11.1% of total positive cases. Detection of anti dengue antibody and knowledge of dengue was found statically significant with p-value of0.00. (Table 8)

Table 8: Relation between knowledge of dengue and IgM detection

Knowledge of dengue	**Total no. of suspected Case and %**	**No. of IgM positive in suspected case and %**	**% of IgM Positive case n(49)**	**p-value**
Yes	172(65.9)	20(11.6)	7.7	
No	89(34.1)	29(32.6)	11.1	0.00*
Total	261(100)	49	18.8	

* People with knowledge of dengue have mentioned mode of transmission and at least one symptom of the disease dengue.

⋆ Statistically significant

4.10 Relation between risk factor and IgM detection

In 261 suspected cases, the highest anti dengue positive cases was found in water logging 15(10.9%) followed by travel to endemic area 10(37%) and family history of febrile illness 3(30%).The highest odds ratio 2.94 was found in travel to endemic area and lowest odds ratio 0.319 in water logging.(Table 9).

Table 9: Relation between risk factor and IgM detection

Risk factor	**Total no. of suspected cases at risk and %**	**No. of IgM positive in suspected case and %**	**p-value**	**Odds ratio**
Water logging	138(52.9)	15(10.9)	0.001*	0.319
Blood transfusion	2(0.76)	0(0.00)	0.805	_
Travel to endemic area	27(10.3)	10(37)	0.01*	2.94
Family history of febrile illness	10(3.83)	3(30)	0.354	1.91

⋆ Statistically significant

4.11Relation between breeding place and IgM detection

In 261 dengue suspected case,the highest anti dengue positive cases was found in flower pot 19(36.5%), followed by open jar 8(26.6%) and lowest in house drain10(20%).The highest odds ratio 3.453 was found in flower pot as breeding place and lowest 1.103 in house drain. (Table 10)

Table 10: Relation between breeding place and IgM detection

Breeding places	**Total no. of suspected case having breeding place and%**	**No. of IgM positive in Suspected cases and %**	**P-value**	**Odds ratio**
Open jar	30(11.5)	8(26.6)	0.239	1.685
House drain	50(19.1)	10(20)	0.805	1.103
Flower pot	52(20)	19(36.5)	0.00*	3.453
Domestic waste	15(5.75)	4(26.6)	0.425	1.624
Kitchen garden	39(15)	11(28.2)	0.102	1.902

⋆ Statistically significant

4.12 Relation between use of preventive measures and IgM detection

In 261 dengue suspected cases, the highest number of anti dengue positive cases was found in spraying 3(18.75%) followed by cover water container 41(17.6%) and the lowest use of mosquito repellant 3(13.6%).Table 11

Table 11:Relation between use of preventive measure and IgM detection

Preventive measures	**Total no. of suspected cases using preventive measures and %**	**Total no. of IgM positive in suspected case and %**	**p-value**
Cover water container	233(89.2)	41(17.6)	0.160
Garbage disposal	218(83.5)	36(16.5)	0.035*
Use of nets	228(87.3)	38(16.6)	0.022*
Spraying	16(6.1)	3(18.75)	0.998
Change stored water	224(85.8)	37(16.5)	0.022*
Mosquito repellant	22(8.4)	3(13.6)	0.519

⋆ Statistically significant

CHAPTER V

DISCUSSION

5.1Discussion

Dengue is one of the most rapidly spreading mosquito-borne viral diseases in the world. Some 1.8 billion (more than 70%) of the population at risk for dengue worldwide live in member states of the WHO South-East Asia Region and Western Pacific Region, which bear nearly 75% of the current global disease burden due to dengue (WHO 2009). Dengue is transmitting in tropical and sub-tropical regions around the world, predominantly in urban and suburban areas. Domestic Dengue Virus Infection (DVI) occurs in more than 100 countries and over 2.5 billion people live in the areas with a risk of dengue virus infection (Gibbons and Vaughn 2002). Up to 100 million cases of DF and 500,000 cases of DHF and several thousand deaths are estimated to occur annually worldwide. Dengue is a climate sensitive vector borne disease, which in recent years has become a public health concern.

The study was a cross-sectional seroepidemiological study done at Narayani Sub Regional Hospital and Bhawani Hospital and Research Centre, Parsa, Birgunj. The present study was carried out during post monsoon period from July- December 2013. Serum samples of 261 clinically suspected caseswere analyzed.

Among suspected dengue cases,134 (51.4%) were males and 127 (48.6%) were female. Among 49 anti-dengue IgM positive cases, 33(67.4%) were males and 16 (32.6%) were females with male to female ratio of 2.06:1. Statistically, there is significant relation between sex and the occurrence of the disease (p=0.013). The result was in accordance to the previous studies in which the number of DV cases was more in males 1.5:1 (Shah et al 2012; Pun et al 2011). In present study, the numbers of male cases were higher than the female that might be due to their greater involvement in outdoor activities. The result is in agreement with Ministry of Health, Bangladesh that reported hospital patients with DF having male to female ratio of 1.5:1 during an outbreak in Chittangong in 1997and Garg et al (2011)who reported male to female ratio 2:1 in Kanpur among 1227 serum samples during 2006 to 2010.

The result is consistent with Sukraraj Tropical and Infectious Disease Hospital, Teku that reported hospital patients with DF having male to female ratio of 2.2:1 during an outbreak in Nepal in 2010 (STIDH 2010).

A. aegypti, the principal mosquito vectors of dengue are peridomestic, day-biting species that lives and breeds in and around the home. *A. aegypti* prefers to lay eggs in artificial water containers such as flower vases, old automobile tires, buckets that collect rainwater, cement cisterns, drums, and barrels and trash in general (Gubler 1998).As male have greater involvement in outdoor activities, this makes males more prone to being bitten and infected by the mosquito in the day time (Shah et al 2012).

The age wise distribution of the suspected patients (range: 1 year to 80 years) revealed the highest numbers of cases; 186(71.3%) from the age group 15-50 years and least numbers of cases; 28(10.7%) from the age group of above 50 years.The age wise distribution of dengue cases revealed that the positive cases were highest, 40 (81.6%) in the age group 15-50 years and least in less than 15 years which comprised 4(8.2%) of the positive cases respectively. Statistically, there is no significant relationship between age and occurrence of disease ($p=0.124$). This is not in accordance to the popular belief that dengue is a pediatric disease (Halstead 2008). Several other studies have been done in Nepal with variable results, the peak reaching 23.08% in 2007 among <15 years age group (Pun et al 2011); 12.3% in 2010 among <15 years age group (Khadka 2011). 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 more in age group greater than 15 years (WHO/SEARO 2006). A study done during 2010 outbreak has reported 95% cases being above 14 years of age (Pun 2011) and 43% reported by Neupane in 2013. The middle age group (15-50 years) 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. The reason for the lower number of isolates 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.

In profession wise distribution of suspected dengue cases, the highest numbers of cases were 90(34.5%) from the profession groups student and the least numbers of cases were 21(8%) from farmers group. Cases below 5 and above 70 years were included in the profession group of others. The highest number of anti-dengue IgM positive cases; 16(32.7%) was revealed in patients involved in student profession group which is 6.2 % of the total suspected cases and farmers group constituted the least number of cases; 4(8.2%) which is 1.6% of the total suspected cases. Statistically there is no significant association of IgM positivity with profession (p=0.899). The higher positivity was observed among students because they may have higher outdoor activity and frequent travel from one place to another. The result was inconsistent to previous similar studies in Nepal (Sah et al 2009; Shah 2010 and Subedi 2013) who reported Agriculture as most affected group and Gupta 2011 reported business as the most affected profession group.

Forty nines cases with anti-dengue IgM antibody were detected by IgM capture ELISA which was 18.8% of the total suspected samples while 40 cases were detected by RDT which was 15.38% of the total suspected cases. Some of the previous studies by Gupta et al(2005) (8.2%), Khadka (8.9%) in 2011 and Shah et al(9.8%) in 2012, Subedi (13.73%) in 2013, Joshi (13.8%) in 2012 and Neupane (11.8%) in 2013 reported lower seropositivity while the others, Sah (27.3%) in 2009, Pun (29.3%) in 2011, and Gupta et al (29.09%) in 2012 reported higher positivity of anti-dengue IgM antibody. The variations in the results of the different years is due to the geographical variation in sampling areas, fluctuations in temperature, rainfall and humidity etc. even though most of the studies were conducted in the post monsoon periods.

Among 261 febrile patients, 49 were positive to anti-dengue IgM by ELISA while the rest was negative. The RDT detected anti-dengue IgM in only 36 patients out of 49 that were positive by ELISA. The remaining 13 patients were negative by RDT. The number of patients that were negative by enzyme immunoassay but positive by rapid test was 4. The sensitivity of RDT was 73.6% and specificity was 98.1% with positive predictive value of 90% and negative predictive value of 94.1%. The measure of agreement (kappa) value of the test was 0.77. The result was in harmony reported in similar studies by Subedi in 2013 with sensitivity of 72% and specificity of 95.8 % and kappa value of 0.73. The test performed poor as kappa value of 0.81 or more is regarded almost perfect for serological diagnosis (WHO 2010). In another study by Pun et al2012, the sensitivity was in accordance while specificity and kappa value varied. In the study, the sensitivity was 70% and the specificity was 76.54% with kappa value of 0.46. The result was also consistent to a Thai study which showed sensitivity and specificity of 79% and 95% respectively (Kittigul and Suankeow 2002), but the result was also inconsistent to the similar studies done by Palmer et al(1999), with sensitivity of 98% and specificity of 100% and Shuenn et al(2000) with sensitivity of 97.9% and specificity of 97.1% respectively. Immunoglobulin M (IgM) capture ELISA (S D, South Korea) was chosen as a gold standard for serological diagnosis of DENV infection. This enzyme immunoassay revealed 97.6% sensitivity along with specificity of 86.6%. The overall performance was graded perfect as given by kappa value of 0.85; the kappa value of 0.81 or more is regarded almost perfect for serological diagnosis (WHO 2010). Therefore, RDT was evaluated with reference to ELISA. The factors responsible for the poor performance of RDT might be storage temperature of the test kit and prevalence of other febrile endemic diseases. The sensitivity of RDT decreases when stored at 35^{o}C but there is no loss at 4^{o}C (Blacksell et al2006). The test kits might be exposed to high temperature and humidity during transportation, which could directly influence the test performance (WHO 2010).

DF can be misdiagnosed by under diagnosis or due to lack of enough knowledge about the disease. Clinicians could face diagnostic dilemma between dengue and other infections, such as influenza, enterococcus, chikungunya, viral hemorrhagic fevers, leptospirosis, malaria and typhoid fever because the clinical manifestations mimic among these conditions. The most common clinical manifestation in the DV cases were fever, headache, retro orbital pain, muscular and joint pain . Relatively less common clinical features were nausea, skin rash, abdominal pain, lethargy, and hemorrhagic manifestations. A similar study was carried out in Chitwan during 2010 outbreak in which the clinical presentations were headache (96%), body ache (93%), nausea (85%), retro-orbital pain (49%), itching (42%), vomiting (39%), loose motion (26%) and skin rash (27%) (Sedhain et al 2010).

Among the positive cases, skin rash, retro orbital pain, joint pain and mucosal bleeding was seen, which are specific feature in case of dengue. Mucosal bleeding was seen in six cases which is the possible threat that may lead patient towards DHF. The severity of these clinical symptoms was different in each patient. Many factors may be attributed to the differences seen, such as infection with different serotypes or infection with more than one serotype, either sequentially or concurrently. Differences in host genetics and immune responses may also play a role in the severity of infection (Ahmed 2010).

Relationship between duration of fever at the time of sample collection was analyzed with the rate of detection of anti-dengue IgM antibody. In 261 suspected cases, the duration of fever in less than 5 days was found in 62(23.75%) cases, while in 5 days and more was found in 199(76.25%) cases. 16.3% of IgM positive cases were detected within less than 5 days while 83.7% of IgM positive cases were detected in the day 5 onwards. Detection rate of anti-dengue IgM and the number of days of onset of fever is not significantly associated ($p=0.175$). The rapidity with which IgM develops varies considerably among patients. Although the date of onset are not always recorded accurately, some patients have detectable IgM on days 2 to 4 after the onset of illness whereas others may not develop IgM for 7 to 8 days after onset. By day 5 of illness, most patients (80%) in Puerto Rico whose cases were subsequently confirmed by HI on paired serum samples or by virus

isolation had detectable IgM antibody in the acute-phase serum in IgM Capture ELISA. Nearly all patients (93%) developed detectable IgM antibody 6 to 10 days after onset, and 99% of patients tested between 10 and 20 days had detectable IgM antibody (Gubler and Sather 1988). In the present study, rate of detection of IgM in suspected patients with fever of less than 5 days duration is 12.9% while in those with duration of fever of 5 and more days is 20.6% but 23.75% of the suspected cases visit the hospitals before 5th day of onset of fever. This shows the poor detection of dengue cases in early days of illness by serology.

Relation between knowledge of dengue was analyzed with rate of detection of anti dengue IgM antibody. Among 261 suspected patient, knowledge of dengue was found in 172(65.9%) and IgM positive in 20(11.6%) which is 7.7% of total positive cases and knowledge of dengue was not found in 89(34.1%) and IgM positive in 29(32.6 %) which is 11.1% of total positive. Detection of anti dengue antibody and knowledge of dengue was found statically significant with p-value of 0.00. Fever was the most mentoined symptoms and mosquito bite as mode of transmission. The majority of the respondents knew the mode of transmission of DF and said that dengue fever is transmitted by mosquito bite. This finding is supported by research conducted by Syedet al(2010) which revealed that 93% people knew that the vector for dengue is a mosquito and other researches supporting this finding are conducted by Sharma et al (2012) and Neupane et al (2014) which revealed that 92% people knew that the vector for dengue is a mosquito.

Relationship between risk factors was analyzed with the rate of anti dengue IgM detection. Among 261 suspected cases, water logging was found in 138(52.9%) and IgM positive in 15(10.9%) of total cases at risk, travel to endemic area was found in 27(10.3%) and IgM detection in 10(37%) of total cases at risk, blood transfusion was found in 2(0.76%) with no IgM positive case and family history of febrile illness was found in 10(3.83%) and IgM detection in 3(30%) of total cases at risk. Detection of anti- dengue antibody and risk factor was found statically significant in case of water logging and travel to endemic area with p-value of 0.001 and 0.01 respectively. Blood

transfusion and family history of febrile illness is not statically significant. The result is in harmony with another study done in Brazilian which plants with temporary water pools on the property, gutter to collect rainwater was found as risk factor for dengue transmission (Heukelbach et al 2001). In the study, water logging was found as major risk factor as *A. aegypti*, the principal mosquito vectors of dengue are peridomestic, day-biting species that lives and breeds in and around the house and prefers to lay eggs in artificial water logging sites (Gubler 1998).Travel to endemic area was found as another major risk factor in the study, as IgM positive cases in the study have travel history to Delhi, Rajasthan and different cities of Bihar which are well known endemic places for dengue virus infection. The result is consistent with Hammond et al (2005) who reported Dengue virus infection is known to be endemic in India.

Relation between breeding places was analyzed with the rate of anti-dengue IgM detection. In 261 suspected cases, flower pot was found in 52(20 %) and IgM positive in 19(36.5%),house drain was found in 50(19.1%) and IgM 8(26.6%),Kitchen garden was found in 39(15%)and IgM positive in 11(28.2%) respectively. Detection of anti-dengue antibody with flower pot is statically significant with p-value (0.000) whereas with others it is statically insignificant. Flower pot (20%), house drain (19.1%) and kitchen garden (15%) was found as the most frequently mentioned breeding places in our study. The result is in consistent with Neupane et al (2014) who reported house drain and kitchen garden as the most common breeding sites of mosquito. However, the result was inconsistent with Benthem et al (2002) who reported water jars (75%) and house drain (68%) as breeding place of mosquitoes. In another study having garden near house (34.4%),animal shelter near house (21.6%) and rubbish around house (29.4%) were reported as breeding places of mosquito (Phuong et al 2008). *A. aegypti*, the principal mosquito vectors of dengue and prefers to lay eggs in artificial water containers such as flower vases, old automobile tires, buckets that collect rainwater, cement cisterns, drums, barrels and trash in general (Gubler 1998). As Parsa district is an industrial district but it lacks proper drainage system in

and around the city which makes favourable breeding sites for *A. aegypti* which is the principal mosquito vector of dengue.

Relationbetween use of preventive measures was analyzed with rate of anti-dengue IgM detection.In 261 suspected cases, covering water container was found in 233(89.2%) and IgM positive in 41(17.6%), spraying 16(6.1%) and IgM positive in 3(18.75%), use of mosquito repellant in 22(8.4%) and IgM positive in 3(13.6%).The result is statically insignificant ($p > 0.05$), whereas garbage disposal was found in 218 (83.5%) and IgM positive in 36(16.5%), use of net in 228(87.3%) and IgM positive in 38(16.6%) and changing stored water in 224(85.4%) and IgM positive 37(16.5%) respectively. The result is statically significant ($p<0.05$). The result is consistent with Neupane et al (2014) who reported garbage disposal, use of mosquito net and covering water container as the best known preventive measures. The study is also in harmony with the study done in Thailand in which use of the mosquito nets(65%), disposal of discarded containers (61%), covering water container (40%) and changing stored water (48%)were reported as best known preventive measures to prevent dengue infection (Benthem et al 2002). In our study, the use of mosquito nets was frequently mentioned as a prevention measure by persons but this measure is not effective for dengue infection as *Aedes* mosquitoes bite mainly during daytime (WHO 1997).However, for small children who sleep during the day, a mosquito net is an important preventive measure for dengue infection. *A. aegypti* is the principal mosquito vectors of dengue and prefers to lay eggs in artificial water containers such as flower vases, old automobile tires, buckets that collect rainwater, cement cisterns, drums, and barrels and trash in general (Gubler 1998). Preventive measures such as covering containers, disposal of discarded containers and changing stored water was effective preventive measures used in the study and use of such preventive measures can decrease the risk of dengue virus infection.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Out of 261 samples collected and tested from two hospitals of Parsa district in 2013, 49 were found to be positive for DVI. The samples were tested for anti-dengue IgM antibody by ELISA and RDT and the IgM positivity was 18.8% and 15.38% respectively. The sero-prevalence of dengue has significantly 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. The diagnosis of DF by molecular and virological tests is a complex process in terms of time and technique in developing countries. Considering the fact, the study has attempted to search the effective serological methods for diagnosis of DF in early stage. Severity of infection varies according to the serotypes of dengue virus. However, clinical features and serological test can be useful for the diagnosis and assessment of risk factor, breeding places and use of effective preventive measures in controlling dengue.

6.2 Recommendation

1.Sero-prevalence of dengue has increased in Nepal infecting all ages therefore awareness programs should be campaigned educating about the disease; mode of transmission and prevention of disease.

2. Risk factors like travel to endemic area and breeding place such as water logging site around house, flower pot should considered and preventive measures should be adopted.

3.Reference laboratories should be initiated for accurate diagnosis of the cases in the rural hospital settings.

4. The diagnostic accuracy of the RDT for the detection of anti-dengue IgM antibody is low, it would be better to use ELISA test rather than RDT for dengue diagnosis.

5. Secondary infections have high risk of developing DHF/DSS. IgG based studies should be done in dengue endemic areas.

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