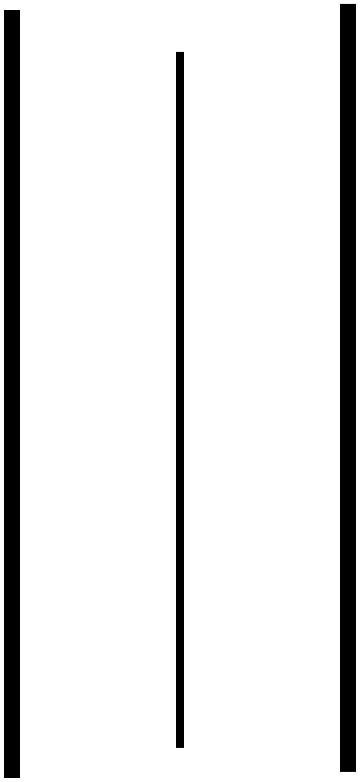
Serological-epidemiological and Molecular Study of Dengue Viruses in Nepal, January, 2008



Submitted to

Nepal Health Research Council Ramshahpath, Kathmandu

Submitted by:

Dr. Basu Dev Pandey,

Principle Investigator

Date: 1st January, 2008

To the Member Secretary,

Nepal Health Research Council

Ramshahpath, Kathmandu, Nepal

Re: Submission of final report

Dear Sir,

As our contract for the regional grant with the NHRC, the study entitled "Serological-epidemiological and Molecular Study of Dengue Viruses in Nepal" has completed successfully. The final report of this study is submitted for your kind attention and necessary action.

Thank you,

Sincerely yours,

Dr. Basu Dev Pandey

Principle Investigator

Acknowledgement:

The PI is grateful to all the doctors, nurses, laboratory staffs of the participating hospitals including Koshi Zonal Hospital, Biratnagar, Narayani sub-reginal hospital Birgunj, Hetauda District Hospital, Makwanpur, Bharatpur Hospital, Chitwan, Lumbini Zonal Hospital, Butwal, Bheri Zonal Nepalgunj, Banke, Nepalgunj Medical College, Nepalgunj, Banke, Bardiya District Hospital, Gulariya, Bardiya, Mahakali Zonal Hospital, Mahendranagar, Kanchanpur in the Teria region for their co-operation for the collection of samples. I also thanks to all the staff of Everest International Clinic and Research Center (EICRC) for their technical help and providing the laboratory facility for the study. I would also like to thank Dr. Bishnu Acharya and Kiran Pandey from EICRC for their technical supervision of overall activities in the laboratory work and Master students; Ramesh Pun and Krishina Panta from Tribhuban University, Kritipur and Om Prakash Shah of National Institute of Science and Technology (NIST) for their hard work in collecting samples and laboratory work. Finally, I am very much grateful to NHRC for providing research grants for the study.

Dr. Basu Dev Pandey

Principle Investigator

ABSTRACT

Objectives: To know the sero-epidemiological, molecular information of dengue virus (DV) and Japanese encephalitis (JE) in Nepal and to characterize these viruses at molecular level

Background: Dengue fever (DF) and more severe forms namely dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are caused by dengue virus, transmitted by *Aedes* mosquito. DF/DHF is primarily a disease in tropical and sub tropical areas of the world. Dengue virus infection occurs in more than 100 countries and over 2.5 billion people live in the areas with a risk of infection. Up to 100 million cases of DF and 500,000 cases of DHF and several thousands deaths are estimated to occur annually worldwide. During the past decades, dengue virus emerged in South Asia and DF/DHF epidemics occurred in Bhutan, India, Maldives, Bangladesh and Pakistan. In Nepal, the first case of dengue was reported on 2004 from Chitwan district. The recent outbreak of 2006 was observed in nine districts of Terai including Banke, Dang and Parsa, posses a serious threat of future epidemic of dengue in Nepal.

Methods: A total of 422 serum samples were collected during viremic period from the patients suspected DF, JE and other viral illness from August to November 2007 in nine districts of Teria region of Nepal and 127 samples were also collected from asymptomatic individuals. IgM ELISA, IgG-ELISA was performed using particle agglutination assay (PA) and IgM ELISA kit on serum samples for both dengue and JE. RNA extraction was performed using QIAGEN RNA EXTRACTION kit from the serum samples collected during viremic phase followed by RT-PCR.

Result: The result showed that 28 % of the cases were positive for dengue-IgM out of 422 collected serum samples. There was no haemorrhagic manifestation observed among the patients. Bardiya districts showed highest percentage of positive dengue antibodies (64%). *Stegomyia* indices seasonal changes related to rain were identified in all the major cities affected during the 2006 DF/DHF outbreak. To know the possible time of interdiction of dengue in Nepal, IgG ELISA was performed and gave only one positive case out of 127 asymptomatic individuals. Among analysed IgM antibody positive cases, 69.06% were male and 30.88% were female and the highest numbers of positive cases were found in an age group 21-30 (29 %). There was no significant difference was observed in relation to age, sex and occupations of the patient and dengue infection.

Two serological methods, PA and ELISA were used to compare their sensitivity and specificity for the detection of IgM antibody of dengue and the result showed that PA assay has sensitivity of 98 % and specificity of 96 %, a positive predicts value of 0.90 and negative predict value of 0.99 in comparison with IgM-capture ELISA. Since PA assay does not required sophisticated instruments and specialized manpower it could be useful and reliable diagnostic test to support clinical diagnosis in district hospitals of Nepal. Molecular diagnosis based on RT-PCR was also performed in an optimize condition for rapid and confirmatory diagnosis and to know the dengue serotype prevalent in Nepal. However, RT-PCR failed to show positive for dengue indicating inappropriate time for collection or transportation. Virus isolation is undergoing with the collaboration of international collaborator.

ACRONYMS

EICRC:	Everest International Clinic and Research Center
JE:	Japanese encephalitis
DF:	Dengue Fever
DHF:	Dengue Haemorrhagic fever
PCR:	Polymerase Chain Reaction
RT-PCR:	Reverse Transcriptase Polymerase Chain Reaction
WHO:	World Health Organisation
EDCD:	Epidemiology and Disease Control Division
DNA:	Deoxyribonucleic Acid
RNA:	Ribonucleic Acid
ELISA:	Enzyme Linked Immunosorbant Assay
PA:	Particle agglutination assay
NIST:	National Institute of Science and Technology.
TU:	Tribhuban University

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CHAPTER I: INTROUCTION

1. Background and significance of the study:

Dengue virus induces clinical illness ranging from a nonspecific viral syndrome [dengue fever (DF)] to severe and fatal hemorrhagic disease [dengue hemorrhagic fever (DHF)] (WHO, 1999). DF/DHF is primarily a disease in tropical and sub tropical areas of the world. Dengue virus (DV) is maintained in a cycle between humans and *Aedes aegypti,* domestic day-biting mosquitoes. Domestic DV infection occurs in more than 100 countries and over 2.5 billion people live in the areas with a risk of dengue virus infection (WHO, 1999, 2002). Up to 100 million cases of DF and 500,000 cases of DHF and several thousands deaths are estimated to occur annually worldwide (WHO, 1999). During the past decades, dengue virus emerged in South Asia and DF/DHF epidemics occurred in Bhutan, India, Maldives, Bangladesh and Pakistan (Islam et al 2006, WHO, 2007, Jamil et al, 2007) . There is limited information available on dengue viral infection in Nepal. In the year 2006, increase in the number of febrile patients was observed in four major hospitals at Terai region; Nepalgunj Medical College, Bheri Zonal Hospital in Nepalgunj, Tribhuban Hospital in Dang and Narayani sub-regional hospital in Birgunj. An outbreak of dengue with the confirmed cases of DF and DHF documented for the first time in several locations of Teria region of Nepal, where *aedes* mosquito persist bordering with India state of Bihar (Pandey et al 2008). On the other hand JE is endemic in 24 districts of Nepal since 1978 and it is expanding to the inner Teria including Kathmandu valley since the year 1995 (EDCD, 2005). The present study intended studies

to know the epidemiological, serological and molecular situation of dengue in the Teria region for future planning and to control activities of flaviviral diseases in Nepal.

2. Statement of the problem:

JE is the public health problem in Nepal and about 12.5 million populations are at risk of infection in the Terai area of Nepal (EDCD, 2005). There is limited information available on dengue infection in Nepal. It is not clear the precise year in which dengue was introduced in Nepal. Sporadic cases were noticed in foreigners in 90's and the first case of DF was reported in the year 2004 from Chitwan district (Pandey et al, 2004). However, no other cases of DF and DHF were reported in Nepalese patients until 2006. In September and October 2006 after the dengue epidemic in India, Nepal's Central and Western Teria and Kathmandu hospital reported 12 laboratory confirmed cases. This is the first report of dengue outbreak in different locations of Teria region of Nepal, where *aedes* mosquito persist bordering with India state of Bihar (EDCD 2006, WHO, 2007, Pandey et al 2008). It was expected that when monsoon start on the coming year the dengue transmission recommence and major outbreaks could occur causing high morbidity and overwhelming the Nepalese health system. In this situation sero-epidemiological surveillance of dengue could be an important steps for the prevention and management of dengue in Nepal.

3. Objectives of the study:

General:

To study the epidemiology, serology dengue viruses from August to September 2007 and to characterize isolated viruses in Nepal at molecular level from Taria region of Nepal

Specific

1. To know the epidemiological situation of dengue viruses during summer season from August to September 2007 in Taria region of Nepal
2. To identify serotypes and isolate dengue viruses from Nepal
3. To know the serological situation of dengue in Nepal
4. To analyse these viruses at the molecular level and determine the serotype of dengue viruses in Nepal
5. To perform phylogenitic analysis from the isolated viruses

4. Preparation of study:

The research team was formed for the data collection, filling of questioners, sample collection and laboratory assays. The research plan was formed under the co-ordination of PI and other supervisors. The research supervisor's team comprises of Dr. Basu Dev Pandey, PI and Dr Bishnu Acharya from EICRC. The other investigators in this study involved are Mr Ramesh Pun, Krishna Panta, Omprakash Shah, Kiran Pandey and Khem Aryal, well oriented about the objectives of the study and experienced in surveillance, serology and molecular assay. All the questioners, reagents and equipment including the sample carrying boxes needed for the study was prepared and instructed each investigators. Study sites were identified according to the information available in the past on dengue and JE in Nepal (EDCD 2005, WHO, 2007). An orientation session was conducted in Kathmandu to explain the aim of the study, appropriate collection techniques of samples before starting the study. The importance of appropriate storage of serum samples and transportation from the filed to the center was explained to the field staff.

CHAPTER 2: LITERATUTE REVIEW

Dengue is the most widespread vector-borne virus disease in the world, and DHF/DSS are rapidly increasing in incidence in many tropical areas (Halstead 1992). This dramatic increase is a result of changes in human lifestyle, increased international travel, urban crowding and global warming. Dengue has been known for at least 200 years, but DHF/DSS has only been routinely recognized since 1950s (Hare 1898 and Craven 1991). The rapid growth and urban crowding of Asian cities, associated with increased habitat for the vector mosquito of dengue, *Aedes aegypti*, which selectively breeds in water containers associated with human habitations. These breeding sites can include water jugs, blocked drain spouts, decorative plant containers, discarded refuse that may retain water and many other items associated with modern life (Gratz 1993). A pattern of dengue transmission then became evident, in South East Asia, first with increasingly frequent epidemics of classic dengue, continuous endemic transmission and appearance of DHS/DSS, becoming more common within the last 20 years (Akiyama 1993).

The same evolutionary pattern is now being repeated in the urban centers of Latin America (Gubler 1993). In the 1960s, a hemisphere-wide *A. aegypti* eradication programme was in existence, and nearly succeeded in total eradication of the vector. However, the decision was made to change the strategy from eradication to control. As a result, *A. aegypti* has now reinfested virtually all areas where it was once eliminated, and has even expanded to new habitats. As a result of this reinfestation, transmission of dengue has recently occurred in almost all parts of Latin America and the Caribbean. It is currently limited to increasingly frequent epidemics of DF, often coupled with sporadic cases or outbreaks of DHF/DSS. Indeed, in 1994 a major outbreak of dengue and DHF occurred in north-eastern Brazil. In Africa, an outbreak of dengue occurred in Comoros in 1993, during which an estimated 60,000 cases occurred (PAHO 1993).

DF and DHF are caused by one of four closely related, but antigenically distinct, virus serotypes (DEN 1-4), of the genus Flavivirus. DF and DHF are primarily disease of tropical and subtropical areas, and the four different dengue serotypes are maintained in a

cycle that involves human infection produces a spectrum of clinical illness ranging from a non specific viral syndrome to severe and fatal hemorrhagic disease.

In Nepal, sporadic cases were noticed in foreigners in 1990's and the first case of DF was reported in 2004 (Pandey *et al*., 2004). In 2006, Nepal's central and western Terai reported 12 laboratory conformed dengue cases. The serotype identified were DEN-1, DEN-3 and DEN-4 indicating dengue is an emerging disease in Teria, Nepal (WHO 2007, Pandey *et al*., 2008). Other report suggest that the prevalence of dengue antibodies in the Southwestern region of Nepal is 10.4 % (Sherchand *et al*, 2001) indicating dengue infections are being misdiagnosed; its importance is underestimated. DV was isolated from Japanese patients traveled to Hetauda, Nepal in 2004. The sequences were identical 98 % homology with Indian DEN 2 virus isolates (Takasaki *et al*, 2007).

The *A aegypti* mosquito is the principal urban vector (Clark & Casals 1965). Infected people are infective for feeding mosquitoes during the febrile phase of their illness, and mosquitoes may then transmit the virus to others at subsequent blood-meals after an incubation period of one week. Infected female mosquitoes may also pass the virus on to progeny by transovarial transmission (Rosen 1983). Once infected, mosquitoes remain infection for life. Thus, a single infected mosquito may transmit the virus to several susceptible humans over its lifetime. An entomological survey conducted by EDCD in Bharatpur, Biratnagar, and Birgunj where Stegomya indexes were estimated, the larvae/pupae density index was between four and five in September to December 2006. Tires living outside the household or the empty lots were found insignificant quantities and proved to be positive for Aedes larvae/pupae in percentages ranging from 17% in Bharatpur to 85% in Birgunj. It indicates that *A. aegypti* has been introduced in the country and that its densities are high enough for successful transmission.

DV infection can be asymptomatic or causes two forms of illness, DF and DHF, although the majority of DV infections are asymptomatic. DF is a self-limited febrile illness (Monath and Tsai 2002) and (WHO 1997). After an incubation period of 2–7 days, a sudden onset of fever occurs. The fever is usually accompanied with retro-orbital or frontal headache, myalgia and bone pain occur. A transient macular rash, nausea, vomiting, and lymphadenopathy develop. These symptoms are accompanied by leucopenia and thrombocytopenia. One to 2 days after defervescence, a generalized

morbilliform maculopapular rash appears. Patients usually recover from the symptoms without complications about a week after the onset of disease.

Some patients infected with DV demonstrate plasma leakage into interstitial spaces, thrombocytopenia, and also hemorrhagic manifestation (Nimmannitya et al., 1997). This severe life-threatening syndrome is called DHF. The incubation period of DHF is similar to that of DF. Illness starts with fever, malaise, vomiting, headache, anorexia, and cough. Rapid clinical deterioration and collapse follows after 2–5 days. In the second phase, the patients demonstrate cold clammy extremities, warm trunk, flashed face, restlessness, irritability, middle gastric pain, and may progress to a rapid weak pulse, hypotention, and narrow pulse pressure. The crisis lasts for 24–36 h and the patients recover rapidly once convalescent starts. The hematological manifestations include an increase in hematocrit, thrombocytopenia, a prolonged bleeding time, and an increased prothrombin time. The temperature returns to normal when capillary leakage occurs. The occurrence of capillary leakage differentiates DHF from DF. The WHO categorizes DHF into four grades, from less severe (grade 1) to severe (grade 4).

DF/DHF	Grade*	Symptoms	Laboratory
DF		Fever with two or more of the following headache,retro-orbital pain, myalgia, arthralgia	Leukopenia 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)

Major manifestations of DHF include (i) plasma leakage through elevated vascular permeability, (ii) hemorrhage, and (iii) thrombocytopenia. Some of the patients infected with dengue virus develop DHF, while most with symptomatic infections end up as DF. The pathogenesis of DHF has been explained by two theories. One theory is based on the virulence of infecting dengue viruses; virulent DV strains cause DHF, while avirulent DV strains cause DF (Pandey *et al*., 2001). The other is based on immunopathogenesis. This theory suggests that DHF is mediated by host immune responses including dengue virus-cross-reactive antibodies that augment infections. These two theories have been considered as opposing each other for a long period of time. However, it appears that they represent different aspects of the pathogenesis of DHF.

A constant finding in DHF/DSS is activation of the complement system, with profound depression of C3 and C5 level. Platelets defect may be both qualitative and quantitative, i.e. some circulating platelets during the acute phase of DHF may be exhausted. Therefore, even a patient with platelets count greater than 100 000 per mm^3 may still have a prolonged bleeding time. 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 (Kurane *et al.,* 1991). This phenomenon is called antibody-dependent enhancement (Halstead 1980).

Infants born to the mothers develop DHF in the primary infections. The levels of maternal DV antibodies in the infants needed to decline to the levels that can enhance DEV infection and lead to DHF (Kliks *et al*., 1989).These observations are consistent with the idea that enhancing antibodies increase the number of dengue virus-infected cells and the levels of viremia and lead to DHF.

Although DHF occurs more frequently in secondary infection than in primary 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 strains cause DHF, while avirulent dengue virus strains cause DF. There are multiple genotypes

in each of four dengue viruses. The introduction of the Southeast Asian genotype coincided with the appearance of DHF in different countries in the Americas, while the original American genotype was only associated with DF, but not with DHF (Rico-Hesse *et al.,* 1997, Pandey and Igarashi 2000, Green and Rothman 2006). Various groups have attempted to define the molecular determinants in of dengue virus virulence. It was demonstrated that non-synonymous amino acid replacements in the preM , NS1, NS2a, NS3, and NS5 by analyzing multiple strains of DEV- 2 was correlated with the severity of disease (Pandey and Igarashi 2000). 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 or chemical mediators rather than by destruction of the small vessels (Rothman and Ennis 1999). However, it is not clearly understood how these cytokines are induced and how these cytokines cause malfunction of vascular endothelial cells and lead to plasma leakage.

Of these cytokines, TNF-α has attracted attention because of its well-known activity in inducing plasma leakage. It was reported that dengue virus-infected monocytes and endothelial cells produce some of multiple cytokines including TNF-α (Kurane and Ennis, 1997). It is likely that both dengue-virus-infected monocytes and activated specific T lymphocytes are responsible for increased levels of cytokines in DHF. Activation of complement is another important clinical manifestation in DHF. It was reported that the levels of C3a and C5a, complement activation products, are correlated with the severity of DHF and the levels of C3a and C5a reached the peak at the time of defervescence when plasma leakage becomes most apparent (Malasit 1987). This is consistent with the assumption that complement activation is also responsible for the pathogenesis of DHF.

DV was first isolated in India in 1945. All four virus types circulate and cause epidemics, but only occasional cases of DHF/DSS have been reported in India (Sharma *et al.,* 1999). Delhi had its largest outbreak of DHF/DSS in 1996 and started the last week of August and continued until the end of November. A total of 8,900 cases were reported, with a death rate of 4.2% (Dar et al., 2006) report the first major outbreak of DHF in Delhi, India. An epidemic of dengue was also observed in Chennai, in 2002 with the reported about 700 cases (Kabilan *et al*., 2005)

Anand *et al*., in 2003 studied the DV circulation and association with epidemics and severe dengue disease were studied in hospitalized children with suspected dengue in Bangkok, Thailand, from 1973 to 1999. Dengue serology was performed on all patients and viral isolation attempted on laboratory confirmed patients. Acute dengue was diagnosed in 15,569 children and virus isolated from 4,846. DEN-3 was the most frequent serotype in primary dengue (49% of all isolates), DEN-2 in secondary and in DHF (37%and 35%, respectively). The highest incidence of the disease was observed in July 1995 in Trang Province on the Andaman Sea side with 192.73 cases per 100 000 population (WHO, 2002). It is not very clear the serotype prevalent in Nepal until now. However, report suggest that DEN-1, DEN-3 and DEN-4 were identified in 9 districts of Nepal in 2006 (WHO 2007). In other report DEV 2 was isolated from Japanese traveled to Hetauda, Nepal in 2004 (Takasaki *et al*, 2007).

Various serological tests are available for the diagnosis of dengue infection. IgM capture ELISA is the gold standard methods for the diagnosis of dengue until now. However, the problem of cross reaction with other flavivirus is one of the important aspects to be considered (Knox *et al*., 2003). Raising titer on paired samples is required for the definite diagnosis. Other flavivirus prevalent in the area should be ruled out. Assays including haemagglutination inhabitation, detection of antigen by ELISA, electron microscopy are also been available.

Virus isolation using suckling mice and use of mosquito cell cultures are also very useful. C6/36 cell culture is one of the best methods for virus isolation (Igarashi 1978). However, these procedures are time consuming and laborious to perform. Polymerase chain reaction (PCR) has been applied to dengue diagnosis with sera, tissue from fatal cases, mosquito pool, infected cell cultures and strain characterization. Several PCR protocols for dengue detection have been described that vary in the RNA extraction methods, genomic location of primers, specificity, sensitivity and the methods to detect PCR products and to determine the serotype (Lanciotti *et al*., 1992). A rapid assay has been developed followed by a second PCR with specific primers allowing serotype identification (DNA products) of different sizes according to the dengue serotype obtained. Reverse transcriptase (RT)-PCR has provided one of the most important steps in the molecular diagnosis of dengue virus. In recent years RT-PCR has been developed

for a number of RNA viruses, including dengue viruses. The technique allows for the multifold biological amplification of viral nucleic acid and has been used to rapidly diagnose viral diseases (Morita 1991).

The development and evaluation of a simple, rapid, and cost-effective one-step, real-time, and quantitative reverse transcription–loop-mediated isothermal amplification (RT-LAMP) assay is used for rapid detection and differentiation of DV serotypes (Parida *et al* 2006) . 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 degrees of specificity and selectivity and with rapidity under isothermal conditions.

In the early febrile phase, it is not possible to distinguish DF from DHF. Their treatments during the febrile phase are the same, i.e. symptomatic and supportive. There is no role of antibiotics and use of brufen is contraindicated (WHO, SEARO, 2002). In children, with signs of some dehydration, oral rehydration solution which is commonly used in the treatment of diarrhoeal diseases and/or fresh juices are preferable. Children who are breastfed should continue to be breastfed in addition to ORS administration. All dengue patients must be carefully observed for complications for at least 2 days after recovery from fever. This is because life threatening complications often occur during this phase. Patients and households should be informed that severe abdominal pain, passage of black stools, bleeding into the skin or from the nose or gums, sweating, and cold skin are danger signs of DHF. If any of these signs is noticed, the patient should be taken to the hospital. The patient who does not have any evidence of complications and who has been a febrile for 2-3 days does not need further observation. Most patients will recover without complication.

Without proper clinical management, case-fatality rates for DHF can exceed 20%. However, with intensive supportive therapy, rates can be reduced to less than 1%. The resurgence of epidemic dengue fever and the emergence of DHF as major public health problems are rooted in the demographic trends of the twentieth century. Several factors have combined to produce epidemiological conditions that highly favors viral transmission by the main mosquito vector, *A. aegypti*: population growth, rural-urban

migration, the inadequacy of basic urban infrastructure and the huge increase in volume of solid waste resulting from the new habits of consumers, for example, discarded plastic containers and other abandoned items which provide larval habitats in urban areas. The species thrives in intimate association with humans and is also the vector of the virus of urban yellow fever, a vaccine-preventable disease. Geographical expansion of this mosquito has been aided particularly by international commercial trade in used tyres which, with accumulated rainwater, are attractive habitats for egg-laying females of the species. Its role in the transmission of dengue and other arthropod-borne viruses in these new epidemiological settings remain to be determined. The magnitude of the public health problem will continue to grow unless more effective measures are taken to reduce viral transmission.

Although research on dengue vaccines for public health use is in process, currently the only method for the prevention and control of the disease is vector control. The global strategy enunciated in 1995 recommended the application of integrated vector-control measures, with community and intersectoral participation.

CHAPTER IV: METHODOLOGY

Based upon the previous information the peak season for JE and dengue usually start from August to November in Nepal (EDCD 2005, WHO 2007). For a systemic study three groups was formed for sample collection in 9 districts of Teria region. Each group spent one to two weeks period in each hospitals depending upon the availability of suspected patients and recommendation of clinician and the staffs in each hospital. PI and other senior researcher supervised their activities in the study.

i) Study area:

The following hospital and districts used for the sample collection based on the information available on the *Aedes* activities and previous dengue outbreak in 2006.

1. Koshi Zonal Hospital, Biratnagar, Morang
2. Narayani sub-reginal hospital Birgunj, Parsa
3. Sagarmatha Zonal Hospital, Rajbiraj, Saptari
4. Hetauda District Hospital, Makwanpur
5. Bharatpur sub regional Hospital, Chitwan
6. Lumbini Zonal Hospital, Butwal, Rupendehi
7. Bheri Zonal Nepalgunj, Banke
8. Nepalgunj Medical College, Nepalgunj, Banke
9. Bardiya District Hospital, Gulariya, Bardiya
10. Mahakali Zonal Hospital, Mahendranagar, Kanchanpur

ii) Research design and Faculty

The study group was composed of medical clinician, epidemiologists, entomologist and research scientist. The faculty exchanged comments on the organization of the study before the research activities initiated. The day previous to

starting the departure all the members met and agreed on the final plan of the time schedule, collection of samples, storage, transportation of samples and division of specific responsibilities for the research activities and sites to visit.

Group 1: Investigator headed by Krishna Prasad Pant, visited to Mahakali Zonal Hospital, Mahendranagar, Kanchanpur and spent two weeks for sample collection.
Group 2: Ramesh Pun and Krishna Prasad Panta visited to Bheri Zonal hospital Nepalgunj, Banke Nepalgunj Medical College, Nepalgunj, Banke, Bardiya District Hospital, Gulariya, Bardiya and for the period of two weeks
Group 3: Ramesh Pun and Krishna Panta jointly visited Lumbini Zonal Hospital, Butwal
Group 4. Dr Basu Dev Pandey PI visited different areas including, Nepalgunj, Chitwan, Hetauda, and Bardiya for the overall supervision.

iii) Epidemiological data collection on dengue and JE by using prepared questioner's sheet.

Prepared questioners were used to collect epidemiological information of the patients after taking consent either from the patients or their guardians (Annex i) .

iv) Collection of samples from the selected sites:

A total of 422 serum samples were collected from 11 sites of Teria region. These samples were collected from febrile patients in the outpatient department with the probable diagnosis of dengue and encephalitis or viral fever patients. The samples were stored in the freezer in the local hospital and transported to EICRC by fastest route using icebox. The samples were stored at the optimal temperature in the laboratory in Kathmandu until use.

V) Laboratory processing of the samples

a) IgM- Capture ELISA:

Serum samples are assayed by IgM-Capture ELISA against anti dengue IgM and anti-JE IgM. Particle agglutination (PA) Assay (Pentax Ltd Tokyo, Japan) and IgM-capture ELISA (Dengue/JE IgM Combo ELISA kit, Panbio Ltd, Brisbane, Australia) was performed. The samples from the asymptomatic patients were also investigated by using IgG ELISA.

b) Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

Sample preparation; Genomic RNA was extracted from 140 µl serum samples using QIAGEN- RNA extraction kit according to the manufacturers instructions and 60 µl was eluted in elution buffer.

Primers used: Initially sets of dengue consensus primers DC-IS and and DC-IC were used which was followed by dengue serotype (D1, D2, D3, D4) specific primers to know the dengue serotype prevalent in Nepal (Table 1).

RT-PCR was performed from the serum samples in febrile patients collected during viremic phase and were negative for IgM-Ab. Ready-To- RT-PCR Go^{TM} beads (Amershan Paharmacia Biotechnology) in a 0.5-ml tube containing 0.5 µM of each primer and 5 µl of RNA template. The MJ Research Mini Cycler was used for RT-PCR. The RT reaction was (42 ^{0}C for 10 min) was followed by 35 cycles of PCR (94^{0}C for 30 sec, 54 C for 30 sec and 72 ^{0}C for 30 sec, for each cycle). The final extension step was to 7 mins. In this study 8 µl of PCR product was subjected to 2% gel electrophoresis and visualized on ultraviolet transilluminator.

vi) Data analysis and management:

Differences between the values and statistical significance of the test were checked by SPSS soft were statistical analysis software 2007**.**

CHAPTER V: RESULT

Total of 422 serum samples were collected from febrile patients admitted in the hospital clinically diagnosed as, DF, JE and viral fever in JE and dengue endemic area. The samples were collected from east to west of Teria region namely in the following hospital: Biratnagar of Morang districts, Narayani sub-regional hospital Birgunj, Parsa, Sagarmatha Zonal Hospital, Rajbiraj, Saptari, Hetauda District Hospital, Makawanpur, Lumbini Zonal Hospital, Butwal, Rupendehi, Bheri Zonal Nepalgunj, Banke, Nepalgunj Medical College, Nepalgunj, Bardiya District Hospital, Gulariya, Seti Zonal Hospital, Dhangadi, Kailali and Mahakali Zonal Hospital, Mahendranagar, Kanchanpur. Another 127 serum samples also collected from the asymptomatic individuals from Chitwan and Saptari.

Fig 1. Map of sample collection sites through out the country

Table: 1. Result of serological study of Dengue virus infection

Samples collection sites	Number of Samples	IgM Capture ELISA		Percentage of Positive samples
		Positive	Negative	
Mahendranagar	54	3	51	5.55
Bardiya	39	25	14	64.10
Nepalgunj	28	4	24	14.28
Butwal	118	38	80	32.20
Chitwan	66	11	55	16.66
Birgunj	60	15	45	25.0
Hetauda	17	6	11	35.29
Biratnagar	40	19	21	47.5
Grand Total	**422**	**121**	**301**	**28.67**

Table 2: Age and Sex wise distribution in patients based on Serological study

Age Group (Years)	Male	Female	Total
0-10	16.36	7.27	23.63
11-20	14.54	10.9	25.4
21-30	18.18	10.9	29.08
31-40	10.9	0	10.9
41-50	3.63	1.81	5.44
Above 51	5.45	0	5.45
Total	69.06	30.88	100

Out of 183 serum samples examined, 55 samples were IgM positive. Among which 38 (69.06%) were male and 17 (30.88%) cases were female. The highest number of positive

cases were found in an age group 21-30 (29.08%), followed by 11-20 (25.4%), 0-10 (23.63%), 31-40 (10.9%) and above 40 (10.89%).

Table 3. Comparison of the result between PA assay and IgM-capture ELISA

		IgM-captured ELISA		
		Positive	Negative	Total
PA assay	Positive	50	5	55
	Negative	1	127	128
Total		51	132	183

Fifty-five (30%) of the 183 sample were IgM positive by the PA and 50 (90%) of the 55 PA IgM-positive samples were also IgM positive by IgM capture ELISA. (Table 3). This assay has sensitivity of 98% and specificity of 96%, a positive predicts value of 90% and negative predictive value of 99% in comparison with IgM-capture ELISA. Five samples were positive by PA assay, but negative by IgM captured ELISA. One hundred twenty seven cases were dengue IgM negative by both IgM-captured ELISA and PA. These results suggest that there is high level of compatibility between the PA assay and IgM-captured ELISA, using these samples.

Table 4. Detection of IgM antibody in relation to the Occupations

	IgM Positive		**Total**
	Negative (%)	Positive (%	
Agriculture	49 (26.7)	16 (8.7)	64(35)
Laboraror	17(9.2)	8 (4.3)	25 (13.6)
services	14 (7.6)	5(2.7)	19 (10.3)
Business	13 (7.1)	3 (1.6)	16 (8.7)
Student	20 (10.9)	15 (8.1)	35 (19)
None	16(8.7)	7 (3.8)	24 (13.1)
Total	**129 (70.7)**	**54 (29.3)**	**100**

Molecular Assay:

We performed molecular assay among 50 febrile patients using dengue consensus and dengue specific primers for all serotype and Japanese encephalitis virus. The expected PCR product was 561 to 639-bp product size using RNA extracted from the serum samples. RNA templates of dengue virus types 1-4 were negative for respective primers. The positive control dengue virus templates were positive from RT-PCR. We could not identify dengue virus genome by using RT-PCR method.

In our study there were different groups on the basis of their occupation: agriculture, labor, service, business, student and none (Table 4). The is no significance difference between occupation of the patients having IgM positive for dengue $P>0.05$.

Table 5. Knowledge about dengue among IgM antibody positive cases:

			IgM Positive		Total
			Negative	Positive	
Knowledge on dengue	No	Number	120	53	173
		Percentage	65.5	28.9	94.4
	Yes	Number	8	2	10
		Percentage	4.3	1.1	5.3
Total		Number	128	55	183
		% of Total	69.8	30	100.0

Among the analysed samples 53 (28.9%) of dengue IgM positive were found in the individuals who does not have knowledge about dengue fever.

In relation to dengue transmission, we asked the patients or visitors about the possible route of infection. Out of 183 serum samples collected from the suspected individuals, 40 (21.8%) of dengue IgM positive were found in the individuals who does not know the route of transmission. About 8% of the individual believe that the dengue is transmitted through the animals (Table 6).

Table 6. Knowledge about transmission cycle in relation to dengue IgM antibody

			IgM antibody		Total
			Positive	Negative	
How does it transmitted?	Mosquito	Number	10	9	19
		Percentage	5.4	4.9	10.3
	Animal	Number	10	5	15
		Total %	5.4	2.7	8.1
	others	Count	109	40	149
		% of Total	59.5%	21.8%	59.7%
Total		Count	127	56	183
		% of Total	70.3%	30.6%	100.0%

Table 7. Knowledge about having practice of mosquito net among the population in relation to detect IgM antibody.

			IgM antibody		Total
			no	yes	
Do you have mosquito net?	no	Count	47	19	66
		% of Total	25.6%	10.3%	35.9%
	yes	Count	80	37	117
		% of Total	43.7%	20.2%	63.9%
Total		Count	127	56	183
		% of Total	69.3%	30.6%	100%

Out of 183 serum samples collected from the suspected individuals, 37(20.2%) of dengue IgM positive were found in the individuals having mosquito net.

CHAPTER VI: DISCUSSION

The global prevalence of dengue has increased substantially recently. Dengue is endemic in ≥100 countries in Southeast Asia, Africa, the Western Pacific, the Americas, Africa and the eastern Mediterranean area with imported cases essentially everywhere. DF/DHF had been considered as a possible public health threat to Nepal after recent epidemics of DF/DHF in India and Pakistan, which claimed more than 100 deaths and several thousand cases (Gupta et al 2006). The first case of DF in Nepal was reported in 2004 (Pandey et al 2004). Further, the first Nepal DEN-2 isolate was obtained in Japan from a Japanese traveler who visited Nepal and developed DF after returning to Japan. The isolated DEN-2 demonstrated 98 % homology with those isolated in India (Takasaki et al 2008). It was reported that the prevalence of dengue virus antibody was 10.4 % in the southwestern region of Nepal (Sherchand et al 2001). These reports suggest that dengue virus has been circulating in Nepal for several years. Thus, it is likely that DF/DHF is misdiagnosed and the importance of dengue virus is underestimated in Nepal, contrast to JE. JE has been a major public health problem in Southwestern Nepal and large epidemic occurs almost every year since 1978 (EDCD, 2005). Nepal has no dengue surveillance programs, and health professionals do not usually consider dengue as a differential diagnosis.

From August to November in 2006, increase in the number of febrile patients was observed in four major hospitals at Teria region; Nepalgunj Medical College, Bheri Zonal Hospital in Nepalgunj, Tribhuban Hospital in Dang and Narayani sub-regional hospital in Birgunj. Patients with severe symptoms were referred to Sukraraj Tropical and Infectious Disease Hospital,Kathmandu for diagnosis and better management. Most of the patients

presented with the clinical features of DF but some showed signs consistent with World Health Organization (WHO) definition of DHF: high fever, rash, echymosis, epistaxis, positive tourniquet test, liver dysfunction and thrombocytopenia (platelets count <100,000) and the outbreak was confirmed (Pandey et al 2008) .

This is the first reported outbreak of dengue in Nepal. The epidemic occurred in areas from east to west of lowland Teria belt bordering with India state of Bihar. It is known that *aedes* mosquito persist in this region. There are constraints for the proper diagnosis of dengue necessitates the regular epidemiological studies due to lack of diagnostic facilities in Nepal. This study was initiated to know the serological and molecular studies in Terai belt of Nepal. Our finding shows that there is considerably higher prevalence (28 %) of dengue infection. The increased incidence and prevalence of dengue in Nepal might be due to cross boarder, transmission as Terai belt of Nepal is linked with the boarder of India.

The previous outbreak of 2006 shows DEN 1, DEN 2 and DEN 3 in different areas. We performed PCR to know the dengue serotype in other part of the country and intended to do phylogenitic analysis. However, we were not able to get positive cases in this study. We tried to collect samples in the viremic, transportation of samples with the utmost precaution. However, the negative result may be due to the delay in the sample collection and inappropriate cold chain maintenance during transportation. We are processing for the inoculation of samples in the mosquito cell lines with the collaboration of international collaborator and we expect to get the result soon. In India they had faced seven outbreaks of dengue virus infection due to various serotypes since 1967, with the last one in 2003 (Broor et al., 1997). The first major epidemic of dengue hemorrhagic fever (DHF), due to dengue-2 virus in 1996 (Dar et al., 1999), led to >10,000 cases (60% of cases reported from India in 1996) with 423 deaths. The report of Takasaki et al 2007 isolated DEN 2 virus from Japanese volunteer visited Nepal in 2004. These report

indicates that all dengue serotyopes are prevalent in Nepal. However, it needs further investigation.

Mohammed *et al*., 2005 reported that there was a febrile illness epidemic in Bangladesh in 2002 where 58 people died out of the 6,132 affected. Two hundred hospitalized patients were analyzed clinically, serologically and virologically to determine the features of this dengue infection. Among the 10- to 70-year-old age group of the 200 clinically suspected dengue patients, 100 (50%) were confirmed as dengue cases by virus isolation and dengue IgM-capture ELISA.

We used IgM Capture ELISA based assay using ELISA CONMBO KIT of panbio and Particle agglutination assay in our study. IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) has become an important tool in the routine diagnosis of dengue; this technique has a sensitivity and specificity of approximately 90% and 98%, respectively, but only when used 5 or more days after the onset of fever. In many regions of India, an increasing number of suspected cases of dengue are seropositive for IgM and IgG antibodies. The existence of IgG antibodies in a patient demonstrates prior infection with dengue and an increased risk of the severe forms of the disease. Outbreaks of dengue are increasingly reported in rural areas, implying that the population at risk is increasing, since dengue is considered to be a predominantly urban disease.

It is not clear exactly when dengue was first introduced in Nepal. In this study we detected the IgG antibody of the dengue from asymptomatic patients. We could not find the significant number of the IgG antibody positive for dengue. This result suggest that the dengue is the recently introduced and is emerging infectious vector born disease which is emerging in Nepal.

In the following year though no outbreak occurred in Delhi, definitely higher number of cases than usual were referred to laboratory for testing. The seasonality of transmission of dengue with increased activity in the post monsoon season was seen in the study; in accordance with the reported patterns of dengue transmission (Reiter 2001). Similar observation was seen in the year 1997 following 1996 epidemic in India (Vajpayee *et al.,* 1999).These findings indicate that during epidemic and non-epidemic

years dengue infections are mostly seen in post monsoon season hence preventive measures should be in full swing at the very onset of the monsoon in Nepal.

In this study, the largest proportion of serologically positive cases was recorded in the post monsoon period. Our findings were in coordination with study by other groups from this geographical region (Sharma *et al.,* 1998). The temperature remains high during the pre monsoon period and continuous rain pour for a couple of days that brings down the temperature during the monsoon period, which may also be responsible for an increase in the relative humidity and decrease in the evaporation rate thus maintaining secondary reservoirs containing rain water. More studies are needed to establish the relationship between the climatic changes and dengue outbreaks, which would help in formulating the strategies and plans to forecast any outbreak in future, well in advance.

All mosquitoes have aquatic larval and pupal stages and therefore require water for Breeding. Rainfall events and subsequent floods can lead to outbreaks of DF and DHF mainly by enabling increased breeding of vector mosquitoes. The timing of rainfall is as important as the amount of rain. The pattern of rainfall may also play a part. Extremely heavy rainfall may flush mosquito larvae away from breeding sites or kill them outright (McMichael et al., 1996). The number of rainy days may influence either the life-cycle of a mosquito or viral replication rates since a certain number of rainy days are generally favorable for mosquito development. If the number of rainy days were too low, there would not be enough water for mosquito larvae to complete their development. Therefore, the transmission of arboviruses may increase under warmer conditions as more vector mosquitoes become infectious within their life-span.

This might be the reason that dengue infection is prevalent in Terai region of Nepal and current research was focused in Terai belt. Entomological study of mosquitoes carried out during eighties revealed the presence of the Aedes albopictus in Terai plains which has been reported regularly. *A. Albopictus* is considered as an inefficient vector for DF transmission. Previously no *Ades aegypti* was recorded in Nepal. Recent outbreak of suspected DF cases prompted to conduct the cross-sectional entomology survey to identify the presence of Aedes aegypti.

The Epidemiology and Disease Control Division (EDCD) conducted entomological survey in Bharatpur, Biratnagar, Birgunj; Nepal between September to December 2006 where Stegomya indexes were estimated, the larvae/pupae density index was between four and five. Tires living outside the household or the empty lots were found insignificant quantities and proved to be positive for Aedes larvae/pupae in percentages ranging from 17% in Bharatpur to 85% in Birgunj. There is no doubt that A. aegypti has been introduced in the country and that its densities are high enough for useful transmission.

Based on the Questionnaire response, the number of IgM positive dengue cases were found in the individuals, who do not know about dengue i.e. 28.9%.The result also showed that higher number of IgM positive dengue cases were among the individuals who do not know the mode of transmission i.e.21.8%. Even the individuals who are aware of vector borne diseases and its prevention by using the mosquito net, there was higher prevalence of dengue infection among them. As *A. aegypti* is a day biting mosquito, transmission might have occurred during the day time when tires stay outside the household and work in the fields. This is further supported by this study as there was higher prevalence of IgM positive dengue cases among the individuals whose occupation is Agriculture.

In this study the prevalence of Dengue IgM antibody is highest in age group between 21-30. Out of 183 serum sample examined by PA, 55 (n=100) samples were IgM positive. Among them 38 (69.06%) were male and 17 (30.88%) cases were female. The highest numbers of positive cases were found in an age group 21-30 (29.08%), followed by 11-20 (25.4%), 0-10 (23.63%), 31-40 (10.9%) and above 40 (10.89%).The ratio of male to female is 2:1.

The serologic diagnosis of dengue infection has advanced considerably over the last 20 years (Gubler DJ, 1991). The ELISA evaluated in this report (PanBio Dengue Duo) proved to be a reliable test to diagnose dengue virus infection. However this test requires sophisticated instruments and trained personnel, and are more useful in the referral diagnostic centre in the developing countries like Nepal. The development of the PA assay, which does not require specific equipments and is relatively economical, would be beneficial for rular areas with limited facilities and where the trained personnel

are nor available. We applied the PA test to the serum samples collected from Terai district of Nepal. The Sensitivity and Specificity if PA test is high and determined to be useful in rural area of Nepal. The discrepancies between the PA and IgM-capture ELISA need to be further evaluated by analyzing the patient information in depth. Serially collected serum sampled could provide more valuable information. Cross-reactivity of anti-flaviviral IgG has well documented; however, IgM is known to be specific. (Burke and Monath 2001). This PA assay has Sensitivity of 98% and Specificity of 96%, a positive predict value of 90% and negative predict value of 99% in comparison with IgM-capture ELISA The data suggest that the PA assay for Dengue IgM is quick, easy to perform and specific. Hence, this assay system is useful especially in the rural areas of Nepal to support the clinically diagnosis, management, and epidemiological studies of dengue.

In addition to the serological diagnosis, optimization of conditions for molecular typing of dengue from serum sample was done by using one step RT-PCR bead reaction involving an complete set of reverse transcription and amplification step using consensus dengue primers targeting a region of the virus genome for identifying the presence of dengue in the suspected sera followed by a second amplification that is serotype specific. The products of these reactions are separated by electrophoresis on an agarose gel, and the different sized bands observed are compared with a standard marker for the relative molecular mass of nucleic acids. Dengue serotypes identification can be done by the size of their bands.

VIII: CONCLUSION

The sero-epidemiological study on dengue viruses was conducted in Terai region of Nepal from August to December 2007 shows that 28 % of the febrile patients were positive for dengue infection. In view of the report of outbreak in 2006 it indicates that dengue is firmly established in Terai region of Nepal. It is expected that more extensive outbreak can occur in the coming year with the start of rainy season. To consider this fact the medical personnel should given orientation about dengue, the treatment guidelines and surveillance system should be established. The capacity of dengue diagnostic laboratory should be strength in the JE endemic area. The sensitivity and specificity of Particle agglutination assay used in this study is higher than ELISA. Hence, it is a novel method that allows rapid and accurate identification of dengue IgG and IgM antibody in serum samples. Due to its easy operation without sophisticated equipment, it will be simple enough to use in small-scale hospitals, primary care facilities and clinical laboratories in developing countries like Nepal for the epidemiological studies. Molecular diagnostics, though more specific, is limited in use due to its high cost and sophistication. In the present study, we have tried to optimize the conditions necessary for the molecular diagnosis of dengue virus using RT-PCR with limited facilities in context of Nepal.

RECOMMENDATION

Based on previous evidence of dengue outbreak on 2006 and the present study, it is evident that dengue is an emerging disease in Teria, Nepal. It is also an endemic disease in India for last several years. It is expected that there will be more serious outbreak in the coming year causing high morbidity and overwhelming the health system. The following recommendations have been made for the responsible authorities to keep alert for preparedness for investigations management and dengue control activities in Nepal.

1. Regular studies by strengthening epidemiological surveillance for planning and response should be conducted in Terai region with increased sample size.
2. Monitoring of incidence and prevalence of dengue infection in Nepal.
2. Clinical management guidelines for DHF; improving emergency preparedness and response should be organized for doctors, nurses and paramedical staff to manage DF and DHF in Teria region JE endemic area of Nepal.

3. Strengthening of national vector-control programmers by entomological surveillance and the monitoring of key human behaviors should be done in Terai region of Nepal through collaborating infrastructure and manpower of vector borne disease center and EDCD.

4. Continuous, surveillance and monitoring of DF and DHF should be strengthened in respect of increased incidence of dengue infection in Terai region of Nepal.
5. Training should be given to the laboratories personnel in the laboratory diagnosis of dengue in the affected region.
6. Community awareness programme should be emphasized and community participatory programme on dengue control should be started.
7. Standard molecular and cell culture based laboratories should be established at least at the central and regional level.
8. Molecular characterization for identifying dengue serotype prevalent in Nepal is recommended.

CHAPTER 1X: REFERENCES

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Annexes i)

DENGUE CASE DETAILS AND LAB FORM

S.N…..

Date of Admission: | | | |

Name of the Health Institution/Community:……

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

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

Occupation: Agriculture ☐ Labour ☐ Sesrvice ☐ business ☐ student if ☐ other specify

Travel history of patients with in 14 days: Before of Fever onset: ☐ yes ☐ No

Do you know Dengue? ☐ Yes ☐ No

How does it transmitted ☐ Mosquito ☐ Animal ☐ Others

Do you have mosquito net ☐ Yes ☐ No

Status of patient: Symptomatic ☐ A symptomatic

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

Fever ☐ Muscles or joint pain: ☐

Headache ☐ Haemorrage ☐

Retro orbital pain ☐ Lymphadenopathy ☐

Skin rash (Red Rashes) ☐ If other than specify:……

Provisional Diagnosis: (From Clinician):…….

Laboraory Diagnosis:

Specimen: Serum Date of collection:……

Particle Agglutination test: ☐ Positive ☐ Negative

Table: 1: Primer information

Target (purpose)	Primer Code	Direction	Primer length	Nucleotide Sequences (5' to 3')
dengue consensus	DC-1S	Sense	28	TCA-ATA-TGC-TGA-AAC-GCG-CGA-GAA-ACC-G
	DC-2C	Antisense	29	TTG-CAC-CAA-CAG-TCA-ATG-TCT-TCA-GGT-TC
DEN1 specific	D1-S	Sense	20	GGA-CTG-CGT-ATG-GAG-TTT-TG
	D1-C	Antisense	20	ATG-GGT-TGT-GGC-CTA-ATC-AT
DEN2 specific	D2-S	Sense	20	GTT-CCT-CTG-CAA-ACA-CTC-CA
	D2-C	Antisense	20	GTG-TTA-TTT-TGA-TTT-CCT-TG
DEN3 specific	D3-S	Sense	20	GTG-CTT-ACA-CAG-CCC-TAT-TT
	D3-C	Antisense	20	TCC-ATT-CTC-CCA-AGC-GCC-TG
DEN4 specific	D4-S	Sense	20	CCA-TTA-TGG-CTG-TGT-TGT-TT
	D4-C	Antisense	20	CTT-CAT-CCT-GCT-TCA-CTT-CT
DEN1 specific	TS1	Antisense	19	CGT-CTC-AGT-GAT-CCG-GGG-G
DEN2 specific	TS2	Antisense	21	CGC-CAC-AAG-GGC-CAT-GAA-CAG
DEN3 specific	TS3	Antisense	22	TAA-CAT-CAT-CAT-GAG-ACA-GAG-C
DEN4 specific	TS4	Antisense	22	CTC-TGT-TGT-CTT-AAA-CAA-GAG-A