ROLE OF MOLECULAR METHODS IN THE DIAGNOSIS OF DENGUE

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PSG INSTITUTE OF MEDICAL SCIENCES & RESEARCH,

PEELAMEDU, COIMBATORE - 641 004

TAMILNADU, INDIA

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PSG INSTITUTE OF MEDICAL SCIENCES & RESEARCH

COIMBATORE

CERTIFICATE

This is to certify that the dissertation work entitled **"Role of Molecular methods in the diagnosis of Dengue"** submitted by Dr.Sangeetha.N, is work done by her during the period of study in this department from June 2009 to April 2012. This work was done under the guidance of Dr. B Appalaraju, Professor and Head, Department of Microbiology, PSG IMS & R.

Dr B Appalaraju
Professor and Head,
Department of Microbiology,
PSG IMS & R.

Dr S Ramalingam
Principal,
PSG IMS & R.

Place: Coimbatore
Date:

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Introduction

INTRODUCTION

Dengue is caused by Dengue Virus (DENV), a mosquito-borne viral disease of humans that in recent years has become a major International Public Health concern. Dengue is transmitted by Arthropods (mosquitoes) and is therefore also referred to as Arbovirus (Arthropod-Borne Virus) occurs in tropical and sub tropical areas of the world.[1]

Globally, 2.5 Billion people live in areas where Dengue Viruses can be transmitted. The geographical spread of both the mosquito vectors and the viruses has led to global resurgence of epidemic Dengue Fever (DF) and emergence of Dengue Hemorrhagic Fever (DHF) in the past 25 years with the development of hyperendemicity in many urban centers of the tropics.[2]

Since 1945, outbreaks of dengue caused by all 4 serotypes have been reported regularly from different regions of India. In 1966, large number of cases with serologically proven Dengue infection, few with hemorrhagic manifestations was noted in Vellore and DENV-3 was isolated from humans and mosquitoes.[3]

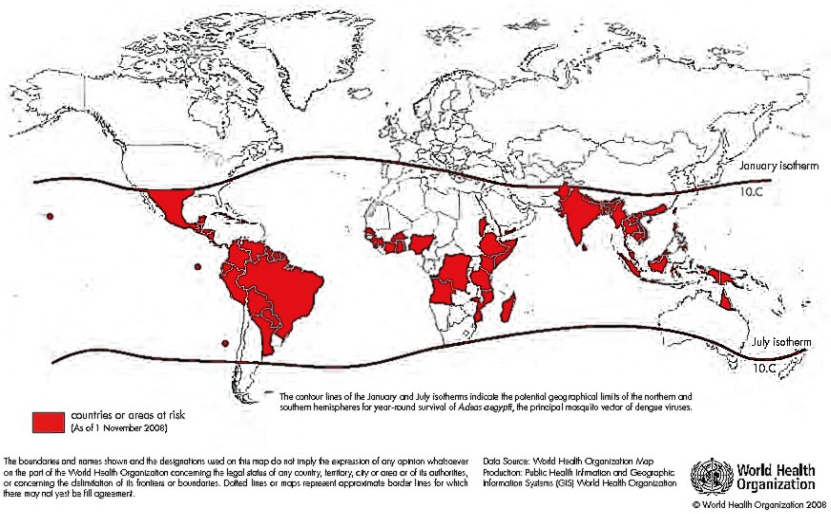
During 1990, an outbreak of dengue was reported from North Arcot district and adjoining districts of Andhra Pradesh.[4] Dengue virus outbreaks regularly reported from Chennai for many years. From October to December 2001 a large outbreak was

investigated. All the four serotypes of dengue virus was detected and isolated during this epidemic.[5] Dengue fever and DHF cases have been consistently noted from Karnataka State including that of Kolar, Bangalore and adjoining areas of Bellary and Mysore. Increasing number of cases DHF is being reported from rural areas in this region. Dengue outbreaks have been noted in Mangalore region with isolation of dengue-2 serotype.[6]

During 1996 one of the largest outbreak reported from North India occurred in Delhi and adjoining areas. During this epidemic mainly dengue-2 virus was isolated.[7,8] Following this 1997, dengue-1 virus activity was seen in Delhi (9) Thereafter, in the year 2003 another outbreak occurred in Delhi. All four dengue serotypes were isolated.[10, 11] However, dengue-3 was reported to predominate in certain parts of North India in 2003 & 2005 epidemic.[12, 13]

Fig -1: Countries at risk of dengue viral transmission

Figure 1.1 Countries/areas at risk of dengue transmission, 2008



CLINICAL MANIFESTATIONS OF DENGUE VIRAL INFECTION:

Clinical spectrum of dengue viral infections, ranging from asymptomatic infections, undifferentiated mild flu like fever and classical Dengue Fever (DF) to the more severe forms like DHF-DSS(Dengue hemorrhagic fever-dengue shock syndrome).DHF-DSS are associated with high rates of morbidity and mortality .[15, 16, 17, 18]

DF is characterized by fever of 3 to 5 days duration, headache, myalgia, joint pain and rash, low white blood cell count, positive tourniquet test or any warning sign in humans are self-limited and from which patients usually recover completely. It is important to maintain hydration because there is no specific antiviral drug available for DF, and most forms of therapy are supportive in nature. DHF-DSS is characterized by the same signs and symptoms as classic DF, but it is

followed by increased vascular permeability and hemorrhage, which may lead to vascular Collapse and death.[19]

Careful clinical management by experienced medical professionals is important in saving the lives of DHF patients. Diagnosis of dengue virus infection on the basis of clinical syndromes is not reliable, and the diagnosis should be confirmed by laboratory studies because more than half of infected individuals either are asymptomatic or have a mild undifferentiated fever.[20,21] Therefore, there is a great demand for the rapid detection and differentiation of dengue virus infection in the acute phase of illness in order to provide timely clinical treatment and etiologic investigation and to control the disease.

At present, Laboratory diagnosis of dengue is done by detecting dengue virus or anti dengue antibodies in blood. Isolation of virus in cell culture or in infant mouse brain remains gold standard for diagnosis[19].But the success rates of these procedures are less because fastidious culture conditions, the low level of transient viraemia and the time consuming procedure. Detection of anti-dengue IgM and IgG antibody in the serum by various serological assays is the commonly used method for presumptive diagnosis of dengue. Limitations for serological diagnosis are cross reactivity with other flaviviruses and unable to detect the infection in the early phase of the illness. Detection of dengue viral

nucleic acid in early phase of the illness by PCR based methods are rapid and sensitive method. Advantage of these molecular methods are serotyping of the dengue virus and by sequencing the amplified products able to detect the potential mutations in the viral genome.[19]

Aims of the Study

AIMS OF THE STUDY

- To study the seropositivity of dengue among suspected cases in a tertiary care Hospital.
- To assess the proportion of primary and secondary dengue infections.
- To evaluate the role of NS1 antigen detection among patients with fever days ≤ 9 days.
- To categorize the NS 1 antigen positive dengue cases according to WHO guidelines.
- To evaluate the role of dengue RT-PCR in diagnosis of fever patients who attending the tertiary care hospital.

Review of Literature

REVIEW OF LITERATURE

The word DENGUE derived from the *Swahili* phrase *ki denga pepo* meaning a sudden seizure by demon.[22]

During Philadelphia epidemic (1780) Benjamin Rush described the dengue fever as break-bone fever. Mosquito-borne transmission of the infection by *Aedes aegypti* was demonstrated in 1903 and its viral etiology 1906. In 1944, Sabin demonstrated the failure of 2 viral strains to cross protect humans, thus establishing the existence of dengue viral serotypes. In 1956, Hammon characterized 2 more serotypes.[23]

DENGUE VIRAL STRUCTURE:

Dengue viruses belong to the *Flavivirus* genus of the *Flaviviridae* family and Flavivirus are enveloped, single-strand positive sense RNA viruses. The Dengue Virus genome contains about 11,000 nucleotide bases, which code for the three different types of protein molecules, they are C (core protein), M (membrane protein), and E (envelope protein) to form the virus particle and seven nonstructural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). The polycistronic coding region is flanked by a non-coding region of about 100 nucleotides at its 5'end and a longer non-coding region at its 3' end. The gene order is 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3', as for other flaviviruses.[24-28]

Lipid envelope contains M (membrane) and E (envelope) glycoprotein. E protein helps in attachment of host cell receptors, endosomal membrane fusion, display of sites mediating hemagglutination and viral neutralization.[29] NS1 antigen expressed on surface of the infected cell and it is also secreted as complement-fixing antigen. Antibodies to NS1 antigen contribute to protective immunity, by antibody dependant cellular cytotoxicity and cell-mediated responses against infected cells.[30]

The serotypes are epidemic potential that present in dengue virus are DENV–1, DENV–2, DENV–3, and DENV–4. Dengue serotypes 1, 3 and 4 show a closer antigenic and genetic relationship to each other than dengue serotype 2.[31] Usually one serotype predominates in an area until increased herd immunity decreases the transmission and then another serotype predominates. DENV-4 generally leads to less severe disease than other serotypes, particularly DENV-2 and DENV-3.[31] Based on sequence analysis of the E/NS1 region and using a cut-off of 6% divergence, each DENV serotype can be divided in different genotypes. [32,33].

Five different genotypes of DENV-2 are

- American,
- Asian (1 & 2),
- American/Asian,
- Cosmopolitan and
- Sylvatic .

The DENV-3 viruses were separated into four genetically distinct subtypes [34] are

- Subtype I consists of viruses from Indonesia, Malaysia, the Philippines and the South Pacific islands
- Subtype II consists of viruses from Thailand
- Subtype III consists of viruses from Sri Lanka**,** India, Africa and Samoa
- Subtype IV consists of viruses from Puerto Rico and the 1965 Tahiti virus dengue genotype example.
- It also had genotype V

VECTOR

The major mosquito vector for urban dengue is *Aedes aegypti* has the ability to produce explosive epidemics. Dengue outbreaks have also been attributed to *Aedes albopictus*, *Aedes polynesiensis* and several

species of the *Aedes scutellaris* complex. Each of these species has a particular ecology, behavior and geographical distribution.[35]

To establish infection, *A.aegypti* needs to feed on individuals with high levels of viraemia. This may select for viral strains of higher virulence and that may leads to severe epidemics. The adult *A. aegypti* mosquitoes prefer to rest indoors, are unobtrusive. They prefer to feed on humans during daylight hours. There are two peaks of biting activity, early morning for 2 - 3hr after daybreak and in the afternoon for several hours before dark. [35]

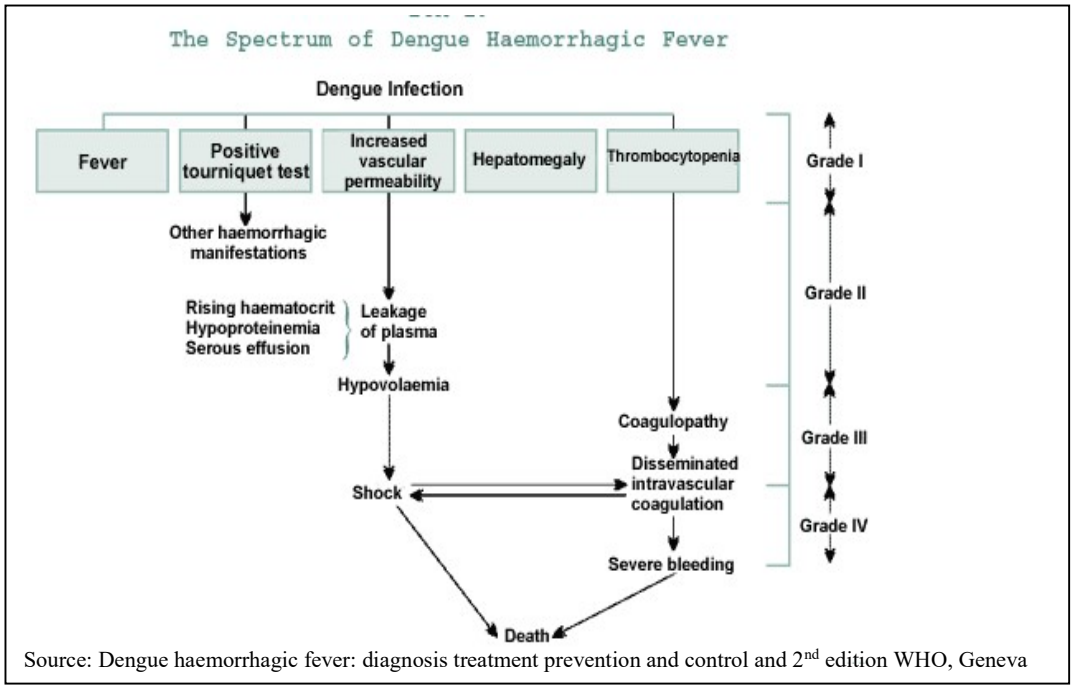
The female mosquitoes are very nervous feeders, disrupting the feeding process at the slightest movement, only return to the same or different person to continue feeding moments later. Because of this behavior, *A. aegypti* females will often feed on several persons during a single blood meal and if infective, it also helps to transmit the dengue virus to multiple persons in a short time, even if they only probe without taking blood. [36-39]

It is not uncommon to see several members of the same household become ill with dengue fever within a 24 - 36hr time frame, suggesting that all of them were infected by a single infective mosquito[40] so this behavior that makes *A. aegypti* such an efficient epidemic vector.

CLINICAL FEATURES OF DENGUE VIRAL ILLNESS:

The existing WHO classification of symptomatic dengue virus infections were grouped into three categories: undifferentiated fever, dengue fever (DF) and dengue hemorrhagic fever (DHF). DHF was further classified into four severity grades, with grades III and IV being defined as dengue shock syndrome (DSS) [41] Currently this classification into DF/DHF/DSS continues to be widely used.

Fig: 2 Spectrum of Dengue haemorragic fever[41]



Source: Dengue haemorrhagic fever: diagnosis treatment prevention and control and 2nd edition WHO, Geneva

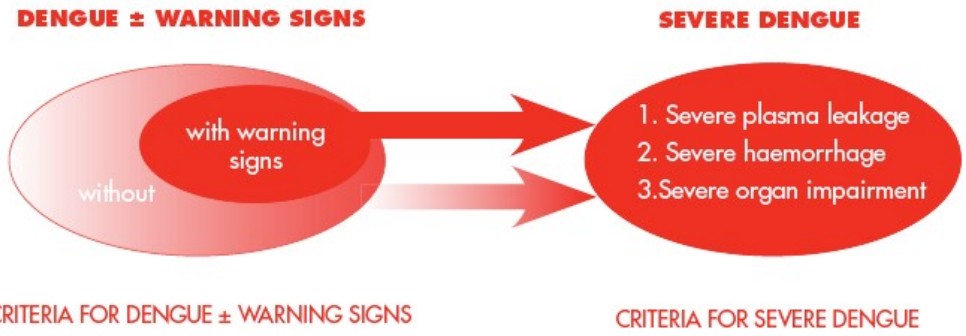
Expert consensus groups in Latin America (Havana, Cuba, 2007), South-East Asia (Kuala Lumpur, Malaysia, 2007), and at WHO headquarters in Geneva, Switzerland in 2008 agreed that:

"Dengue is one disease entity with different clinical presentations and often with unpredictable clinical evolution and outcome";

The model of classifying dengue fever depending on the severity of the disease has been suggested by an expert group (Geneva, Switzerland, 2008) and is currently being tested in 18 countries by comparing its performance in practical settings to the existing WHO case classification.

Dengue infection has a wide clinical spectrum that includes both severe and non-severe clinical manifestations. [42,43]After the incubation period 4-10 days, the illness begins abruptly and is followed by the three phases -- febrile, critical and recovery.

Fig: 3 Dengue clinical features[42]



Probable dengue
live in /travel to dengue endemic area.
Fever and 2 of the fallowing criteria:

- Nausea, vomiting
- Rash
- Aches and pains
- Tourniquet test positive
- Leukopenia
- Any warning sign

Laboratory-confirmed dengue
(important when no sign of plasma leakage)

Warning signs*

- Abdominal pain or tenderness
- Persistent vomiting
- Clinical fluid accumulation
- Mucosal bleed
- Lethargy, restlessness
- Liver enlargement >2 cm
- Laboratory: increase in HCT concurrent with rapid decrease in platelet count

*(requiring strict observation and medical intervention)

Severe plasma leakage
leading to:

- Shock (DSS)
- Fluid accumulation with respiratory distress

Severe bleeding
as evaluated by clinician

Severe organ involvement

- Liver: AST or ALT >= 1000
- CNS: Impaired consciousness
- Heart and other organs

FEBRILE PHASE:

The Febrile phase involves high-grade fever, often over 40°C (104°F), and is associated with generalized pain and a headache; this usually lasts for 2–7 days. It is often accompanied by facial flushing, skin erythema, generalized body ache, myalgia, arthralgia and Anorexia, nausea, vomiting is also common[42]. It can be difficult to distinguish dengue clinically from non-dengue febrile diseases in the early febrile phase. A positive tourniquet test in this phase increases the probability of dengue.[44,45] In addition, these clinical features are indistinguishable between severe and non-severe dengue cases. Therefore monitoring for warning signs and other clinical parameters is crucial to recognizing progression to the critical phase.

Mild hemorrhagic manifestations like petechiae and mucosal membrane bleeding (e.g. nose and gums) may be seen. [44,46] Massive vaginal bleeding (in women of Child bearing age) and gastrointestinal bleeding may occur during this phase but is not common[46].The liver is often enlarged and tender after a few days of fever. [44]The earliest abnormality in the full blood count is a progressive decrease in total white cell count, which should alert the physician to a high probability of dengue.

CRITICAL PHASE:

Around the time of defervescence, when the temperature drops to 37.5–38˚C or less and remains below this level. It happens on 3–7th day of illness. During this period an increase in capillary permeability in parallel with increasing haematocrit levels may occur.[47,48] This marks the beginning of the critical phase. The period of clinically significant plasma leakage usually lasts for 24–48 hours and the degree of plasma leakage varies. Pleural effusion and ascites may be clinically detectable depending on the degree of plasma leakage and the volume of fluid therapy.

Shock occurs when a critical volume of plasma is lost through leakage. It is often preceded by warning signs. The body temperature may be subnormal when shock occurs. With prolonged shock, the consequent organ hypo perfusion results in progressive organ impairment, metabolic acidosis and disseminated intravascular coagulation.[42]

DENGUE WARNING SIGNS:

Persons who improve after defervescence are said to have non-severe dengue. Those who deteriorate will manifest with warning signs. This is called as dengue with warning signs .Cases of dengue with warning signs will probably recover with early intravenous rehydration. Some cases will deteriorate to severe dengue. [42]

RECOVERY PHASE:

If the patient survives after the 24–48 hours of critical phase, a gradual reabsorption of extra vascular compartment fluid takes place in the following 48–72 hours. General well-being, Appetite improves and Gastrointestinal symptoms will become normal. Haemodynamic status stabilizes and diuresis ensues. Some patients may have a rash of "isles of white in the sea of red" [49]Some may experience generalized pruritus. Bradycardia and electrocardiographic changes are common during this stage. [42]

SEVERE DENGUE:

Severe dengue should be considered if the patient is from an area of dengue risk presenting with fever of 2–7 days plus any of the following features: [42]

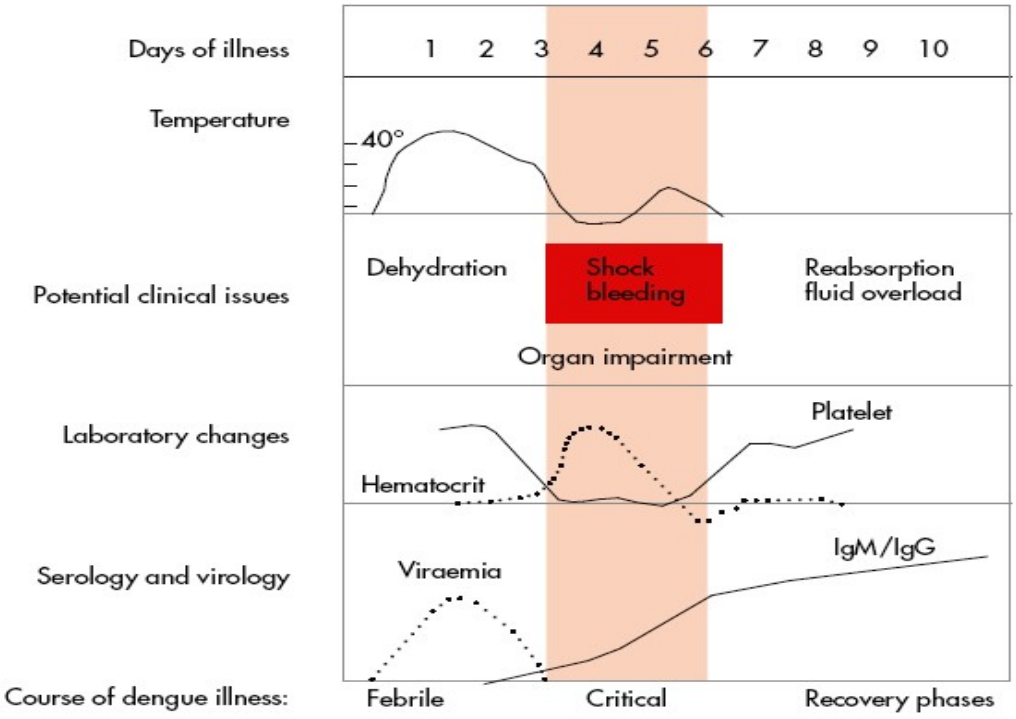
- Evidence of plasma leakage, such as:
  - High or progressively rising haematocrit;
  - Pleural effusions or ascites;
  - circulatory compromise or shock (tachycardia, cold and clammy extremities, capillary refill time greater than three seconds, weak or undetectable pulse, narrow pulse pressure or, in late shock, unrecordable blood pressure).

Significant bleeding.

- An altered level of consciousness (lethargy or restlessness, coma and convulsions).
- Severe gastrointestinal involvement (persistent vomiting, increasing or intense abdominal pain, jaundice).
- Severe organ impairment (acute liver failure, acute renal failure, encephalopathy or encephalitis, or other unusual manifestations and cardiomyopathy)
- Other unusual manifestations.

COURSE OF DENGUE ILLNESS:

Fig: 4 Course of dengue illness[42]



PATHOGENESIS OF DENGUE VIRAL INFECTIONS:

Most commonly accepted theory for dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) is secondary infection or immune enhancement hypothesis (antibody- dependent enhancement)[50,51,52] According to this hypothesis, patient experiencing a second infection with a heterologous dengue virus serotype have a significantly higher risk for developing DHF and DSS.

Preexisting heterologous dengue antibody recognizes the infecting virus and forms an antigen-antibody complex. This antigen-antibody complex is then bound to and internalized by immunoglobulin Fc receptors on the cell membrane of leukocytes, especially macrophages. Because of the heterologous antibody, the virus is not neutralized and the virus starts replicating inside the macrophage. Thus, it is hypothesized the prior infection through a process known as antibody-dependent enhancement (ADE), it enhances the infection and replication of dengue virus in the mononuclear cells.[53-56] This mononuclear cell secretes vasoactive mediators in response to dengue infection, which causes increased vascular permeability leading to hypovolemia and shock.

The occurrence of DHF in primary infections could be related to viral strain and serotype; dengue viral virulence has long been proposed as an alternative to the immune enhancement hypothesis. [57-59] Interestingly,

in many studies have found that DENV-2, DENV-3 causes DHF in primary infections to a greater extent than DENV-1. [60,61] Although the role of viral genetic variants had been implicated in dengue severity many years ago[57-59] it was not until the combined application of viral evolution and epidemiologic analyses that dengue virus evolutionary trees (or "phylogenies") were developed that indicated that specific genotypes within a serotype are associated with disease of greater or lesser severity.[32, 61, 62, 63]

Thus, for serotypes 2 and 3, the genotype originating in Southeast Asia and the genotype originating in the Indian subcontinent respectively, have associated with more outbreaks of DHF and/or DSS and more severe forms of primary infections.[64]

DIAGNOSIS OF DENGUE VIRAL INFECTIONS:

Laboratory diagnosis methods for confirming dengue viral infection may involve detection of the virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques. After the onset of illness, the virus can be detected in serum, plasma, circulating blood cells and other tissues for 4-5 days. During the early stages of the disease the virus isolation, nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis.[19]

VIRUS ISOLATION AND CHARACTERIZATION:

From clinical samples, isolation of dengue virus carried out with both cultured mosquito cell lines and mammalian cell cultures. Mammalian cell cultures used for the study of dengue viruses are

- LLCMK2 (monkey kidney)
- Vero (monkey kidney)
- BHK21 (baby hamster kidney) line

Sensitivity of these cells lines vary to different dengue virus types and strains.[65]

Mosquito cell lines used for dengue virus isolation are[66]

- AP61 (*Aedes pseudoscutellaris*)
- Tra-284 (*Toxorynchites amboinensis*)
- C636 (*A. albopictus*)
- AP64 (clone of an *A. pseudoscutellaris* cell line)
- CLA-1 (clone of an *A. pseudoscutellaris* cell line)

Advantages of Mosquito cell lines are [67, 68]

- More sensitive than vertebrate culture systems for the recovery of dengue viruses
- Relatively easy to maintain and grow at room temperature
- They can be kept for at least 14 days without a change of medium.
- Mosquito cell cultures can be carried into the field and inoculated directly with human sera from patients

Blood samples for viral isolation should be collected during febrile period of illness within fifth day after the onset of the illness. These acute plasma or serum samples stored at -70ºc and repeated freeze-thawing should be avoided [69, 70]

Virus isolation by cell culture and from mosquitoes remains the "gold standard" test. For rapid diagnosis it has replaced by the Reverse transcription (RT)-PCR method. This is mainly due to its lower sensitivity and the fact that a longer time for detection is required if indirect immunofluorescence is performed to identify the isolated virus with dengue or serotype-specific monoclonal antibodies.[41, 71, 72]

However, the molecular method based on RT-PCR has been combined with the cell culture method to improve the sensitivity and reduce the time needed to identify the cultured viruses.[73]The latter method was reported to detect the cultured virus at day 1 (versus day 4 by the indirect immunofluorescence method), if 10^4 viruses/ml were inoculated into the culture.

Viral isolation is important for laboratories interested in studies of the viral genome, various virulence factors and pathogenesis of dengue viral infections.[19.] Limitations of dengue viral culture are need expertise and facility for cell culture technique and fluorescent microscopy not available in the majority of the labs. The virus isolation alone cannot help

to differentiate between the primary and secondary dengue viral infections.[42]Currently, inoculation of the C636 cell line with acute febrile serum sample from the patients is the method of choice for dengue virus isolation.[70]

DETECTION OF VIRUS NUCLEIC ACID:

Molecular detection of dengue viral genome is more sensitive and rapid method than traditional virus isolation method. By using PCR, we able to detect the dengue viral genome from[70]

- Directly in clinical sample,
- Dengue virus infected mosquito cell culture supernatants,
- Infected mosquito larvae.

Most widely used method is two step nested RT-PCR developed by Lanciotti RS *et al*[74] and a modification of this same method to single tube format by Harris E *et al*.[75]These assays used the dengue virus core to premembrane gene regions as the target sequence for dengue virus detection. They had the advantage of detecting and differentiating the four dengue virus serotypes by analyzing the unique sizes of the amplicons in the agarose gel.

More recently, several investigators have reported on fully automatic real-time RT-PCR assays for the detection of dengue virus in

acute-phase serum samples.[76,77]The advantages of real-time PCR, when compare to conventional RT-PCR methods are[19]

- Rapidity,
- Ability to provide quantitative measurements,
- Lower contamination rate,
- Higher sensitivity,
- Higher specificity
- Easy to standardization.

Therefore, real-time PCR has gradually replaced the conventional PCR as the new gold standard for the rapid diagnosis of dengue virus infection with acute phase serum samples.

To avoid the false-negative results caused by sequence variations among different strains and potential mutants, we should use multiple primers and probes targeted at different gene regions.[19]

To avoid the false-positive results, great care should be taken for preventing the sample and/or reagent contamination during the performance of the RT-PCR. Molecular diagnosis are costly, it needs expertise and expensive laboratory equipment.[42]Important disadvantage for nucleic acid amplification is sample should be collected in the early febrile phase of the illness for both primary and secondary dengue viral infections.[78,79]

Nucleic acid sequence based amplification (NASBA) is one of the newer molecular techniques. The amplification procedure is done entirely in isothermal temperature and is conducted at 41°C. Wu and coworkers[79]reported on the detection of dengue viruses by the NASBA assay, which had high degrees of sensitivity and specificity. It is also useful in epidemiological studies in the field.

Manmohan Parida *et al* [80] described a one-step, real-time, and quantitative dengue virus serotype-specific reverse transcription–loop-mediated isothermal amplification (RT-LAMP) assay targeting the 3'noncoding region for the rapid detection and differentiation of dengue virus serotypes.

The RT-LAMP assay is very simple and rapid; the amplification can be obtained in 30 min under isothermal conditions at 63°C by employing a set of four serotype-specific primer mixtures through real-time monitoring in an inexpensive turbidimeter.

NS1 Antigen Detection:

Among the nonstructural proteins, NS1 is a highly conserved glycoprotein which appears essential for virus replication. During acute phase of dengue viral infections, NS1 protein is found associated with intracellular organelles or is transported through the cellular secretory

pathway to the cell surface.[81, 82, 83] A soluble hexameric form of the flavivirus, NS1 proteins were released from infected mammalian cells but not from vector-derived mosquito cells.[84,85] The hexameric form of dengue virus NS1 protein was also found circulating in the sera of patients during acute phase of the illness.[86]

Hua xu [87]et al showed in their study that NS1 antigen was detectable from first day up to day 18 after onset of symptoms, with a peak at days 6 to 10 in the dengue fever patients.

Since 2002 detection of the NS1 protein, a non-structural protein secreted by the dengue virus, has offered a new approach for diagnosis during the acute phase illness (mainly the first five days).[88,95]

Several commercial NS1 antigen detection kits using immunoenzyme, ELISA or immunochromatographic techniques (i.e. rapid tests) are now available. These tests can easily performed by the most clinical laboratories. These tests offer excellent specificity but their sensitivity is thought to vary depending on the viral serotype [89, 90, 91] and depending upon primary or secondary infections. [92, 93,94]

It can be used in laboratories with limited equipment and yield results within a few hours. Rapid dengue antigen detection tests can be used in field settings and provide results in less than an hour.

Disadvantages of these assays are not type-specific and expensive, they are under evaluation for diagnostic accuracy and cost-effectiveness in multiple settings. Another limitation of antigen detection is not as sensitive as viral isolation or RNA detection.[42]

A study conducted by *Koraka et al* (2003)[96] showed the detection of dengue NS1 antigen in patient samples from areas where dengue is endemic was low. It is due to the presence of immune complexes formed following a secondary dengue infection. *Veasna Duong et al* [97]showed the low NS1 antigen ratio was associated with more severe forms of dengue viral diseases.

SEROLOGICAL DIAGNOSIS:

Two patterns of serological response can be observed in acute dengue viral infections are primary and secondary dengue viral infections. A primary response is seen in individuals who are not immune to flaviviruses. A secondary seroresponse pattern occurs in an individual with an acute dengue virus infection who had a previous flavivirus infection.[19, 98]

The serological diagnosis of dengue viruses is complicated by the existence of cross-reactive antigenic determinants shared by all four dengue virus serotypes and some other flaviviruses.[98]Serological

detection of dengue virus-specific antibodies can be done by following methods. They are

- Hemagglutination inhibition (HI) test,
- Neutralization test,
- Indirect immunofluorescent-antibody test,
- ELISA,
- Complement fixation,
- Dot blotting,
- Western blotting,
- Rapid immunochromatography test (for which many commercial kits are available)

HEMAGGLUTINATION INHIBITION (HI) TEST:

Traditionally, the hemagglutination inhibition (HAI) assay has been used to classify dengue infections as primary (gradual increase in antibody to moderate titer) or secondary (rapid increase to high titer). However, the HAI assay has various practical limitations, and since it usually requires paired sera, it cannot helps to an early diagnosis. Furthermore, the variable potency of hemagglutinins made in different laboratories has compromised the general applicability of this assay.[19]

A four-fold increase was considered positive for acute flavivirus infection. The infection was diagnosed as primary if titers a week or more after onset of illness were less than 1:2,560 or as secondary if antibody titers were greater than or equal to 1:2,560.[19]

ENZYME-LINKED IMMUNOSORBENT ASSAY:

Enzyme-linked immunosorbent assays (ELISAs) for dengue viral antibody detection have been developed during the past several years. ELISA is inexpensive and is quick and simple to perform. The detection of IgM antibody to dengue virus by ELISA has become one of the most important and useful methods for dengue diagnosis.[19]

In primary dengue infections, IgM antibodies develop rapidly and detectable on days 3 to 5 of illness in half of the hospitalized patients. Anti-dengue virus IgM, levels peak at about 2 weeks of postinfection and then decline to undetectable levels over 2 to 3 months.[46, 99,10,] anti-dengue virus IgG appears shortly afterwards. In secondary dengue infections, the kinetics of IgM production are similar to those observed in primary infections, but IgM levels are significantly lower.[46, 99,100] In contrast to primary infection, secondary infection with dengue virus results in the earlier appearance of high titers of cross-reactive IgG antibodies before or simultaneously with the IgM responses [19, 99, 100]

Table: 1 Methods used for the laboratory diagnosis of dengue viral infections [101]

Methods	Detected entity	Limit of detection	Time required
Virus isolation 1. Conventional 2. Shell vial	Viable virus Viable virus	1-10 infectious viral particle 1-10 infectious viral particle	days-weeks days
Direct detection of antigen in clinical specimen	Structure or non-structure viral antigen	1. Immunofluorescence: 1-10 infected cells 2. ELISA:10^4PFU	1-3 hours hours
Direct detection of nucleic acid 1. RT-PCR /NASBA 2. Real time RT-PCR	Viral RNA Viral	1-1000 PFU/ml 1-50 PFU/ml;1-250 copies /per assay	hours hours
Serologic diagnosis	IgG/IgM antibodies		10 min-hours for testing

A stepwise approach to the management of dengue [42]

Step I. Overall assessment

I.1 History taking including information on symptoms, past medical and family history

I.2 Physical examination which includes full physical and mental assessment

I.3 Investigations, including routine laboratory and dengue-specific laboratory

Step II. Diagnosis, assessment of disease phase and severity

Step III. Management

III.1 Disease notification

III.2 Management decisions. Depending on the clinical manifestations and other circumstances, patients are divided into 3 groups.

Patients may:

– be sent home (Group A);

– be referred for in-hospital management (Group B);

– require emergency treatment and urgent referral (Group C).

Dengue vaccines [42]

One of the dengue vaccines is a chimeric tetravalent vaccine in which the structural genes (prM and E) of each of the four dengue viruses were inserted individually to replace those of yellow fever virus in the backbone of the yellow fever 17D vaccine.[102]

Strains of the four serotypes of the dengue virus, each attenuated by passage in primary dog kidney cells and initially prepared as candidate vaccines in fetal rhesus monkey lung cells (FRhL). Each attenuated dengue virus was rederived by transfecting cells with purified viral RNA. These 2 vaccine undergoing phase 1 and 2 clinical trials in various countries.

Other two dengue vaccines under trials are Subunit vaccine based on E protein and a tetravalent replication-defective-recombinant adenovirus (cAdVaX) containing 4 serotypes of dengue.[42]

Prevention and Control of Dengue

Dengue prevention and control activities are Environmental management, Chemical control, Municipal services, Entomological Monitoring and surveillance. Regular monitoring of the delivery of these services and evaluation of the impact of interventions are important activities for effective programme management.[42]

Materials & Methods

MATERIALS & METHODS

SAMPLE COLLECTION

Blood samples from 1727 patients with clinical features suggestive of dengue, were included in this study. The samples were received from both In and Out patients attending the PSG hospitals, Coimbatore. The samples were collected aseptically and serum was separated by centrifugation technique and stored at -20˚C until processing .

STUDY PERIOD

This Cross-sectional study was done from May 2010 to June 2011.

INCLUSION CRITERIA

The clinical basis for diagnosing the patients as having dengue fever was based on standard criteria like presentation of febrile illness of 10-12 days duration, with features like headache, myalgia, arthralgia, rash, hemorrhagic manifestations and thrombocytopenia.[41,42]

EXCLUSION CRITERIA

Patients with clinical evidence of urinary tract infection, pneumonia, abscess or any other apparent cause of fever were excluded.

ETHICAL CONSIDERATIONS

This study was reviewed and approved by Institutional Ethical Committee, PSG IMS&R, Coimbatore. All data were handled confidentially and anonymously

Methods

All 1727 samples were subjected to IgM & IgG capture ELISA (PANBIO). Seventy eight serum samples which were collected less than or equal to 9 fever days were subjected to NS1 Antigen detection by PANBIO early ELISA. Two step nested RT- PCR was done for 30 NS1 Antigen positive samples by using primers designed by Robert S.Lanciotti et al.[74]

IgM Capture ELISA (PANBIO)

PRINCIPLE

Serum antibodies of the IgM antibodies, when present, combine with anti-human IgM antibodies attached to the polystyrene surface of the microwell. A Dengue 1-4 Antigens is diluted with Antigen Diluent.An equal volume of the Horse radish peroxidase (HRP) Conjugated Monoclonal Antibody (MAb) is added to the diluted antigen, which allows the formation of antigen-MAb complexes. Residual serum is removed from the wells by washing.then complexed antigen-MAb is added to the assay plate. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine / hydrogen peroxide

(TMB Chromogen) is added. The substrate is hydrolysed by the enzyme (HRP) and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. Colour development is indicative of the presence of anti-dengue IgM antibodies in the test sample.

Materials provided in the kit

1. Anti-human IgM coated microwells
2. Dengue1-4 antigens (Recombinant)
3. Wash buffer (20x)
4. Sample diluent
5. Antigen diluent
6. HRP Conjugated Monoclonal antibody tracer
7. TMB Chromogen
8. Positive Control
9. Calibrator
10. Negative Control
11. Stop solution

Materials required but not provided in the kit

1. Micropipettors with disposable pipette tips
2. Deionised water
3. Microplate washing system

4. Microplate reader with 450nm filter
5. Timer,Graduated cyclinder,Flask, test tubes & plastic tubes or vials for diluting antigen

Procedure

1. Required number of sample wells was taken along with 5 wells for 1 positive control, 1 negative control, 3 micro wells for calibrator.
2. The positive control, negative control, calibrator and serum sample were diluted with sample diluent, by adding 10 µl of each with 1000µl sample diluent in a clean glass test tube.
3. 100µl of diluted serum, positive control, negative control and calibrator were added into the respective wells.
4. The plate was covered and incubated for one hour at 37ºc ± 1ºc after incubation, the wells were washed for 6 times with diluted wash buffer.
5. Dilute the antigen 1/250 using the antigen diluents (it is recommended, as a minimum, to dilute 10µl of antigen into 2.5ml of antigen diluents)
6. The required volume of diluted antigen with equal volume of MAb tracer was mixed in a clean glass or plastic vial.
7. 100µl of antigen-MAb tracer solution was added into respective wells.

8. The plate was covered and incubated for one hour at 37ºc ± 1ºc after incubation the wells were washed for 6 times with diluted wash buffer.
9. 100µl TMB chromogen was added into each well
10. The wells were incubated for 10 minutes at room temperature (20-25ºc). A blue colour was developed.100µl of stop solution was added into each well. The individual wells were mixed well. The blue color will change to yellow color.
11. Within 30 minutes the absorbance of each well was read at a wave length of 450nm with a reference filter of 600-650nm

CALCULATIONS

1. Calculate the average absorbance of the triplicates of the Calibrator and multiply by the calibration factor. This is the Cut-off Value.

   (IMPORTANT NOTE: The calibration factor is batch specific and is detailed in the specification sheet. calibration factor value should obtain before commencing calculations)

2. An index value can be calculated by dividing the sample absorbance by the Cut-off Value (calculated in step (1) above).

Alternatively,

3. Panbio Units can be calculated by multiplying the index value (calculated in step (2) above) by 10.

$$\text{Index Value} = \frac{\text{Sample Absorbance}}{\text{Cut} - \text{off Value}}$$

INDEX	PANBIO UNITS	RESULT
<0.9	<9	Negative
0.9 – 1.1	9-11	Equivocal
>1.1	>11	Positive

Dengue IgG capture ELISA(panbio)

PRINCIPLE

Serum antibodies of the IgG antibodies, when present, combine with anti-human IgG antibodies attached to the polystyrene surface of the microwell. A Dengue 1-4 Antigens is diluted with Antigen Diluent.An equal volume of the horse radish peroxidase (HRP) Conjugated Monoclonal Antibody (MAb) is added to the diluted antigen, which allows the formation of antigen-MAb complexes. Residual serum is removed from the wells by washing. Then complexed antigen-MAb is added to the assay plate. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine / hydrogen peroxide (TMB Chromogen) is added. The substrate is hydrolysed by the enzyme (HRP) if present, and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB becomes yellow. Colour development is indicative of the presence of anti-dengue IgG antibodies in the test sample.

Materials provided in the kit

Same as in the IgM capture ELISA (PANBIO) except

1. Anti-human IgG coated microwells

Materials required but not provided in the kit

Same as in the IgM capture ELISA (panbio).

Procedure

1. Required number of sample wells was taken along with 5 wells for 1 positive control, 1 negative control, 3 micro wells for calibrator.
2. The positive control, negative control, calibrator and serum sample were diluted with sample diluent, by adding 10 µl of each with 1000µl sample diluent in clean glass test tubes.
3. 100µl of diluted serum, positive control, negative control and calibrator were added into the respective wells.
4. The plate was covered and incubated for one hour at 37ºc ± 1ºc after incubation, the wells were washed for 6 times with diluted wash buffer.
5. Dilute the antigen 1/250 using the antigen diluents (it is recommended, as a minimum, to dilute 10µl of antigen into 2.5ml of antigen diluents)
6. The required volume of diluted antigen with equal volume of MAb tracer was mixed in a clean glass or plastic vial.

7. 100µl of antigen-MAb tracer solution was added into respective wells.
8. The plate was covered and incubated for one hour at 37ºc± 1ºc.after incubation, the wells were washed for 6 times with diluted wash buffer.
9. 100µl TMB chromogen was added into each well
10. The wells were incubated for 10 minutes at room temperature (20-25ºc). A blue colour was developed.100µl of stop solution was added into each well. The individual wells were mixed well. The blue color will change to yellow color.
11. Within 30 minutes the absorbance of each well was read at a wave length of 450nm with a reference filter of 600-650nm.

CALCULATIONS

1. Calculate the average absorbance of the triplicates of the Calibrator and multiply by the calibration factor. This is the Cut-off Value.

   (IMPORTANT NOTE: The calibration factor is batch specific and is detailed in the specification sheet. Obtain the calibration factor value before commencing calculations)
2. An index value can be calculated by dividing the sample absorbance by the Cut-off Value (calculated in step (1) above).

Alternatively,

3. Panbio Units can be calculated by multiplying the index value (calculated in step (2) above) by 10.

$$\text{Index Value} = \frac{\text{Sample Absorbance}}{\text{Cut} - \text{off Value}}$$

INDEX	PANBIO UNITS	RESULT
<1.8	<18	Negative
1.8-2.2	18-22	Equivocal
>2.2	>22	Positive

Dengue NS1 antigen detection-Panbio Dengue EARLY ELISA kit

PRINCIPLE

Serum dengue NS1 antigen, when present, binds to anti-NS1 antibodies attached to the polystyrene surface of the microwell. Residual serum is removed from the wells by washing, and horse radish peroxidase (HRP) Conjugated Anti-NS1 Monoclonal Antibody (MAb) is added. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine / hydrogen peroxide (TMB Chromogen) is added. The substrate is hydrolysed by the enzyme (HRP) and the chromogen changes to a blue colour. After stopping the reaction with

acid, the TMB becomes yellow. Colour development is indicative of the presence of dengue NS1 antigen in the test sample.

Materials provided in the kit

1. Anti-NS1 antibody coated micro wells
2. HRP conjugated anti-NS1 MAb
3. Wash buffer(20x)
4. Sample diluents
5. TMB Chromogen
6. Positive control
7. Calibrator
8. Negative control
9. Stop solution

Materials required but not provided in the kit

1. Micropipettors with disposable pipette tips
2. Deionised water
3. Micro plate washing system
4. Micro plate reader with 450nm filter
5. Timer
6. Graduated cylinder
7. Flask, test tubes & plastic tubes or vials for dilutions

Procedure

1. Required number of sample wells was taken along with 5 microwells for 1 positive control, 1 negative control, 3 for calibrator
2. The positive control, negative control, calibrator and serum sample were diluted with sample diluent, by adding 75µl of each with 75µl sample diluent
3. 100µ1 of diluted serum, positive control, negative control and calibrator were added into the respective wells.
4. Plate was covered and incubated for 1 hour at 37ºc ± 1ºc .after incubation,the micro wells were washed for 6 times by using diluted wash buffer
5. 100µl of HRP conjugated anti-NS1 MAb solution was added into respective wells.
6. Again the plate was covered and incubated for 1 hour at 37ºc ± 1ºc. after incubation the micro wells were washed for 6 times by using diluted wash buffer.
7. Then 100µl of TMB was added into each well.
8. The micro wells were incubated at room temperature (20-25º) for 10 minutes. A blue color will develop.Then100µl of stop solution was added into each well. Mixed it well. The blue color will changed to yellow.

9. Within 30 minutes absorbance of each well were read at a wavelength of 450nm with a reference filter of 600-650nm.

CALCULATIONS

1. Calculate the average absorbance of the triplicates of the Calibrator and multiply by the calibration factor. This is the Cut-off Value.

   (IMPORTANT NOTE: The calibration factor is batch specific and is detailed in the specification sheet. Obtain the calibration factor value before commencing calculations)

2. An index value can be calculated by dividing the sample absorbance by the Cut-off Value (calculated in step (1) above).

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3. Panbio Units can be calculated by multiplying the index value (calculated in step (2) above) by 10.

$$\text{Index Value} = \frac{\text{Sample Absorbance}}{\text{Cut} - \text{off Value}}$$

INDEX	PANBIO UNITS	RESULT
<0.9	<9	Negative
0.9 – 1.1	9-11	Equivocal
>1.1	>11	Positive

Two step nested RT-PCR

Principle

Extracted Dengue viral RNA from the serum sample was converted to cDNA by using reverse primer D2 and reverse transcriptase enzyme. Then cDNA was amplified by using universal dengue viral D1and D2 primers. Identification of the four serotypes was achieved by nested amplification of a primary product generated with D1 & D2 (74)

The expected size of the RT-PCR products is 511 bp by using universal D1 and D2 dengue viral primers. Expected size of the amplicons for each dengue serotypes are,

482-bp (D1 and TS1 for dengue serotype - 1)

119-bp (D1 and TS2 for dengue serotype - 2)

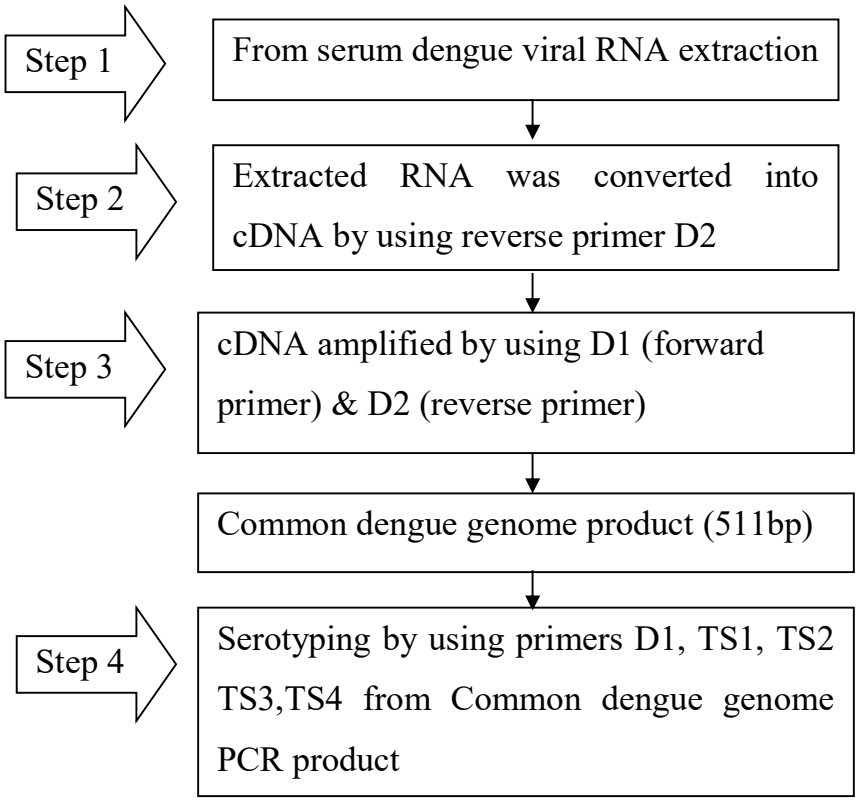
290 bp (D1 and TS3 for dengue serotype - 3)

392-bp (D1 and TS4 for dengue serotype - 4)

Table: 2 Primers used in the present study

D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'
TS1	5'-CGTCTCAGTGATCCGGGGG-3'
TS2	5'-CGCCACAAGGGCCATGAACAG-3'
TS3	5'-TAACATCATCATGAGACAGAGC-3'
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'

Steps in two step nested RT-PCR



STEP 1: Viral RNA extraction procedure (invitrogen Pure Link Viral RNA kit)

Materials Needed

- Cell-free samples (such as plasma or serum samples)
- Heat block set to 56ºC
- 96-100% ethanol
- Sterile 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge capable of centrifuging >10,000 rpm

Components supplied with the kit

- Wash Buffer (W5)
- Sterile, RNase-free Water (E3)
- Viral Spin Column in Collection Tubes
- Wash Tubes and Recovery Tubes
- Appropriate amount of Lysis Buffer (L22)

Preparation of Lysate

1. In a sterile micro centrifuge tube 25 µl Proteinase K (included with the kit) was added.
2. Then 200 µl of serum sample (equilibrated to room temperature) was added into the micro centrifuge tube.
3. Add 200 µl Lysis Buffer (containing 5.6 µg Carrie RNA). Close the tube lid and mixed it by vortexing for 15 seconds.
4. Then the tube was incubated at 56ºC for 15 minutes.
5. Briefly centrifuged the tube to remove any drops from the inside of the lid.
6. Proceeded immediately to Binding and Washing Step,

Binding and Washing Step

1. 250 µl 96-100% ethanol was added to the lysate tube to obtain a final ethanol concentration of 37%, and then the lid was closed. It was mixed by vortexing for 15 seconds.

2. The lysate was incubated with ethanol for 5 minutes at room temperature.
3. The tube was briefly centrifuged to remove any drops from the inside of the lid.
4. The above lysate was transferred with ethanol (~675 µl) onto the Viral Spin Column.
5. The lid was closed and centrifuged the column at ~6,800rpm. The collection tube was discarded with the flow through.
6. The spin column was placed in a clean Wash Tube (2 ml) included with the kit and 500 µl Wash Buffer (W5) with ethanol was added to the spin column.
7. The lid was closed and the column was centrifuged at ~6,800rpm. The flow-through was discarded and the spin column placed back into the Wash Tube.
8. 500 µl Wash Buffer (W5) with ethanol was added into the spin column.
9. The lid was closed, then centrifuge at ~6,800rpm. Now the wash Tube containing the flow-through was discarded.
10. Again the spin column was placed in another clean, Wash Tube (2 ml) included with the kit.
11. The column was centrifuged at maximum speed in a micro centrifuge for 1 minute to dry the membrane completely. The wash tube was

discarded with the flow through. Immediately proceeded to the Elution Step.

Elution Step

1. The Viral Spin Column was placed in a clean 1.5-ml recovery Tube supplied with the kit.
2. 10-50 µl of Sterile, RNase-free water was added to the center of the column. The lid was closed. The tube was incubated at room temperature for 1 minute.
3. The column was centrifuged at maximum speed for 1 minute.

   Now the Recovery Tube contains purified viral nucleic acids.

   The spin column was removed and discarded.
4. The purified viral RNA was immediately used for CDNA Synthesis in STEP 2

STEP 2: CDNA Synthesis Procedure (Stratagene AffinityScript Multiple

Temperature cDNA Synthesis Kit)

Materials Required

1. Extracted dengue viral RNA in the step 1.
2. Reverse primer D2

Reagents provided in the kit

Affinity Script RT Buffer

dNTP mix

RNase Block Ribonuclease Inhibitor

AffinityScript Multiple Temperature Reverse Transcriptase enzyme

RNase-free water (provided in the kit)

Procedure:

1. 5 µl of extracted dengue viral RNA in the step 1 and 1µl of reverse primer D2 was added in a sterile PCR tube.
2. Then the reaction was incubated at 65°C for 5 minutes.
3. The reaction tube was kept at room temperature (~10 minutes) to allow the primers to anneal to the RNA.

Then the following components were added to each reaction, in order, to get a final reaction volume of 20 µl:

- µl of 10× Affinity Script RT Buffer
- 0.5 µl of dNTP mix (25 mM each dNTP)
- 0.5 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)
- 1 µl of AffinityScript Multiple Temperature RT
- 11µl of distilled water

The reaction components were mixed gently, and then the tube was placed in a temperature-controlled thermal block at 42–55°C. the reaction

was incubated for 60 minutes. The reaction was terminated by incubating at 70ºC for 15 minutes.

Step 3: Amplification procedure (SIGMA-ALDRICH ReadyMix Taq PCR Reaction Mix Kit)

Reagents provided in the kit

1. ReadyMix Taq PCR Reaction Mix
2. Water, PCR quality(provided in kit)

Reagents not provided in the kit

1. cDNA (prepared in the step 2)
2. Forward primer-D1 (5'-TCAATATGCTGAAACGCGCGAGAAACCG-3')
3. Reverse primer-D2 (5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3')

Procedure

The following reagents was taken in a sterile PCR tube

12.5µl ReadyMix Taq PCR Reagent Mix

1 µl Forward Primer

1 µl Reverse Primer

0.5 µl Template cDNA

10µl Water provided in the kit

The above mixture was subjected to PCR amplification by using Eppendorf thermocycler.

PCR cycle

Each cycle comprised of an initial denaturation at 94ºC for10 minutes and 40 cycles of denaturation at 94°C for 30 sec, annealing at 55ºC for 1min and amplification at 72º C for 2 min with a final extension at 72 ºC for 5 minutes.

Detection of PCR products by gel electrophoresis

At the end of the reactions, a 1.5% gel was prepared with agarose and the PCR products were run using the electrophoresis kit to look for amplified products.

1.5% Gel preparation

1. To 20 ml of 1X TBE buffer, 300mg of agarose was added and the contents were heated in a microwave until it formed a clear solution.
2. To this 1µl of ethidium bromide was added and the solution was poured into a trough with comb.
3. The solution was allowed to set for approximately 30 minutes.
4. Once set, the comb and the tape around the trough was removed.
5. The trough was placed in an electrophoresis tank containing 1X TBE buffer. The trough should just immerse in the buffer.
6. Loading dye was mixed with each amplified DNA separately in sterile PCR tubes.

7. 1 µl Set up 100bp DNA Ladder (GeNei) was added to lane no 1 and the lane 2, 3, 4 were loaded with 15µl of the amplified DNA products.
8. Electrodes were connected and run at 100v for 30 minutes until the loading dye was seen at 3/4th of the gel.
9. The trough was removed and gel was viewed under UV illuminator for the presence of bands.

Step 4: Serotyping of the ampilified dengue common genome

Procedure

The following reagents were taken in a sterile PCR tube

12.5µl ReadyMix Taq PCR Reagent Mix1 µl D1

Primer (5'-TCAATATGCTGAAACGCGCGAGAAACCG-3')

1 µl TS1 Primer (5'-CGTCTCAGTGATCCGGGGG-3')

1 µl TS2 Primer (5'-CGCCACAAGGGCCATGAACAG-3')

1 µl TS3 Primer (5'-TAACATCATCATGAGACAGAGC-3')

1 µl TS4 Primer (5'-CTCTGTTGTCTTAAACAAGAGA-3')

1 µl Template DNA (1 µl dengue common genome amplified DNA in step 3 was diluted in 100µl of sterile distilled water)

6.5µl Water provided in the kit

The above mixture was subjected to PCR amplification by using Eppendorf thermocycler.

PCR cycle

Each cycle comprised of an initial denaturation at 94ºC for10 minutes and 40 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 1min and amplification at 72º C for 2 min with a final extension at 72 ºC for 5 minutes.

Detection of PCR products by gel electrophoresis

1. At the end of the reactions, a 1.5% gel was prepared with agarose and the serotype specific PCR products were run using the electrophoresis kit to look for amplified product.
2. 1 µl Set up 100bp DNA Ladder (GeNei) was added to lane no 1 and the lane 2,3,4 were loaded with 15µl of the serotype specific amplified DNA products.

Results

RESULTS

A total of 1727 serum samples were tested for dengue IgM & IgG antibodies by using dengue IgM & IgG capture ELISA. A result of Seven hundred and six samples (706) was found to be positive for dengue antibodies (fig-5). Dengue seropositivity was 41%.

Out of 706 positive Dengue patients, 209 (29.60%) had Primary dengue (positive IgM antibody) and 497 (70.39 %) had Secondary dengue (positive either for both IgM&IgG or IgG alone) viral infections. This study shows that the number of secondary dengue infections were much higher than primary dengue infections (fig-6).

Fig-5: DENGUE SEROLOGY

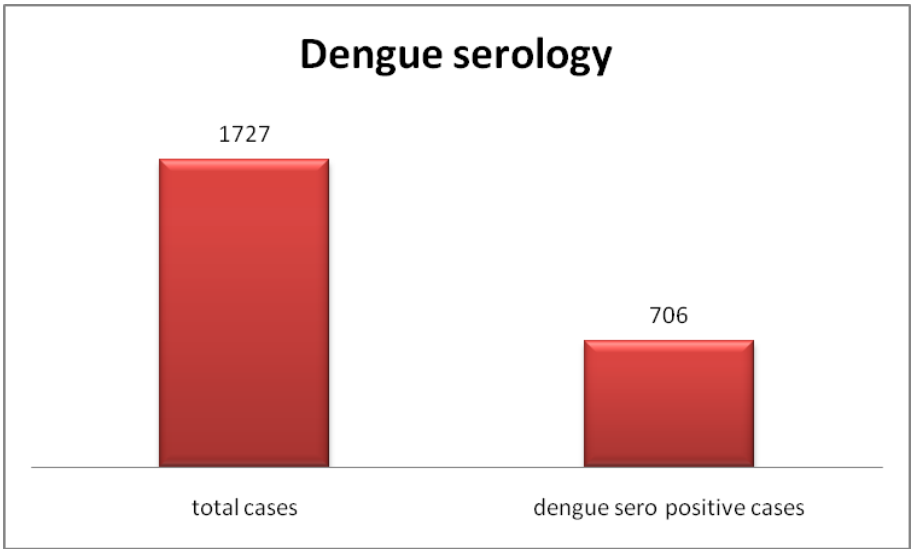
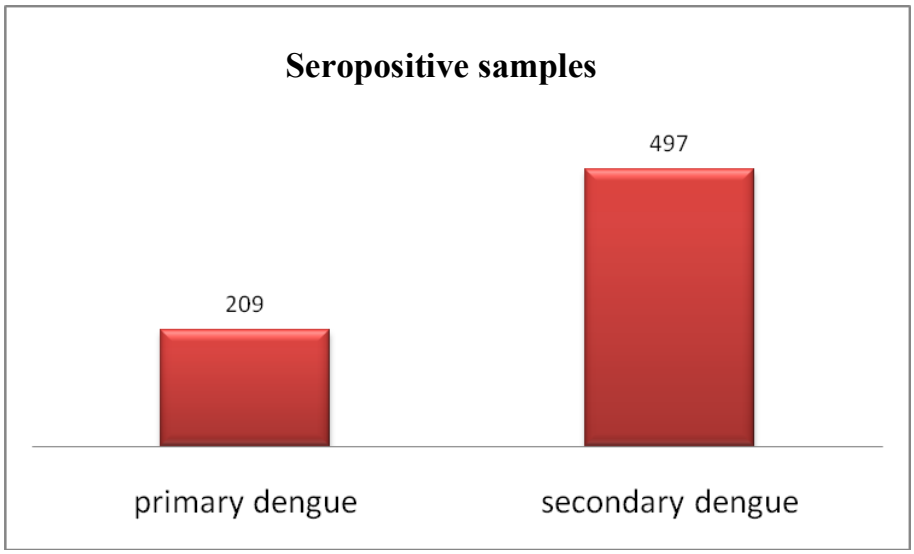


Fig- 6: PRIMARY & SECONDARY DENGUE INFECTIONS



Among the seropositive cases, 388 were males and 318 were females with a male female ratio of 1.2: 0.97 as shown in fig-7.

Fig-7: MALE-FEMALE RATIO OF DENGUE SEROPOSITIVE CASES

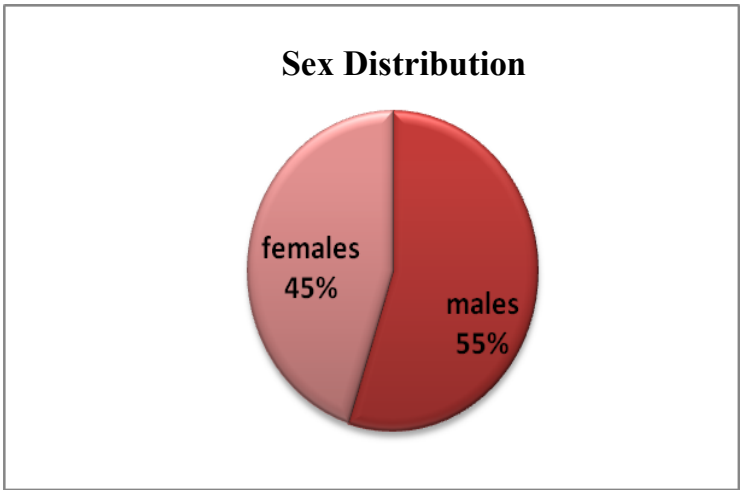
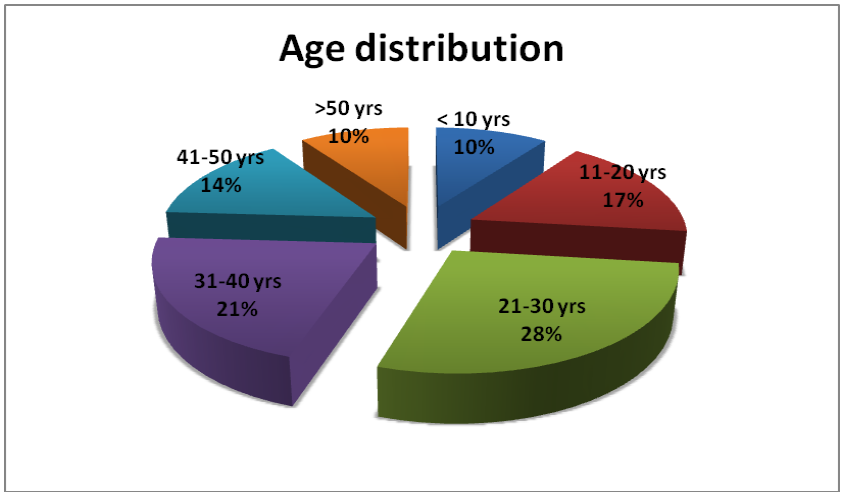
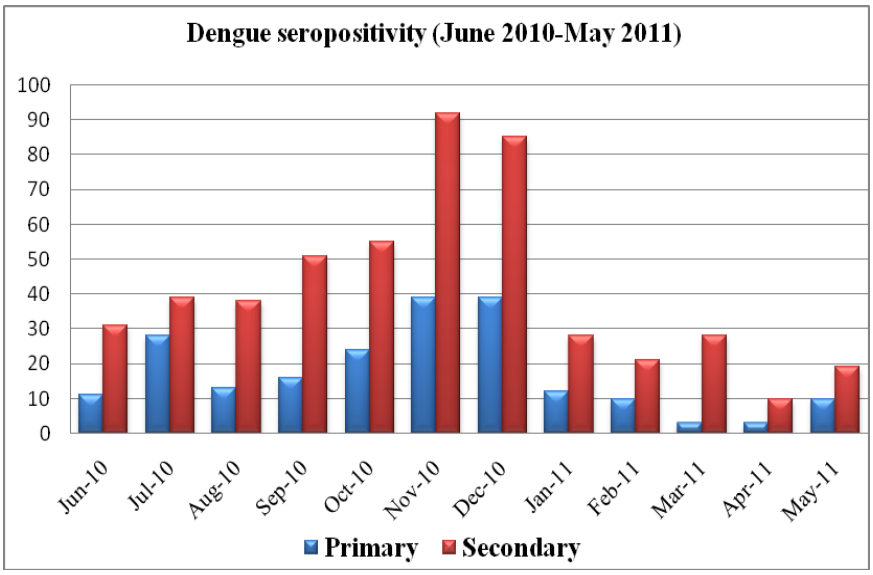


Fig-8: AGE DISTRIBUTION OF DENGUE SEROPOSITIVE CASES



As shown in (fig-8) most commonly affected dengue patients were seen in age group 21-30 yrs (28.36%) followed by 31-40 yrs (21%).

Fig-9: DENGUE SEROPOSITIVE CASES (JUNE 2010 – MAY 2011)



More number of cases was observed during November and December (36%) as shown in fig-9.

RESULTS OF NS1 antigen ELISA:

From 78 patients who had ≤ 9 days fever, serum samples were collected for NS1 antigen detection. NS1 antigen detection was done by using PANBIO early ELISA kit. Clinical data was collected for NS1 antigen positive patients. Results of NS1 antigen are shown in Table:2.

Table: 2 Results of the NS1 antigen ELISA test

	Primary dengue	Secondary dengue	Negative for IgG / IgM Antibodies	Total
Number of samples tested	22	49	7	78
NS1 antigen positive samples (%)	17 (77)	20 (41)	2 (29)	39 (50)

The study shows that NS1 antigen was found in 39 (50%) patients out of 78 patients, 17(77%) out of 22 primary dengue and 20(41%) out of 49 secondary dengue patients had NS1 antigen positivity.NS1 antigen was also positive in 2(29%) out of 7 antibody negative patients.

TWO STEP NESTED RT-PCR RESULTS:

Thirty NS1 antigen positive serum samples were used for two step nested RT-PCR for detecting the dengue viral RNA. Dengue serotype-2 RNA was found positive in three samples.

Table-3: NESTED RT-PCR RESULTS

Samples tested for dengue PCR	Two step nested RT-PCR positive	NS 1 antigen positivity	IgM antibody positive	IgG antibody positive
30	3	3	2	nil

As shown in table-3 dengue RNA was found in 2 IgM positive plus NS1 antigen positive and 1 antibody negative plus NS1 antigen positive serum samples. Common clinical features and hemorrhagic manifestations collected for NS1 antigen positive dengue patients.

Fig-10: Common Clinical features in NS1 antigen positive Dengue Patients

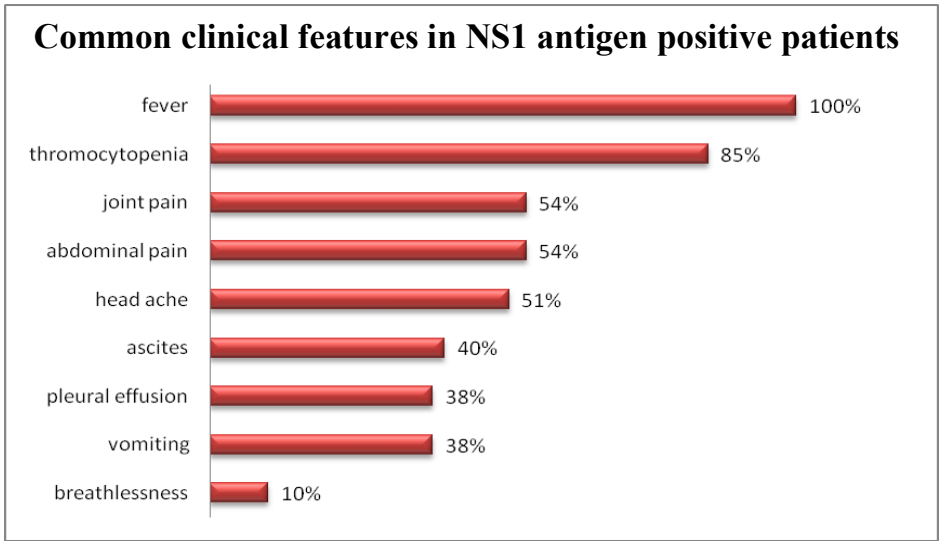


Table-4: Common clinical features in NS1 antigen positive dengue patients

Common clinical manifestation	Primary dengue (17)	Secondary dengue (20)
fever	17 (100)	20(100)
Thrombocytopenia (<1,50,000/cumm)	14 (82.35%)	17(85%)
Head ache	8 (47.05%)	10 (50%)
vomiting	6 (35%)	8 (40%)
Abdominal pain	9 (52%)	12 (60%)
Joint pain	6 (35%)	14 (70%)
breathlessness	1(5%)	3(15%)
Ascites	6 (35%)	9(45%)
Pleural effusion	7(41%)	10(50%)
elevated liver enzyme	3(17%)	9(45%)

(Note: NS1 antigen positive but IgM and IgG antibodies negative samples were excluded from this table)

This case study shows that Common clinical feature in NS1 antigen positive patients was fever (100%) and thrombocytopenia (85%).

Thrombocytopenia (< 1,50,000/cumm) seen in both primary(82.35%) and secondary dengue infections (85%).

Another significant finding of this present study is suggestive of plasma leakage like ascites (40%) and pleural effusion (38%) also present in primary dengue cases.

Table-5: Hemorrhagic Manifestations seen in NS1 Antigen Positive Patients

Hemorrhagic manifestation	Primary dengue (17)	Secondary dengue (20)
Petechiae	10 (59%)	15 (75%)
Bleeding gums	2 (12%)	8 (40%)
malena	10 (59%)	15 (75%)
hematuria	nil	2 (10%)

Hemorrhagic manifestations like Petechiae, malena and bleeding gums also present in primary dengue patients.

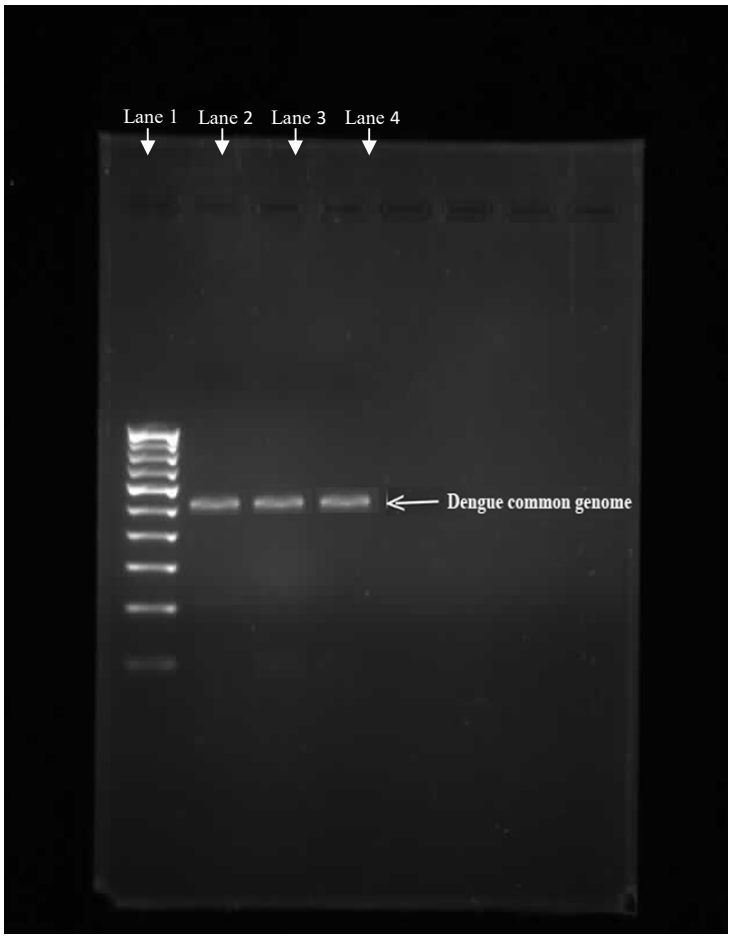
Table-6: Categorization of dengue cases in NS1 antigen positive cases[41]

Category of dengue	No of cases (39)	Percentage (%)
Dengue fever	30	77
Dengue hemorrhagic fever	6	15
Dengue shock syndrome	3	8

Table-7: Results of Two step NESTED RT-PCR positive dengue cases

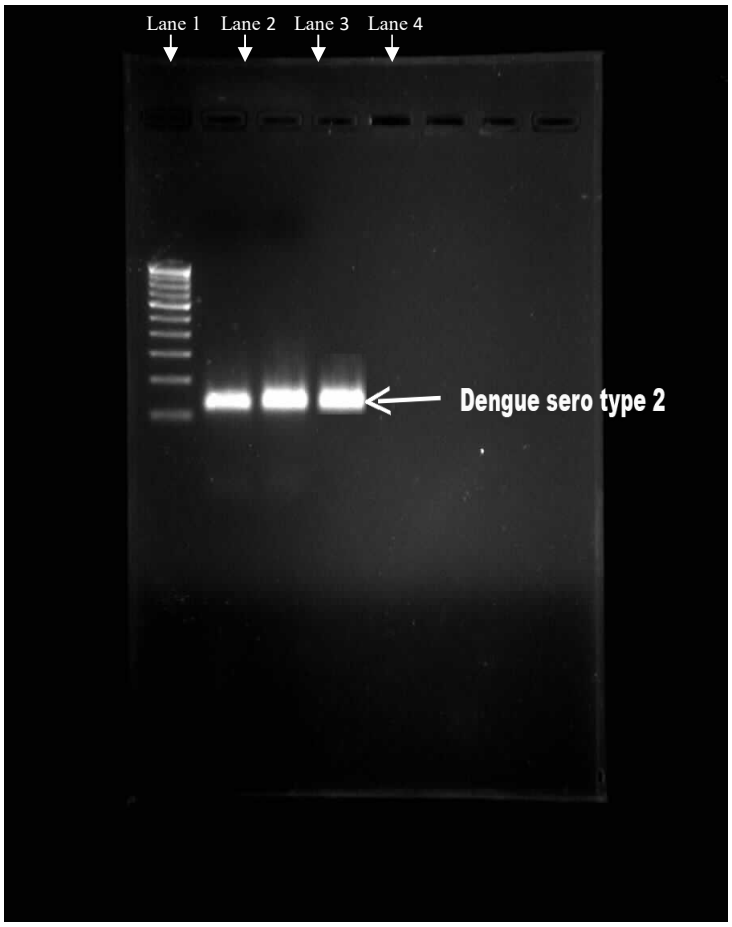
Age /sex	Fever duration	NS1 antigen	Antibody status	Other findings
44/f	3 days	positive	Antibody negative	Head ache, vomiting, thrombocytopenia
24/m	5 days	positive	IgM Antibody positive	Fever, Petechiae , joint pain, thrombocytopenia
32/m	6 days	positive	IgM Antibody positive	Fever, head ache, bleeding gums, thrombocytopenia

Fig-11: Dengue Common Genome



- Lane 1 shows 100 bp DNA ladder
- Lane 2, 3, 4 shows 511 bp of dengue common genome

Fig-12: Dengue Sero Type 2



- Lane 1 shows 100 bp DNA ladder
- Lane 2, 3, 4 shows 119 bp of dengue sero type 2

Fig-13: Dengue antibody and NS1 antigen ELISA micro title plates

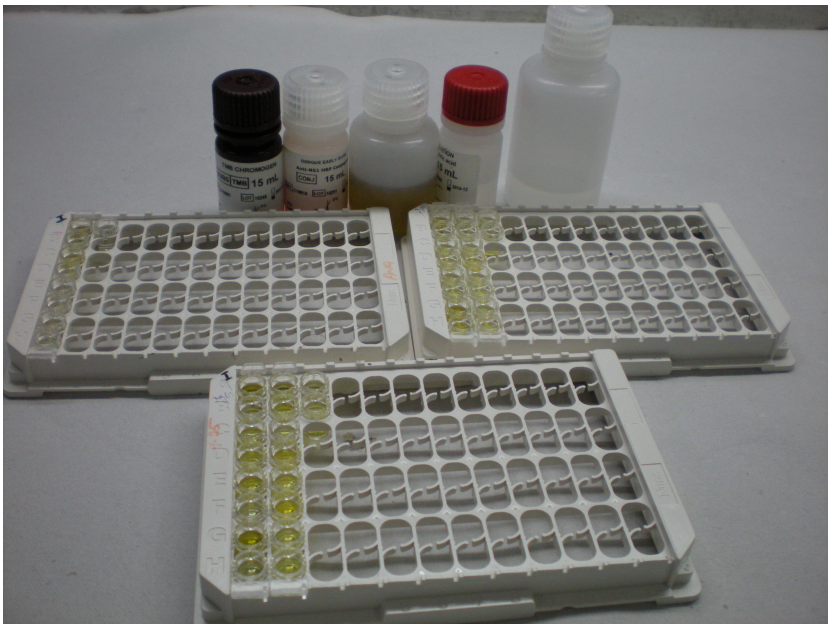


Fig-14: Dengue RNA extraction Kit

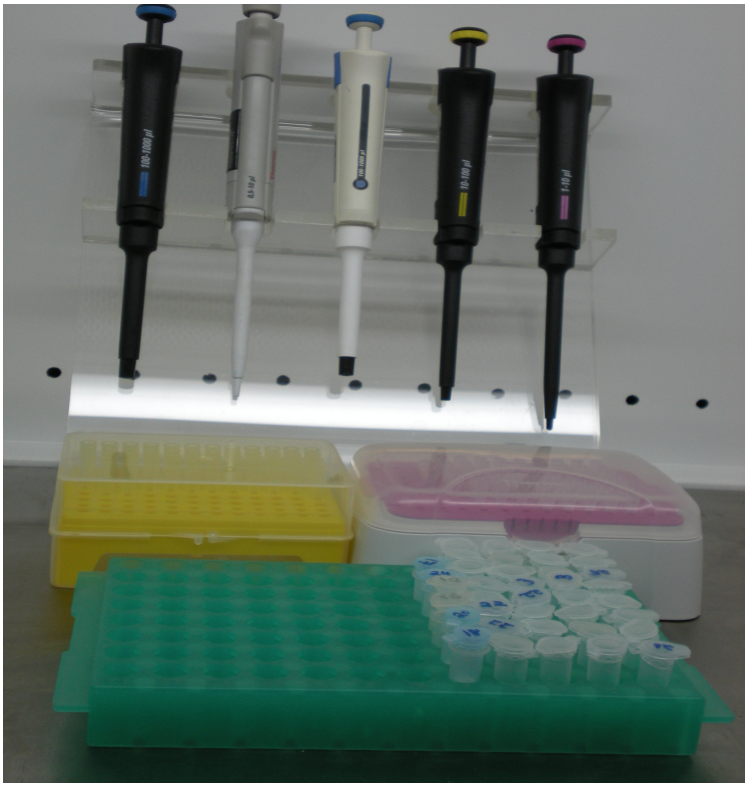


Fig-15: Thermocycler

Fig-16:Gel Documentation System

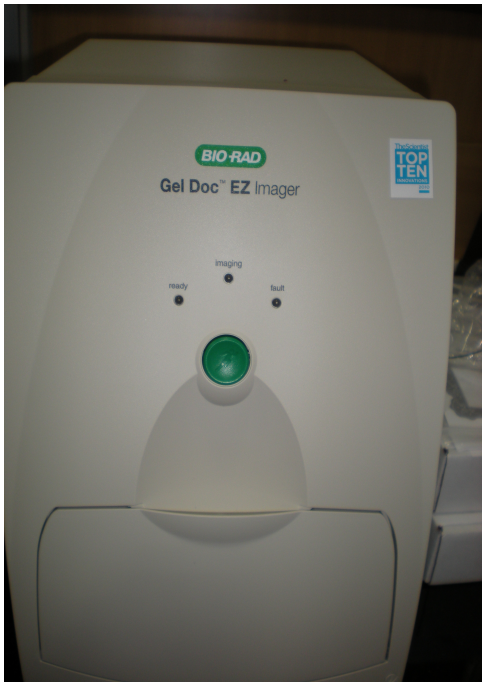
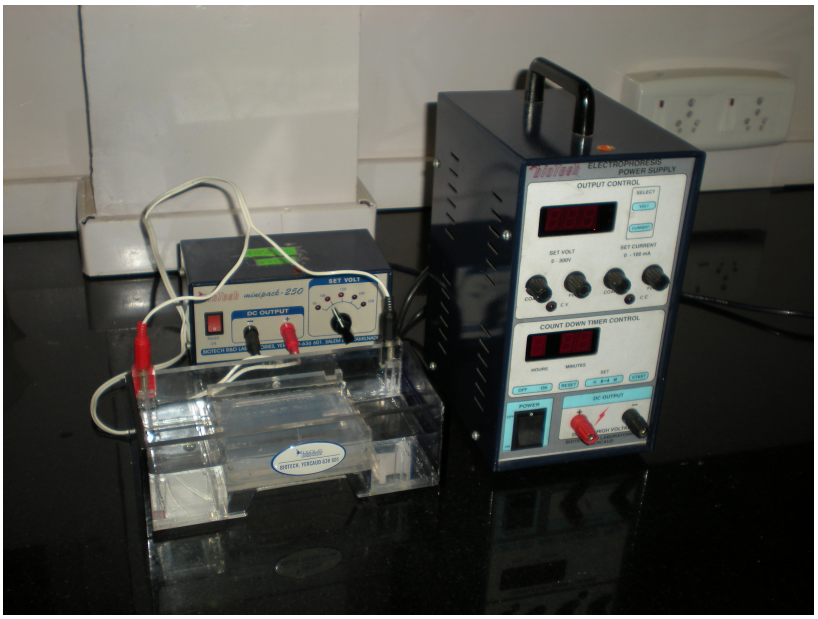


Fig-17: Electophoresis Kit



Discussion

DISCUSSION

Dengue is one of the major mosquito borne viral disease currently transmitted in more than 100 countries. The WHO has estimated that 50-100 million cases of dengue viral infections occur yearly, with more than 500,000 cases require hospitalization and more than 15,000 deaths.[103]

There is also increase in dengue incidence all over India in past few years. Early diagnosis of dengue viral infections reduces morbidity. Dengue antibody detection in febrile patients is a valuable tool for early diagnosis.

A study conducted by king institute, Chennai[104] during 2006-2008 showed 43% dengue seropositivity and a 16.47% belonged to secondary dengue infections. A study conducted by Atul Garg et al[105] between 2006-2010 maximum seropositive cases were observed during 2010 and 92% were secondary dengue infections serepositivity in their study during 2010 was 46.5%.In our study seropositivity was 41% with 29.60% primary dengue and 70.39% Secondary dengue cases .

Detection of dengue IgM antibodies among the suspected cases throughout the year indicates active dengue virus activity suggesting endemicity. Increase number of secondary infections especially in a

country like ours where multiple serotypes are prevalent raises concern over probable increase in the incidence of the more serious DHF/DSS.

Male-to-female ratio in our study group was 1.2:0.97 which is concurrent with other studies.[10,108,109] Commonly affected age group by dengue viral infections were 21-30yrs (28.36%) and 31-40 yrs (21%) followed by 11-20 yrs (17%)and <10 yrs (10%). This was similar to the study conducted by Preeti Bharaj *et al*[106] in 2008, in which the common age group involved was 20-40 years (35.4%), followed by 0-20 years group (20.8%). Ekta gupta[13] *et al*, in 2006,in her work also showed that the maximum number of cases in a 3 year study period was seen in the 21-40 years age group.

Numbers of dengue cases are more during monsoon and post monsoon season. Tamilnadu receives rainfall from North East monsoon during October to December.In our study period as shown in fig-9 dengue cases started increasing during September and October followed by sudden rise in number of cases during November and December (36%).

In a Laboratory based study on dengue fever surveillance conducted by John Victor[107] *et al* in 2007, the data on month wise incidence of dengue in Tamilnadu for the past nine years, revealed that the number of cases increased from June to December. During 2005-2006

PM Ukey [108]*et al* in their study period in Nagpur observed that dengue cases occurring during September to November.

This finding confirms that the active transmission period of dengue corresponds to the heavy rainfalls during monsoon followed by a post monsoon period. During this period mosquito breeding was found to be more. It may be because this season is very favorable for high breeding of the vector, i.e., *Aedes aegypti*. This seasonal outbreak of dengue transmission is very important at local level for effective control measures.

The hexameric form of dengue virus NS1 protein was found to be circulating in the sera of patients during the acute phase of the illness. It has both group specific and type specific determinants[83].The NS1 antigen capture ELISA has been shown to be useful in determining dengue infection in acute phase sera during both primary and secondary dengue infections, although the sensitivity of detection is higher in primary infections. [92,110]

Hua xu[87]*et al* showed in their study that NS1 antigen was detectable from first day up to day 18 after onset of symptoms, with a peak at days 6 to 10 in the dengue fever patients.

Lapphra *et al* [111]in their study showed that dengue NS1 antigen negative in patients with Japanese encephalitis and Yellow fever infections. It implies that there is no cross-reaction of dengue NS1 antigen with other flaviviruses.

A study conducted by Lima MdRQ *et al* [112]in Brazil showed Panbio early ELISA NS1 antigen kit showed sensitivity of 72.3% and a specificity of 100%.

In our study NS1 antigen positivity was found in 39 (50%) patients out of 78 patients studied.NS1 antigen was also positive in 2 out of 7 (29%) antibody negative patients. these serum samples were collected from patients who had fever days ≤ 9 days

S Datta and C Wattal [113]found 71.42% NS1 antigen positivity among the samples that were collected ≤ 5 fever days in their study.

Studies revealed that the detection rate of NS1 antigen is higher in acute primary dengue than in acute secondary dengue infection.[92, 94, 95].A possible explanation for reduced NS1 antigen sensitivity during secondary dengue infection is in the presence of anti-DENV antibody, plasma NS1 antigen sequestered in immune complexes and the target epitopes are not accessible for its detection. However, Alcon[88] *et al* showed NS1 antigen detection rate in primary dengue patients and

secondary dengue patients were almost similar when samples were collected during acute phase of the illness.

In our study common clinical features and hemorrhagic manifestations were analyzed for NS1 antigen positive patients. Following clinical features were commonly observed fever (100%),thrombocytopenia (85%), abdominal pain(54%), Petechiae(69%),malena(64%)jointpain(54%),headache(51%),Ascites(40 %),Pleuraleffusion(38%), vomiting (38%),elevated liver enzymes (31%) and Bleeding gums (26%).

In a study conducted by Vinod H. Ratageri[114] *et al* (2003-2004) the common clinical features noted in order of frequency were fever (100%), Thrombocytopenia (82%) vomiting (82%), pain abdomen (61%), restlessness (65%), headache (22%) and hepatomegaly (87%).The common bleeding manifestations were GI bleeding (22%) and petechiae (18%). Pleural effusion was present in 70% cases and 54% showed ascites. These results were almost similar to our present study.

According to the WHO guidelines[41], 39 dengue NS1 antigen positive cases in the present study were categorized into dengue fever 30(77%), dengue hemorrhagic fever 6(15%) and dengue shock syndrome 3(8%).Similar results observed in a study conducted in Karnataka [118]

where 82.8% patients had dengue fever, 9.8% had dengue hemorrhagic fever and 7.3% had dengue shock syndrome.

Using the primers designed by Lanciotti RS[74] *et al* we were able to detect dengue viral genome of DENV-2 serotype, in 3 out of 30 serum samples which showed NS1 antigen positivity. Using the same primers Paramasivan R[115] et al during 2007 in Madurai, found DENV -3 serotype in 2 out of 25 samples and Ratho RK[116] *et al* in Chandigarh, detected DENV-2 serotype in patient samples.

During 2006 Preeti Bharaj[106] *et al* in New Delhi amplified dengue viral genome in 48 samples out of 69 serum samples. All these serum samples were collected from patients with < 7 days history of fever.

During 2003-2005 Ekta gupta[13] *et al* in New Delhi amplified dengue viral genome in 27 serum samples. All these serum samples were collected from < 5 fever days patients.

Velathanthiri[117] *et al* during 1999-2001 in Srilanka, performed dengue RT-PCR in 226 patients. Dengue viral RNA was detected in 26 patients. All these patients had duration of fever days between 3-5 days. DENV–2 was predominant serotype was isolated.

In our study, dengue serotype-2 was detected in three samples with history of fever days between 3 - 6 days as shown in (tab: 7).This could

be due to the fact that, viremia declines soon after the production of antibodies. There is also a possibility that patients reporting to tertiary care hospital usually present at the late febrile period. The time by which the chances of detection of dengue viral RNA is very low. Hence, much earlier samples, very soon after fever occurs, should be collected and subjected for dengue RT-PCR for detection of the dengue viral genome and for serotyping studies.

Summary

SUMMARY

- Between June 2010 to May 2011, a total of 1,727 serum samples from equal number of patients, suspected dengue fever patients were tested for IgM and IgG antibodies using dengue capture ELISA.
- Seven hundred and six (41%) of patients were found to have dengue antibodies and 29.60% were primary dengue and 70.39 % were secondary dengue.
- Males were affected more than females in this study. Male-to-female ratio in our study group was 1.2:0.97.
- Commonly affected age group by dengue viral infections were 21-30yrs (28.36%) followed by 31-40 yrs (21%).
- More number of cases was observed during November and December (36%) corresponds to monsoon and post monsoon period.
- NS1 antigen positivity was found in 39(50%) out of 78 patients with ≤ 9 fever days.
- Seventeen(77%) out of 22 primary dengue and 20 (41%) out of 49 secondary dengue patients had NS1 antigen positivity NS1 anti-gen detection was more in primary cases than secondary dengue cases.

- NS1 antigen was also positive in 2 out of 7 (29%) antibody negative patients.
- NS1 antigen yield was more when samples were tested in the earlier phase of fever.
- According to the WHO guidelines, 39 dengue NS1 antigen positive cases in the present study were categorized into dengue fever 30(77%), dengue hemorrhagic fever 6(15%) and dengue shock syndrome 3(8%).
- The most Common clinical feature in NS1 antigen and sero positive febrile patients was thrombocytopenia (85%) followed by joint pain (54%)
- Dengue PCR was positive in 3 out of 30 patients with 3-6 fever days. The PCR positive samples were also positive for NS1 antigen.
- The PCR detected dengue virus belongs to serotype-2.

Conclusion

CONCLUSION

Early diagnosis of dengue fever leads to better management, less morbidity and mortality. There are more secondary dengue infections than primary dengue infections indicating circulation of more than one serotype. It was more common in males and in the age group 21-30yrs and the disease was found to be more common during November and December correlating with high mosquito breeding.NS1 antigen and PCR will be useful when samples were collected very early during the course of the fever. The prevalent serotype of dengue in this area was found to be dengue serotype-2.

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ABBREVIATIONS

BHK21	-	Hamster kidney cells
cDNA	-	complementary DNA
DENV	-	Dengue virus
DF	-	Dengue Fever
DHF	-	Dengue haemorrhagic fever
dNTP	-	Deoxynucleotide Triphosphate
DSS	-	Dengue Shock Syndrome
ELISA	-	Enzyme linked immunosorbent assay
LLCMK2	-	Rhesus monkey kidney cells
MAb	-	Monoclonal antibody
NASBA	-	Nucleic acid sequence-based analysis
RNA	-	Ribonucleic acid
rpm	-	revolutions per minute
RT-PCR	-	Reverse-Transcriptase Polymerase Chain Reaction
Vero	-	African green monkey kidney cells
WHO	-	World Health Organisation

Appendix

APPENDIX

Wash buffer (1x) for IgM & IgG capture ELISA and NS1 antigen ELISA, 1 ml of 20x wash buffer (provided in the kit) diluted with 19ml of distilled water

TBE BUFFER

- Tris (89mM)
- Boric acid (89mM)
- EDTA (2mM)

ETHIDIUM BROMIDE – 10mg/ml in distilled water.

LOADING DYE

- Bromophenol blue – trace
- Sucrose – 40mg
- Distilled water – 1ml

Dissolve sucrose in distilled water and add a trace of bromophenol blue to it. Store the solution in refrigerator

Master Chart

Master Chart for NS1 Antigen Detection

S. No	Ip/op number	Age/sex	Fever duration	Other clinical features	Platelet count & liver enzymes	Ns1 antigen status	IgM antibody	IgG antibody	RT-PCR
1	I10050366	32/f	8	Bleeding gums, petechiae headache	1,50,000/cumm, Elevated liver enzymes	negative	positive	positive	negative
2	I10050295	12/f	7	Mylagia, joint pain, pleural effusion	1,50,000/cumm Elevated liver enzymes	positive	positive	negative	negative
3	I10050801	26/m	9	Hematuria, abdominal pain	1,50,000/cumm Elevated liver enzymes	positive	positive	positive	negative
4	I10051263	12/m	3	Mylagia,ascites	<1,50,000/cumm, Normal enzymes	positive	positive	negative	negative
5	I10051448	17/m	9	Headache vomiting	1,50,000/cumm Elevated liver enzymes	negative	positive	positive	negative
6	I10051559	43/m	5	Bleeding gums, petechiah headache, hypotension	<1,50,000/cumm,Elevated liver enzymes	positive	positive	positive	negative
7	O0748427	27/f	5	Headache, vomiting	<1,50,000/cumm,Elevated liver enzymes	negative	positive	positive	negative
8	I11003182	6/f	5	Headache, vomiting, Bleeding gums hypotension	<1,50,000/cumm,Elevated liver enzymes	positive	positive	negative	negative
9	I11003318	19/m	9	Vomiting, nausea	Normalplatelet, elevated liver enzymes	negative	negative	negative	negative
10	I11003282	41/m	9	Rash, joint pain, head ache	Elevated liver enzymes, <1,50,000/cumm	negative	positive	positive	negative

11	I1100356 8	4/f	6	Abdominal pain, rash, ascites	Elevated liver enzymes,< 1,50,000/ cumm	positive	positive	positive	negative
12	I1100398 1	46/m	7	Headache vomiting, joint pain	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
13	I1100398 6	15/M	8	Headache vomiting joint pain	,<1,50,000 /cumm,nor mal enzymes	negative	positive	negative	negative
14	I1100389 5	44/f	3	Headache, vomiting, joint pain	<1,50,000/ cumm.nor mal liver enzymes	positive	Negative	Negative	positive
15	I1100519 7	30/F	4	Petechiae, mylagia, pleural effusion	Elevated liver enzymes,< 1,50,000/c umm	positive	positive	negative	negative
16	I1100572 1	29/M	9	Headache, hematuria, mylagia	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
17	I1100574 7	7/F	9	Bleeding gums, joint pain,ascites	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
18	I1100603 4	37/M	8	Headache, vomiting, fever	Normalpla telet,enzy mes	positive	positive	negative	negative
19	I1100608 8	7/f	8	Head ache, breathlessn ess	<1,50,000/ cumm, normal enzymes	negative	positive	negative	negative
20	I1100575 8	47/m	6	Bleeding gums, joint pain	<1,50,000/ cumm, normal enzymes	negative	positive	negative	negative
21	I1100764 3	22/f	4	Headache,j oint pain	<1,50,000/ cumm, normal enzymes	positive	positive	negative	negative
22	I1100800 6	47/m	3	Headache, hematuria, mylagia	<1,50,000/ cumm, Elevated liver enzymes	positive	positive	positive	negative
23	I1100623 7	36/m	3	Bleeding gums, joint pain	<1,50,000/ cumm, Elevated liver enzymes	positive	positive	positive	negative
24	I1100676 8	15/m	4	Headache, joint pain	<1,50,000/ cumm, normal enzymes	positive	positive	negative	negative
25	I1100670 4	40/m	7	Headache, vomiting, joint pain	Normalpla telet,enzy mes	negative	positive	negative	negative

26	I1100773 4	26/f	9	Rash, head ache, mylagia	<1,50,000/ cumm, Elevated liver enzymes	negative	positive	positive	negative
27	I1100688 1	32/m	6	Joint pain, hypo tension, pleural effusion, hematuria	<1,50,000/ cumm, Elevated liver enzymes	positive	positive	positive	negative
28	I1100773 0	11/f	5	Headache, vomiting, joint pain	Normalpla telet,enzy mes	positive	positive	negative	negative
29	I1102504 8	31/m	3	Headache ,joint pain	<1,50,000/ cumm, Elevated liver enzymes	positive	positive	negative	negative
30	O011631 6	13/f	6	Headache, joint pain	Normalpla telet,enzy mes	negative	negative	negative	negative
31	I1024295	32/m	7	Joint pain, pleural effusion	<1,50,000/ cumm, Elevated liver enzymes	positive	positive	positive	negative
33	I0104691 9	24/f	8	Mylagia, Joint pain, petechia	<1,50,000/ cumm, Elevated liver enzymes	negative	positive	positive	negative
34	O010470 3	42/f	9	Vomiting, nausea	<1,50,000/ cumm, Elevated liver enzymes	negative	negative	positive	negative
35	I1104612 0	33/f	5	Joint pain, hypo tension, pleural effusion	<1,50,000/ cumm, Elevated liver enzymes	positive	positive	positive	negative
37	I1104690 2	26/f	4	Headache, vomiting, joint pain	Normalpla telet,enzy mes	negative	positive	negative	negative
38	I1100800 6	24/m	5	Petechiae joint pain,	<1,50,000/ cumm	positive	positive	negative	positive
39	I1108007	47/m	6	Headache, vomiting, joint pain, mylagia	<1,50,000/ cumm Elevated liver enzymes	negative	positive	positive	negative
40	I1101003 8	35/m	7	joint pain, mylagia	<1,50,000/ cumm Elevated liver enzymes	positive	positive	positive	negative
41	O001077 1	28/f	8	Headache, vomiting, joint pain, mylagia	<1,50,000/ cumm, Elevated liver enzymes	negative	positive	negative	negative

42	O0010608	13/m	8	Head ache, joint pain	<1,50,000/cumm Elevated liver enzymes	positive	positive	negative	negative
43	I10009929	52/f	9	bleeding gums Ascites, pleural effusion, head ache	<1,50,000/cumm Elevated liver enzymes	positive	positive	positive	negative
44	O0105197	10m	7	Head ache, joint pain	Normalplatelet,enzymes	positive	positive	positive	negative
45	I11003182	24/f	5	joint pain, mylagia, pleural effusion	<1,50,000/cumm ,Elevated liver enzymes	positive	positive	negative	negative
46	I10045123	42/f	6	Headache, vomiting, joint pain, mylagia	<1,50,000/cumm ,Elevated liver enzymes	positive	positive	positive	negative
47	O1046329	6/f	3	Fever, vomiting	Normalplatelet,enzymes	negative	negative	negative	negative
48	I01046233	27/m	4	Fever, vomiting	Normalplatelet,enzymes	positive	positive	negative	negative
49	O0016316	55/m	9	Fever, vomiting, icterus	Elevated liver enzymes,<1,50,000/cumm	negative	negative	positive	negative
50	I11010326	23/m	9	Fever, mylagia, joint pain	<1,50,000/cumm,elevated liver enzymes	positive	positive	positive	negative
51	I11024894	49/m	9	Vomiting, icterus	3,45,000/cumm	negative	negative	positive	negative
52	I11046885	35/f	9	Fever, vomiting	<1,50,000/cumm, Normal enzymes	negative	positive	positive	negative
53	I11046614	17/f	5	bleeding gums Ascites, pleural effusion, head ache	<1,50,000/cumm,elevated liver enzymes	positive	positive	negative	negative
54	O0045789	31/f	6	Ascites, pleural effusion, head ache	<1,50,000/cumm,elevated liver enzymes	negative	positive	positive	negative
55	I11047014	37/m	8	head ache, malena	Elevated liver enzymes, <1,50,000/cumm	negative	positive	positive	negative
56	O1046320	22/m	6	head ache, bleeding gums	<1,50,000/cumm	negative	positive	negative	negative

57	I1101003 8	32/m	6	head ache, bleeding gums	<1,50,000/ cumm	positive	positive	negative	positive
58	O110349 9	48/f	7	Fever, vomiting, nausea	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
59	I1102488 0	37/f	3	head ache, bleeding gums	Elevated liver enzymes,< 1,50,000/c umm	positive	positive	positive	negative
60	O006572 3	26/f	2	Headache, vomiting	<1,50,000/ cumm	positive	positive	Negative	negative
61	I1101051 5	19/f	4	Head ache,	<1,50,000/ cumm,nor mal enzymes	negative	negative	Negative	negative
62	I1008010	26/m	7	Headache, vomiting, hematuria	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
63	O007845 2	31/m	8	Ascites, pleural effusion, head ache	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
64	I1107646	24/f	5	Head ache, vomiting	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
65	I0100553 2	40/m	4	bleeding gums Ascites, pleural effusion, head ache	<1,50,000/ cumm,nor mal enzymes	negative	positive	positive	negative
67	I0104688 5	15/m	9	Fever, vomiting, nausea	Normal liver enzymes,n ormal platelet	negative	negative	negative	negative
68	O011608 8	11/f	9	head ache, bleeding gums	Elevated liver enzymes,< 1,50,000/c umm	positive	positive	positive	negative
69	O110764 3	25/f	9	head ache, bleeding gums, pleural effusion, ascites	Elevated liver enzymes,< 1,50,000/c umm	positive	positive	positive	negative
70	I0011623 7	37/m	9	Mylagia, abdominal pain	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
71	I0112489 4	8/f	6	Ascites, pleural effusion, head ache	Elevated liver enzymes,< 1,50,000/c umm	positive	positive	negative	negative

72	I0112495 6	20/f	9	Ascites, mylagia	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
73	I0112488 0	43/m	3	pleural effusion, head ache	Elevated liver enzymes, <1,50,000/ cumm	positive	positive	positive	negative
74	I0112512 2	29/m	4	Ascites, mylagia, bleeding gums	Normal enzymes, platelet count	positive	positive	negative	negative
75	I0112487 2	23/F	5	abdominal pain, joint pain, pleural effusion	Elevated liver enzymes,< 1,50,000/c umm	negative	positive	positive	negative
76	I0111032 7	26/M	5	Headache, mylagia	2,oo,ooo/c umm	positive	negative	negative	negative
77	I0112403 3	56/f	7	Fever, vomiting, nausea	Normal liver enzymes,n ormal platelet	negative	positive	positive	negative
78	I0112504 8	36/m	9	Headache, mylagia	<1,50,000/ cumm,nor mal liver enzymes	positive	positive	positive	negative