A COMPARATIVE STUDY OF RAPID IMMUNO CHROMATOGRAPHY TEST WITH ELISA FOR DETECTION OF DENGUE NS1 ANTIGEN, IGM AND IGG ANTIBODY

Dissertation submitted to

THE TAMIL NADU DR.M. G. R MEDICAL UNIVERSITY

CHENNAI- 600032

In partial fulfillment of the requirement for the degree of

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M. D. (MICROBIOLOGY)

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

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CERTIFICATE

This is to certify that the Dissertation "**A COMPARATIVE STUDY OF RAPID IMMUNO CHROMATOGRAPHY TEST WITH ELISA FOR DETECTION OF DENGUE NS1 ANTIGEN ,IGM AND IGG ANTIBODY**" presented herein by **Dr.Nagalakshmi. R** is an original work done in the Department of Microbiology,Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D.(Branch IV) Microbiology under my guidance and supervision during the academic period of 2012-2015.

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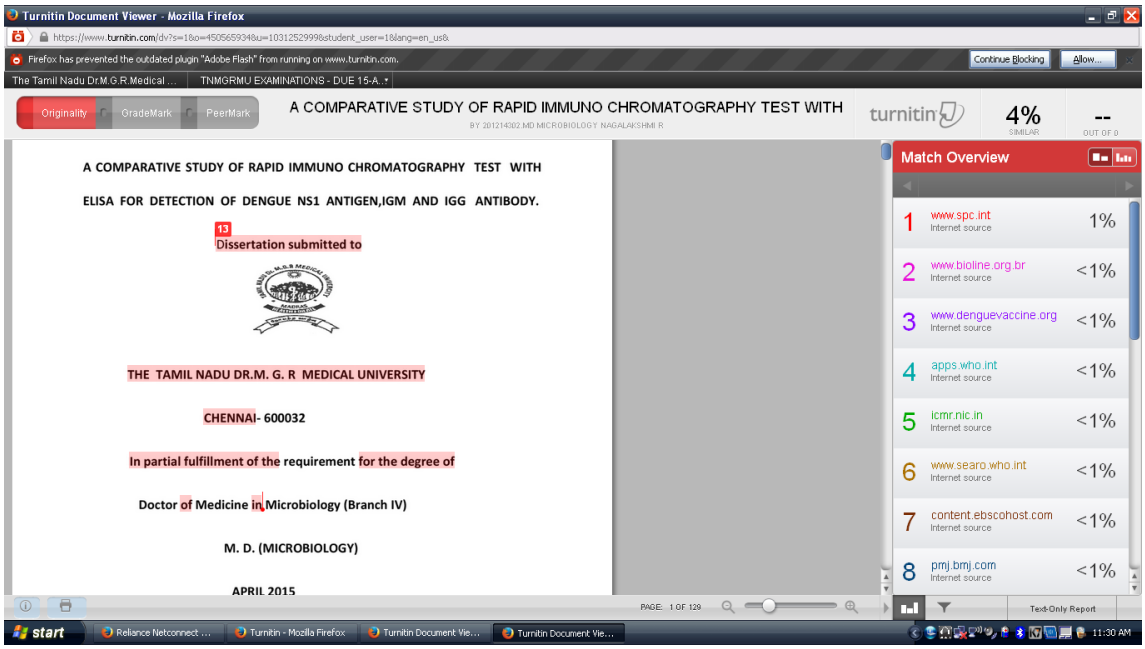
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DECLARATION

I, solemnly declare that the Dissertation titled **"A COMPARATIVE STUDY OF RAPID IMMUNO CHROMATOGRAPHY TEST WITH ELISA FOR DETECTION OF DENGUE NS1 ANTIGEN ,IGM AND IGG ANTIBODY"** is done by me at Tirunelveli Medical College Hospital, Tirunelveli .

The Dissertation is submitted to The Tamilnadu Dr. M.G.R. Medical University, towards the partial fulfillment of requirements for the awardof M.D Degree (Branch IV) in Microbiology.

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ABBREVIATIONS

CDC - Centre For Disease Control

CF - Complement Fixation

DF - Dengue Fever

DHF - Dengue Haemmorrhagic Fever

DSS - Dengue Shock Syndrome

ELISA - Enzyme linked immunosorbent assay

GAC ELISA - IgG Capture ELISA

HI - Haemagglutination Test

ICT - Imunochromatography Test

IFN - Interferon

IgA - Immunoglobulin A

IgM - Immunoglobulin M

IgG - Immunoglobulin G

IL-2 - Interleukin

MAC ELISA - IgM capture ELISA

NS1 - Non- Structural Protein 1

NT - Neutralisation Test

PRNT - Plaque Reduction Neutralization Test

RDT - Rapid Diagnostic Test

RT-PCR - Real Time Polymerase Chain Reaction

Th - T-helper

TNF - Tumor Necrosis Factor

WHO - World Health Organisation

CONTENTS

A COMPARATIVE STUDY OF RAPID IMMUNO CHROMATOGRAPHY TEST WITH ELISA FOR DETECTION OF DENGUE NS1 ANTIGEN ,IGM AND IGG ANTIBODY.

Aim

To analyse the efficacy of Immunochromatography with ELISA for detection of Dengue parameters (NS1 antigen, IgM and IgG antibodies) in Dengue suspected cases.

Materials and Methods

A total of 100 blood samples obtained from adults with fever >4 days presenting with thrombocytopaenia (<1,00,000) were taken for the study from the Department of Medicine, Government Medical College Hospital, Tirunelveli after obtaining informed written consent. The samples were tested by Rapid Immunochromatography (ICT) and ELISA methods for NS1 Ag, IgM and IgG Ab and the results were evaluated for efficacy.

Results

Out of 100 adults tested by rapid ICT, 38% were positive for Dengue infection(NS1 antigen, IgM and IgG antibodies)in the serum sample. The same samples were tested by ELISA (Positive for any one of the three parameters)the positivity was found to be 59%. 37 samples positive by ICT were positive by ELISA also. Rapid ICT had a sensitivity of 87.5%, Specificity of 98.8% for NS1 antigen when evaluated against NS1 antigen

ELISA as gold standard.Rapid ICT had a very low sensitivity of 28.6% and 47.1% and an excellent specificity of 98% and 100% for IgM and IgG antibody detection when evaluated against MAC ELISA and GAC ELISA as gold standard respectively.13.6% showed primary infection and 64.4% showed secondary infection.

Conclusion

Out of the total 100 cases studied,the rapid immunochromatography test has very less sensitivity and specificity is satisfactory .All samples should be subjected to both antigen(NS1) and antibody (IgM and IgG) testing to increase the positivity rate and to prevent the positive cases being missed. Cases with higher degrees of suspicion are to be subjected to diagnostic tests with higher sensitivity & specificity like ELISA and PCR. The commercially available rapid immunochromatographic test device can be used as a screening device during Dengue outbreaks. It should not be used as a standalone device for diagnosis of Dengue. Further molecular studies are essential to know the accurate information of Dengue serotypes which will be helpful in formulating vaccines in future.

Key words : Dengue, Immunochromatography, ELISA, NS1 Antigen, IgM and IgG Antibodies.

1.INTRODUCTION

Dengue is the most rapidly spreading mosquito-borne viral disease in tropical and subtropical regions of the world caused by the bite of Aedes mosquito [1]. Dengue virus belongs to the broad group Arboviruses, family Flaviviridae, subfamily Flavivirinae and genus Flaviviruses. Dengue virus has a positive sense, ssRNA viral genome. It is becoming a global public health emergency[1].Though disease is usually seen in endemic areas, many epidemics involving continents and pandemics have been witnessed by the world. One such epidemic was being experienced in India while this study was being performed. Pertaining to weak immunity, this disease usually affects children. But nowadays, large numbers of adult cases are also being seen.

Dengue manifests in three forms, Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). A full spectrum of disease ranging from a subclinical infection toa mild self limiting disease, the Dengue fever (DF) and a severe disease that may be fatal, the Dengue haemorrhagic fever/Dengue shock syndrome (DHF/DSS) is caused by all the four serotypes (DEN 1-4) . The WHO 2009 classification divides Dengue fever into two groups: uncomplicated and severe. Four main characteristic manifestations of Dengue illness are *(i)* continuous high fever lasting 2-7 days; *(ii)* haemorrhagic tendency as

shown by a positive tourniquet test, petechiae or epistaxis; *(iii)* thrombocytopenia and *(iv)* evidence of plasma leakage manifested by haemoconcentration , pleural effusion and ascites, *etc* .DSS being the most serious form of illness.

Some 1.8 billion (more than 70%) of the population at risk for Dengue worldwide live in member states of the WHO South-East Asia Region and Western Pacific Region, which bear nearly 75% of the current global disease burden due to Dengue. It causes high mortality during the early phases of outbreaks . The Asia Pacific Dengue Strategic Plan for both regions (2008--2015) has been prepared in discussion with member countries and development partners in response to the increasing risk from Dengue. This strategic plan aims to aid countries to reverse the rising trend of Dengue(1). Based on this, the present study is aimed to detect, characterize and contain outbreaks rapidly and to stop the spread to new areas.

In 2013, Dengue ranks the most important mosquito-borne viral disease in the world.Dengue outbreaks put forth a huge burden on populations, health systems and economies in most tropical countries of the world. The emergence and spread of all four serotypes of Dengue viruses from Asia to the Americas, Africa and the Eastern Mediterranean regions represent a global pandemic threat.

The incidence of Dengue has increased 30-fold during the past five decades. Some 50–100 million new infections are estimated to occur annually in more than 100 endemic countries, with a documented further spread to previously unaffected areas[2].The true numbers are probably far worse, since severe under reporting and misclassification of Dengue cases have been documented[3].

The Dengue virus is found in serum or plasma, circulating blood cells and selected tissues especially those of the immune system ,after the onset of illness (2 to 7 days), roughly corresponding to the period of the fever[4]. Dengue diagnosis is achieved either by virus isolation or by viral RNA identification through RT-PCR or by serological detection of Dengue specific IgM and IgG antibodies .Virus isolation and RT- PCR are time consuming and costly laboratory methods .Thus detection of Dengue antigen or antibodies is feasible for diagnosing Dengue in most cases. NS1 antigen is a hexameric form of Dengue virus protein was found circulating in the sera of patients during the acute phase of the illness. Detection of Dengue NS1 antigen indicates early Dengue infection[2].

Since viral isolation is laborious, expensive and is only available in reference laboratories, serological diagnosis remains the mainstay of diagnosis during the epidemic. Serological detection of antibodies based on

capture ELISA has become the new gold standard for the detection of Dengue virus infections[5].

Early diagnosis is useful in triaging patients and have a central role in Dengue case management and plays a vital role in forecasting an early warning of an epidemic and in undertaking effective vector control measures.

Since there is no specific antiviral therapy or immune prophylaxis available, timely and rapid diagnosis plays a vital role in patient management and implementation of control measures . Rapid assessment with a balanced reliability is of great importance in peripheral health settings where the present study was being conducted.

In this context, this study was carried out to evaluate the performance of a rapid immunochromatographic test device for the detection of IgM, IgG antibodies and NS1 antigen to Dengue infection against the serological detection of antibodies based on capture IgM ,IgG ELISA and detection of antigen by NS1 ELISA was taken as the reference standard.

2.AIM AND OBJECTIVES

- To detect Dengue specific NS1 antigen ,IgM and IgG antibodies by Enzyme Immunosorbent Assay (ELISA).
- To detect Dengue NS1 antigen ,IgM and IgG antibodies by rapid Immuno-chromatographic (IC) card test.
- Comparison of two diagnostic tests for early/rapid diagnosis of acute Dengue infection.

3. REVIEW OF LITERATURE

3.1 HISTORY

The word Dengue was derived from Swahili phrase 'Ki-dinga pepo', meaning "cramp-like seizure caused by an evil spirit'. In Spanish ,"Dengue" means fastidious or careful, which describes the gait of a person suffering bone pain due to Dengue fever .The disease was also known as "Dandy fever" in slaves of West Indies. The first case of probable Dengue fever was recorded in a Chinese medical encyclopedia from the Jin Dynasty (265 – 420 AD) which referred to a "water poison" associated with flying insects[6].The term 'break bone fever' was coined during the Philadelphia epidemic in 1780, by Benjamin Rush because of the symptoms of myalgia& arthralgia[7].

The viral etiology and the transmission by the mosquitoes were deciphered in the 20th century. During 1944 to 1956, it was shown that same clinical syndrome was caused by four distinct types of viruses. DEN1 was first isolated from Hawaii in 1944, DEN2 from New Guinea also in 1944, DEN3 & 4 from the Philippines in 1956. The other two serious forms of the disease, Dengue Hemorrhagic Fever(DHF) & Dengue Shock Syndrome (DSS) were documented for the first time in 1956 [8].

3.2 PREVALANCE

3.2.1.Global prevalence

The incidence of Dengue has grown dramatically around the world in recent decades. Over 2.5 billion people that is, around 40% of the world's population are now at risk from Dengue. WHO currently estimates there may be 50–100 million Dengue infections worldwide every year.

More than 70% of the population at risk for Dengue worldwide live in member states of the WHO South-East Asia Region and Western Pacific Region, which bear nearly 75% of the current global disease burden due to Dengue [1]which includes asymptomatic cases. Every year hundreds of thousands of severe cases arise, including 20,000 deaths[9]**;** 264 disability-adjusted life years per million population per year are lost[10]**,** often affecting very poor populations .An estimated 5,00,000 people with severe Dengue require hospitalization each year, a large proportion of whom are children. About 2.5% of those affected die [2]. Estimates suggest that annually 100 million cases of Dengue fever and half a million cases of Dengue haemorrhagic fever(DHF) occur in the world with a case fatality in Asian countries of 0.5%–3.5%.

Factors Responsible for the Increased Incidence of Dengue [11]

- unprecedented growth of population
- unplanned and uncontrolled urbanization,
- lack of effective mosquito control in areas where Dengue is endemic
- increased air travel.
- decay in public health infrastructures in most countries and lack of resources.

A study was carried out in Bangladesh by Sultana N et al [12], to find out the seroprevalence of Dengue fever in Chittagong .Among 1181 serum samples tested from suspected Dengue patients, 533 (45.13%) were positive for IgM and IgG antibodies by ICT.

3.2.2.Prevalence in India

Dengue infection is the most rapidly spreading mosquito-borne viral disease in the world and an estimated 50 million Dengue infections occur annually. Case fatality rates for the South-East Asian region are 1%, but in India, Indonesia and Myanmar, focal outbreaks have reported rates of 3%-5% [13].

The global epidemiology of Dengue has been changing during the last 50 years[11]. The number of countries reporting Dengue has increased in recent years and also the number of disease severity in the form of DHF/DSS is also being increasingly reported. The first

Dengue epidemic in India occurred in Kolkata during 1963-64[14] and ever since , the epidemiology of Dengue virus has been changing . India is endemic for Dengue , having witnessed several DF/DHF outbreaks in the last decade [15,16].The last major DF outbreak in India occurred between September and October 2006 involving more than 12,000 cases and nearly 184 deaths, of which approximately 3366 cases and 65 deaths were reported from Delhi alone [17]. In 2006 the number of cases reported as compared to 2005 shows some reduction whereas the case fatality rate has remained above 1%. In 2009, in India 15535 cases were reported with 96 deaths while in 2010, 28292 cases of Dengue were reported with 110 deaths. In 2013, till August, 22092 cases were reported with 74 deaths.

The case fatality rate in patients with Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) can be as high as 44%. Hence early and rapid laboratory diagnosis of Dengue is vital. Appropriate clinical management can save the lives of patients suffering from DHF and DSS and mortality can be reduced to less than 1%.

According to a study conducted by Saini *et al*[18], the prevalence of Dengue seropositivity among clinically suspected cases in a tertiary care hospital in Maharastra was 30.6% .

Recent study by Gargi ghosh *et al* [21] highlighted that out of 1782 samples received from clinically suspected cases of Dengue over a

period of 2 years, 320 showed positive by Dengue Rapid Immunochromatography method.

Sharma Y *et al*[19] revealed that during the study period (2006–2010), a total of 8138 serum samples were tested out of which, 1600 (19.66%) were positive for Dengue specific IgM antibodies by MAC ELISA. A similar study by Garg *et al*[20] reported that the seroprevalence of Dengue virus infection at Kanpur, North India was 19.7% by IgM and IgG anti Dengue antibody detection by ELISA.

Manisha Patankar *et al*[22] stated that the seroprevalence of Dengue in Gujarat was 21% by NS1 ,MAC-ELISA and RT-PCR.

Chakravarti *et al*[23]in 2011 reported that the seroprevalence of Dengue infection in central part of India was 60.1% tested by ELISA.

Based on the study conducted by SirajA.Khan *et al*[24] in a hilly region of Andhrapradesh, out of 164 samples, 107 (65.2%) were found to be Dengue positive.

3.2.3.Prevalence in Tamilnadu

In southern India , epidemic of Dengue has been reported in Tamil Nadu, Kerala ,Karnataka, and Andhra Pradesh . During the past years, there has been an improvement in the reporting of Dengue fever cases in Tamil Nadu, since the laboratory diagnostic facilities have considerably increased. A total of 128 cases and 5 deaths were reported in 1998 which

increased to 1600 cases and 12 deaths in 2003 and 1150 cases and 8 deaths in 2005.[25]

Recent statistics as per "NVBDCP INDIA 23" reported that during 2007 , a total of 707 cases and 2 deaths were reported which increased to 1072 cases and 7 deaths in 2009 and 2501 cases and 9 deaths in 2011.During 2012 epidemic ,12826 cases and 66 deaths were reported which eventually decreased to 4294 cases and no deaths in 2013. This is mainly due to increase in the testing facilities in Tamil Nadu.

Vijayakumar *et al*[13], in a five year study conducted at Christian Medical College Vellore reported that the seroprevalence was found to be 55.1% during 2003 tested by various methods such as Dengue blot tests , ICT and IgM capture E LISA assay.

A Chennai based study by P Gunasekharan *et al*[26] stated that out of 1593 samples tested ,686 (43.0%) were positive for IgM antibodies detected by MAC-ELISA and ICT during a period of 2006-2008.

A Hospital based study at Perambalur conducted by M Anuradha *et al*[27] (2013-2014) observed that out of 151 Dengue suspected cases , 60 were positive by using Dengue ELISA kit. The incidence of Dengue viral infection was 39.74%.

Paramasivam *et al*[28] in a study stated that during an outbreak of Dengue fever in certain rural areas of Kanyakumari district, Tamil Nadu , out of the

76 samples tested , 15 (20%) were found positive for Dengue virus specific IgM Antibodies by ELISA.

Based on the study conducted by N Satish *et al*[29] at a Hospital in Vellore, estimated that the prevalence of Dengue among the study population was 25%.

3.2.4. Prevalent serotypes in India

Samples isolated from Gujarat showed that DENV2 was the predominant serotype during the epidemics of 1988-89 [30]. With time, Dengue virus outbreak reached different states of India. In 1992 Jammu also saw an outbreak of DENV2[31]. DENV2 serotype was isolated during an outbreak in Haryana as well [32.] DENV2 was also prevalent in Northern India where outbreaks were seen in Delhi, Gwalior and Lucknow[33,34]. However, DENV1 was the dominant serotype isolated during an outbreak in New Delhi 1997 [35]. The Gwalior outbreaks of 2003-04 were dominated by DENV3 [36,37], and was also prominent in 2004-05 [38]. This co-circulation of serotypes in the same area might be the reason behind the large number of DHF cases reported this year [39]. In 2006, all four serotypes of Dengue viruses were found in co-circulation.

A study by Cecilia *et al*[40] in Pune , Maharastra reported that from May 2009 to September 2010, 56 cases was serotyped by the multiplex RTPCR test. 13 cases of DENV-1, 21 of DENV-2, 20 of DENV-3 and 2 cases of DENV-4 were detected.

3.2.5.Recent Trends In India.

Rapid growth of population and sudden climatic changes in India were found to contribute to the rise in DF/DHF cases [41]. During 1997 to 2004, the causative agent of most DF/DHF cases was of DENV1 serotype but later in 2005, DENV3 became the leading source of Dengue outbreaks[42]. According to the WHO in 2006, the total number of reported cases reached 12,317, while 5,534 cases occurred in 2007 owing greatly to preventive measures taken by both the public and private sectors. In 2009, however, DF cases again reached 11,476 by November. The first cases were reported in July 2009 with the greatest number of cases seen in October. These trends demonstrate that DENV has penetrated deep into India, with DENV2 and DENV3 predominating among different DENV serotypes.

3.2.6.Prevalent serotypes in Tamilnadu

The prevalence of Dengue vector and silent circulation of Dengue viruses have been detected in rural and urban Tamil Nadu.

A study conducted by Bhuvaneshwari CK *et al*[25] on clinically suspected cases of Dengue attending Thanjavur Medical College and Trichy Hospital (2011) revealed that DENV‑2 and DENV1 had dominated ,although three serotypes were found to be co- circulating as detected by RT‑PCR.

Myers RM *et al*[43] during a Dengue outbreak in Vellore, southern India, in 1968 documented that there is circulation of all the four serotypes of the Dengue viruses in Tamilnadu.

3.3. VIRION STRUCTURE

Flaviviruses possess an isometric core, 30–35 nm in diameter, that contains a nucleocapsid or core (C) protein complexed with single-stranded positive-sense RNA. This core is surrounded by a lipid bilayer containing an envelope (E) protein and a membrane (M) protein, giving a total virion diameter of about 45 nm. The flavivirus E protein is a class II fusion protein. The M protein is first synthesized as a precursor protein, prM. The virion structure of Dengue virus (DENV) virus has recently been solved using cryo-electron microscopy[44].

3.4.CLASSIFICATION AND GENOME STRUCTURE

There are four serotypes (DEN 1–4), classified according to biological and immunological criteria .The viral genome is approximately 11 kb in length. It has been shown that there is abundant genetic variation within each serotype in the form of phylogenetically distinct clusters of sequences dubbed subtypes or genotypes . At present , three subtypes can be identified in DENV-1 , six in DENV-2 (one of which is only found in non-human primates), four in DENV-3 and four in DENV-4 (with another exclusive to non-human primates)[45]. The mature virions consist of three structural

(core , membrane associated , and envelope) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins. The envelope protein is involved in the main biological functions of the virus. It binds to receptors on host cells, allowing the virus to be transported through it. In addition, the envelope protein is associated with haemagglutination of erythrocytes, induction of neutralizing antibodies and protective immune responses. Non-structural proteins (NS1–NS5), expressed as both membrane associated and secretory forms, have also been implicated in the pathogenesis of severe disease.

NS1 gets expressed on the surface of infected cells. Preliminary evidence suggests its involvement in viral RNA replication. Plasma levels of secreted NS1 (sNS1) relate well with viral titre being more in DHF patients than DF only. Moreover, elevated free sNS1 levels within 72 hours of onset of illness identify patients at risk of developing DHF. Very high levels of NS1 protein are detected in acute phase samples from patients with secondary Dengue infections but not primary infections.This suggests that NS1 may contribute to formation of circulating immune complexes, which are thought to have an important role in the pathogenesis of severe Dengue infections. [46]

A study by Thomas *et al* demonstrated that the Dengue virus NS1 was detected in 47 (67.1%) of 70 patients positive for Dengue by RT-PCR and it was found to be that there was increase in the plasma virus loads in

NS1-positive patients and the sensitivity of NS1 testing also increased with virus load.

3.5. MOSQUITO VECTORS IN DENGUE INFECTIONS

Dengue is transmitted by the mosquitoes belonging to the genus Aedes (Aedes aegypti, Aedes albopictus, and Aedes polynesiensis). A. aegypti is the most important vector, but A.albopictus and A.polynesiensis may also act as vectors depending on the geographic location[2].A.albopictushas also been found to transmit Dengue in Thailand, India, Singapore, Samui island and Mexico. Aedes aegypti, a day biting and a container breeding mosquito is found in tropical and subtropical areas[47]. They breed indoors, mainly in living rooms and bedrooms that increases man-vector contact and reduces the contact with insecticides sprayed outdoors ,thus contributing to difficulty in controlling this vector[48]. Aedes aegypti can breed in small collections of water such as flower vases or coconut shells and also in polluted water[49]. Eggs of these vectors resist dessication and hence they survive for long periods. The factors responsible for high mosquito densities in endemic areas are improper garbage disposal and inadequate drainage of wastewater facilitates. Both the factors are consequences of unplanned urbanization. During the rainy season, there is a significant increase in the mosquito larval populations which may be a reason why epidemics of Dengue tend to coincide with the rainy season[50].

Ambient temperature and relative humidity also has an effect on viral propagation in mosquitoes; rates being highest in climates resembling the rainy season .Environmental temperatures also affect the time to acute viraemia in female mosquitoes, being shorter with rises in temperature [51].

After biting an infected human, Dengue viruses enter an adult female mosquito following which it replicates in the midgut, reaches the haemocoel and haemolymph, and then gain its entry to different tissues of the insect. After viral replication in the salivary glands, the infected mosquito transmits the virus to another human. Viral particles are shown within the epidermal cells ,nervous system, salivary glands, foregut, midgut, ovary and internal body wall lining cells of the mosquito by ultrastructural studies .In contrast, they are found to be absent from the muscle, the hindgut, and malphigian tubules. Infected mosquitoes take longer time to complete a blood meal when compared to uninfected one which contributes to the efficiency of A .aegypti as a Dengue viral vector. This increased time corresponds to Dengue virus infection of organs known to control or influence activities associated with feeding[52].

Several studies suggest the existence of transovarial Dengue virus transmission in Aedes infected female mosquitoes, allowing propagation of virus to their progeny. Such a process would allow it to act as a reservoir for virus maintenance during inter epidemic periods (without human or other vertebral host participation)[53].Reports also suggest that Dengue viruses may

be transmitted sexually from the male to female mosquitoes, but not vice versa[54].

3.6.PATHOGENESIS OF DENGUE FEVER/DHF[27]

Dengue is caused by anyone of the Dengue viral serotypes. In general, infection with one serotype gives protective immunity against that particular serotype but not against other serotypes in future. More severe form of infection occurs when infected for a second time with a different serotype. This phenomenon is as a result of a process known as antibody dependent enhancement, where antibodies against the first serotype boost up the infection with the second serotype .Only 2–4% of individuals with a secondary Dengue infection develop severe disease[55].

When an infected mosquito bites,the Dengue virus enters the body and replicates within cells of the mononuclear phagocyte lineage(monocytes, macrophages and B cells). In addition, infection also involves the mast cells, dendritic cells, and endothelial cells. Dengue infection has an incubation period of 7–10 days followed by a viraemic phase where the patient becomes febrile and infective .Subsequently ,the patient may either recover or progress to the leakage phase ultimately leading to DHF and/or DSS. Severity of Dengue infections correlates with the peak plasma viraemia . Patients with uncomplicated Dengue fever or DHF/Dengue shock syndrome show differences in antibody, cytokine, and T-cell responses .

3.6.1.Antibody responses to the Dengue virus .

Antibody dependent enhancement plays a crucial role in the pathogenesis of severe Dengue infections. During secondary Dengue infections, antibodies that is already present in the patient form complexes with the Dengue virus. The Fc portion of these antibodies bind to FccRI and FccRII possessing cells and results in increased number of cells being infected by the Dengue virus. Antibody dependent enhancement occur only in the presence of subneutralizing concentrations of antibodies to Dengue virus [56,57].

3.6.2.Cytokine responses in Dengue infections.

During the initial 3 days high levels of TNF-α, IL-2, IL-6 and IFN-γ are seen. IL-13 and IL-18 is increased during severe infection. Cytokines IL-2 and IFN-γ are of Th1 type and IL-5 and IL-4 are of Th2 type. Thus, Th1 responses are seen during the first 3 days and Th2 responses occur later .During severe Dengue infections, increased levels of IL-13 and IL-18 have also been reported, with highest levels seen in patients with grade IV DHF. Serum IL-12 levels are increased in patients with Dengue fever, but in patients with grade III and IV DHF,the level becomes undetectable. TNF-α, IL-6, IL-13, IL-18, and cytotoxic factor levels are increased in patients with DHF when compared to patients with DF [58,59].These cytokines have a key role in causing increased vascular permeability and shock during Dengue infections[60].High levels of TNF-α correlate with haemorrhagic

manifestations . Increased levels of IL-10 correlates with platelet decay and in addition it down regulates platelet function contributing to platelet defects that are associated with Dengue infections.

3.6.3.Cellular immune responses in Dengue infections

In Dengue fever and DHF ,suppression of T-cell responses can occurs which persists for at least two weeks after the onset of fever[61]. In one study, 6% of patients presents with respiratory tract infections or diarrhoea after Dengue infections. This suppression was found to be due to a primary defect within antigen presenting cells. As the levels of IL-10 are increased in DHF, they downregulate antigen presenting cell responses and induce unresponsiveness in T-cells.

3.7.CLINICAL FEATURES

Infection with Dengue virus may be subclinical or may cause illness ranging from a mild undifferentiated fever to a severe, life-threatening condition (DHF or DSS). The symptoms lasts for more than a week and even longer in some patients.

3.7.1.Undifferentiated fever

This usually follows a primary infection but may also occur during the early phase of a secondary infection. Clinically it mimics many other viral infections and is frequently not diagnosed.

3.7.2.Dengue fever

Dengue fever is most commonly seen in adults and older children. It occurs during both primary and secondary infections. Typically symptoms start with sudden onset of high fever, which could be biphasic lasting 3 to 7 days [62,63] associated with chills ,rigor and intense headache (especially behind the eyes),fatigue ,muscle and joint pain (ankles ,knees and elbows) ,unpleasant metallic taste in mouth ,loss of appetite, vomiting, diarrhea and abdominal pain .Dermatological manifestations presents as flushed skin on face and neck, macular papular rash on the arms and legs with severe itching and peeling of skin and hair loss .Young patients may present with coryza , diarrhea, rash, seizure, vomiting, headache, and abdominal pain[64].

Hemorrhagic manifestations are uncommon in Dengue fever. However, minor bleeding (nose or gums), heavy menstrual periods, petechiae/purpura, gastrointestinal bleeding may be seen [65,66].

A positive tourniquet test has been reported in many individuals with Dengue fever [67]. Recovery from Dengue fever is usually uneventful, but may be prolonged especially in adults [2].

3.7.3.Dengue hemorrhagic fever

Dengue hemorrhagic fever (DHF) usually follows a secondary Dengue infection. In infants, it may follow a primary infection due to maternally acquired Dengue antibodies [68]. Typically, DHF is characterized

by high fever, hemorrhagic phenomena, features of circulatory failure and hepatomegaly [69].

DHF is divided into four grades according to severity.

3.7.3.1.Grading of Dengue hemorrhagic fever

Grade	
I	No shock, only positive tourniquet test
II	No shock, has spontaneous bleeding other than a positive tourniquet test
III	Shock
IV	Profound shock with un-measurable blood pressure and/or pulse.

The clinical course of DHF is divided into three phases, namely,

- febrile phase
- leakage phase and
- convalescent phase.

The febrile phase begins with sudden onset of fever - lasts for 2-7 days usually high grade (39°C), intermittent and associated with rigors accompanied by generalized constitutional symptoms, facial flush, rash and bleeding manifestations.The liver is often enlarged, soft and tender but jaundice is not observed. Splenomegaly is rarely observed in small infants .Generalized lymphadenopathy is noted in about half of the cases.

Plasma leakage phase /critical phase is characterized by tachycardia and hypotension ,sweating and restlessness ,cold extremities ,pleural

effusions (usually right side) and ascites[70]. Thrombocytopenia and hemoconcentration are detectable before the subsidence of fever and the onset of shock.

Hemorrhagic manifestations occur after fever has settled[68].The commonest site being gastrointestinal tract (which manifests as hematemesis or melena) followed by epistaxis[71].Vaginal bleeding has been reported in females despite high platelet counts.Complications such as pericardial effusions, myocarditis occur that is associated with increased morbidity and mortality. In less severe cases of plasma leakage, patients recover spontaneously after a short period of fluid and electrolyte replacement. In severe cases with high plasma leakage, patients may develop full blown circulatory shock characterized by prolonged capillary refill time and narrow pulse pressures. Convalescent phase is characterized by bradycardia ,confluent petechial rash with erythema and pallor .

A one year study conducted by Nizal MG *et al*[72] analyzed that among 1466 cases of Dengue , 1342 (91.5%) cases were Dengue fever and the remaining 124 (8.5%) were Dengue hemorrhagic fever in Neger Sembilan,Malaysia in 2010.

3.7.4.Dengue shock syndrome

Presents with tachycardia, hypotension ,cold blotchy skin, congested peripheries and circumoral cyanosis. It is important to distinguish DSS from septic shock to avoid inappropriate use of antibiotics[73]. Patients with DSS die due to multiorgan dysfunction and disseminated intravascular coagulation.DSS is associated with very high mortality (9.3-47%) .DSS may be accompanied by encephalopathy due to metabolic or electrolyte disturbances. Adequate urine output and the return of appetite are considered to be good prognostic signs.

A Hospital based 5 year study in New Delhi by Sharma Y *et al*[19]revealed that out of the total 1600 cases admitted between 2006 and 2010, 279 (58.9%) had DF, 178 (37.6%) had Dengue hemorrhagic fever, and 16 (3.38%) had Dengue shock syndrome.

3.7.5.The differential diagnosis of Dengue fever and DHF

Dengue fever: Infectious mononucleosis, Chikungunya viral Infections**,** Coxsackie and other enteroviral infections**,** Rickettsial infections**,** Rubella**,** Parovirus B19 infections **,**Influenza **,** Leptospirosis etc…

DHF : Leptospirosis, Chikungunya viral infections, Hanta viral infections, other hemorrhagic fever, Meningococcal septicemia, Yellow fever,Kawasaki disease etc…..

Singh NP *et al* [74]observed that fever was present in all the cases with an average duration of 4.5±1.2days with headache (61.6%), vomiting

(50.8%) backache (57.8%) and abdominal pain (21%) being the other presenting complaints. Hemorrhagic manifestations in the form of a positive tourniquet test (21%), gum bleeding and epistaxis (40%), hematemesis (22%), melena (14%) and skin rashes (20%) were also observed. Hepatomegaly and splenomegaly were observed in 10% and 5% of cases, respectively. Laboratory investigations revealed thrombocytopenia (platelet count of <100,000 /µl) in 61.39%of cases, hemoconcentration (Hct>20% of expected for age and sex) and leucopenia (WBC <3,000/mm2) were observed in 68% and 52% of the cases, respectively.

A recent study by Gargi Ghosh *et al*[21]proposed that 71% of the patients presented with only fever with chills , followed by fever with chills and rashes 13%, fever with chills and arthralgia 5% and fever with myalgia 3%. Sub-conjunctiva lhaemorrhage, a presumptive sign of DHS was seen only in 2% of the patients in this study whereas the rest showed other insignificant associated signs and symptoms.

3.8.RISK FACTORS

3.8.1.Age

DF is considered as a childhood disease and is an most important cause of paediatric hospitalization in Southeast Asia .However, in India, DF affects all age groups.

Gubler *et al* [11] studied that Dengue affects all age groups worldwide and poses a pediatric public health threat in some parts of the world.

Based on the study by Sumitasood *et al* [75],the mean age group that was mostly affected in the study was the 16-30 years .

Sharmila *et al*[76] stated that the age wise distribution of IgM positive cases in three year study indicates that children (<10 yrs) were more commonly affected.

According to a study by Garg *et al*[20] among clinically suspected fever cases at a teaching hospital in North India reported that the most affected age group was 0 to 15 years of age (Pediatric population), followed by 16 to 30 years group.

3.8.2. Sex

A study by Ekta gupta *et al*[77] revealed that in overall DF/DHF cases males predominated over females(M: F ratio) .Year wise distribution of Dengue cases shows that males were more frequently affected when compared to females.

Smitasood *et al*[76]reported that a higher prevalence of Dengue infection was found in males than in females ,the ratio being 2.43:1.

Khan *et al*[78] revealed that female patients comprised 67.2% of the positive cases when compared to males in a hilly region in Arunachal Pradesh . A similar study was carried in Kolkata by Sarkar D *et al*[79]observed that females are more affected than males.

3.8.3.Climatic Factors

Study by Gargi ghosh *et al*[21] revealed that there was a peaking of serologically positive case from July to October in each of the years of the study. The peak incidence of Dengue cases in a study by EktaGupta *et al*[77],(2006) was in the 2nd and 3rd week of October. A study by Saini *et al*[18] observed that the transmission of Dengue virus infection increases in post monsoon period . This is mainly due to the presence of stagnant water after rain fall which favours the breeding of mosquito. There was also a increase of cases during the month of May which may be due to severe drought in the area that year and people have tendency to store the water for long period.

Based on the study by Sharma *et al* [19], prevalence of Dengue for the past 5 years reported that there is an increase in the number of cases from July to October that is during monsoon and post-monsoon period every year , confirming the active transmission period.

3.8.4.Socio-demographic factors

Yadava and Narasimaham *et al* [80], 1992 stated that the disease was mainly restricted to urban and semi-urban areas of the country earlier because of the availability of favourable sites for breeding of the mosquito vector species, *A. aegypti*, .Thus ,rural areas were found to be largely free of the vector species .

Rakesh *et al*[81],1997 observed that introduction of safe drinking water in rural areas led to water storage practices providing breeding sites for vectors .

3.8.5.Travellers

In a study to estimate the incidence and prevalence of Dengue virus infection in Australian travellers to Asia Ratnam *et al* [82]reported the incidence of 3.4 infections per 10,000 days of travel and seroprevalence of Dengue infection was 4.4% and a greater number of prior trips to Asia was a predictor for Dengue seroprevalence detected by IgM and IgG ELISA. All travellers experienced subclinical Dengue infections and had travelled to India and China . This significant attack rate of Dengue infection can be used to advise prospective travellers to Dengue-endemic countries.

3.9.LABORATORY DIAGNOSIS AND DIAGNOSTIC TESTS.

Accurate and efficient diagnosis of Dengue is of main importance for early detection ,case confirmation , differential diagnosis with other infectious diseases, outbreak control, pathogenesis, academic research, surveillance activities ,vaccine development and clinical trials. Dengue virus infection can be confirmed by various laboratory diagnostic methods. This involves virus isolation and identification, detection of viral nucleic acid, antigens or antibodies, or a combination of these techniques. After the onset of infection, the virus are found to be detected in serum, plasma, circulating blood cells and other tissues for 4–5 days.Virus isolation, nucleic acid or

antigen detection can be used in the diagnosis of infection during the early stages of the disease but serology is the method of choice at the end of the acute phase of infection.

Depending on the host's immune status, antibody response to infection differs [82]. When a person who have not been infected previously with flavivirus or immunized with a flavivirus vaccine (e.g. for Japanese encephalitis, yellow fever, tick-borne encephalitis) ,develops primary antibody response if the person is being subsequently infected with Dengue virus. This primary response is characterized by a slow increase of specific antibodies. The first immunoglobulin to appear are IgM antibodies .These antibodies are detectable in 50% of patients in 3-5 days after the onset of illness, increasing to 80% by 5th day and 99% by day 10 . IgM levels reaches its peak in about two weeks after the onset of symptoms and then declines to undetectable levels over a period of 2–3 months.

In general, serum IgG antibodies is detectable at low titres at the end of the first week of illness. Thereafter ,it increases slowly, with serum IgG still detectable after several months, and may be even for life [83,84]. In a secondary Dengue infection, there is a rapid rise in antibody titre which react against many flaviviruses. IgG isotype is the dominant immunoglobulin detectable at high levels, even in the acute phase the infection .IgG are found to persists for periods lasting from 10 months to life. Early convalescent stage IgM levels are significantly lower in

secondary infections than in primary ones and may be undetectable in some cases, depending on the test used [85].

Kuno G *et al*[86], Shu PY *et al*[87]and Falconar*et al*[88]observed that to distinguish primary and secondary Dengue infections, IgM/IgG antibody ratios are more commonly used now than the haemagglutination -inhibition test**.**

A range of laboratory diagnostic methods are available to support management of patient and control of disease. The choice of diagnostic method depends on the purpose of the test for which it is done, the type of laboratory facilities and availability of technical expertise, costs, and the time of sample collection.

Generally, tests with high sensitivity and specificity are technically more complex and requires technical expertise, whereas rapid tests may compromise sensitivity and specificity for the ease of performance and speed. Although virus isolation and nucleic acid detection are more specific than serologic methods for antibody detection ,they are found to be more labour-intensive and costly.

Methods used for diagnosis of Dengue infections are:

1. Virus isolation

2. Viral nucleic acid detection

3. Serological methods

- Haemagglutination inhibition (HI)

- ELISA
- Immunochromatography
- Neutralization test (NT)
- Complement fixation (CF)

4. Viral antigen detection

3.8.1. Virus isolation and identification.

Detection of Dengue virus by culture is the definitive diagnostic test.Its practical considerations limit its use. Dengue virus can be isolated from serum, plasma, circulating blood cells like leucocytes and also be isolated from postmortem specimens such as liver, lung, spleen, lymph nodes, thymus, cerebrospinal fluid and pleural/ascitic fluid. Blood should be collected during the febrile period, if possible before the fifth day of illness before the formation of neutralizing antibodies.Immune complex formation in secondary Dengue patients is due to the presence of large quantities of neutralizing antibodies which interferes with virus isolation. Serum can be placed at 4–8° for a short time if less than 24 hours.

Race MW *et al*[89] and Singh KRP *et al*[90] reported that the mosquito cell line C6/36 ,a clone obtained from *Ae. albopictus* has become the choice of host cell used for routine Dengue virus isolation and in addition, cell line obtained from *Ae.pseudoscutellaris* AP61 can also been used successfully. Cordeiro M.T *et al*[91] reported that mammalian cell cultures like Vero cells, LLCMK2 and others have also been employed , but with less efficiency.

Henchal EA *et al*[92] observed that Dengue virus in general is identified using immunofluorescence techniques with serotype-specific monoclonal anti-Dengue antibodies on mosquito head squashes or infected cells.Some strains of the virus ,when present in low concentration cannot be easily identified. Plaque assay is considered as the gold standard methodology for Dengue virus quantification.

Payne *et al*[93]proposed that an indirect immunofluorescence assay can be used as an alternative to virus culture. Kao Cl*et al*[94] reported that Flow cytometry has recently been a useful method for Dengue virus 1 (DEN-1) identification and allows the virus to be detected 10 hours earlier than with an immunofluorescence assay by using an anti nonstructural glycoprotein (NS1) monoclonal antibody.

3.8.2. Viral nucleic acid detection:

Dengue viral genome, which possess RNA, can be detected by reverse transcriptase polymerase chain reaction (RT – PCR) assay.It offers better specificity and sensitivity when compared to virus isolation with a advantage of more rapid turnaround time. But again the high cost and requirement of technical expertise is the question n the utility of PCR.

Lanciotti *et al*[95]developed that some laboratories routinely used nested PCR assay for Dengue virus identification .This PCR assay comprises a two-step reaction which involves an initial reverse transcription and amplification step that uses a universal Dengue primers

which mainly targets a region of the virus genome (C-prM) and is followed by a second amplification that is serotype specific. The resulting amplified product are separated by agarose gel electrophoresis, and by the size of the bands Dengue serotypes are identified .

Chien LJ *et al*.[96] and Johnson BW *et al*[97] proposed that the real-time RT-PCR assay is a one-step assay system using primer pairs and probes that are specific to each Dengue serotype. The reaction products are detected by a fluorescent probe enables without need for electrophoresis. Many real-time RT-PCR assays have been developed either as 'singleplex' or 'multiplex' by their ability to detect one or four serotype from a single sample at a time respectively. . The advantage of multiplex assay is that all the four serotypes can be determined by a single reaction without the possibility for contamination during manipulation of the sample.

Vaughn DW *et al*[98] reported that the fourplex real-time RT-PCR assays found to be less sensitive than nested RT-PCR assay even though they are faster. This assay determines the viral load in a given sample, which is important in determining the severity of Dengue disease. Current PCR tests are 80–90% sensitive and >95% specific in detection of Dengue virus in acute phase sera[99].

3.8.3. Serological methods

3.8.3.1. Haemagglutination Inhibition test (HI)

This is a simple, sensitive and reproducible test and has the advantage of using locally prepared reagents. HI test requires paired sera that are obtained upon hospital admission (acute) and discharge[92](convalescent). This test is based on the ability of Dengue virus antibodies to inhibit this agglutination[100]. A fourfold or greater rise in antibody titre is suggestive of a flavivirus infection and not diagnostic of Dengue infection. However, a single antibody titre>1/2560 is accepted as indicating secondary Dengue infection if supported by a clinical history suggestive of Dengue. Based on this principal, Immunochromatography is formed**.** These tests are mainly used in research purposes.

Cordeiro*et al*[91], 2007 proposed that quantification of HI antibodies (total: IgM and IgG)are done by a microtechnique that serves to characterize the type of immune response according to WHO criteria .

3.8.3.2.ELISA

MAC ELISA

MAC-ELISA is one among the classic serological test for Dengue. This assay utilizes all the four serotypes (DEN 1–4) of Dengue-specific antigens for the capture of anti DengueIgM-specific antibodies in serum samples. The antigens that are mostly used for this assay are derived from Dengue virus envelope protein. Specificity of these antigens and cross-

reactivity with other circulating flaviviruses limits the usage of this test. These limitations are taken into consideration when working in regions where there is co-circulation of multiple flaviviruses. IgM detection is not useful for the determination of Dengue serotypes owing to cross-reactivity of the antibody, even during primary infections.

Guzman and Kouri*et al*,[101] 2004 reported that specific IgM antibodies are produced during both primary and secondary infection. Anti-Dengue IgM can be detected, generally from day 5 of the disease and may last for 60 to 90 days. Hence, the detection of IgM in any serum sample is indicative of recent or active infection within the last 2-3 months. The antibody production for DENV usually varies from those having primary and secondary infections. During the primary infection, IgM is usually detected from day 5 after the onset of illness, while in secondary infection ,IgM are detected in a titre lower than that of the primary infection. Vorndam and Kuno *et al*,[82] 1997 observed that during a secondary infection, about 20% of patients do not have IgM at detectable levels .

According to the Pan American Health Organization (PAHO)[84] guidelines ,IgM antibodies are detectable by fifth day of illness in 80% of all Dengue cases, and by day 6–10 of illness in 93–99% of cases, and then may remain detectable for more than 90 days. MAC-ELISA has become a significant tool in the routine diagnosis of Dengue. This assay has a

sensitivity and specificity of 90% and 98% respectively when used ≥5 days after the onset of symptoms.

Vasquez S *et al.*[102] 2006 proposed that serum, blood on filter paper, and saliva (not urine)can be used for IgM antibody detection,if the samples are taken within the appropriate time (≥5 days after the onset of fever) .The different commercial kits are available which have variable sensitivity and specificity .

N Sathish *et al*[29]observed that MAC-ELISA showed a high sensitivity (96%) as compared to Rapid ICT (73%) and the specificity was low (81%) when compared to Rapid (95%) .

Hunsperger *et al*[103]evaluated six commercially available anti-Dengue IgM ELISA kits which showed mean sensitivities of ELISAs were 61.5%–99.0%, and specificities were 79.9%–97.8%.

Similar study was conducted by Blackshell *et al*[104]on seven commercially available IgM ELISA and reported that the sensitivity and specificity was found to be ranging from 85 to 89% and 88 to 100%, respectively .

IgG ELISA.

The classic IgG ELISA are mainly used for the detection of a past Dengue infection.This assay uses the same antigens as that of the MAC-ELISA. IgG ELISA is usually performed with multiple dilutions of the sera tested to determine an end-point dilution. This assay correlates with the

haemagglutination assay that was used in the past.Generally,IgG ELISA lacks specificity within the flavivirus sero-complex groups.

Cardosa*et al*[105] demonstrated that the IgG response to premembrane protein is specific to individual flaviviruses. No cross-reaction was observed when sera were tested from individuals infected with Dengue virus or Japanese encephalitis virus. IgG avidity ELISAs can be used to distinguish between a primary or secondary Dengue infection , and is more useful than the HI test for this purpose.

Guzman and Kouri *et al* [101], 2004observed that IgG is detected at low levels from day 7 after the onset of illness in primary infection. On the contrary, during the secondary infection, IgG antibodies are detected at high levels even in the acute phase.

Inoue *et al*[106] revealed that the results of DEN IgG was highly correlated with that of the DEN HI test with a titer of 1:29,000 as the cut-off value for the diagnosis of Dengue secondary infection 90.9% sensitivity and 92.9% specificity .

IgM/IgG ratio.

The IgM/IgG ratio is also used to differentiate primary from secondary Dengue infection. A primary and secondary Dengue virus infection is considered when the capture IgM/IgG ratio is > 1.2 and < 1.2 respectively.

A recent study by Falconar *et al.*[88] shows that the ratio differ variably depending on whether the patient has a serological classical or non- classical Dengue infection. The adjusted ratios of > 2.6 and less < 2.6 correctly classified 100% of serologically classical and 90% of serologically non-classical Dengue infections.

3.8.4. Viral antigen detection

ELISA, rapid immunochromatography and dot blot assays are directed against nonstructural protein (NS1) that detects Dengue infection upto day 6 after the onset of infection. Detection of NS1 antigens do not distinguish the virus serotypes.

Young *et al.*[107], 2000 reported that NS1 protein exists in hexameric form and is highly conserved in all four DENV serotypes. This antigen was found circulating in the blood of patients from the day 1-9 after the onset of fever . As it is present in the serum during the acute phase of the infection, NS1 antigen is considered a marker used for early diagnosis of the disease, with a greater sensitivity in the first 5 days of disease. Detection of NS1 by ELISA is rapid and as sensitive as RT-PCR.

Based on the study by Hang *et al.*[108], 2009,false-negative results may occur most probably due to the formation of immune complexes of NS1 antigen with IgG, especially in secondary infections, where there is non-availablility of target antigen to the monoclonal antibody from ELISA.

McBride *et al*[109]2009 observed that NS1 antigen had a sensitivities of 97.5% and 59.1% for primary and secondary infections, respectively.

Srivastava *et al*[110]compared the evaluation of NS1 Ag by ICT with reference to NS1 capture ELISA in which the sensitivity, specificity, positive predictive value and negative predictive value were 62.5% ,100%, 100% and 88.15 % respectively.

A study by Kulkarni RD *et al*[111] determined the association of platelet counts and Dengue serum markers (NS1, IgM and IgG) tested by rapid immunochromatography test.

3.8.5.Neutralisation test

During the primary infection, relatively monotypic neutralizing antibodies are detected whereas in the secondary infections, there is a production of high titre of neutralizing antibodies of two or more of the four DENV serotypes.

Vorndam and Kuno *et al*,[82]1997 observed that in combinations of sequential or secondary infections, the highest titer for neutralizing antibodies present in the serum of convalescent patient is targeted against the virus that infected earlier .This phenomenon may hamper the interpretation of the serological test sometimes, known as "original antigenic sin",

A study by Roehring *et al.*[112], 2008 shows that the neutralizing test by plaque (PRNT)reduction method is considered as "gold-standard" to

determine DENV serotype-specific immunity, though newer techniques are being currently developing to measure neutralizing antibodies . This PRNT technique is recommended by the WHO to assess the efficacy of Dengue vaccines under development .

Morens *et al* [113], 1985 proposed that the neutralizing activity in serum is determined by their ability to reduce the largest number of viral plaques. The sample that shows reduction of 50% or more in plaque formation is considered as positive. PRNT is the most specific serological tool for the determination of Dengue antibodies.This test is mainly used to determine the infecting serotype in convalescent sera, measures the neutralizing antibodies titre in the serum of the infected individual and determines the protection level of the individual against the infecting virus. The assay is mainly based on the principle of virus interaction and antibody which results in virus inactivation in such a way that it no longer infect and replicate in cell culture . Some variability are found in this assay which attributes to the differences in interpretation of the results. The cell lines and virus seeds used as well as the dilution of the sera accounts for these differences.

The microneutralization assay is based on the same principle as that of PRNT. For determining the end-point dilution, this assay uses a colorimetric measurement of virus-induced lysis of cells instead of counting the number of plaques per well as in PRNT. This assay utilizes small amounts of reagents and is also suitable for testing large numbers of

samples. During secondary infections, this assay shows a poor correlation with PRNT results which limits its use.

3.8.6.Immunochromatograpy

Immunochromatographic tests are used for the detection of Dengue virus NS1 antigen, IgM, IgG, and IgA antibodies. These RDTs are commercially available nowadays and have found wide application because of their ease of use and rapidity of results. These RDTs are presented in the form of a lateral flow cassette which allow the sample to flow in a horizontal plane or in a vertical plane where the sample in a tube is drawn by capillary action. The Dengue RDTs have the advantage that they are less time consuming (15–20 minutes),does not require any special equipment or training.

Stuart D Blacksell *et al*[104] compared the accuracy of 8 Commercial Rapid Immunochromatographic Assays for the Diagnosis of Acute Dengue Virus Infection and found that the assay sensitivities were low, ranging from 6.4% to 65.3%and specificities ranged from 69.1% to 100% .

Similar study by V. Tricou *et al*[114] showed sensitivity and specificity of 75.5% and 100% respectively.A multicentre evaluation of Dengue IgM antibody RDTs by Hungsper *et al,* sponsored by WHO showed sensitivity ranging from 21% to 99% and specificity ranging from 77% to 98% when compared with reference ELISAs [103].

Saini *et al*[18]showed that 198 cases were positive for NS1 antigen and 40 cases were considered as primary infection (84.6%)which showed the presence of Dengue specific IgM antibodies /with or without NS1 Ag . 43 cases (15.3 %) were categorized as secondary infection which is indicated by the presence of Dengue specific IgG antibodies with or without any other parameter by Immunochromatography .Sekaran *et al*[115]reported the sensitivity and specificity in diagnosing acute Dengue infection in the SD Duo NS1/IgM were 88.65% and 98.75%, respectively.

Hunsperger *et al*[103]evaluated four commercially available RDT kits for detection of IgM antibodies that showed Mean sensitivities and specificities to be 20.5%–97.7%, and 76.6%–90.6% respectively.

Hasan *et al* [116]in his study revealed that the sensitivity / specificity of Dengue IgG, IgM, IgA and rapid antigen by ICT were compared with ELISA IgM (gold standard) and these were 33.3/100%, 16.67/88.89%,50.0/77.78%, 23.3/88.8% respectively.

3.8.7.Comparison of diagnostic methods

Lam *et al*[117] evaluated rapid immunochromatographictest and ELISA for the detection of IgM and IgG antibodies produced during Dengue infection. Both assays showed excellent sensitivity(100%) diagnosing primary and secondary Dengue infection and specificity in both assays was 89% .

A study by Gargi ghosh *et al*[21]revealed that the, sensitivity and specificity of NS1 ICT were found to be 91.2% and 78.4%, while that of NS1 ELISA were found to be 75% and 88% with RT-PCR as standard reference.

In a study by Datta *et al*[118], the performance of NS1 antigen (Ag) assay was evaluated in comparison to MAC-ELISA and to determine their detection rate when performed together in a single sample. Out of 600 early convalescent sera were screened by both the assays, the sensitivity of NS1 and MAC-ELISA were 23.3% and 39.1% respectively. The detection rate increased to 53.3% when both the assays were used together on a single sample. NS1 Ag assay holds promise in early diagnosis of Dengue infection. When used in combination with MAC-ELISA on a single sample it significantly improves the diagnostic algorithm without the requirement of paired sera.

Lanciotti *et al*[95]compared and analyzed the efficacy of RT-PCR assay and virus isolation for the identification and typing of Dengue viruses from human serum.The RT-PCR assay demonstrates sensitivities of 94% with Dengue type 1 virus, 93% with Dengue type 2 virus, and 100% with Dengue type 3 and 4 viruses, compared with virus isolation.

Chungue *et al*[119] studied on comparison of IgG-ELISA to the haemagglutination inhibition test (HI). and found that the sensitivity, and specificity between the 2 techniques were good. The coefficients of

correlation between IgG-ELISA and HI results, using Dengue 1-4 antigens, were highly significant .

3.9.TREATMENT

The management of Dengue virus infection is essentially supportive and symptomatic. No specific treatment is available.

3.9.1.Management of Dengue fever

Fever is treated with anti-pyretics (paracetamol). Nonsteroidal anti-inflammatory drugs such as ibuprofen or aspirin should be avoided. Tepid sponging is helpful. Antiemetic such as domperidone and a proton pump inhibitor (pantoprazole) can be used. Increased oral fluids are adviced during the early phase of the disease; intravenous fluids are administered in presence of severe vomiting or dehydration.

3.9.2.Management of DHF

Cautious administration of IV fluids are adviced to avoid respiratory distress secondary to massive pleural effusions/ ascites or pulmonary edema. Intravenous fluid therapy should be stopped when the packed cell volume(PCV) falls to 40% generally heralding the convalescent phase.

3.9.3.Management of DSS

Significant hemorrhagic manifestations requires platelet transfusions. Electrolyte abnormalities, metabolic acidosis and hypoglycemia should be corrected. Laboratory indicators should be looked for monitoring disseminated intravascular coagulation (DIC).Depending upon clinical and

laboratory parameters of the individual ,Fresh frozen plasma, platelet concentrates, or cryoprecipitate are administered .

3.10.PREVENTION OF DENGUE

There is no availability of vaccine and there is no specific medications to treat a Dengue infection. This makes prevention the most important step. Prevention is by avoiding mosquito bites if living in or travel to an endemic area.The best way is to reduce mosquito beeding places like artificial containers that hold water in and around the home. Outdoors, clean water containers like animal watering containers, flower planter dishes or cover water storage barrels,standing water indoors such as flower vases and should be cleaned at least once a week. The adult mosquitoes bite inside as well as around homes, during the day and at night when the lights are on. To protect from bites repellents are used while indoors or out. Windows and door screens are to be secured and be without holes. If someone in the house is ill with Dengue, extra precautions are taken to prevent mosquitoes from biting the patient and to bite other household contacts .Mosquito nets and repellents are adviced to eliminate mosquitoes found indoors.

3.11 VACCINE

There is no Dengue vaccine available against Dengue at present. Research is in progress to develop a safe and effective tetravalent Dengue vaccine. Many Dengue vaccines, e.g.live attenuated, recombinant vaccines

and inactivated whole virus are in developmental process and some were in phase 2 field trials. In the absence of Dengue vaccine , prevention and containment of Dengue outbreak requires an effective long-term vector control with community participation and aggressive epidemiological surveillance.

4.MATERIALS AND METHODS

The present study was conducted at the Department of Microbiology , Tirunelveli Medical College , Tirunelveli from May 2013 to March 2014 to evaluate the efficiency of rapid immunochromatographic test(ICT) in Dengue NS1 antigen, IgM and IgG antibody detection and also to compare its performance against NS1 antigen, IgM and IgG antibodies detection by Enzyme Linked Immunosorbent Assay (ELISA) respectively.

4.1 Study group

Atotal of 100 serum samples were collected from clinically suspected Dengue cases in adults.

4.2 Inclusion criteria

- Serum samples of patients with acute onset of fever of >4 days, clinically suspicious of Dengue virus infection
- Adults requiring hospitalization

4.3 Exclusion criteria:

- Patients with any other proven febrile illnesses like Malaria, Typhoid etc. are excluded.
- Children <12 years of age.

4.4 Ethical clearance

Ethical clearance was obtained from the college ethical committee before the commencement of the study.

4.5 Consent

Informed consent was obtained from reliable informants of patients who participated in the study.

4.6 Questionniare

Symptoms regarding the onset of fever, duration, presence of other clinical features like chills, rigor, vomiting,retro-orbital pain, myalgia and other features of haemorrhagic manifestations,leucopenia,thrombocytopenia and rising haematocrit were recorded in the questionnaire.

History of previous hospitalization, mosquito bite, domestic water storage and stagnant water around the living area was also included in the questionnaire.

4.7Study sample

A total of 100 non-duplicate serum samples were collected from the study group. Around 5 ml of blood sample was collected from suspected cases in the acute phase of the illness. Serum was separated and collected in serum vial .The specimen was properly labeled with serial number , name of the patient and date of collection.

4.8 Storage of sample

Sample were immediately tested for Dengue NS1 antigen , IgM and IgG antibodies by rapid immunochromatographic method and then stored for ELISA at -80 ^{o}C.

4.9 METHODS

All 100 samples were tested for Dengue parameters (NS1 antigen, IgM andIgG antibodies) by both rapid immunochromatography and ELISA.

4.9.1 Immunochromatography

All the 100 samples were tested by rapid immune chromatography card test (SD BIOLINE DENGUE NS1 +Ab COMBO).

Test principle

In general, ICT is a lateral flow assay that contains a chromatographic pad with three zones - sample pad , conjugate pad and capture line.Colloid gold is impregnated in the conjugate pad. When the specimen is applied to the sample pad, it flows laterally by capillary action. On reaching the conjugate pad it forms antigen-antibody complex by binding to the antibody conjugate in the pad .This complex then flows laterally to reach the capture line where it is captured by second antibody. The presence of coloured line indicates a positive result.

This rapid test cassette contains two windows, one for detection of NS1 antigen and IgM/IgG antibody each. The Dengue NS1 window contains two pre coated lines, ‘‘T” as NS1 antigen test line and ‘‘C” as control line. Dengue IgG/IgM rapid test is designed to simultaneously detect and differentiate IgG and IgM antibodies to Dengue virus in the sample. This device detect all four Dengue serotypes by using a mixture of

recombinant Dengue envelope proteins. Dengue IgG/IgM test device contains three precoated lines, ‘‘G” as Dengue IgG test line, ‘‘M” as Dengue IgM test line and ‘‘C” as control line on the surface of the device.

Materials provided

The rapid immunochromatographic test kit contains the following items to perform the assay

- Dengue NS1 antigen and Dengue IgG/IgM combo device
- Assay diluents for Dengue IgG/IgM test
- 10μl capillary pipette for Dengue IgG/IgM test
- Disposal dropper for Dengue NS1 antigen

Active ingredients of main components

Dengue NS1 antigen test device

- 1st test strip included: Gold conjugates (as main component):mouse monoclonal anti-Dengue NS1-gold colloid
- Test line (as main component): Mouse monoclonal anti-Dengue NS1
- Control line(as main component): Goat anti-mouse IgG.

Dengue IgG/IgM test device

- 1st test strip included: Gold conjugates (as main component): Recombinant Dengue virus envelope protein gold colloid
- Test line ‘‘G”(as main component): Mouse monoclonal anti-human IgG

- Test line ‘‘M” (as main component): Mouse monoclonal anti-human IgM
- Control line (as main component): Rabbit anti-Dengue IgG
- Assay buffer included: 100 Mm Phosphate buffer and Sodium azide.

Kit storage

The Dengue duo test kit was stored at 1~ 30 ^{0} C.

Procedure of the test

Dengue NS1 antigen test device

- 100µl or 3 drops of the specimen were added to the sample well(S) with a help of a disposable dropper.

Dengue IgG/IgM test device

- With a capillary pipette, 10 µl of specimen was added to the square sample well marked as ″S″.
- 4 drops of assay diluent was added to the round shaped assay diluent well

Interpretation of the test

Dengue NS1 antigen test device

Negative result *:* The presence of each one colour line within the result window indicates a negative result.

Positive result: The presence of two coloured lines (‘‘T” band and ‘‘C’’band) within the result window no matte which line appears first,indicates a positive result.

Invalid result *:* If the colour is not visible within the result window after performing the test,the result was considered invalid.

Dengue IgG/IgM test device

Negative result : The control line is only visible on the test device.

IgMPositive: The control line(C) and IgM line (M) are visible on the test device. This is positive for IgM antibodies to Dengue virus. This is indicative of a primary Dengue infection.

IgGPositive : The control line and IgG line (G) are visible on the test device. This is positive for IgG antibodies. This is indicative of secondary or past Dengue infection.

IgG and IgMPositive : The control line,IgM(M),IgG(G) are visible on the test device. This is positive for both IgM and IgG antibodies. This is indicative of late primary or early secondary Dengue infection.

Invalid : The control line fails to appear within the result window after performing the test, the result is considered invalid.

4.9.2. NS1 antigen ELISA

All 100 samples were tested for detection of NS1 antigen by ELISA(PANBIO DENGUE EARLY ELISA KIT)

Principle of the test

Serum Dengue NS1 antigen, when present, binds to anti-NS1antibodies attached to polysterene surface of the microwells. Residual serum is removed by washing. And HRP conjugated anti-NS1 MAb is

added. After incubation, the microwells are washed and a colourless substrate system, tetramethyl benzidine/hydrogen peroxide (TMB chromogen) is added. The substrate is hydrolysed by the enzyme and the TMB changes to a blue colour. After stopping the reaction with acid, the TMB turns yellow. Colour development is indicative of the presence of Dengue NS1 antigen in test sample.

Materials provided

- Anti-NS1 Antibody Coated Microwells(12×8 wells)
- HRP Conjugated Anti-NS1 MAb – 15 ml,one bottle. Horseradish – peroxidase conjugated Anti-NS1 monoclonal antibody with preservative(0.1 % Proclin^{TM}).
- Wash buffer (20×) – one bottle, 60 ml of 20 × concentrate of phosphate buffered saline (p^H 7.2 – 7.6) with Tween 20 and preservative (0.1 % Proclin^{TM}).
- Sample diluent – one bottle 22 ml. Tris buffered saline (p^H 7.2 – 7.6) with preservative(0.1 % Proclin^{TM}).
- TMB Chromogen (TMB) – One bottle ,15 ml. A mixture of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in citric acid citrate buffer (p^H 3.5 – 3.8).
- Positive control – one purple –capped vial, 1.2ml recombinant antigen (contains 0.1 % Proclin^{TM} and 0.005% gentamycin sulphate).
- Calibrator - two orange – capped vials , 1.5ml recombinant antigen

(contains 0.1 % Proclin^{TM} and 0.005% gentamycin sulphate).

- Negative control - one white –capped vial, 1.2 ml human serum (contains 0.1% sodium azide and 0.005 % gentamycin sulphate)
- Stop solution – one red –capped bottle, 15ml.1M Phosphoric acid.

Test procedure

- All reagents was equilibrated to room temperature (20-25^{0}C)before commencing the assay.
- Control and Sample predilution
  1) Required number of micro wells were removed from the foil and was inserted into the strip holder. Five microwell are required for Positive control(P),Negative control (N),Calibrator (CAL)in triplicate.
  2) 75 µl of Sample diluent was added to 75 µl of Positive control,Negativecontrol,Calibrator and serum samples. Mixed well. The final dilution of the sample is 1 in 2.

ELISA Procedure

- 100 µl of diluted test samples and controls were pipetted into their respective microwells.
- The plate was covered and incubated for 1 hour at 37^0 C.
- Washed six times wth diluted wash buffer
- 100 µl of HRP conjugated Anti-NS1 MAb was pipetted into each well.

- The plate was covered and incubated for 1 hour at 37^0 C.
- Washed six times wth diluted wash buffer.
- 100 µl of TMB was pipette into each well .
- Incubated at room temperature (20-$25^{0\,C}$) for 10 minutes, timing from the first addition.there was development of blue colour.
- 100 µl of Stop solution was pipette to all wells and mixed well. The blue colour changed to yellow.
- The absorbance of each well was read within 30 m wavelength of 450 nm with a reference filter of 600-650nm.

Calculations

- The calibration factor is batch specific and the calibration factor value was obtained before commencing calculations.
- The average absorbance of the calibrator triplicates was calculated and multiplied by the calibration factor. This gives the Cut-off Value.
- An Index value was calculated by dividing the sample absorbance by the Cut-off value.
- Alternatively ,Panbio units can be calculated by multiplying the Index value by 10.
- Index value = Sample Absorbance / Cut – off value
- Panbio Units = Index value × 10

Interpretation of results

Diagnosis of Dengue infection : This early ELISA kit assesses the presence of Dengue NS1 antigen in the patient's serum. A positive result (> 11 Panbio units) is indicative of either an active primary or secondary Dengue infection. It does not differentiate between primary and secondary infection.

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

Negative result : No detectable Dengue NS1 antigen. This result does not rule out Dengue infection. If this sample is negative and Dengue infection is still suspected, a follow up sample should be taken and tested,using serology, no later than 14 days l after the initial sample was taken.

Equivocal result : Equivocal samples should be repeated. Samples that remain equivocal after repeat testing should be repeated by an alternative method or another sample should be collected.

Positive result : Presence of detectable Dengue NS1 antigen. Dengue serology assays should be performed on follow – up samples to confirm Dengue infection.

4.9.3. IgG CAPTURE ELISA

All 100 samples were tested for detection of IgG antibodies by CAPTURE ELISA(SD DENGUE IgG CAPTURE ELISA KIT).

Principle of the test

IgG Capture ELISA is an Enzyme Linked Immunosorbent Assay for the qualitative detection of IgG antibodies against Dengue virus antigen in human serum. This kit contains a microplate, which is pre-coated with mouse monoclonal anti-human IgG antibodies on well. During first incubation with withmicroplate,anti – Dengue IgG antibody in patient serum is bound to mouse monoclonal anti-human IgG antibodies on well,and then is bound to mixture of Dengue antigen and mouse monoclonal anti- Dengue HRP Conjugate. Following this incubation,all unbound materials are removed by aspiration and washing. The residual enzyme activity found in the wells will thus be directly proportional to the mouse monoclonal anti-Dengue IgG antibody concentration in patient serum and evidenced by incubating the solid –phase with a substrate solution (Working TMB). Colorimetric reading will be performed by using a spectrophotometer at 450nm.

Materials provided

- Coated microplate : 96 wells (12×8 wells),coated anti-human IgG antibodies.
- Dengue antigen : Dengue 1~4 antigens pool.

- Sample diluents :Tris buffer saline with preservative NaN_3(0.05%)
- Mouse monoclonal anti-Dengue IgGonjugate(2×) : Anti-Dengue Antibodies conjugated to horseradish peroxidase(HRP) with preservative Thiomersal (0.01 %)
- Conjugate diluents : Phosphate buffered saline,BSA and stabilizers with preservative Thiomersal(0.01%).
- Positive control : Inactive Anti-Dengue IgG/IgM antibody positive human serum with preservative Proclin 300(0.01%)
- Negative control : Anti-Dengue IgG/IgM antibody negative human serum with preservative Proclin 300(0.01%).
- Working TMB : Hydrogen peroxidase Tetramethylbenzidine (TMB)
- Washing solution (20 x) : PBS –Tween 20
- Stopping solution : 1.6 N sulfuric acid.
- Adhesive plate sealer.

Active ingredients of materials and reagent provided

- Coated microplate : includes mouse monoclonal anti-human IgG antibodies(as a main component)
- Anti-Dengue IgG HRP conjugate (2x) : consists of mouse monoclonal anti-Dengue IgG antibodies conjugated solution to horseradish peroxidase (as a main component) with Thiomersal.
- Conjugate diluent : consists of phosphate buffered saline,BSA and Thiomersal.

- Sample diluent ; includes Tris,NaCl,NaN_3
- Positive control : consists of inactive anti-Dengue IgG/IgM antibody positive human serum with proclin 300.
- Negative control : includes anti-Dengue IgG/IgM antibody negative human serum with proclin 300.
- Working TMB : contain Hydrogen peroxidase with tetramethylbenzidine(TMB)
- Washing solution (20X) : contains phosphate buffer solution,Tween 20 and Thiomersal.
- Stopping solution : contains 1.6N sulfuric acid.

Kit storage

IgG capture ELISA assay kit was stored at $2\sim8^0$ C.

Test procedure

Sample predilution

The test strip wells and other reagents was prepared and brought to room temperature.

With the suitable test tubes 3 negative controls, 2 positive controls and serum sample were diluted with sample diluent in the ratio of 1 in 100 and mixed well.

ELISA procedure

Dengue antigen

A bottle of Dengue antigen powder was diluted using 1.5 ml of the Conjugate diluent.

Anti-Dengue HRP Conjugate 1 : 1 was diluted with diluted Dengue antigen and gently mixed and left at room temperature for 60 minutes.

Assay plate

- 100 µl of diluted controls and serum sample were added to their respective wells of the microplate.
- The microplate was then covered with adhesive plate sealer and incubated at 37^0C for 60 minutes.
- The wells were washed 5 times with diluted washing solution using an automatic washer.
- The diluted Anti-Dengue HRP conjugate solution was mixed well before transfer and 100 µl of the diluted Anti – Dengue HRP conjugate solution into the appropriate wells of microplate.
- The microplate was then covered with adhesive plate sealer and incubated at 37^0C for 60 minutes.
- The wells were washed 5 times with diluted washing solution using an automatic washer.

- 100 µl of working TMB was pipette into each value and incubated for 10 minutes at room temperature. Blue colour developed in the wells.
- 100 µl of stopping solution was pipette into each wells and mixed well and the blue colour changed to yellow.
- The absorbance of each well was read within 30 minutes at a wavelength of 450nm with a reference filter of 620nm.

Interpretation of the test

Calculations

The mean absorbance of the negative controls was calculated ,then the cut-off value was calculated by adding 0.300.

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

Test results :

1) absorbance of sample < cut-off = anti-Dengue IgG negative
2) absorbance of sample ≥ cut-off = anti-Dengue IgG positive

Negative result : no evidence of a past Dengue infection. If a recent Dengue infection is suspected,this can be confirmed by testing a further specimen 7 ~ 14 days after.

Positive result : presence of detectable anti-Dengue IgG antibodies indicates evidence of past or recent infection.

4.9.4. DENGUE IgM CAPTURE ELISA

All 100 samples were tested for detection of IgM antibodies by CAPTURE ELISA (PANBIO DENGUE IgM CAPTURE ELISA KIT).

Principle of the test

Serum antibodies of the IgMclass,whenpresent,combine with anti-human IgM antibodies attached to polysterene surface of the microwell test strips. A concentrated pool of of Dengue 1-4 antigens is diluted to the correct working volume with antigen diluents. The antigens are produced using aninsect cell expression system and immunopurified utilizing a specific monoclonal antibody. An equal volume of HRP conjugated Monoclonal Antibody (MAb) is added to the diluted antigen,which allows formation of antigen –MAbcomplexes.Residual serum is removed from the assay plate by washing,and complexed antigen-MAb is addaed 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 hydrolyzed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with the acid,the TMB becomes yellow. Colour development is indicative of the presence of anti-Dengue IgM antibodies in the test sample.

Materials provided

Anti-human IgM coatedmicrowells (12x8 wells).

Dengue 1-4 antigens(recombinant)- 150µl vial

Wash buffer(20x) -60 ml of 20x concentrate of phpsphate buffered saline (p^H 7.2-7.6) with Tween 20 and preservative(0.1% proclin)

Sample diluents – 50 ml(2 bottles): contains Tris buffered saline (p^H 7.2-7.6) with preservative(0.1% proclin) and additives.

Antigen diluents- 50 ml:includes Phosphate buffer containing preservatives(0.1% proclin and 0.005% gentamycin)

HRP Conjugated Monoclonal Antibody Tracer – 7ml. Includes Horseradish peroxidase conjugated monoclonal antibody tracer with preservative (0.1% proclin)and protein stabilizers .

TMB Chromogen(TMB) – 15 ml . A mixture of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in a citric-acid citrate buffer (p^H 3.5 – 3.8)

Positive control - 200 µl human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate)human serum

Calibrator – 400 µl human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate)

Negative control - 200 µl human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate)

Stop solution - 15ml,contains 1M Phosphoric acid.

Test procedure

- All reagents was equilibrated to room temperature (20-25^{0}C) before commencing the assay.

- Control and Sample predilution

    1) Required number of micro wells were removed from the foil and was inserted into the strip holder. Five microwell are required for Positive control(P),Negative control (N),Calibrator (CAL)in triplicate.
    2) 90 µl of Sample diluent was added to 10 µl of Positive control, Negative control, Calibrator and serum samples. Mixed well. The final dilution of the sample is 1 in 10.

ELISA Procedure

Antigen

Antigen was diluted 1/250 using antigen diluent. 10 µl of antigen was diluted into 2.5ml of antigen diluent which was sufficient for upto five strips. A volume of 0.5ml of diluted antigen was required per strip.

Required volume of diluted antigen was mixed with an equal volume of MAb Tracer in a clean vial and gentlt mixed the antigen-MAb Tracer solution and left at room temperature.

Assay plate

Within 10 minutes after the MAb Tracer was mixed with diluted antigen,

- 100 µl of diluted test samples and controls were pipetted into their respective microwells.
- The plate was covered and incubated for 1 hour at 37^0 C.
- Washed six times with diluted wash buffer

- 100 µl of antigen- MAb complexes from the antigen vial was pipetted into each well.
- The plate was covered and incubated for 1 hour at 37^0 C.
- Washed six times with diluted wash buffer.
- 100 µl of TMB was pipette into each well .
- Incubated at room temperature (20-$25^{0\,C}$) for 10 minutes , timing from the first addition.there was development of blue colour.
- 100 µl of Stop solution was pipette to all wells and mixed well. The blue colour changed to yellow.
- The absorbance of each well was read within 30 m wavelength of 450 nm with a reference filter of 600-650nm.

Calculations

- The calibration factor is batch specific and the calibration factor value was obtained before commencing calculations.
- The average absorbance of the calibrator triplicates was calculated and multiplied by the calibration factor. This gives the Cut-off Value.
- An Index value was calculated by dividing the sample absorbance by the Cut-off value.
- Alternatively ,Panbio units can be calculated by multiplying the Index value by 10.
- Index value = Sample Absorbance / Cut – off value
- Panbio Units = Index value × 10

Interpretation of results

Diagnosis of Dengue infection : This early ELISA kit assesses the presence of Dengue NS1 antigen in the patient's serum. A positive result (> 11 Panbio units) is indicative of either an active primary or secondary Dengue infection.

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

Negative result : No detectable Dengue IgM antibody. The result does not rule out Dengue infection. An additional sample should be tested in 7-14 days if early infection is suspected. Other Dengue assays should be performed to rule out acute infection.

Equivocal result : Equivocal samples should be repeated. Samples that remain equivocal after repeat testing should be repeated by an alternative method or another sample should be collected.

Positive result : Presence of detectable Dengue IgM antibody. Dengue serology assays should be performed to confirm Dengue infection.

Figure 1 : Dengue Rapid ICT Kit

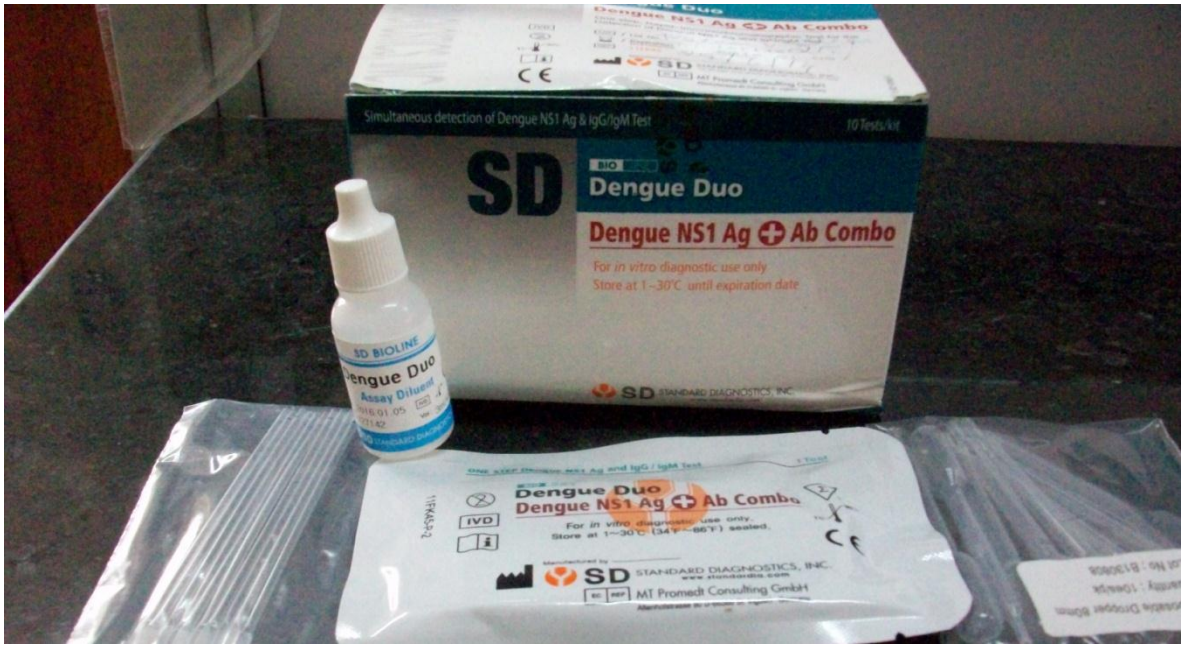


Figure 2: Card Test Positive For All The Three Parameters(NS1,IgM , IgG)

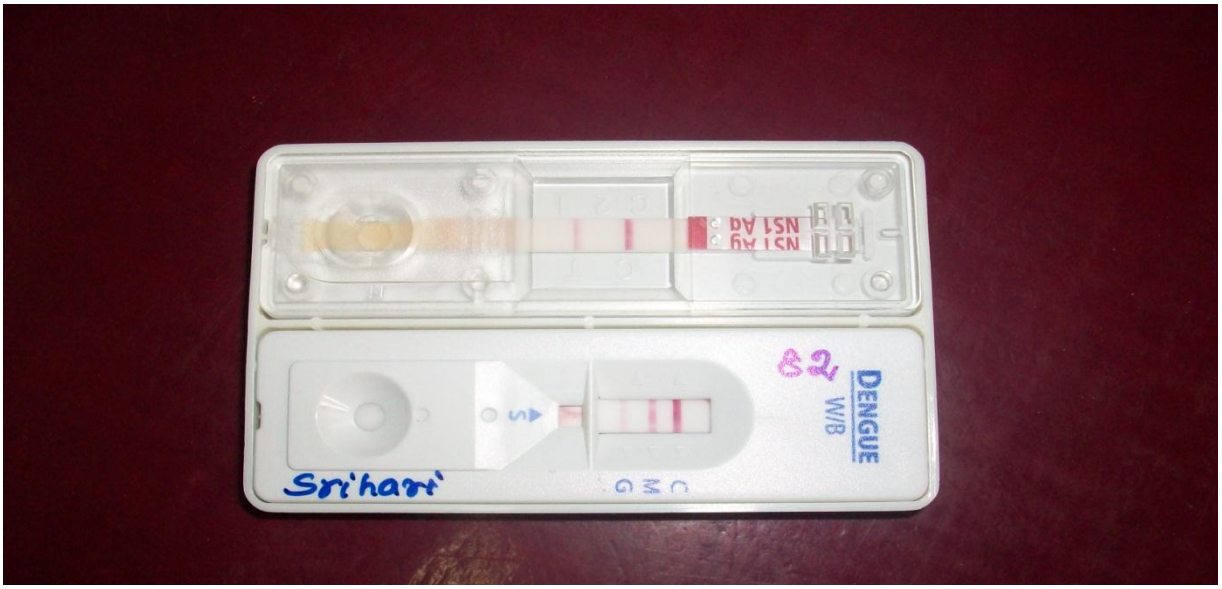


Figure 3 : Card Test Negative For Three Parameters

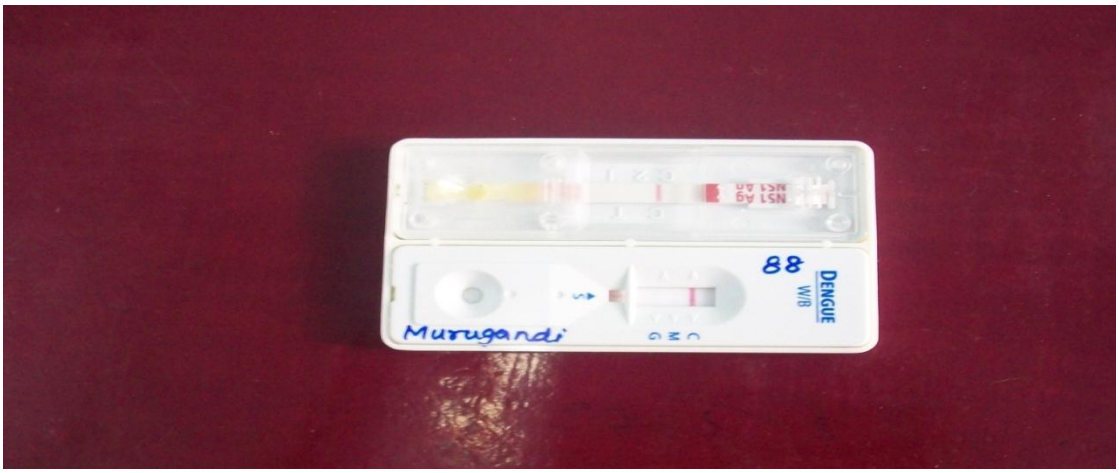


Figure 4 : Card Test Positive For NS1 Antigen.

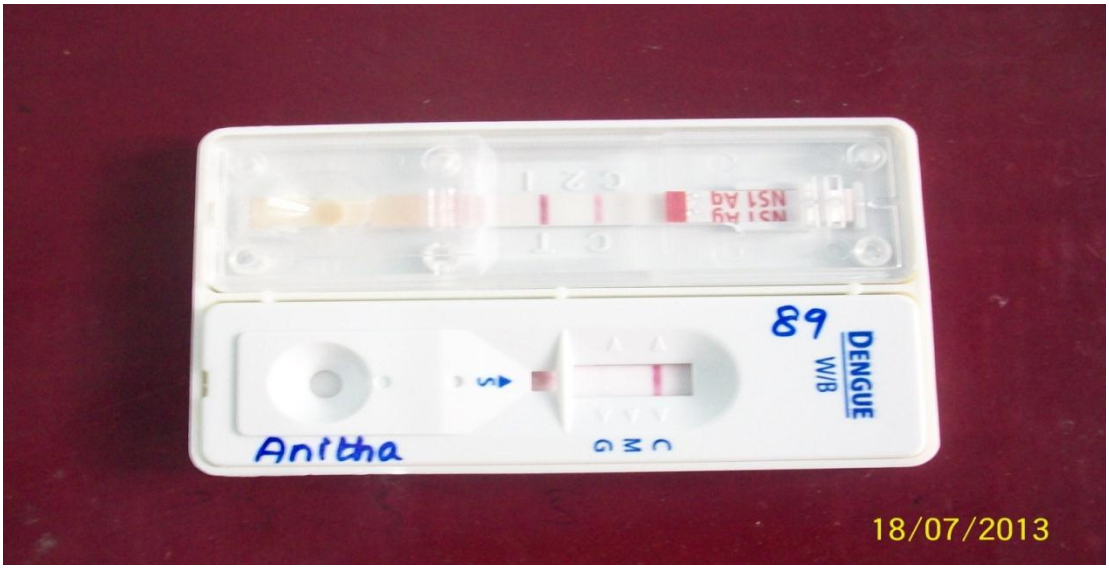


Figure 5 : Card Test Positive For IgM And IgG Antibodies.

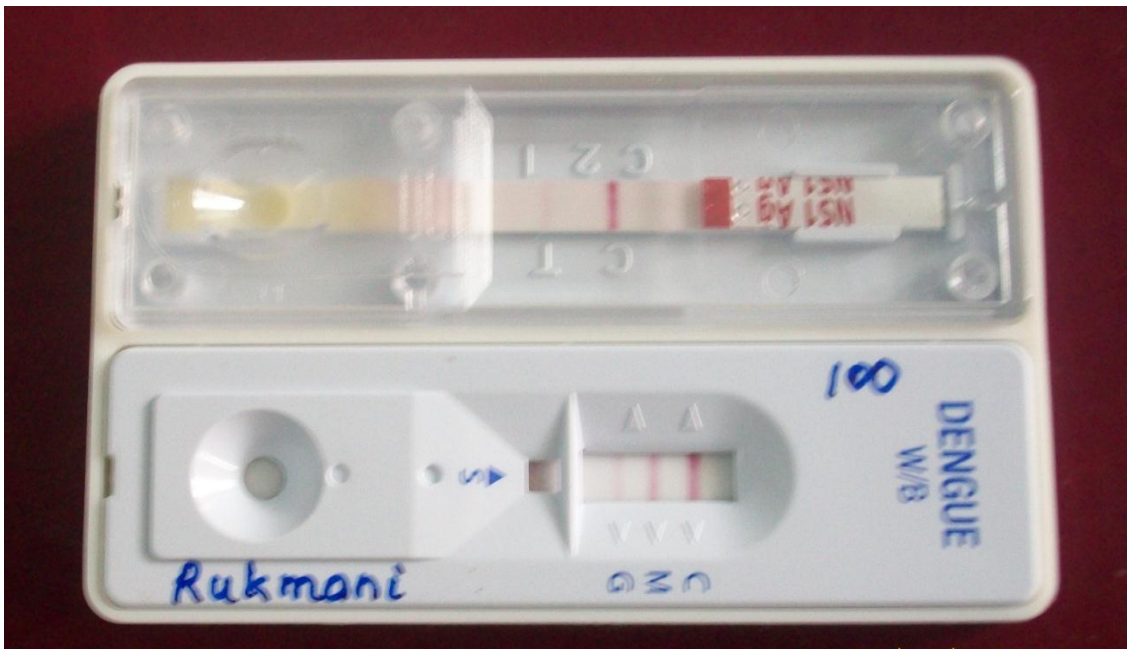


Figure 6 : Dengue Elisa Kit

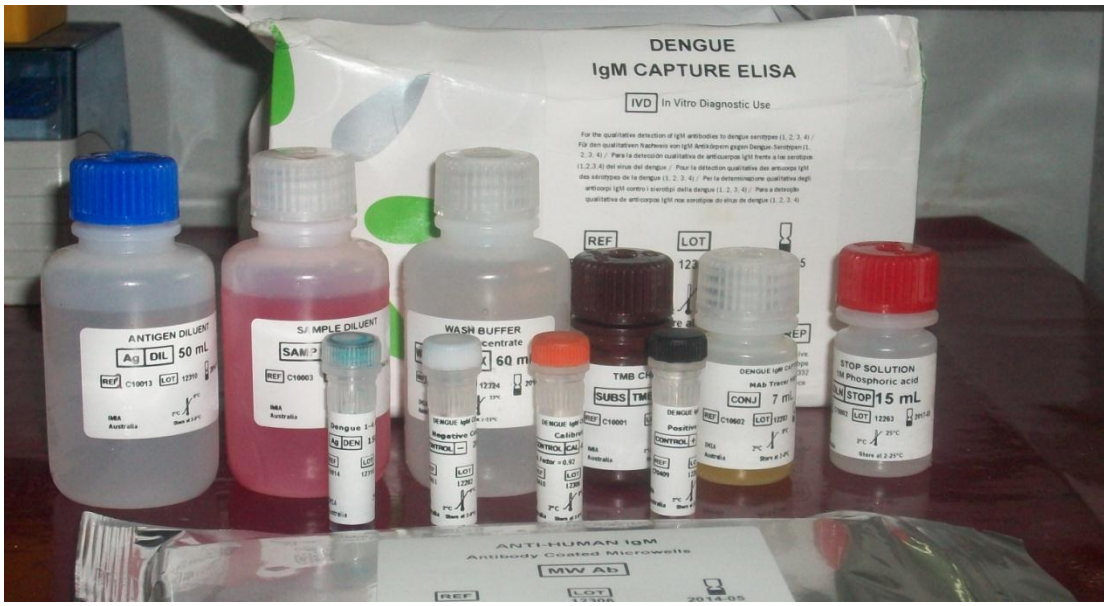


Figure 7 : Microtire Plate

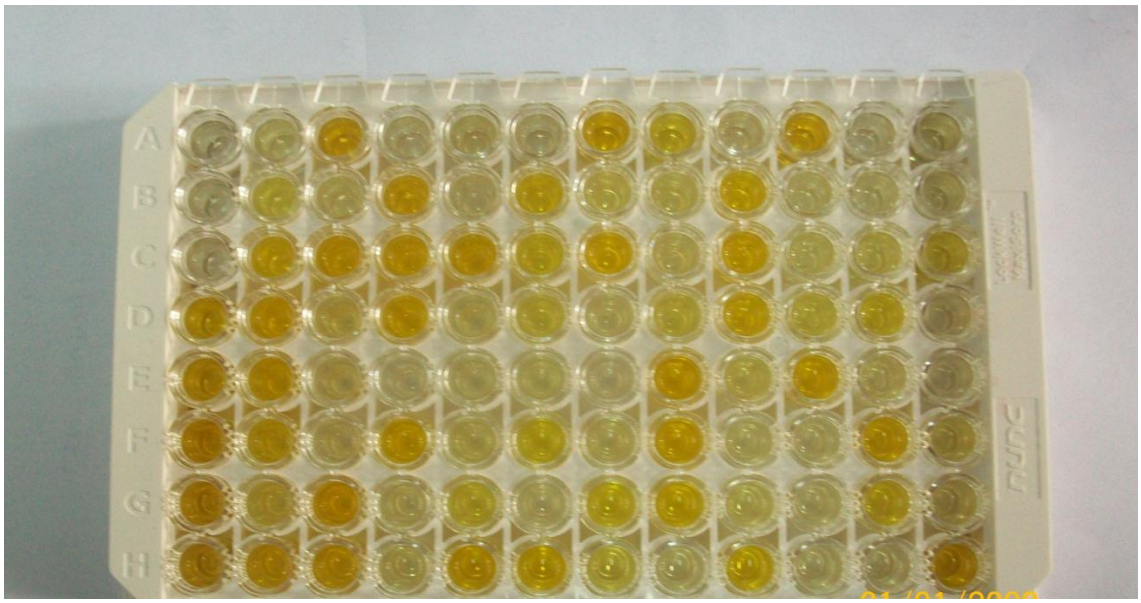
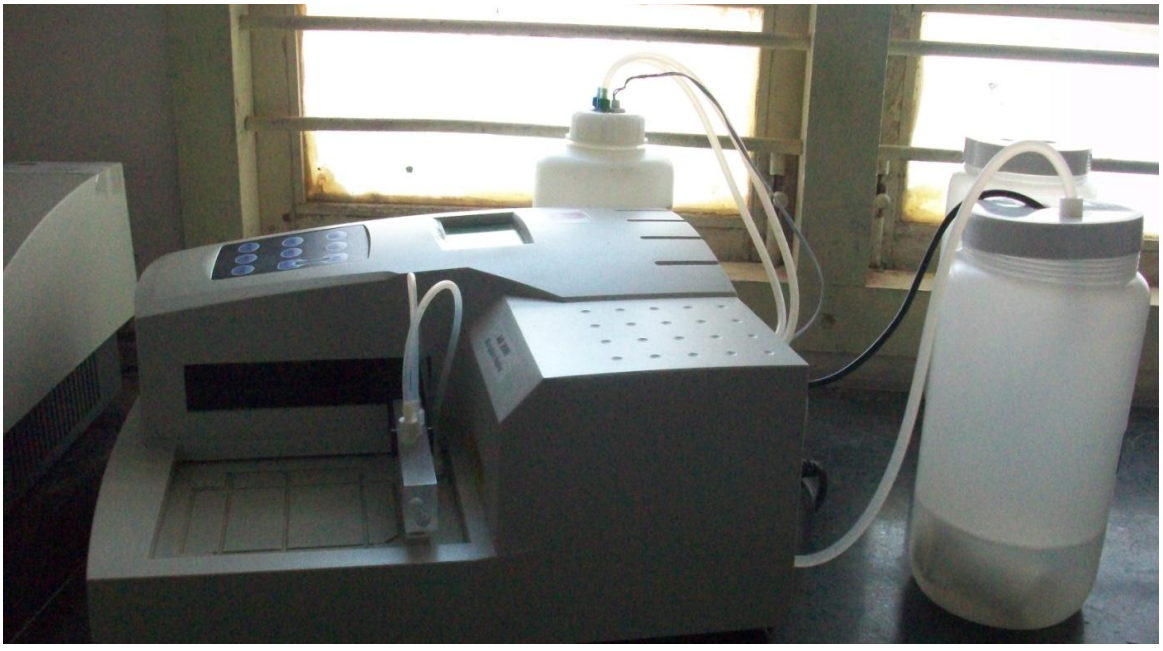


Figure 8 : Elisa Reader

Figure 9 : Elisa Washer



5. RESULTS

5.1 The Study Group

A total of 100 adults aged above 12 years with complaints of fever more than 4 days with thrombocytopenia (i.e.suspected Dengue cases) were analyzed. This study was conducted at the Department of Microbiology , Tirunelveli Medical College Hospital ,Tirunelveli over a period of one year from May 2013 to March 2014.

5.2 Statistical Analysis

All the results obtained were analysed statistically for their completeness , consistency and accuracy by the parameters like mean and percentages. Kappa value was calculated to measure the degree of agreement between two diagnostic methods - Rapid ICT and ELISA . The above statistical procedures were performed by IBM SPSS Statistics 20. Chi-square test and Fisher Exact test were used in calculating the P-value. The P-Values of less than 0.05 were considered as statistically significant ($P<0.05$).

5.3 Result Analysis

The selected 100 study subjects were analyzed based on age and sex, The results of the analysis are tabulated in Table 1.

Table 1.Age and sex wise distribution in study group

Age (in years)	Male		Female		Total	
	No.	%	No.	%	No.	%
11-20`	24	40.6%	18	43.9%	42	42%
21-30	16	27.1%	11	26.8%	27	27%
31-40	12	20.3%	6	14.6%	18	18%
41-50	4	6.8%	4	9.8%	8	8%
51-60	1	1.7%	2	4.9%	3	3%
61-70	2	3.4%	0	0	2	2%
Total	**59**	**59%**	**41**	**41%**	**100**	**100%**

Of the 100 patients, 59(59%) were males and 41(41%) were females. A total of 42(42%) cases were in the age group of 11-20 of which 24(40.6%) cases were males and 18(43.9%) cases were from females. 27(27%) cases belong to the age group of 21-30 years ,of which 16(27.1%) cases belong to males 11(26.8%) cases belongs to female.Out of 18(18%)cases in the age group of 31-40 years,12(20.3%) and 6(14.6%) were from males and females respectively.A total of 8(8%) belongs to the age group of 41-50 years,of which 4(6.8%) and 4(9.8%) were from males and females respectively.3(3%)case were in the age group of 51-60 years,among which 1(1.7%) and 2(4.9%) were males and females respectively.Only 2(2%) cases belongs to 61-69 years,of which 2(3.4%) were males and no females belongs to this age group.(Fig 10)

Table 2.Distribution of associated clinical features in study group.

Clinical features	No. of patients (n =100)	Percentage
Vomiting	55	55%
Headache	55	55%
Myalgia	73	73%
Bleeding manifestations	19	19%
Abdominal pain	11	11%

The above table shows that among the 100 cases studied, 55% of cases were associated with vomiting , 55% had headache , 73 % had myalgia, 19% had bleeding manifestations and 11% had abdominal pain.(Fig 11)

Figure 10 .Age and sex wise distribution in study group.

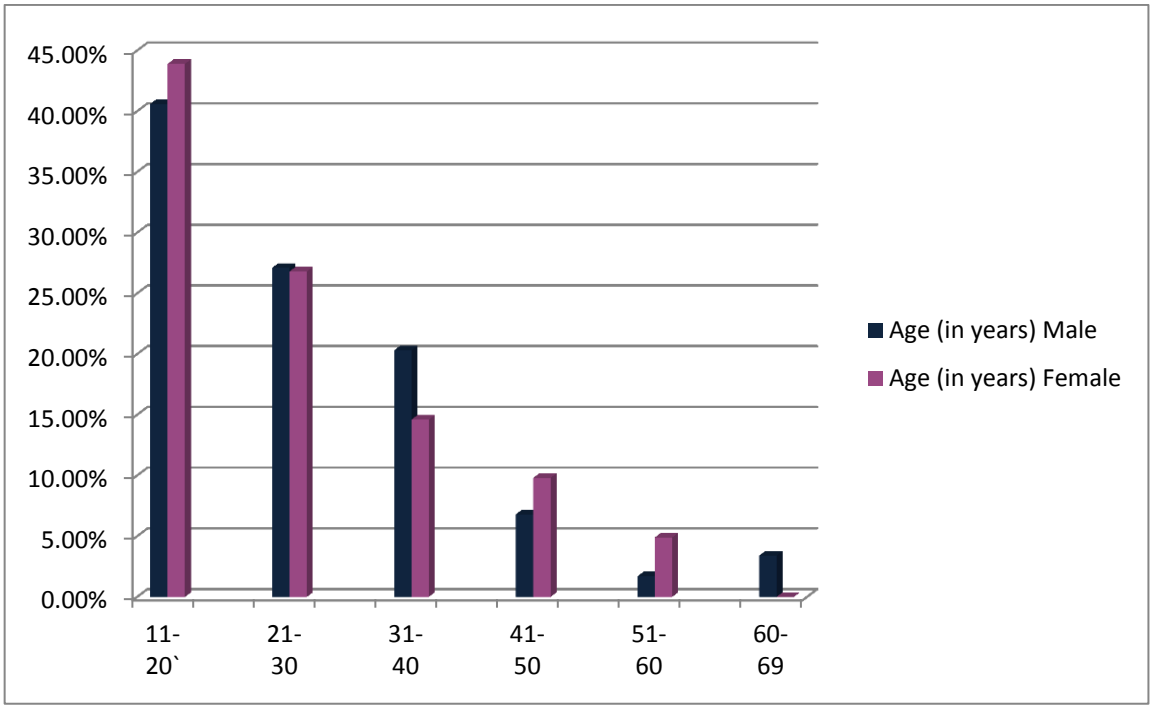


Figure 11. Distribution of associated clinical features in study group.

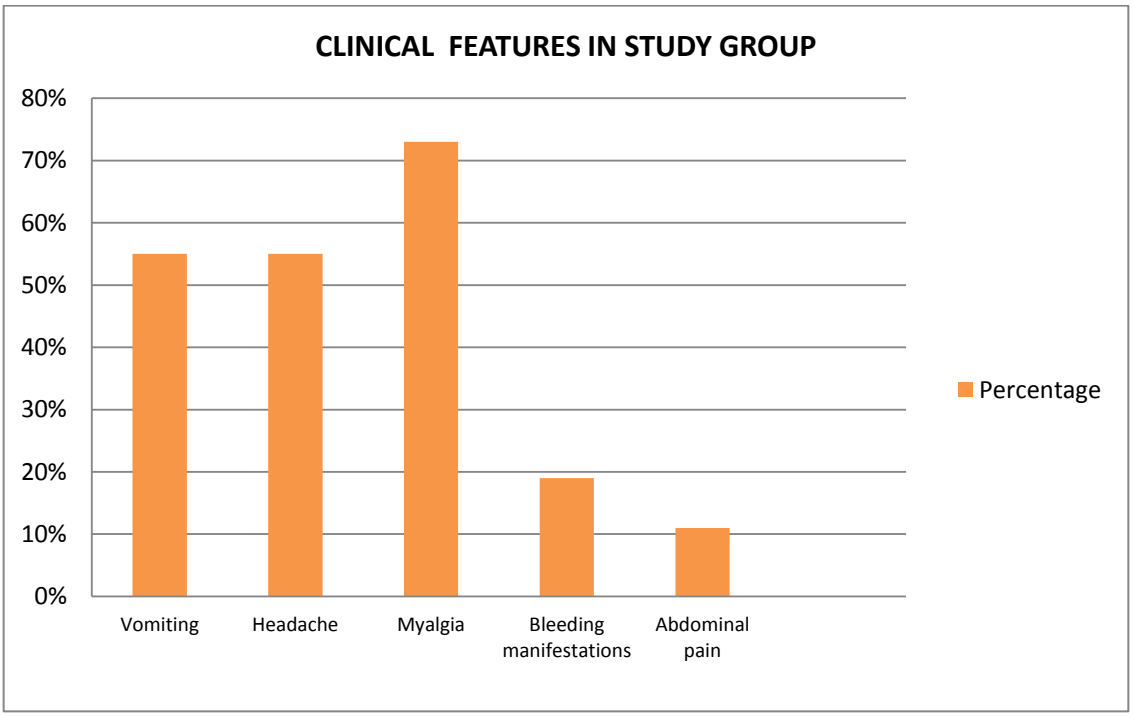


Table 3. Laboratory findings among the study group

Test	No.of patients (n=100)	Percentage
Thrombocytopenia	100	100%
Elevated Haematocrit	37	37%
Leucopenia	31	31%
Elevated liver enzymes	11	11%

The table above (figure) shows that out of 100 cases studied, haematocrit (Hct) was found to be elevated in 37% and 31% presented with leucopenia and 11% had elevated liver enzymes . Thrombocytopenia was present in all the study group.(Fig 12)

Table 4. Ultrasonography findings among the study group.

USG findings	No. of patients (n=100)	Percentage
Ascites	13	13%
Pleural effusion	9	9%
GB wall edema	9	9%

The ultrsonography findings from the above table 4(fig 13) shows that among the 100 patients studied, 13% had ascites, 9% had pleural effusion ,and 9% presented with GB wall edema .

Figure 12 . Haematological findings among the study group

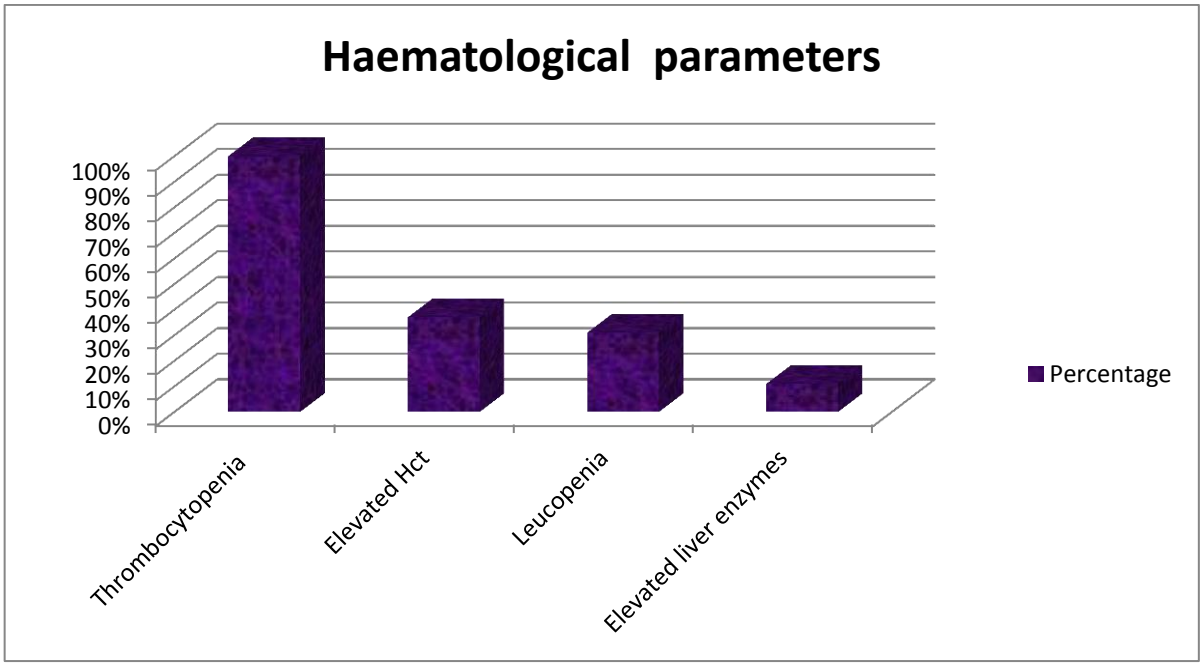


Figure 13. Ultrasonography findings among the study group.

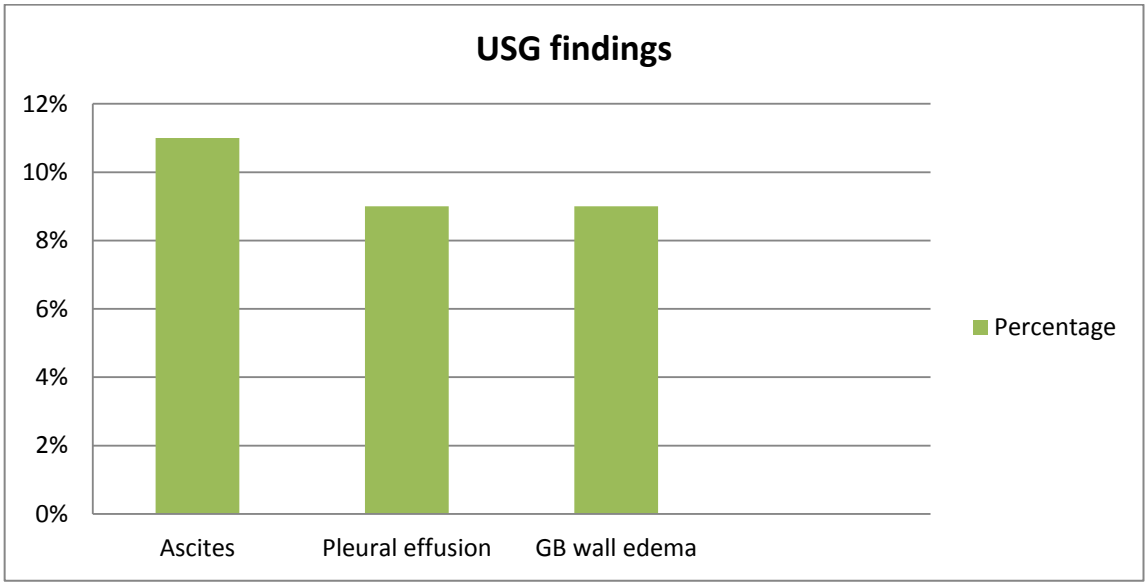


Table 5.Detection of Dengue cases by rapid ICT and ELISA (by any one of the parameters)

Test	Sample tested	Positive		Negative	
		Cases	%	Cases	%
ELISA	100	59	59%	41	41%
ICT	100	38	38%	62	62%

All the 100 samples were tested by both rapid ICT and ELISA for Dengue parameters(NS1 antigen, IgM and IgG antibodies). Out of this ,ELISA was positive for 59% of samples while 38% were positive by ICT as shown in table 5(figure 14).

Figure 14 .Detection of Dengue cases by any one of the parameters.

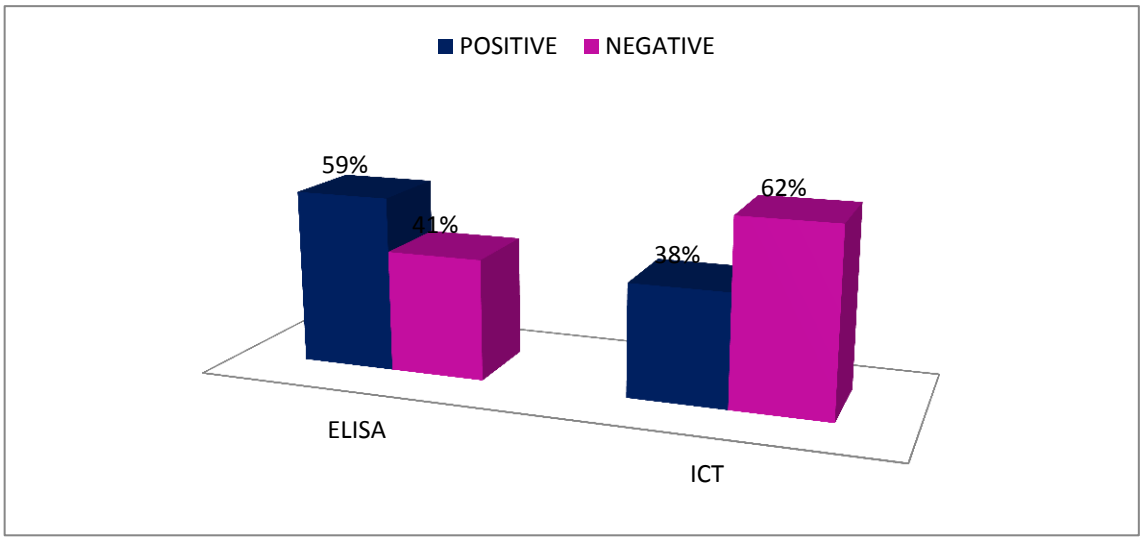


Table 6. Comparison of ICT and ELISA.

ICT	ELISA	
	Positive	**Negative**
Positive	37	1
Negative	22	40
Total	59	41

Detection of Dengue positive cases by rapid immunochromatographic card test was evaluated for its sensitivity and specificity against ELISA as reference test for NS1 antigen,IgM and IgG antibody detection.

Sensitivity = 62.7%

Specificity =97.6%

Positive predictive value =97.4%

Negative predictive value =64.5%.

From the above table , sensitivity of rapid ICT was 62.7% when evaluated against ELISA , a reference test. Specificity of ICT was 97.6% compared to ELISA and positive and negative predictive value were 97.4% and 64.5% respectively.

Table 7: Detection of Dengue parameters by ICT and ELISA

DENGUE PARAMETERS (alone or in combination)	NO. OF POSITIVES	
	ICT	ELISA
NS1 Antigen	15(15%)	16(16%)
IgM Antibody	15(15%)	49(49%)
IgG Antibody	24(24%)	51(51%)

From a total of of 100 cases, 15(15%) cases was positive for NS1 antigen,15(15%) was positive for IgM antibody and 24(24%) was positive for IgG antibody by rapid ICT. ELISA was able to detect 16(16%),49(49%) and 51(51 %)cases with NS1 Ag,IgM and IgG Ab respectively.(Fig 15)

Fig 15 :Dengue parameters detected by ICT and ELISA

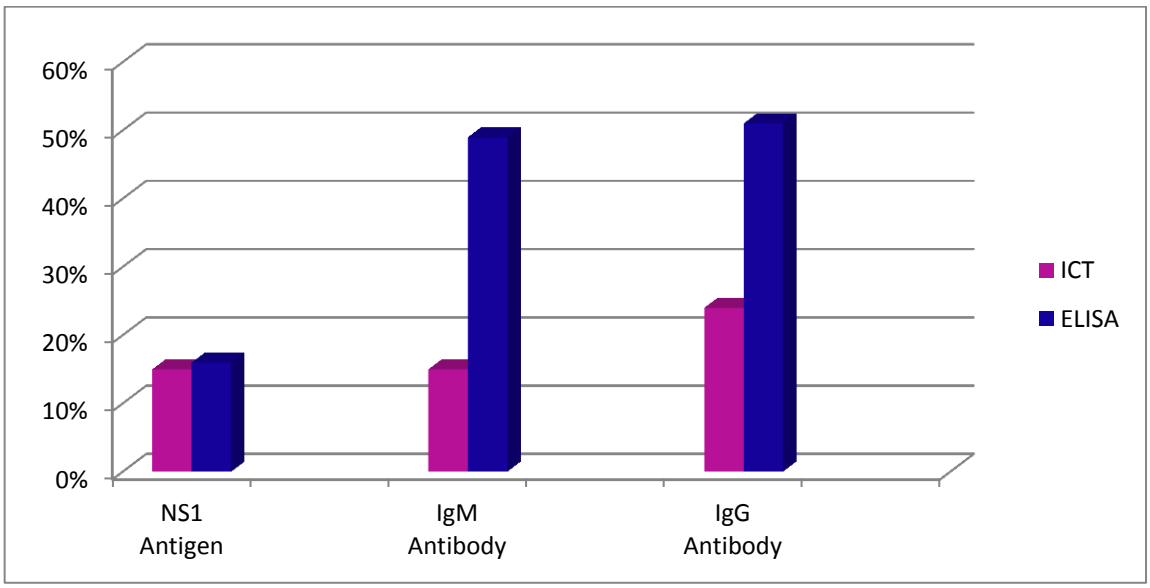


Table 8. Distribution of positive cases by ICT and ELISA

PARAMETERS	NO.OF POSITIVES	
	ICT	ELISA
NS1 only	9(23.7%)	2(3.4%)
IgM only	4(10.5%)	5(8.5%)
IgG only	12(31.6%)	8(13.6%)
NS1+IgM+IgG	3(7.9%)	13(22%)
NS1+IgM	1(2.6%)	1(1.7%)
NS1+IgG	2(5.3%)	0(0%)
IgM+IgG	7 (18.4%)	30(50.8%)
Total	38	59

The above table (fig 16) shows that among 38 ICT positive cases , 9 (23.7%) showed positive for NS1 only , 4 (10.5%) for IgM only, 12 (31.6%) for IgG only , 3 (7.9%) for NS1+IgM+IgG ,1 (2.6%) for NS1+IgM , 2 (5.3%) for NS1+IgG and 7 (18.4%) were positive for IgM+IgG.

The above table shows that among 59 ELISA positive cases , 2 (3.4%) showed positive for NS1 only , 5 (8.5%) for IgM only , 8 (13.6%) for IgG only , 13 (22%) for NS1+IgM+IgG ,1 (1.7%) for NS1+IgM, 0 (0%) for NS1+IgG and 30 (50.8%) were positive for IgM+IgG.

Table 9 : Distribution of primary/secondary infection in positive cases.

	Primary Dengue infection (NS1 ,IgM or NS1+IgM positive)		Secondary Dengue Infection (NS1+IgG,IgM+IgG or IgG positive)		Late stage of primary/early stage of secondary infection (NS1+IgM+IgG positive)	
	NO.	%	NO.	%	NO.	%
ICT	14	36.7%	21	55.3%	3	7.9%
ELISA	8	13.6%	38	64.4%	13	22%

The above table shows the distribution of primary and secondary infections among Dengue positive cases. ICT was able to differentiate 14(36.7%) and 21(55.3%)as primary and secondary infection respectively. Among the 59 ELISA positive cases 8(13.6%) showed primary infection and 38(64.4%) showed secondary infection. 3(7.9%) of ICT positive cases and 13(22%) of ELISA positive cases showed positive for all the three parameters(NS1+IgM+IgG) which indicates that the patient may be either in the late stages of primary infection or in early stages of secondary infection.(Fig 17)

The presence of high titre of IgG early in the course of the disease is considered as a criterion for secondary infection.

Fig 16 :Distribution of positive cases by ICT and ELISA

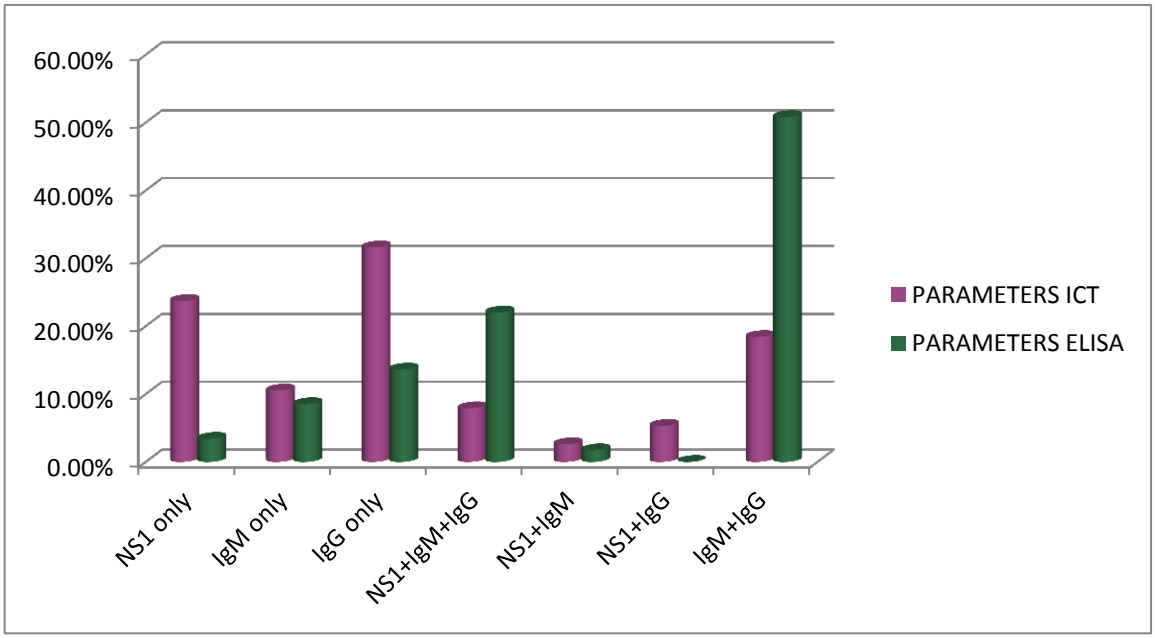


Figure 17 : Distribution of primary/secondary infection

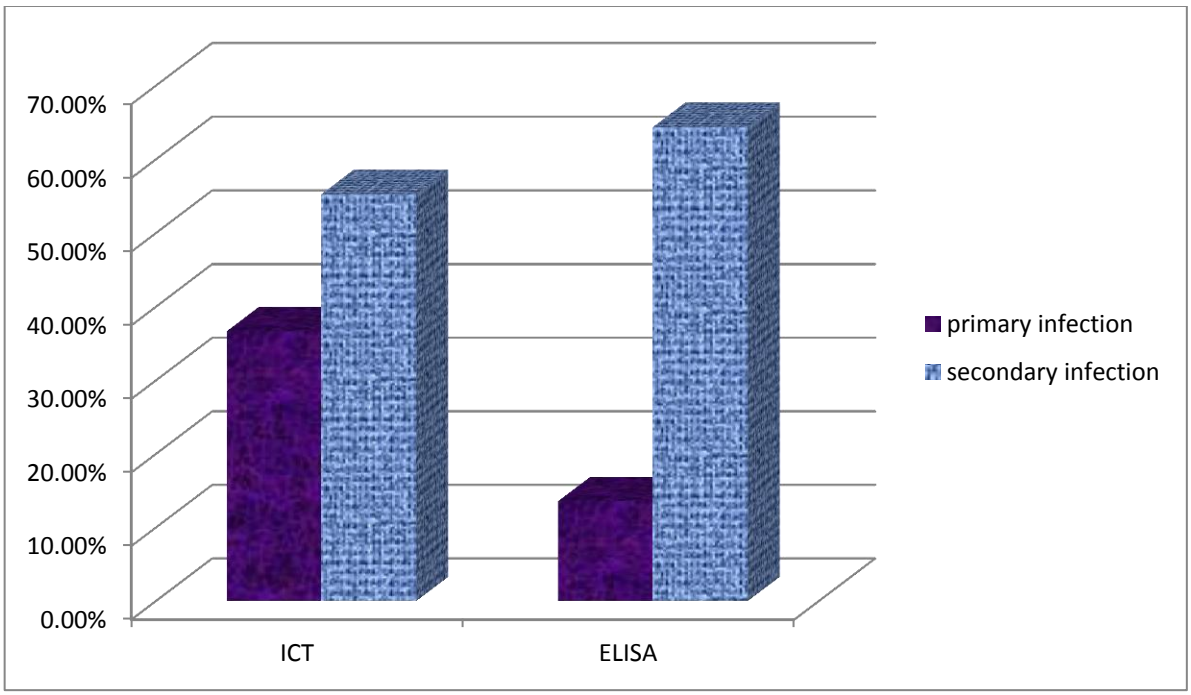


Table 10. Comparison of NS1 antigen by ICT and ELISA.

ICT	ELISA	
	Positive	**Negative**
Positive	14	1
Negative	2	83
Total	16	84

Detection of Dengue NS1 antigen by rapid immunochromatographic card test was evaluated for its sensitivity and specificity against NS1 antigen ELISA as reference test.

Sensitivity = 87.5%

Specificity =98.8%

Positive predictive value =93.3%

Negative predictive value =97.6%

From the above table , sensitivity of rapid ICT was 87.5% when evaluated against NS1 antigen ELISA , a reference test. Specificity was 98.8% compared to ELISA and positive and negative predictive value were 93.3% and 97.6% respectively.

Table 11. Comparison of IgM antibody by ICT and ELISA.

ICT	ELISA		TOTAL
	Positive	**Negative**	
Positive	14	1	15
Negative	35	50	85
Total	49	51	100

Detection of Dengue IgM antibody by rapid immunochromatographic card test was evaluated for its sensitivity and specificity against IgM Capture ELISA as reference test.

Sensitivity = 28.6%

Specificity =98%

Positive predictive value =93.3%

Negative predictive value =58.8% .

From the above table , sensitivity of rapid ICT was 28.6% when evaluated against IgM Capture ELISA , as a reference test. Specificity was 98% compared to ELISA and positive and negative predictive value were 93.3% and 58.8% respectively.

Table 12. Comparison of IgG antibody by ICT and ELISA.

ICT	ELISA		TOTAL
	Positive	**Negative**	
Positive	24	0	24
Negative	27	49	76
Total	51	49	100

Detection of Dengue IgG antibody by rapid immunochromatographic card test was evaluated for its sensitivity and specificity against IgG Capture ELISA as reference test.

Sensitivity = 47.1%

Specificity =100%

Positive predictive value =100%

Negative predictive value =64.5% .

From the above table , sensitivity of rapid ICT was 47.1% when evaluated against IgG Capture ELISA , a reference test. Specificity was 100% compared to ELISA and positive and negative predictive value were 100% and 64.5% respectively.

Table 13.Association of urban and rural area in positive cases.

	Positive		Negative		Total	
	No.	**%**	**No.**	**%**	**No.**	**%**
Urban	35	59.3%	23	56.1%	58	58%
Rural	24	40.7%	18	43.9%	42	42%
Total	**59**	**100**	**41**	**100**	**100**	**100**

The above table shows that out of 59 ELISA positive cases 35(59.3%) cases belong to urban area and 24(40.7%) to rural area . In Dengue negative cases , 23(58%) cases reside in urban and 18(43.9%) in rural area. The association of cases living in urban and rural area was found to be statistically not significant. (Fig 18)

Figure 18 .Association of urban and rural area in positive cases.

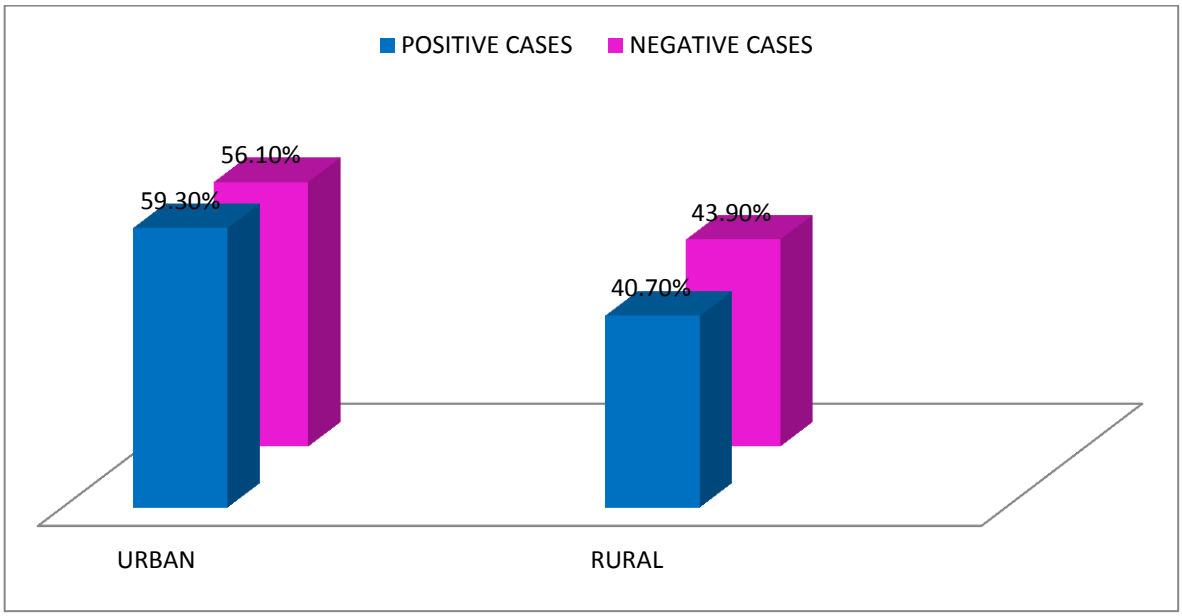


Table 14. Sex wise distribution among positive cases.

Sex	Positive cases	
	No.	%
Male	35	59.3%
Female	24	40.8%
Total	**59**	**100**

Sexwise distribution of Dengue positive cases in the above table shows that males were more affected than the females(fig 19). Out of 59 positive cases detected by ELISA,35(59.3%) were males and 24(40.8%) were females . The ratio of male to female was found to be 1.5:1.

Figure 19. Sex wise distribution among positive case

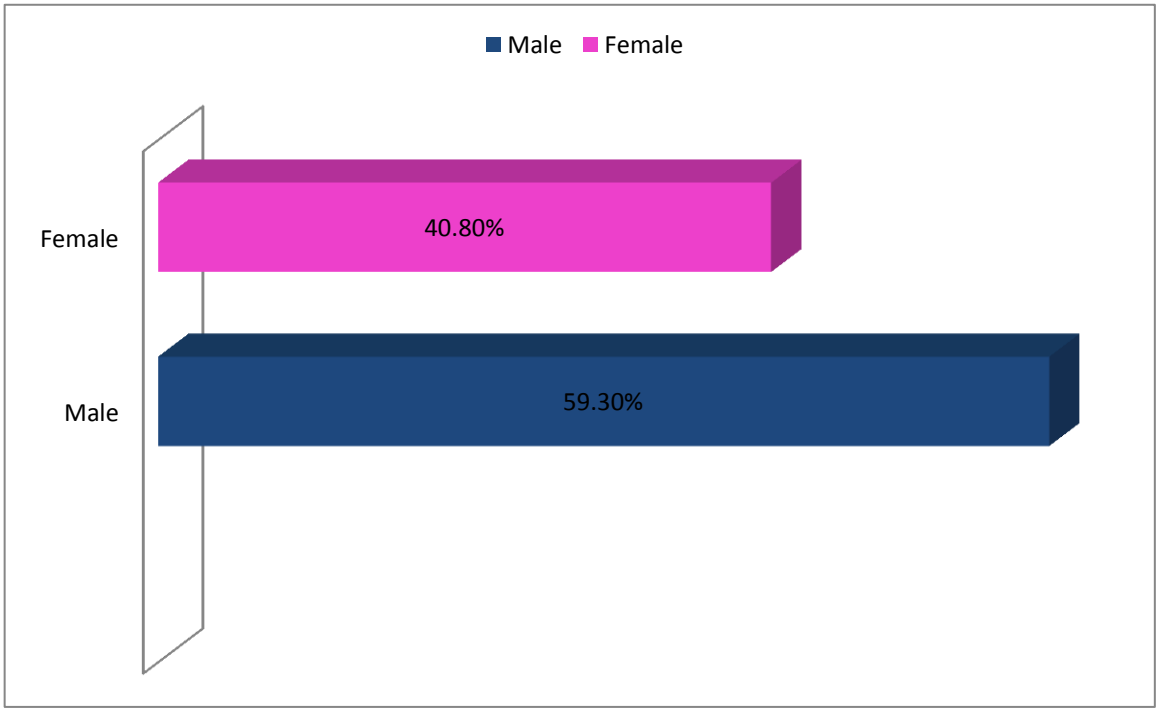


Table 15. Age wise distribution among positive cases.

Age (in years)	Positive cases	
	No.	%
11-20	28	47.5%
21-30	17	28.8%
31-40	9	15.3%
41-50	4	6.8%
51-60	1	1.7%
61-70	0	0
Total	**59**	**100**

The above table (Fig 20) shows that majority of positive cases are in the age group of 11-20 years . Out of 59 Dengue positive cases shown by ELISA, 28 (47.5%) were in the age group of 11-20 years . This is followed by a higher incidence in the age group of 21-30 years (28.8%).

Figure 20. Age wise distribution among positive cases.

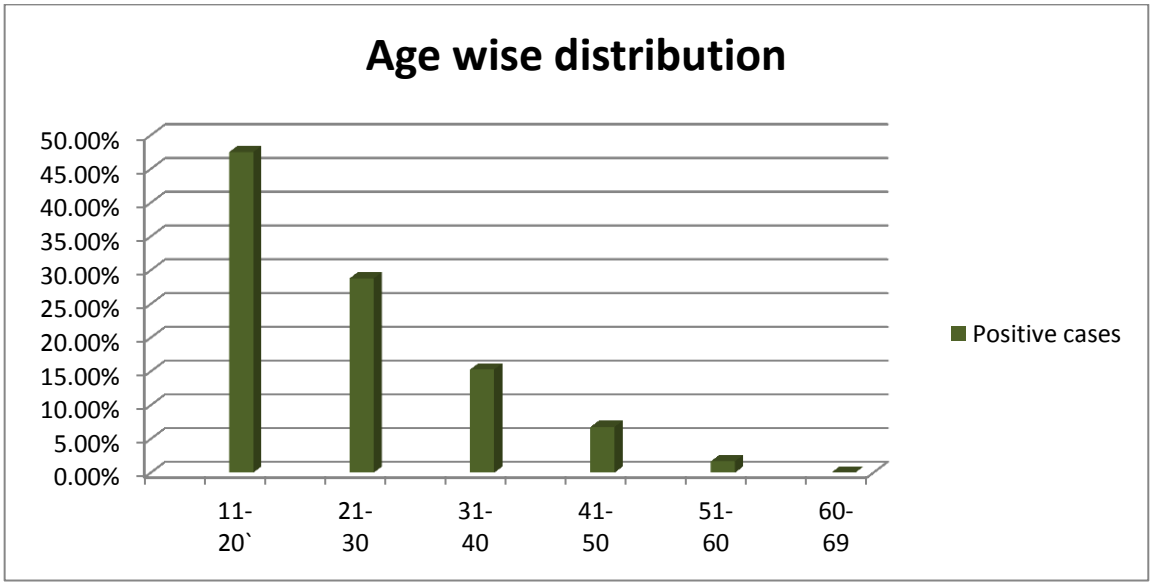


Table 16 : Association of clinical features in Dengue positive cases.

Clinical features	Dengue positive cases (n=59)	Dengue negative cases (n=41)	Total	Test of Significance(P<0.05)
Vomiting	38 (64.4%)	18(43.9%)	56	Significant
Myalgia	46 (78%)	27(65.9%)	73	Not significant
Headache	34 (57.6%)	22(53.7%)	56	Significant
Bleeding manifestations	10 (16.9%)	9(22%)	19	Not significant

The above table (Fig 21) shows the association of clinical presentation in Dengue positive cases. Vomiting was present in 38(64.4%) of positive cases ,myalgia in 46(78%),headache in 34(57.6%) and bleeding manifestations in 10(16.9%) of cases. Among the clinical features vomiting and headache was found to be statistically significant.

Table 17 : Laboratory findings in Dengue positive cases.

Haematological tests	Dengue positive cases (n=59)	Dengue negative cases (n=41)	Total	Test of Significance(P<0.05)
Thrombocytopenia	9(100%)	41(100%)	100	Significant
Leucopaenia	23(39%)	8(19.5%)	31	Significant
Elevated Haematocrit	26(44.1%)	11(26.8%)	37	Significant
Elevated Liver Enzymes	10(16.9%)	1(2.4%)	11	Significant

Association of laboratory findings in Dengue positive cases are shown in the above table. The association of thrombocytopenia, leucopaenia, elevated haematocrit (Hct) and elevation of liver enzymes among Dengue positive and negative cases were found to be statistically significant.(Fig 22)

Table 18 : Association of USG findings in Dengue positive cases.

USG findings	Dengue positive cases (n=59)	Dengue negative cases (n=41)	Total	Test of Significance(P<0.05)
Ascites	11 (18.6%)	2(4.9%)	13	Significant
Pleural effusion	6 (10.2%)	3(7.3%)	9	Not significant
GB wall edema	8 (13.6%)	1(2.4%)	9	Significant

USG findings such as ascites 11(18.6%),pleural effusion6(10.2%) and GB wall edema 8(13.6%) in Dengue positive cases are shown in the above table. Among the Dengue positive and negative cases ascites and GB wall edema was found to be significant statistically.(Fig 23)

Figure 21: Association of clinical features in Dengue positive cases

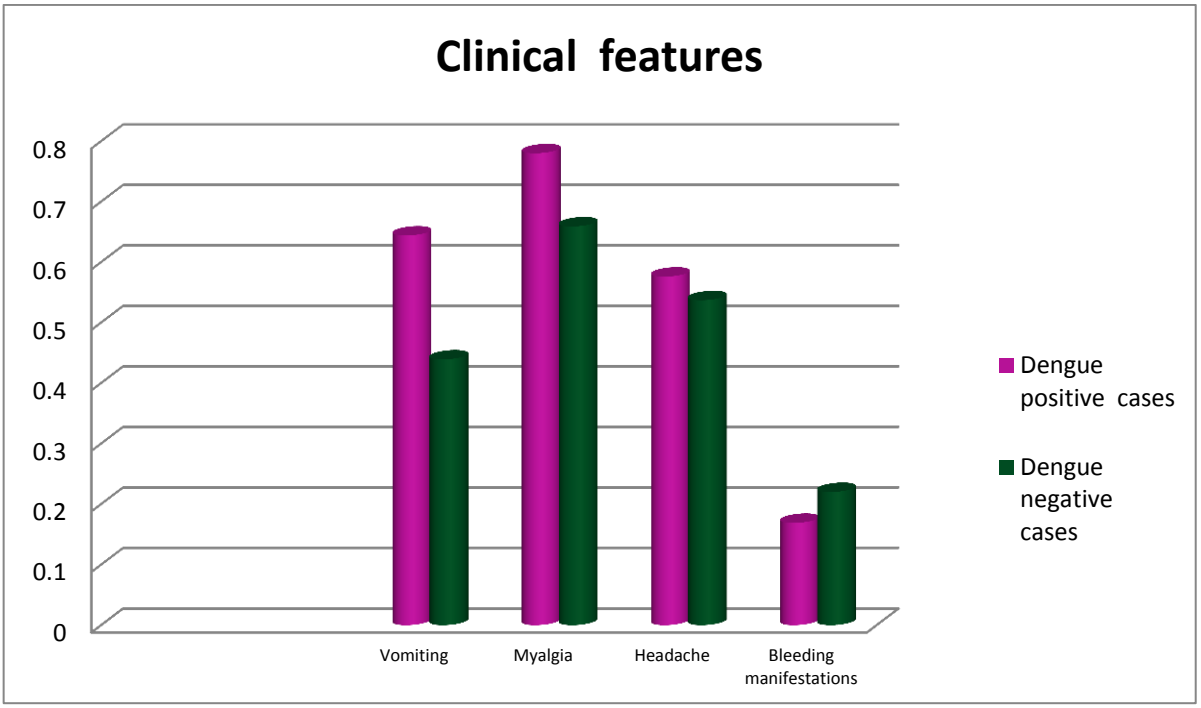


Figure 22: Laboratory findings in Dengue positive cases.

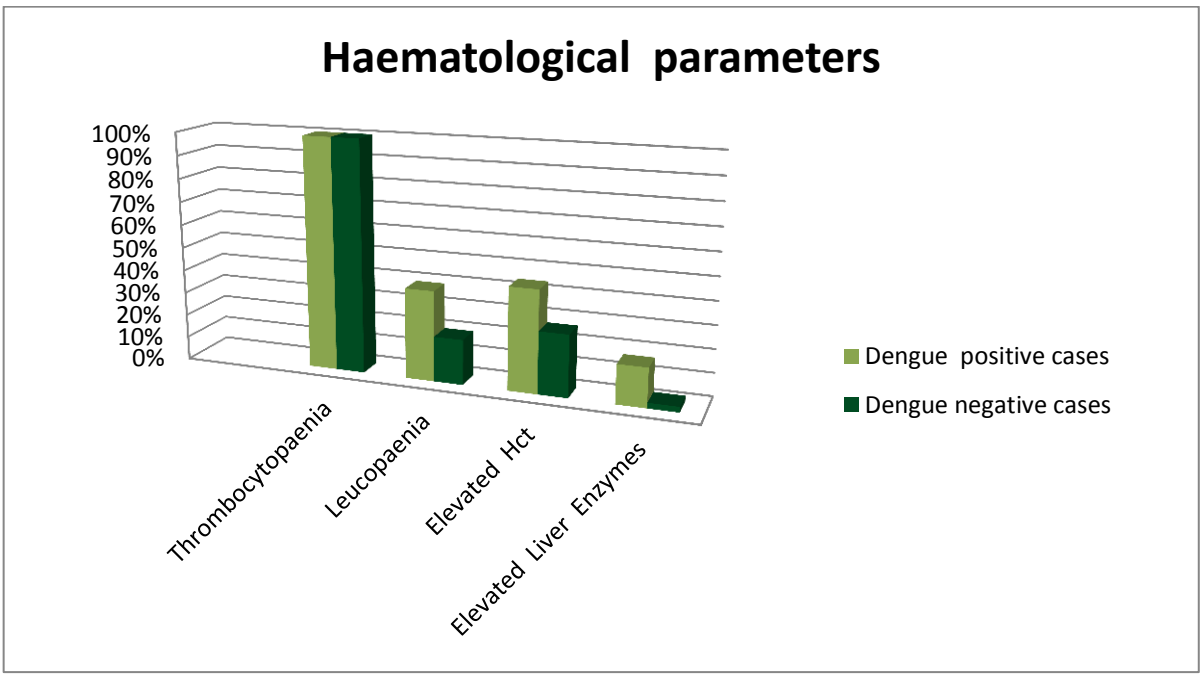


Table 19 :Distribution of laboratory and USG findings in Primary/Secondary Dengue cases

Findings	Primary Dengue infection	Secondary Dengue infection	Late stage of primary/early stage of secondary infection	Total positives
Thrombocytopenia	8(13.6%)	38(64.4%)	13(22%)	59
Leucopaenia	3(13%)	16(69.6%)	4(17.4%0	23
Elevated Hct	4(15.4%)	17(65.4%)	5(19.2%)	26
Elevated Liver Enzymes	1(10%)	7(70%)	2(20%)	10
Ascites	2 (18.2%)	6(54.6%)	2(18.2%)	11
Pleural effusion	2(33.3%)	2(33.3%)	2(33.3%)	6
GB wall edema	2(25%)	5(62.5%)	1(12.5%)	8

The above table(Fig 24) shows the distribution of laboratory and USG findings among primary,secondary and during late stage of primary or early stage of secondary Dengue infection. All the findings were found to be higher in secondary Dengue infection when compared with the others.

Figure 23: Association of USG findings in Dengue positive cases.

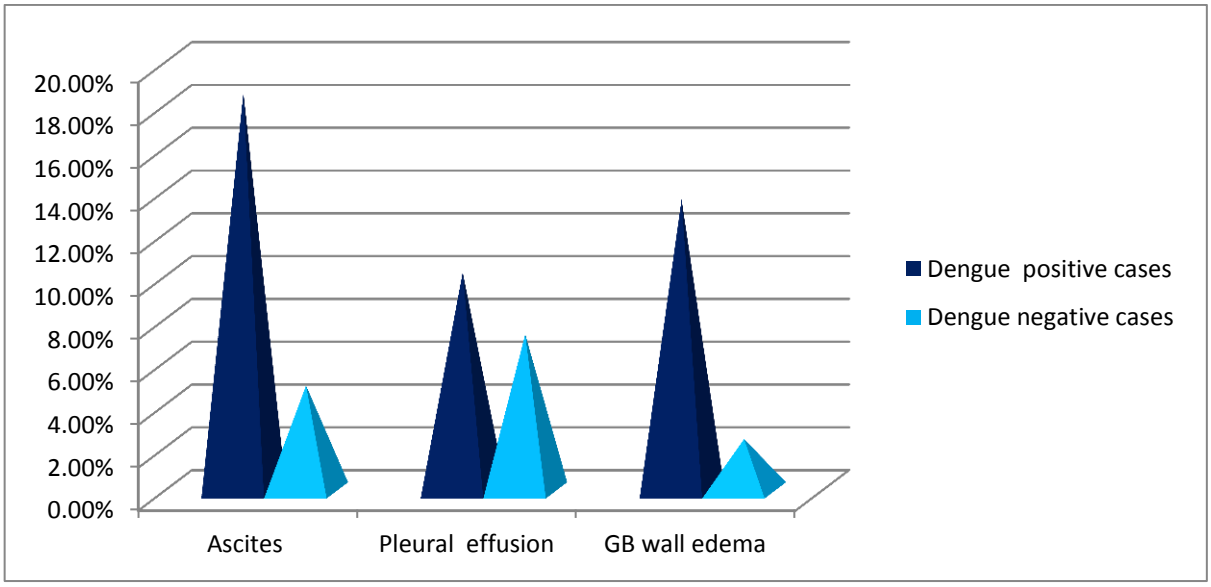
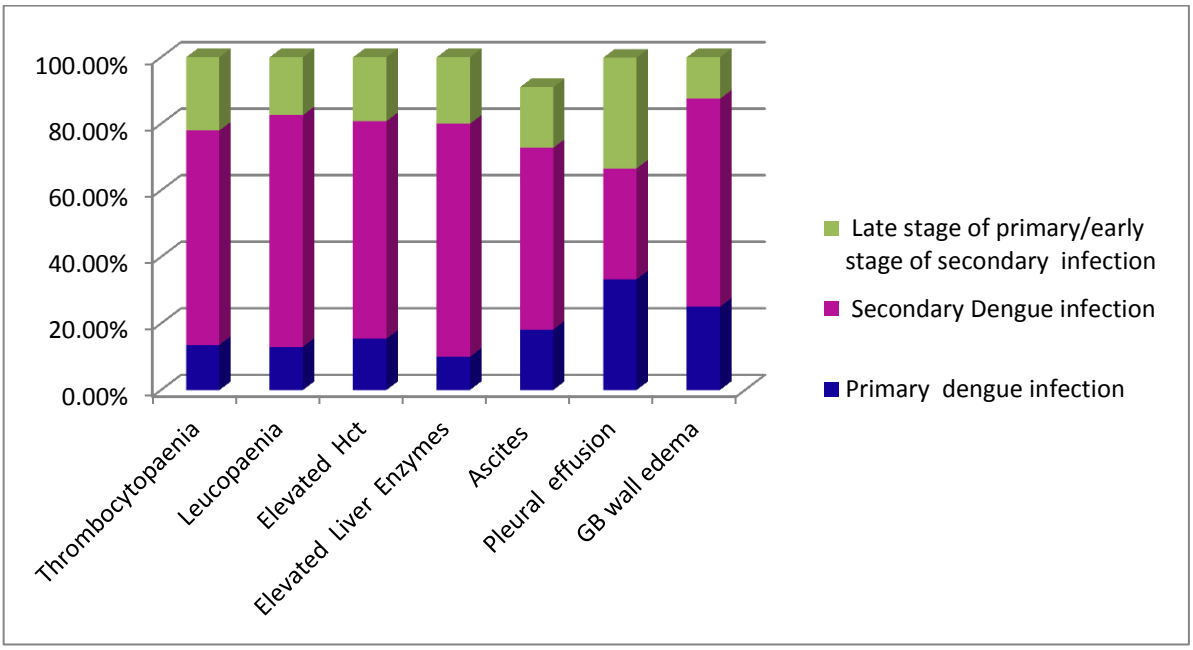


Figure 24:Distribution of laboratory and USG findings in Primary/Secondary Dengue cases



6. DISCUSSION

Dengue is one of the major re-emerging viral infections. Though the disease is usually seen in endemic areas, many epidemics involving continents and pandemics have been witnessed by the world.For the past few decades, there has been a significant raise in the number of cases , disease severity and geographic spread .This is mainly due to drastic change in the environment which in turn is due to rapid urbanization and increase in transportation across several parts of India .Dengue fever has been known to be endemic in India for more than 20 years as a benign and self-limited disease.During recent years, severe form of Dengue infection known as DHF manifests more frequently which leads to increase in the number of hospitalization of the affected patients .Outbreaks of Dengue fever are reported almost every year in India .Detection of all four Dengue serotypes in India has now rendered India hyper endemic .An early diagnosis of the disease aids in effective management and prevents further complications like DHF and DSS .With this background, the present study attempts to evaluate the efficacy of rapid immunochromatography test against ELISA in diagnosing acute Dengue infection .

6.1 Age and sex wise distribution in study group

The present study shows that out of 100 suspected cases, males were found to be predominant than the females in the ratio of 1.4 : 1.

Male preponderance were observed in similar studies conducted by Manisha Patankar *et al*[22] and Gargi Ghosh *et al* [21]. This may be the representation of those who visited the hospital to seek healthcare rather the truely infected population.

The suspected cases who were seeking medical attention belonged to the age group of 11-20 years in the present study. This result is similar to the study conducted by Gargi Ghosh *et al* [21].

Demographic characteristics like age distribution and gender differences are important for the successful planning of public health programmes and effective control of communicable diseases[120].

6.2 Distribution of associated clinical features ,laboratory and USG findings in study group.

The current study shows that among the 100 Dengue suspected cases ,55% was associated with vomiting , (55%) with headache, 73 % had myalgia, 19% had bleeding manifestations , 11% had abdominal pain and 13% presented with URI .The laboratory and USG findings shows that haematocrit (Hct) was found to be elevated in 37% and 31% presented with leucopenia and 11% had liver enzymes elevated , 13% had ascites,9% had pleural effusion,9% with GB wall edema and 8% presented with hepatomegaly which was similar to the study conducted by NP Singh *et al* [74]and in contrast to a study by Ghargi ghosh *et al*[21].

6.3 Prevalence of Dengue infection

In the present study Dengue viral infection accounts for 59% of hospitalization in adults with acute febrile illness .The prevalence is comparable to a study conducted by Chakravarti *et al*[23](60.7%)in 2011.

Vijayakumar *et al* [13]in his five year study conducted at Christian Medical College , Vellore observed that the prevalence of Dengue infection was 43.5% in 1999 and 55.1% in 2003 whereas in the intervening years the percentage was just above 25 %.

In contrast there was a high prevalence of Dengue infection (65.2%) in a study conducted by Siraj A. Khan *et al*[24] in a hilly region of Andhra Pradesh.

6.4 Comparison of ICT and ELISA.

The sensitivity of rapid ICT was 62.7% when evaluated against ELISA , as a reference test. Specificity of ICT was 97.6% compared to ELISA and positive and negative predictive value were 97.4% and 64.5% respectively.

The sensitivity of the rapid ICT in present study correlates well with that of a study conducted by Pramiladevi *et al*[121] (68.75%) but with lower specificity than that of the present study. Similar study by Scott R. Fry *et al*[122] showed sensitivity and specificity of rapid ICT as 69.2% and 96% respectively.

Hence all the samples negative by ICT have to be confirmed by a more sensitive test like ELISA or PCR.

6.5 Detection of Dengue parameters by ICT and ELISA

From a total of of 100 cases, 15(15%) cases were positive for NS1 antigen,15(15%) positive for IgM antibody and 24(24%) cases positive for IgG antibody by rapid ICT. ELISA was able to detect 16(16%),49(49%) and 51(51 %)cases positive for NS1 Ag,IgM and IgG Ab respectively.

The present study shows that ELISA was able to detect more IgM and IgG positive cases than the ICT whereas there was no much of difference between ICT and ELISA for NS1 antigen detection . Hence ICT can be used in NS1 detection in the early stages of the diseases.

6.6 Distribution of positive cases by rapid ICT and ELISA.

The present study shows that among 38 ICT positive cases , 9 (23.7%) showed positive for NS1 only , 4 (10.5%) for IgM only , 12 (31.6%) for IgG only , 3 (7.9%) for NS1+IgM+IgG ,1 (2.6%) for NS1+IgM and 2 (5.3%) for NS1+IgG.

In contrast , a study by Bala *et al*[133]showed that out of 78 ICT positive cases 26 cases (33.33%) was positive for NS1 antigen only, 7 cases (8.97%) positive for IgG + IgM , 7 cases (8.97%) and 27cases (34.6%) were positive for NS1 +IgG and NS1+IgM+IgG respectively.

Results reported in another comparative study by GargiGhosh *et al*[21] showed that of 320 samples positive for one or more Dengue parameters by ICT,165 (51.56%) were positive for NS1 only, 73 (22.81%) were positive for only IgM, and 39 (12.2%) were positive for IgG only ,3 (0.93%) were positive for NS1 + IgM + IgG, 33 (10.31%) and 7 (2.19%) for NS1 + IgM and IgM + IgG respectively.

In the present study , distribution of positive cases by NS1 antigen ELISA,MAC and GAC ELISA shows that among 59 ELISA positive cases , 2 (3.4%) showed positive for NS1 only , 5 (8.5%) for IgM only , 8 (13.6%) for IgG only , 13 (22%) for NS1+IgM+IgG ,1 (1.7%) for NS1+IgM , 0 (0%) for NS1+IgG and 30 (50.8%) were positive for IgM+IgG.

In a similar study by Chakravarti *et al*[123] in 2011 observed that there is a gradual increase in positivity toward the end of the acute phase of the disease when combining NS1 antigen ELISA (detectable in patient sera from Day 1 onwards) and Dengue IgM ELISA (detected from Day 3 onward).

The present study shows an increase in positivity when a single sample obtained from the patient was subjected to a combination of antigen(NS1) and antibody(IgM and IgG) detection rather than subjecting to detection of single parameter when one or more biomarkers are likely to be missed.

6.7 Distribution of primary/secondary infection in positive cases.

The present study shows that the distribution of primary and secondary infections among Dengue positive cases. ICT differentiates 14(36.7%) and 21(55.6%) as primary and secondary infection respectively. Among the 59 ELISA positive cases 8(13.6%) had primary infection and 38(64.4%) had secondary infection. 3(7.9%) of ICT positive cases and 13(22%) of ELISA positive cases showed positive for all the three parameters NS1+IgM+IgG.

Hossain *et al.*[124]and Sultana N *et al*[12] also reported in a similar study where secondary infections were more prevalent than the primary infections.

Present study shows that majority of ELISA positive cases had secondary infection which may be due to the sequential circulation of different Dengue serotypes during various outbreaks of Dengue infection. Prior sensitization by a heterologous Dengue serotype increases the risk of acquiring more severe form of the disease in secondary infection than the primary infection. Thus detection of Dengue secondary cases at the early stage of infection is of major importance to avoid fatal out comes.

The rapid ICT provides just a hint about the primary or secondary infection but lacks in giving quantitative information.

6.8 Comparison of NS1 antigen by ICT and ELISA.

The sensitivity of ICT in detecting NS1 antigen was low (87.5%) while the specificity was 98.8% when evaluated against NS1 antigen ELISA. Similar low sensitivity was reported by Philippe Dussart *et al* [125]which showed 81.5% but with a specificity of 100% .

Based on the study by Hang *et al.*[108], 2009,false-negative results may occur due to the formation of immune complexes of NS1 antigen with IgG, especially during secondary infections, when there is non- availablility of target antigen to the monoclonal antibody from ELISA.

Dussart *et al*[125] stated that rapid tests are being used for the detection of Dengue NS1 antigen and it provides a promising alternative for those tests that are based on antibody detection .

6.9 Comparison of IgM antibody by ICT and ELISA.

The sensitivity of ICT was very low (28.6%) while the specificity was high (98.8%) when evaluated against MAC ELISA. Similar low sensitivity and high specificity was reported by Hunsperger *et al*[103](sensitivities ranged from 21% to 99% and specificities ranged from 77% to 98%),Hasan *et al* [126](Sensitivity / specificity was 16.67/88.89%) and Stuart D. Blacksell *et al*[104](sensitivity and specificity ranged from 6.4% to 65.3% and 69.1% to 100% respectively).

Vorndam and Kuno *et al*,[82] observed that during a secondary infection, about 20% of patients do not have IgM at detectable levels.

Vasquez S *et al*.[102] stated that different kits used for IgM detection have variability in their sensitivity and specificity.The sensitivity and specificity of IgM based assays is strongly influenced by the quality of the antigen used and can vary greatly between commercially available products[127].

6.10 Comparison of IgG antibody by ICT and ELISA.

The sensitivity of ICT was low (47.1%) while the specificity was 98 % when evaluated against GAC ELISA. Similar low sensitivity and high specificity was reported by Hasan *et al*[126] (sensitivity / specificity was 33.3/100%).

IgG is a less reliable marker in the diagnosis of Dengue infection. Both clinical and sub-clinical infections produce IgG that persists for several years which affects the interpretation of test results[23].It is highly likely that IgG levels could be higher in endemic areas because of bites from infected mosquitoes.The results of the study by Tran ThiThanhNga *et al*[128]confirms that rapid tests that are based on antibody detection still have limited value for diagnosing acute Dengue infection.

6.11 Association of urban and rural area in positive cases.

The present study shows that out of 59 ELISA positive cases 35(59.3%) cases belong to urban area and 24(40.7%) to rural area .

Aedes aegypti, the main vector species of Dengue fever/Dengue haemorrhagic fever (DHF) is commonly found in urban areas which can be

attributed to the availability of breeding sites such as water storage containers due to poor management of water and limited supply of drinking water, non degradable tyres, long lasting plastic containers and ineffective community participation in eliminating the breeding places. However, new foci are a major concern for public health by affecting the epidemiology of this disease such as rural and semi-urban areas, which may be linked to climate change.

6.12 Sex wise distribution among positive cases.

Sexwise distribution of Dengue positive cases in the present study shows that males are more affected than the females. Out of 59 positive cases detected by ELISA,35(59.3%) were male and 24(40.8%) were female in the ratio of 1.5:1.

Smitasood *et al*[75],ManishaPatankar *et al* [22] and Ahmad Nizal MG *et al*[72]also showed similar results where males are more predominant than the females.

High prevalence amongst males is probably due to more outdoor activities in comparison to females which results in more exposure to day biting mosquitoes. Thelower infection rates in females of Asian community might be attributed to lower reporting rate and the fact that they remained stationed at home and are less exposed to this vector born infection.[120]

In contrast , studies by Paramasivan *et al*[28] and Siraj.A khan *et al*[78] reported that females were more predominant than the males.Siraj.A

khan *et al*[78] stated that the vector mosquitoes (*Aedes sp.*) are mainly domestic and peridomestic in nature and females/house wives have a increase chance of exposure to mosquito bites.

6.13 Age wise distribution among positive cases.

The present study shows that the majority of positive cases are in the age group of 11-20 years . Out of 59 Dengue positive cases shown by ELISA, 28 (47.5%) were in the age group of active adults 11-20 years.

Aneela Altaf Kidwai *et al*[120] ,Sharma *et al* [19] and Manisha Patankar *et al*[22] also reported the same results in which active adults forms the majority of the positive cases. As active adults are doing more outdoor work activities , there are more chances of them being infected.

Sharma *et al* [19]proposed that exposure to multiple serotypes over a period of time results in immunity development,i.e., multitypic immunity in adults. The shift in the age preponderance may be due to the accumulation of multitypic immunity in the adult population. It is suggested that over a period of time , as the co-circulation of multiple Dengue serotypes increases in a particular geographic area , adults have a lower chance of remaining susceptible to infection. This results in the young population susceptible to Dengue infection.Therefore, monotypically immune individuals are more likely to be from younger age groups.

6.14 Association of clinical features in Dengue positive cases.

The current study shows that vomiting was an associated feature in 38 (64.4%) myalgia 46(78%) of 59 Dengue positive cases as shown by a similar study Ahmad Nizal MG *et al*[72] . In the present study, among 59 ELISA positive cases , 34 (57.6%) presented with headache as an associated feature similar to Sanjay Kumar Mandal *et al*[129].Headache and retro-orbital pain are well known features in Dengue fever that occurs mostly from systemic inflammatory mediators. The current study shows that bleeding manifestations was an associated feature in 10 (16.9%) of 59 Dengue positive cases which is similar to Sanjay Kumar Mandal *et al*[129]. Bleeding diathesis , a known feature of Dengue fever is due to low platelet count and leakage from blood vessels.Although thrombocytopenia was a constant finding, no correlation could be established between the platelet count and bleeding manifestations, indicating that other features, such as a disturbance in platelet function and capillary fragility, contribute to the bleeding diatheses.[74]

6.15 Association of laboratory findings in Dengue positive cases.

The current study shows that among the Dengue positive cases, thrombocytopaenia and leucopenia were present in 59(100%) and 23(39%) of cases respectively. Haematocrit and liver enzymes were elevated in 26(44.1%) and 10(16.9%) of cases respectively.

Similar results were obtained in a study by NP Singh *et al*[74] that showed thrombocytopenia (with a platelet count <100,000/µl) was found in about 61.39% of cases and the liver enzymes were elevated in 16% of cases (16 of 100). The factors responsible for thrombocytopenia are bone marrow suppression, immune mediated clearance and spontaneous aggregation of platelets to virus infected endothelium .Elevation of Liver enzymes could be due to virus induced damage of the hepatocytes , shock , hypotension or associated liver disease[74].

6.16 Association of USG findings in Dengue positive cases.

Present study shows that the USG findings such as ascites , pleural effusion and GB wall edema are present in 11 (18.6%),6 (10.2%) and 8 (13.6%) of positive cases respectively.

In contrast , a study by Sanjay Kumar Mandal *et al*[129] showed ascites and pleural effusion in 8.1% and 18.9% of cases respectively. Another contrast study by P M VenkataSai *et al*[130] reported that nearly all Dengue positive cases were detected with GB wall edema(100%) during the first week of fever.

Ascites and pleural effusion results from capillary leak syndrome. Ultrasound features of thickened gall bladder wall, ascites and pleural effusion strongly favours the diagnosis of Dengue fever. In a study by VenkataSai *et al*[130] **,**ultrasonographic yield was found to increase in the later stages of the disease. Repeat ultrasonography performed on the 5th to 7th day

detected pleural effusion, ascites ,hepatomegaly and GB wall edema in significantly higher number of patients with Dengue fever.

6.17 Association of laboratory and USG findings in Primary/Secondary Dengue cases.

The laboratory and USG findings were found to be significantly higher in numbers in patients with secondary Dengue infection when compared to primary infection.It seems to be almost specific for diagnosing Dengue Hemorrhagic Fever. These sonographic findings are more common in higher grades of clinical severity of Dengue fever. Hence, ultrasonographyis useful for early prediction of the disease severity and thus help in more meticulous management of the patients which enables to reduce morbidity and recommend close follow up. During an epidemic the ultrasound findings of Gall Bladder wall thickening with or without polyserositis in a febrile patient should suggest the possibility of Dengue Fever / Dengue Hemorrhagic Fever[131].

A rising HCT is a marker of plasma leakage in Dengue infection and helps to differentiate between DF and DHF but it can be masked in patients with concurrent significant bleeding and in those who receive early fluid replacement. The frequency and degree of elevation of the liver enzymes are higher with DHF compared to DF[132].

Hence, the baseline HCT , leucopenia and liver enzymes should be looked for as early as possible in all patients with suspected Dengue and must be monitored as the disease progresses.

The current study shows that among the distribution of ELISA positive cases 24(40.8%) and 35(59.2%) cases presented as DF and DHF respectively .This study is in accordance with the study conducted by Nizal MG *et al*[72]and in contrast to Sharma Y *et al*[19].The present study shows more prevalence of secondary infection than that of primary infection.DHF occurs more commonly in secondary infection.As the present study shows more prevalence of secondary infection, more cases could have presented with DHF,a severe form of the disease when compared to DF which is mild and self-limiting.None of the cases presented with DSS .

As there is no prevention in the form of any vaccine for Dengue, early diagnosis and treatment is recommended for preventing complications. Immuno chromatographic tests for Dengue NS1 antigen and IgM and IgG antibody show a high specificity with poor sensitivity, especially with respect to the detection of IgM and IgG. The rapid diagnostic test is convenient, easy to use and the results are obtained within 15 minutes for ambiguous results. All the three parameters when detected, differentiates primary and secondary Dengue infection and this reduces the need for paired sera in diagnosing secondary infection,as secondary infection leads

to complications like DHF/DSS and subsequent mortality. Thus, while the rapid test can be more feasible in small peripheral laboratories and in field situations but in well-established laboratories in India, considering its moderate performance, this device is not used as an stand alone test in Dengue diagnosis but can be supplemented with more sensitive and specific test such as ELISA on those samples to ensure both rapidity as well as quality of reported results..Highly suspicious cases should be subjected to investigations with higher sensitivity & specificity, though the results take little more time.

7. SUMMARY

The present study aimed at detecting the prescence of Dengue NS1 antigen,IgM and IgG antibodies in serum samples of adults admitted with febrile illness suspicion of Dengue infection . 100 cases of hospitalized adults were included in the study and samples were tested by both rapid Immunochromatographic test and ELISA for the three parameters(NS1 antigen,IgM and IgG antibodies).The rapid card test was evaluated against ELISA in detection of Dengue infection in serum samples.The factors associated with in children were analysed.

- Of the 100 samples tested 48 % of adults with acute febrile illness belonged to the age group of 11-20 years.
- 59% were male and 41% were female in the study group.
- Vomiting, myalgia, headache, abdominal pain, bleeding manifestations and URI were the associated clinical features in adults with fever.
- Out of 100 adults tested by rapid ICT, 38% were positive for Dengue infection(NS1 antigen,IgM and IgG antibodies)in the serum sample.
- When the same samples were tested by ELISA(Positive for any one of the three parameters), positivity was 59%.
- 37 samples positive by ICT were positive by ELISA also.

- Rapid ICT had a sensitivity of 87.5%, Specificity of 98.8% for NS1 antigen when evaluated against NS1 antigen ELISA as gold standard.
- The positive predictive value of ICT was 93.3% and the negative predictive value was 97.8 % for NS1 detection.
- Rapid ICT had a very low sensitivity of 28.6%, Specificity of 98% for IgM antibody when evaluated against MAC ELISA as gold standard.
- The positive predictive value of ICT was 93.3% and the negative predictive value was 58.8 % for IgM detection.
- Rapid ICT had a low sensitivity of 47.1% and had a very good specificity of 100% for IgG antibody when evaluated against GAC ELISA as gold standard.
- The positive predictive value of ICT was 100% and the negative predictive value was 64.5 % for IgG detection.
- Adults from urban area showed positivity (59.3%) higher than those from rural area (40.7%)
- Maximum number of positive cases (47.5%) occurred in the age group of 11-20 years.
- Male preponderance(59.3%) were present among the positive cases
- Vomiting was present in 64.4% of Dengue positive cases.

- Myalgia was observed in 78% of positive cases.
- Headache was found to be associated in 57.6% of positive cases.
- Bleeding manifestation was present in 16.9% of positive cases.
- USG findings among the positive cases showed ascites (18.6%), pleural effusion (10.2%), and GB wall edema (13.6%).
- 13.6% showed primary infection and 64.4% showed secondary infection
- DF and DHF was presented in 40.8% and 59.3% of positive cases respectively. No case presented with DSS in the current study.

8. CONCLUSION

- A total of 100 cases were studied.
- The rapid immunochromatography test has very less sensitivity and specificity is satisfactory .
- The PPV is satisfactory but NPV is less satisfactory.
- The rapid test can be used as a screening tool in situations of outbreaks but not as a diagnostic test.
- All samples should be subjected to both antigen(NS1) and antibody (IgM and IgG) testing to increase the positivity rate and to prevent missing of positive cases.
- Cases with higher degrees of suspicion are to be subjected to diagnostic tests with higher sensitivity & specificity like ELISA and PCR.
- The commercially available rapid immunochromatographictest device can be used as a screening device during Dengue outbreaks. It should not be used as a standalone device for diagnosis of Dengue. It is recommended that highly suspicious cases should be subjected to tests with higher degree of accuracy.
- Further molecular studies are essential to know the accurate information of Dengue serotypes which will be helpful in formulating vaccines in future.

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NAME		AGE		SEX		HOSPITAL NO.	

ADDRESS :

CONTACT NO :

FEVER	
Duration	
Rigor	
Vomiting	
Retro orbital Pain	

Back PAIN	
MYALGIA	
ARTHRALGIA	

MACULOPAPULAR RASH	
STARTED ON	
SPREAD	

HAEMORRHAGIC MANIFESTATION	
Perechiae	
Echymosis	
Bleeding guns	
GIT bleeding	
Haematuria	

RESPIRATORY SYMPTOMS	
Sore throat	
Rhinitis	
Cough	

SYMTOMS OF CNS INVOLVEMENT	
Altered Consciousness	
Focal neurological deficit	
Fits	

SYMPTOMS OF HEPATITIS	
Jaundice	

SYMPTOMS OF SHOCK	
Hypothermia / Sweating	
Restlessness	
Somnolence	

Previous episode :

Family h/o dengue :

Past h/o hospitalization :

H/O Mosquito bite

Presence of Stagnant water around the living area.

Proper domestic water storage.

General Examination.

Built		Petechiae		Weight	
Anaemia		Rash		Temperature	
Jaundice		Conjunctival congestion		Heart rate	
Cyanosis		Pedal edema		RR/Sa O2 %	
Lymphadenopathy		Torniquet test		Blood Pressure	

Systemic Examination

CVS	
RS	
CNS	
Abdomen	

Investigations:-

Hb				RBS	
Tc				Sr.Protein	
Dc				Urea	
ESR				Creatinine	
HCT%				Urine deposits	
Platelets					
LFT					
Blood Culture		Typhoid		Leptospirosis	
Malaria		Chikungunya		JE	
USG					
CXR					

Serology

Tests	IMMUNOCHROMATOGRAPHY	ELISA
NS_1 IgM IgG		

MASTER CHART

																Rapid ICT			ELISA			OD VALUE		
S.No	Age	Sex	Fever duration	vomiting	headache	myalgia	URI	abdominal pain	bleeding manifestation	ascites	pleural effusion	GB wall edema	↑ LFT	↑ HCT	Leucopenia	Thrombocytopenia	NS 1	IgM	IgG	NS1	IgM	IgG	NS1 Cut off (0.361)	IgM Cut off (0.607)	IgG Cut off (0.397)
1	13	MALE	7	P	N	P	N	P	N	P	P	P	N	N	+	+	-	-	-	-	+	+	0.028	2.4	2.44
2	25	FEMALE	5	N	N	P	N	P	N	P	N	P	P	P	-	+	-	-	+	-	+	+	0.059	1.71	2.27
3	23	FEMALE	5	P	N	P	N	P	N	N	N	N	P	N	+	+	-	-	+	-	+	+	0.008	1.06	2.31
4	48	MALE	6	P	N	P	P	N	malena	N	N	N	N	P	+	+	-	-	-	-	-	-	0.001	0.57	0.235
5	42	FEMALE	3	N	P	P	N	N	N	N	N	N	N	N	+	+	-	+	-	-	+	+	0.008	1.636	1.665
6	20	MALE	4	P	P	P	N	N	N	N	N	N	N	P	+	+	-	-	-	-	+	+	0.006	2.283	1.938
7	15	MALE	4	N	N	P	N	N	N	P	N	P	P	N	+	+	-	-	-	-	+	+	0.008	0.742	2.229
8	16	MALE	3	P	N	P	N	N	N	N	N	N	N	P	-	+	-	-	+	-	+	+	0.008	2.305	2.15
9	15	MALE	3	P	P	P	P	N	N	N	N	N	N	P	+	+	-	-	+	-	+	+	0.001	2.325	1.511
10	20	FEMALE	3	N	N	N	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	+	0.005	0.185	1.492
11	17	MALE	4	P	N	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	+	+	0.054	2.193	2.275
12	23	FEMALE	4	P	P	P	N	N	N	P	N	N	N	P	-	+	-	-	+	-	+	+	0.036	2.313	2.336
13	22	FEMALE	11	N	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.058	0.228	0.21
14	13	FEMALE	5	P	P	P	N	N	N	N	N	N	N	P	-	+	-	-	+	-	-	+	0.044	0.54	2.328
15	20	MALE	5	N	N	N	N	N	Haematuria	N	N	N	N	P	-	+	+	-	-	-	-	-	0.038	0.267	0.306
16	48	MALE	7	N	N	N	N	N	malena	N	P	N	N	N	-	+	-	-	-	-	-	-	0.032	0.272	0.212
17	58	MALE	30	N	N	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.047	0.18	0.166
18	35	MALE	4	N	P	P	P	N	N	N	N	N	N	P	-	+	-	+	-	-	+	+	0.044	2.272	2.325
19	21	MALE	4	P	P	P	N	P	malena	N	N	N	N	P	+	+	+	-	-	+	+	+	2.361	2.198	2.305
20	26	FEMALE	7	N	P	P	N	N	N	N	N	N	N	N	+	+	-	-	-	-	-	-	0.034	0.164	0.105
21	58	FEMALE	7	P	P	P	N	N	N	N	N	N	N	P	-	+	+	-	-	+	+	+	2.127	2.23	2.363
22	22	FEMALE	4	P	P	P	N	N	N	N	N	N	N	P	-	+	-	-	-	-	+	+	0.116	2.301	2.31
23	20	FEMALE	4	N	N	P	N	N	bleeding gums	N	N	N	P	N	-	+	+	-	-	+	+	+	2.381	0.795	2.224
24	38	FEMALE	6	P	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.024	0.159	0.086
25	23	FEMALE	4	P	P	P	P	N	malena	N	N	N	P	P	-	+	+	-	-	+	+	+	2.286	2.417	2.381
26	23	MALE	7	P	P	P	P	N	N	N	N	N	P	N	-	+	-	-	-	-	-	-	0.027	0.203	0.184
27	70	MALE	7	P	P	P	N	P	N	N	N	N	N	P	-	+	-	-	-	-	-	-	0.042	0.181	0.247
28	33	MALE	7	P	P	P	P	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.03	0.223	0.223
29	32	FEMALE	6	P	P	P	N	N	N	N	N	N	P	N	-	+	-	-	-	-	-	-	0.031	0.177	0.1
30	27	FEMALE	4	P	P	P	N	P	N	N	N	N	P	N	+	+	-	-	-	-	+	+	0.031	2.158	2.219
31	34	MALE	7	N	P	P	N	N	malena	N	N	N	N	N	-	+	-	-	-	-	+	-	0.033	0.662	0.208
32	14	FEMALE	5	P	P	P	N	N	N	P	P	N	P	N	+	+	-	-	-	-	-	-	0.034	0.337	0.164
33	18	FEMALE	4	P	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.032	0.169	0.275
34	13	FEMALE	4	N	N	P	N	N	N	N	N	N	N	N	+	+	-	-	-	-	-	+	0.039	0.416	1.727

35	35	FEMALE	8	P	P	P	N	N	malena	N	P	N	P	P	-	+	-	-	+	-	+	+	0.041	2.153	2.279
36	40	MALE	2	N	N	P	N	N	N	N	N	N	N	P	-	+	+	-	-	+	-	-	2.413	0.196	0.086
37	15	FEMALE	5	P	P	P	N	N	N	N	N	N	N	N	+	+	-	-	-	-	+	+	0.032	0.994	1.75
38	32	MALE	4	P	P	P	N	N	N	N	N	N	N	P	-	+	+	-	-	+	+	+	2.334	2.057	1.145
39	25	MALE	3	P	N	P	N	N	N	N	N	N	N	P	-	+	-	+	-	-	+	+	0.03	0.808	1.588
40	21	FEMALE	3	P	P	P	P	P	malena.bleeding gums	N	N	N	P	P	-	+	-	-	-	-	-	-	0.031	0.211	0.234
41	36	FEMALE	5	P	N	N	P	N	N	N	N	N	N	N	+	+	-	-	-	-	+	+	0.026	0.887	1.231
42	52	FEMALE	10	N	N	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.064	0.203	0.143
43	31	FEMALE	7	P	P	P	N	N	N	N	N	N	N	N	+	+	-	-	-	-	+	+	0.034	1.053	2.082
44	26	MALE	3	N	P	P	N	N	N	N	N	N	N	N	-	+	+	+	+	+	+	+	2.364	2.302	2.304
45	40	FEMALE	5	N	N	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.028	0.144	0.302
46	27	FEMALE	3	P	N	P	N	N	N	P	N	P	N	N	-	+	-	-	-	-	+	+	0.029	2.144	2.296
47	22	MALE	7	N	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.029	0.157	0.16
48	14	MALE	3	P	N	N	N	P	malena,haemetemesis	N	N	N	N	N	-	+	-	-	-	-	-	-	0.035	0.136	0.077
49	16	MALE	4	P	P	P	N	N	malena	N	N	N	P	P	+	+	-	-	-	-	-	-	0.025	0.174	0.21
50	25	FEMALE		P	N	P	N	N	Bleeding gums	N	N	N	N	N	+	+	-	-	-	-	+	+	0.038	1.372	1.097
51	48	MALE	4	P	P	P	N	N	N	N	N	N	N	P	-	+	-	-	-	-	-	+	0.035	0.15	1.551
52	18	MALE	4	N	P	P	N	N	N	N	N	N	N	P	+	+	-	+	+	-	+	+	0.025	2.29	1.126
53	73	MALE	10	N	N	N	P	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.028	0.449	0.295
54	28	MALE	5	P	N	P	N	N	N	N	N	N	N	N	-	+	-	-	-	+	-	-	0.852	0.148	0.22
55	16	MALE	7	P	N	P	N	N	N	N	N	N	N	N	-	+	-	-	+	-	-	+	0.033	0.172	1.515
56	14	FEMALE	6	P	N	N	N	N	N	P	P	N	N	N	-	+	+	-	+	+	+	+	2.434	2.243	2.362
57	17	MALE	4	N	N	N	N	N	N	N	N	N	N	N	+	+	-	-	+	+	+	+	1.938	1.172	2.377
58	18	FEMALE	7	N	N	N	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	+	0.037	0.401	1.891
59	16	FEMALE	4	N	N	N	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.024	0.18	0.167
60	30	MALE	4	N	P	P	P	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.027	0.221	0.135
61	22	FEMALE	6	P	N	N	N	N	N	N	N	N	N	N	-	+	-	-	+	-	+	+	0.042	0.806	2.227
62	42	MALE	4	N	N	N	N	N	N	N	N	N	N	P	-	+	-	-	+	-	+	+	0.03	0.635	2.307
63	17	MALE	3	P	N	P	N	N	N	N	N	N	N	P	+	+	+	-	-	+	+	+	1.412	1.956	2.053
64	19	MALE	4	N	N	N	N	N	N	N	N	N	N	P	-	+	-	-	-	-	-	-	0.21	0.296	0.297
65	48	FEMALE	7	P	P	N	P	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.208	0.21	0.181
66	22	MALE	7	P	N	N	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.025	0.208	0.298
67	33	MALE	5	N	P	N	N	N	N	N	N	N	N	P	+	+	+	-	+	+	+	+	2.244	1.703	1.843
68	25	FEMALE	7	P	P	P	N	N	N	N	N	N	N	P	-	+	-	+	+	-	+	+	0.024	1.33	2.327
69	28	MALE	5	N	P	P	N	N	malena	N	N	N	N	P	+	+	-	-	-	-	-	-	0.039	0.173	0.176
70	34	FEMALE	5	N	N	N	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.02	0.167	0.25
71	29	MALE	7	N	P	P	N	N	N	N	N	N	N	P	-	+	-	+	+	-	-	+	0.047	0.175	2.323
72	14	FEMALE	4	P	P	P	N	N	Bleeding gums	P	N	P	P	N	+	+	-	+	+	-	+	+	0.049	1.775	0.145
73	31	MALE	7	P	N	P	N	N	N	N	N	N	N	P	-	+	-	-	-	-	-	-	0.024	0.304	0.164
74	14	FEMALE	4	N	N	N	N	N	N	N	N	N	P	N	+	+	-	-	-	-	-	-	0.027	0.174	0.187
75	58	FEMALE	5	N	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.028	0.353	0.115
76	17	MALE	3	P	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.026	0.263	0.18

77	35	MALE	4	P	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.05	0.156	0.398
78	16	MALE	6	N	P	N	N	N	N	N	N	N	N	N	+	+	+	+	-	+	+	+	2.315	2.325	1.133
79	20	MALE	5	N	N	P	N	N	N	N	N	N	N	P	+	+	-	+	+	-	+	+	0.175	2.254	0.205
80	25	MALE	7	N	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.04	0.452	2.315
81	25	MALE	4	N	P	P	N	N	N	N	N	N	N	P	-	+	-	-	-	-	-	+	0.028	0.532	1.213
82	13	MALE	5	P	N	P	N	N	N	N	N	N	N	N	+	+	+	+	+	+	+	+	2.437	2.289	0.154
83	35	MALE	10	P	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.068	0.152	0.284
84	19	MALE	7	P	P	P	N	N	N	N	N	N	N	N	+	+	-	-	-	-	+	-	0.065	0.742	0.229
85	18	MALE	4	N	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.062	0.23	0.91
86	24	MALE	5	N	P	N	N	N	N	N	N	N	N	N	-	+	-	-	-	-	+	+	0.072	1.907	0.145
87	50	MALE	14	P	N	N	N	N	malena	N	N	N	N	N	+	+	-	-	-	-	-	-	0.056	0.241	0.12
88	38	MALE	7	P	N	N	P	N	N	N	N	N	N	N	+	+	-	-	-	-	+	-	0.06	0.61	0.374
89	13	FEMALE	4	N	N	P	N	N	N	N	N	N	N	N	-	+	+	-	-	+	+	-	0.671	1.474	0.276
90	45	FEMALE	7	P	P	P	N	N	N	N	N	N	N	N	+	+	-	-	-	-	-	-	0.079	0.192	2.272
91	20	FEMALE	10	N	P	N	P	N	N	N	N	N	N	N	-	+	-	-	+	-	+	+	0.022	1.901	2.241
92	26	MALE	6	P	P	P	N	P	malena,haematuria	p	p	p	p	P	-	+	-	-	-	-	-	-	0.035	0.356	0.356
93	19	FEMALE	4	P	N	N	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.037	0.423	0.267
94	22	FEMALE	4	N	N	N	N	N	Bleeding gums	N	N	N	N	N	+	+	-	+	+	-	+	+	0.038	2.254	1.758
95	23	MALE	5	N	N	N	N	N	N	N	N	N	N	P	-	+	-	-	-	-	-	-	0.03	0.152	0.21
96	19	MALE	5	P	P	P	N	P	malena,bleeding gums	P	P	P	P	N	-	+	-	-	-	-	+	-	0.024	0.805	0.256
97	14	MALE	3	N	P	N	N	N	N	P	N	N	P	N	-	+	-	+	+	-	+	+	0.2	2.217	2.435
98	17	MALE	7	P	P	P	N	P	malena	P	P	P	N	P	-	+	-	+	-	-	+	-	0.085	2.432	0.345
99	48	FEMALE	4	P	P	P	N	N	N	N	N	N	N	N	-	+	-	-	-	-	-	-	0.025	0.452	0.26
100	32	FEMALE	5	N	P	P	N	N	N	P	P	P	N	P	-	+	+	+	+	+	+	+	0.498	2.153	2.65