Serological & Molecular Characterization of Dengue Virus in a Tertiary Care Hospital of North Karnataka.

Thesis submitted to Faculty of Medicine of

BLDE (Deemed to be University), Vijayapur, Karnataka, India

For the Award of the Degree of

Doctor of Philosophy

in

Medical Microbiology

By

Pramod S. Manthalkar

(Reg. No.11PHD008)

PhD Research fellow

Department of Microbiology

Shri B.M. Patil Medical College

& Research centre, Vijayapura, India.

March 2020

BLDE (DEEMED TO BE UNIVERSITY)

Vijayapura, Karanataka, India

Certificate

This is to certify that the **Mr. Pramod S Manthalkar** carried out the work on **"Serological & Molecular Characterization of Dengue Virus in a tertiary care hospital of North Karnataka"** for the award of **Doctor of Philosophy in Medical Microbiology**, submitted to **BLDE (Deemed to be University),** Vijayapura, India, for the requisite period, under the regulations in force and this thesis is the bonafide record of the work done by him under my supervision and guidance. This work is original and has not formed before the basis for the award to the candidate of any degree, diploma, associateship, fellowship or any other title.

I further certify that the entire thesis represents the independent work of **Mr. Pramod S.Manthalkar**, and all the experimental techniques employed in this work are actually undertaken by the candidate himself under my supervision and is worth submitting for the award of the said degree.

Vijayapura

Date: 15/03/2020

Dr. B.V.Peerapur.

Guide

Former Professor & HOD,

Shri B.M. Patil Medical college

& Research Centre, Vijayapura,

Karnataka.

BLDE (DEEMED TO BE UNIVERSITY)

Vijayapura, India

Certificate

This is to certify that the thesis entitled **"Serological & Molecular Characterization of Dengue Virus in a tertiary care hospital of North Karnataka"** submitted to **BLDE (Deemed to be University)**, Vijayapura, India, for the fulfillment of the requirements for the degree **of Doctor of Philosophy in Medical Microbiology** is a bonafide record of the original research work carried out by **Mr. Pramod S. Manthalkar,** from 2012-2020 under our supervision and guidance in the Department of Microbiology, Shri B.M. Patil Medical College & Research Center, Vijayapura.

This study represents the independent work conducted by him and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

Dr. A.Y. Takpere

Professor & HOD

Department of Microbiology

HeadDepartment of Microbiology

Shri B.M. Patil Medical College

& Research Centre Vijayapura.

Dr. Arvind V. Patil

Dean, Faculty of Medicine

BLDEU's Shri B.M. Patil Medical College

& Research Centre Vijayapura,

Karnataka

BLDE (DEEMED TO BE UNIVERSITY)

Vijayapura, India

Declaration

I hereby declare that the thesis entitled **" Serological & Molecular Characterization of Dengue Virus in a tertiary care hospital of North Karnataka"** submitted to **BLDE (Deemed to be University)**, Vijyapura, India, for the fulfillment of the requirement for the degree of Doctor of Philosophy in Medical Microbiology, is a bonafide record of the original research work done by me under the supervision and guidance of **Dr. B.V. Peerapur**, Former Professor and Head, Department of Microbiology, Shri B.M. Patil Medical College & Research Centre Vijayapura.

This study represents the independent work conducted by me and has not previously formed the basis for award of any degree, diploma, associateship, fellowship or other similar title.

Pramod S. Manthalkar

Vijayapura.

March 2020.

Acknowledgment

I sincerely take an opportunity to acknowledge gratitude to all the people without whom this thesis would not have been possible.

Firstly, I express my sincere gratitude to my guide, **Dr. Baswaraj .V. Peerapur**, for his masterly directions, constant support and eminent guidance, at every stage of this study. He has placed a full faith and undoubted trust in me which helped me a lot in carrying out my research work successfully.

I express my thanks to Professor **Kusal K Das**, Dept of Physiology, BLDE University's Shri B.M. Patil Medical College, Hospital & Research Centre Vijayapura, for his great ideas, untiring guidance, affectionate & constant encouragement to complete this research work.

I extend my gratitude to **Dr. M.S Biradar** vice-canceller, BLDE, **Dr. J.G. Amberkar**, Registrar, BLDE, **Dr. Arvind V. Patil**, Principal Shri B.M. Patil Medical College, Hospital & Research Centre Vijayapura, for their kind support.

I extend my thanks to **Dr. Aparna Takpere**, Professor and Head, Department of Microbiology, BLDEU's Shri B.M. Patil Medical College, Hospital & Research Centre Vijayapura, for her kind support.

I owe my most sincere gratitude to the vice- principal, Shri B.M. Patil Medical College, Hospital & Research Centre Vijayapura, **Dr. Tejashwini Vallabha** for her support.

I extend my thanks to **Dr. Nilima Dongre** Professor Dept of Biochemistry for her timely guidance.

I am thankful to **Dr. Akram Naikwadi** Professor Dept. of Pharmacology for his kind guidance when needed.

I would like to thank **shri. Satish Patil**, Deputy Registrar BLDE, for his support throughout the work.

I owe my thanks to the Professor and Head, Dept. of Microbiology, Bidar Institute of Medical Sciences, Bidar Karnataka. **Dr. Chandrakant C** for his support during the work.

I would like to express my sincere gratitude to District surveillance officer and staff, District surveillance unites, Bidar for coordination and support during the study.

I also acknowledge the help provided by all my colleagues of Dept of Microbiology, I extend my thanks to all the non- teaching staff of the department for assistance during the work.

I extend my warm thanks to my Father Late. **Shri. Sidram N. Manthalkar**, Mother **Smt. Latika S.M**, my sisters **Nayana** and **Shailaja**, brother **Pritish** and my better half **Archana** for their constant support and help, whenever I needed.

Mr. Pramod S Manthalkar

B.L.D.E. UNIVERSITY'S
SHRI.B.M.PATIL MEDICAL COLLEGE, BIJAPUR-586 103
INSTITUTIONAL ETHICAL COMMITTEE

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 29-12-2011 *at* 10-30 am *to scrutinize the Synopsis/Research projects of postgraduate/undergraduate student/Faculty members of this college from Ethical Clearance point of view. After scrutiny the following original/corrected & revised version synopsis of the Thesis/Research project has been accorded Ethical Clearance.*

Title "Serological and molecular characterization of Dengue virus in North Karnataka"

—x—x—x—

Name of P.G./U.G. student/Faculty member Mr. Pramod. S. Manthalkar, Dept of microbiology.

Name of Guide/Co-investigator Dr. B.V. Peerapur Prof & HOD. Microbiology

DR.M.S.BIRADAR,
CHAIRMAN
INSTITUTIONAL ETHICAL COMMITTEE
BLDEU'S, SHRI.B.M.PATIL
MEDICAL COLLEGE, BIJAPUR.

Following documents were placed before E.C. for Scrutinization
1) Copy of Synopsis/Research project.
2) Copy of informed consent form
3) Any other relevant documents.

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: **Mr.Pramod S Manthalkar** Reg No.: **11PHD008**
2. Title of the Thesis: **"Serological and Molecular Characterization of Dengue Virus in North Karnataka".**
3. Department: **Microbiology**
4. Name of the Guide & Designation: **Dr. Basawaraj V Peerapur, Prof & HOD**
5. Name of the Co Guide & Designation: **Not Applicable**.

The above thesis was verified for similarity detection. The report is as follows:

Software used: **URKUND** Date: **13/02/2020**

Similarity Index (%): **Two (2%)** Total word Count: **21795**

The report is attached for the review by the Student and Guide.

The plagiarism report of the above thesis has been reviewed by the undersigned. The similarity index is below accepted norms.

The similarity index is above accepted norms, because of following reasons: **Not Applicable**

The thesis may be considered for submission to the University. **The software report is attached.**

Signature of the Guide
Name & Designation

Professor & Head
DEPT. OF MICROBIOLOGY
Raichur Institute of Medical Sciences
Karnataka Govt. Autonomous Medical Institution, RAICHUR-584 102.

Signature of Co-Guide
Name & Designation

Signature of Student

Verified by (Signature)
Name & Designation

Librarian
B.L.D.E. University's
Shri B.M. Patil Medical College
Bijapur.

INDEX

List of Tables

List of Figures and Graphs:

Abstract:

Dengue is a viral disease, caused by *Flavivirus*, transmitted by mosquito vector. Dengue virus infection may vary from mild Dengue fever to severe complications like, DHF and DSS. Dengue fever is the major cause of high mortality and morbidity in Tropical and Subtropical regions. 50 to 100 million people are affected every year globally. Dengue virus is a RNA virus and has got four different serotypes. Dengue presents with Fever, Arthralgia, Retro-orbital pain, Myalgia and in severe cases it may present with Nausea, Vomiting and bleeding tendency. Severity in dengue infection is because of the partial protection against the other serotypes and in multiple serotype infection the antibody dependent enhancement of the infection is seen. Laboratory diagnosis plays an important role in diagnosis of disease, which may help in proper treatment and immediate action, as there is no treatment available and vaccines are still under trial. There are various methods for detection of the Antigen and Antibodies of dengue virus in patient's serum, which may help in diagnosis of the disease.

This study was aimed to detect the incidence of the Dengue virus infection and to evaluate the proper dengue diagnostic test and to know the incidence of multiple serotype infection.

Material and Methods:

1000 Dengue suspected patients as per WHO criteria were considered, serum samples were collected from the patients and subjected for the detection of Antigen and antibodies by rapid and ELISA method, all the NS-1 positive serum samples were subjected for detection of the viral RNA, serotyping was done to know the serotype present in the serum.

Result:

Out of 1000 samples, 462 serum samples were positive for Ag or Ab or both by rapid or ELISA. All the serum samples were subjected for testing by Rapid card method and ELISA. Male patients were more infected compared to women but significant difference was not observed. Age groups of 15-30 years were more infected followed by 30-45 age groups. More number of Dengue cases were seen in the month of October i.e. Post monsoon months, cases started in the month of June and ended in December. More number of cases was from Bidar city followed by Humnabad, Bhalki, Aurad and Baswakalyan. All the patients were suffering from fever, followed by headache, myalgia, body ache, arthralgia, nausea, vomiting and abdominal pain. All the NS-1 positive samples were subjected for RT PCR for the detection of the Dengue virus serotype. DENV-1, 2 and DENV3 were identified from the samples and detailed clinical correlation was done.

Conclusion:

ELISA is the most useful tool for detection of Dengue virus antigen or antibodies from patient's serum. DENV-1, DENV-2 and DENV-3 are circulating with changing trend of serotype was observed, with severe clinical features in DENV-2 and 3 serotype infection. Serotype-1 showed unique conserved site Nco1.

Introduction:

Dengue is one of the vector borne disease found throughout the world ranging from 17- 20 % of the total infectious diseases.[1] The most common vector borne diseases are transmitted by the bite of mosquitoes, like Malaria, Chikungunya, Japanese encephalitis, Dengue, Rift valley fever, yellow fever and Zika viral disease. Arthropod borne viral diseases are endemic in many areas.[2] Tropical and subtropical regions are commonly affected by arthropod borne diseases infecting 25 to 80 million people throughout the world [3], with 5 lakh cases of DHF and 30,000 deaths in a year [4]. Since 2009 India is experiencing concurrent infections with such Arthropod borne viral diseases, especially Chikungunya and Dengue. Constant outbreaks have been observed throughout India, especially Dengue which have led to large number of mortality and morbidity.[5]

Dengue is one of the mosquito transmitted *flaviviral* disease, due to its painful signs and symptoms i.e. Joint pain, back ache and body ache it was also termed as break bone fever.[6] Dengue virus is a ss RNA virus with 11Kb genome length and is a enveloped virus with core protein, membrane protein.[6] Dengue virus is having four different serotypes, depicted as Dengue serotype -1(DENV-1), Dengue serotype-2(DENV-2), Dengue serotype-3(DENV-3) and Dengue serotype -4 (DENV-4).[6,7] Infection with a single serotype induce lifelong immunity but infection with second serotype at the same time or during secondary infection, gives partial protection or no protection and may lead to Antibody dependent immune enhancement, which may lead to severe consequences like Dengue shock syndrome (DSS) or Dengue haemorrhagic fever (DHF).[8]

Dengue infection may range from flu like illness with Dengue fever to severe forms like DSS or DHF. In DF there is sudden onset of fever, headache, arthralgia, myalgia, retro-orbital pain and maculapapular rash.[8]

Aedes aegypti is circulating and transmitting the disease throughout the globe, many attempts are being made to eradicate the mosquitoes, especially in endemic areas, but still not succeeded.[9] Dengue virus vaccine is still under trial and none has been licensed. Specific treatment is not available for dengue virus infection till date.[10]

There are different methods of Laboratory diagnosis of DF other than clinical findings, which are must for confirmation of Dengue viral infection. They are virus isolation, genome detection (amplification), Antigen & Antibody detection by serology.[11] as isolation of virus needs skilled persons and laboratory; it is lengthy and time consuming procedure[12]. Genome detection by amplification technique, for the identification of serotype and sequencing is done; amplification is done by Polymerase chain reaction (PCR) and other methods like NASBA.[13] Detection of Antigen and antibodies is done in all laboratories especially for identification of acute infection. NS-1 antigen detection and IgM antibody detection is commonly used test for detection of dengue infection. Nowadays we can also identify the primary and secondary dengue infection on the basis of IgM to IgG ratio[14]. Also we can detect the early NS-1 antigen. NS-1 is a nonstructural protein of Dengue virus which is found in circulation during the acute phase, can be detected in the serum or plasma and helpful in early diagnosis of the disease, while IgM may appear only after 3 to 5 days of infection.

For detection of NS-1, IgM and IgG in the patient's serum there are many commercially available kits, which are rapid and less time consuming and ELISA is

more specific but time consuming. To know which one is better for the diagnosis of the disease, we have also compared the rapid card method with ELISA.

Platelet count is another diagnostic criteria associated with the Dengue virus infection, as the platelet count depletes in Dengue virus infection. Platelet transfusion becomes important in cases with DHF and DSS. Most of the times platelet count is not done, hence delay in transfusion may lead to complication and shock. Early estimation of platelet number will help in supportive treatment. We have also studied the association of platelet count with NS-1, IgM and IgG positivity.

Large number of cases of Dengue and Chikunguniya were reported from Karnataka and South India from the year 2006.[15] many outbreaks were seen from then onwards. Due to similar clinical manifestations and lack of diagnostic facilities much of the cases remained undiagnosed. This study was conducted to know the incidence of DENV infection in North Karnataka. Detailed clinical history was taken from the patients suspected of DF infection; hematological parameters were studied in detail to see the changes in blood picture as the platelet count is depleted in Dengue. This was supported by serodiagnosis by detection of IgM, IgG antibodies and NS1 Antigen.

As the pattern of antigen and antibody appearance in the serum varies depending on the stage of the disease and because of which misdiagnosis may occur, to overcome this problem detail analysis of the antigen appearance and antibody appearance in the serum was done by Rapid and ELISA and was compared to know the specificity and sensitivity of these methods.

In India since 2006 many places including Delhi, Vellore, Madhya Pradesh, Andhra Pradesh & Lucknow have experienced the multiple serotype circulation and

monotype infection, also multiple infection in a single patient with severe clinical manifestations with shock and hamemorrhage.

Moreover further confirmation was done by molecular technique i.e. RT- PCR (polymerase chain reaction), from Blood samples for correlation and to identify which serotype and genotype is prevalent in North Karnataka. To know whether multiple serotype infection is present, and which serotypes are circulating.

2.1 HISTORY:

Word "Dengue" is derived from "Ka-Dinga pepo" which means cramp like seizure, from this Christie named it as Dinga[16]. The slave trade of the New world, where it was named as Dandy fever or "The Dandy" by St. Thomas epidemic in 1827[(17)]. In 1653 first ever known epidemic of Dengue was recorded in French West Indies, and the same type of illness was seen in China in 992 AD.[17]

In Chinese Medical Encyclopedia Dengue was termed as water poison associated with flying insects in 992 AD.[17] Asia, America and Africa came across the epidemic of Dengue in 1780. The first known clinical case was detected in 1789 in Philaelphia which was termed as Break bone fever by Benjamin Rush, because of the myalgia and arthralgia. The name Dengue came into use generally in 1828 after the outbreak in Cuba. In 1940's the etiological agent virus was proved.[18]

Cleland and Bancroft first described the transmission of Dengue virus by *Aedes* mosquito, but the virus carrying *Aedes aegypti* is from Africa or Asia is debatable.[19]

Craig and Ashburn[20] first found the filterable agents from human blood. Incubation period was first established by Siler et al. Kimura and Hotti[21] in 1943 first isolated the virus in mice. Sabin and Schlersinger in 1944 isolated the virus in suckling mice in Calcutta.[22] William Hammon in 1956 showed the presence of different serotypes and genotypes of Dengue virus.[23]

Sabin and Albert isolated different strains of dengue virus by inoculating, infectious human serum in human volunteers.[22]

The first major epidemic of Dengue hemorrhagic fever was seen in 1954; it started from Philippines and involved the entire globe.

Since last 40 years dengue is epidemic in more than 100 countries and is spreading very fast. Brazil, Paraguay, Thailand and Pakistan experienced the recent outbreak in the year 2011, by 2013 it involved Brazil, Singapore and Thailand and Central America had a worst outbreak in 2008.[24,25]

Dengue type 3 has affected most of the countries involving Cook Island Malaysia, Fiji and Vanuatu.

Table no 1: Year wise distribution of Dengue virus serotypes in different countries.

Sr no	Country	Year	Dengue serotype isolated
1.	Indonesia, Thailand	1983	DENV- 1,2,3,4
2	Thailand	2000	DENV-1,2,3,4
3	Peru	2002	DENV-1,2,3
4	Nicaragua, India	2006	DENV-1,2
5	Hong Kong	2008	DENV-1,2,3,4
6	Martinique	2008	DENV-2,4
7	Taiwan	2009	DENV-2,3
8	Thailand	2010	DENV-1,2,3
9	India	2010	DENV-1,2,3,4
10	Vietnam	2010	DENV-1,2,3,4

2.2 EPIDEMIOLOGY OF DENGUE VIRUS INFECTION

Dengue virus has four serotypes and all the serotype are prevalent all over the world, major infections are due to no long lasting cross protective immunity to other serotype, Sabin first showed that cross protection occurs between two serotypes and lasts for few months.

Geographical distribution of dengue around the world:

1779-1780

Three continents Asia, Africa and North America have experienced the first epidemic in the year 1779-1780, this was the first incidence which proved that Dengue Virus and its vector are worldwide in distribution and are evident since 200 years.[26]

1780

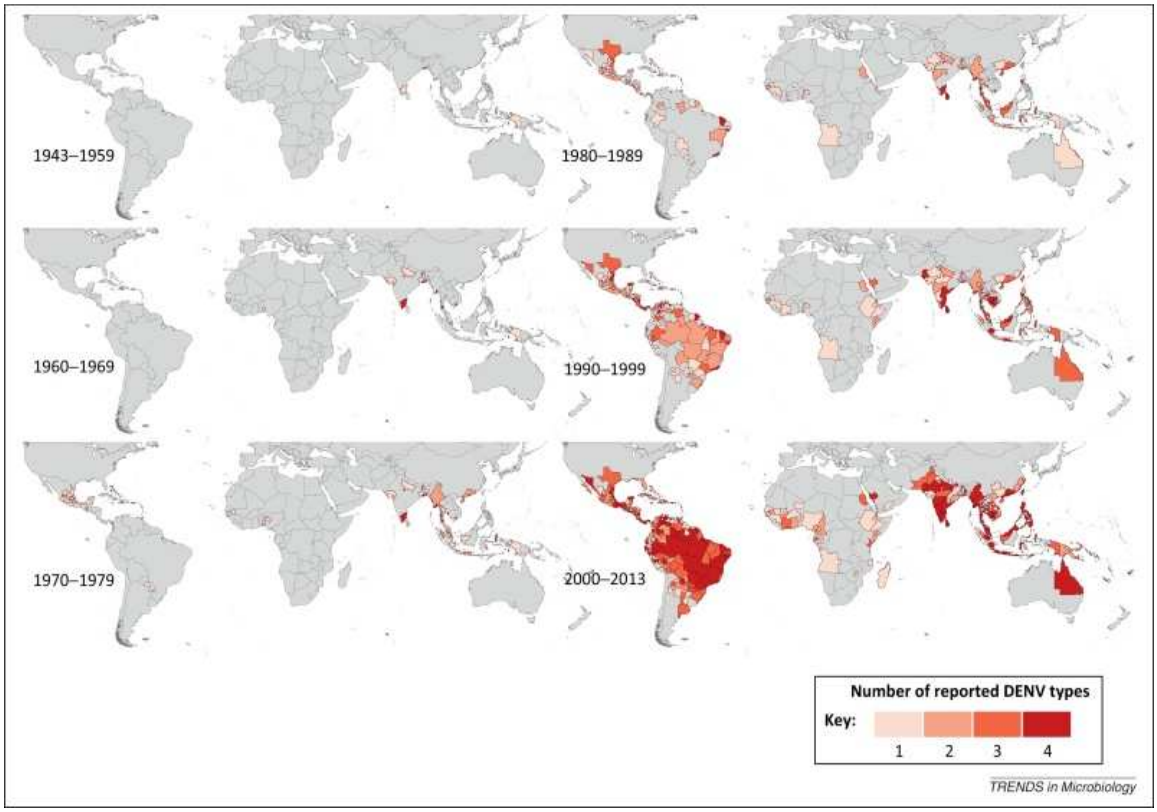
Philadelphia have experienced the epidemic in the year 1780, then onwards many epidemics were seen in America up to early 20th Century, in the year 1945 USA experienced the last outbreak in New Orleans.[17]

1953- 54.

Manila, Philippines were the first to witness Dengue Hemorrhagic fever (DHF) in the year 1953-1954, it was also known as " Thai or Phillipine Hemorrhagic fever" which was later on seen in Bangkok, Thailand in 1958 and Singapore, Thailand and Vietnam in 1960[27]. Due to increase in trade, shipping vessels and transportation, disease spread was throughout the tropical. World war 2 boosted the spread of Dengue fever which further appeared as epidemic in Central and South America, hence the control centers

were shifted their. The campaigns were discontinued in 1970 due to low incidence. In 1980 again the incidence of the Dengue increased, which lead to pre-campaign in 1995.[17]

Figure no:1 Global spread of different serotype of Dengue from 1943 to 2013.



Epidemiology of Dengue fever in Asia:

Incidence of Dengue was more prior in 1970, which later on subsided but last five decades Asia have witnessed new outbreak. Due to troop movement, transportation, rapid urbanization, change in ecology at the end of Second World War lead to increased transmission of Dengue, due to increased density of *Aedes aegypti* mosquitoes. Few years later from this emerged the threat of Dengue Hemorrhagic fever in this region.[28]

Dengue serotype 3 has caused havoc since last twenty years, because of DHF epidemics in East Africa, Latin America and Sri Lanka. The emergence of DHF in Sri Lanka in 1989 was due to new DENV-3, subtype III variant, which was closely related and belonged to DENV-3 subtype III which originated in the Indian Subcontinent. Bhutan and Nepal witnessed the outbreak in the year 2004. [29]

Dengue serotype 1 and Dengue serotype -2 were first isolated from the epidemic in Pakistan in the year 1994.[30] The major outbreak was experienced by Pakistan in the year 2004 , in this serotype 2 was more prominent. There after several studies were reported from Pakistan and other endemic areas where DENV-1 and DENV-2 was predominantly circulating in the Pakistani population. [31]

Majority of the South East Asia has experienced multiple serotypes Dengue infection especially in children below 7 years of age, monsoon or rainy season were epidemic's trend in the tropical areas.[9]

Epidemiology of DF, DHF and DSS in India:

First Clinical Dengue –like illness epidemic was recorded in the year 1780 from Madras. And the first proven case of dengue fever was seen in Calcutta an Eastern Coast of India in the year 1963-1964.[26,32] Trend of northward spread was seen then on which hit Delhi in the year 1967[33], while Kanpur in 1968.[34,35] DENV – 4 was responsible for the outbreak of 1968 in Kanpur.[35] In 1969 the virus serotype isolated was DENV -2 and DENV-4. Vellore witnessed the epidemic due to serotype DENV-3 in 1966.[36] DENV-3 was also isolated form Calcutta and Tamil Nadu in 1968. Prevalence of all the four serotype i.e. DENV-1,2,3& 4 was seen in Vellore during 1968.[36] Further it was replaced by serotype -2, Haryana , Lucknow , Gwalior, Gujrat had serotype -2 prevalence, while Delhi showed the presence of DENV-2 and DENV

-1[37,38,39,40,41,42] Rajasthan had serotype -1 and serotype -3 in circulating in the population. Phylogenetic analysis revealed the details of viral movement.[43] It showed the isolates of Dengue virus from Delhi and Gwalior in 1966 belonged to DENV -2 and genotype IV.[44,45] Serotype 2 and FJ10/11 isolated from China were closely related. Early two isolates of Dengue serotype 2 were classified in genotype V which was further replaced by genotype IV in last decade.[43]

Delhi has witnessed many epidemics during last few decades, since 1988[46] then after a gap of eight years in 1996 and in 2003[46]. Outbreaks from Mangalore in the year 1993[47] and Chandigarh in 2002.[48]

Spread of DHF began from Delhi and Lucknow in 1996, there was an outbreak of DHF/DSS in these areas, which spread rapidly throughout the country. DHF was spreading in India since 1988. In 2004 there was sudden shift in the serotype in Northern part of India where serotype 2 was replaced by dengue serotype 3 subtype IV. In 2005 major Dengue epidemic was seen during August – November 2005 affecting more than 15 districts of West Bengal including Kolkata.[21]

In Delhi the major serotype circulating in 2003 to 2005 was Dengue serotype 2 with predominance of genotype four, there was co-circulation of Dengue serotype 2 and 3 simultaneously which led to server form of disease, like many cases of Dengue hemorrhagic and Dengue shock were seen during this co-circulation. Change in serotype as well as change in the genotype of the virus and replacement was seen.[49]

Co-circulation and mix infection with other falviviral agents led to many complications and Delhi, Vellore have seen the mix infection of Chikungunya and dengue together from the year 2007 to 2009.[50]

Dengue -1 isolated from Vellore in 1957[51] belonged to the American African genotype. Whereas few reports say that the Dengue virus serotype and genotype are of African lineage including I,II,III type. These serotypes have come to India from Singapore and III has come from Africa. These two strains are constantly present in the circulation leading the mix infections.[51]

Table no 2: Distribution of Dengue serotype in India

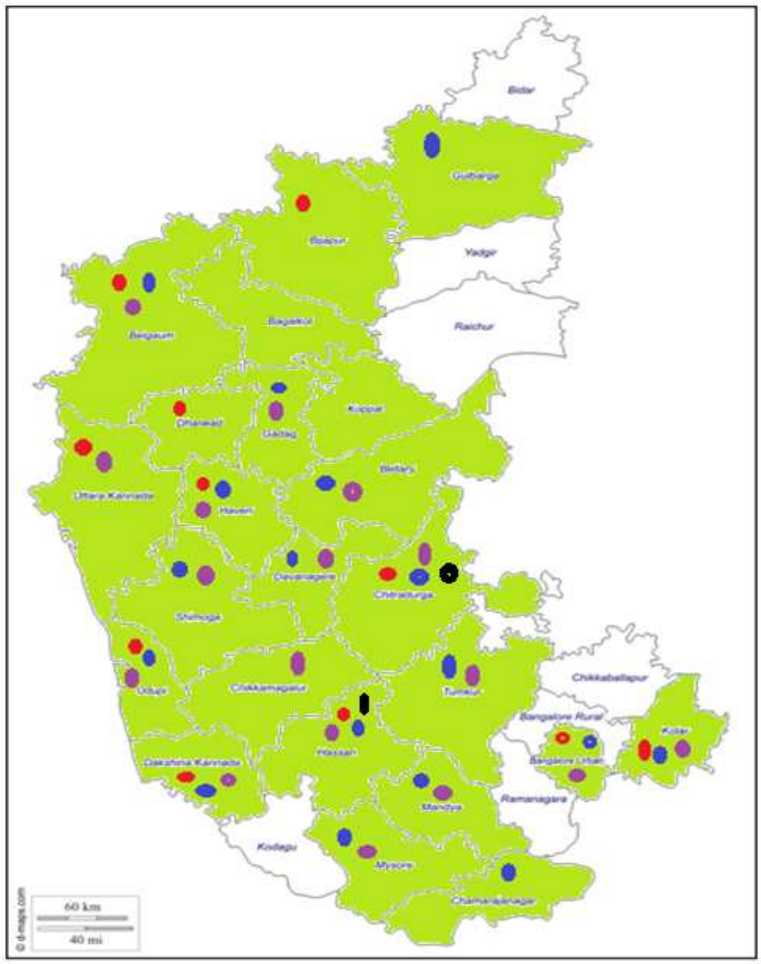
Sr no	Place of study	Year of study	Dengue virus serotype isolated
1.	Vellore [50]	1964	DENV-2
2	Vellore [51]	1966	DENV-3
3	Vellore [36]	1968	DENV-1,2,3,4
4	Kanpur [34]	1968	DENV-4
5	Kanpur [52]	1969	DENV-2,4
6	Hardoi[53]	1970	DENV-2
7	Kolkata [54]	1983	DENV-3
8	Jalore[39]	1985	DENV-3
9	Delhi [53]	1988	DENV-2
10	Gujarat [37]	1988	DENV-2
11	Kolkata [21]	1990	DENV-3
12	Mangalore[47]	1993	DENV-2
13	Ludhiana [56]	1996	DENV-1,2,3,4
14	Lucknow [57]	1996	DENV-2
15	Delhi [58]	1996	DENV-2
16	Delhi [58]	1996	DENV-2
17	Delhi [59]	1996	DENV-2
18	Haryana[60]	1996	DENV-2
19	Delhi [60]	1997	DENV-1
20	Dharmapuri, TN[61]	2001	DENV-2
21	Gwalior[42]	2001	DENV-2
22	Chennai [62]	2001	DENV-3
23	Delhi & Gwalior [44]	2003	DENV-3
24	Kanayakumari[63]	2003	DENV-3
25	Delhi [45]	2003	DENV-3
26	Delhi [64]	2003-2004	DENV-3
27	Delhi [65]	2003-2005	DENV-1,2,3,4
28	Kolkata [66]	2005	DENV-3
29	Entire country[67]	1956-2005	DENV-2
30	Delhi [65]	2006	DENV-3
31	Delhi [65]	2006	DENV-1,3
32	Delhi [68]	2006	DENV-1,2,3
33	Delhi [69]	2006	DENV-1,2,3,4

34	Madhurai[70]	2007	DENV-3
35	Andhra Pradesh[71]	2007	DENV-1,4
36	Gwalior &Delhi[72]	2001-2007	DENV-1,
37	Delhi[73]	2008	DENV-1,2,3
38	Ernakulam[74]	2008	DENV-2,3
39	Different parts of the country [75]	2003-2008	DENV-3,
40	Delhi [73]	2007-2009	DENV-1,2,3,4
41	Maharashtra Pune[76]	2009-2010	DENV-4

Karnataka has experienced large number of outbreaks of dengue, which were found in North and South Karnataka in the year 2006 to 2008. There was co-circulation of all dengue serotypes leading to many sporadic cases. All the dengue serotypes have been detected from Banglore, Mysore, Mandya, Hassan, Dharwar, where most of the cases remain unnoticed and due to lack of facilities, serotype prevalence was not studied. Moreover cases of Dengue declined then onwards in many places of North Karnataka, but they remain endemic for vector borne diseases like Dengue, Chickungunya and Malaria[50,51,69,75.]

Figure no 2: Karnataka Map showing distribution of Dengue virus serotype

DEN 1 DEN 2 DEN 3 DEN 4



2.3 DENGUE VIRUS

Dengue is the most important and wide spreading arthropod borne viral disease transmitted in human beings, which has got four different serotypes i.e. Dengue virus serotype-1 (DENV-1), Dengue virus serotype-2 (DENV-2), Dengue virus serotype-3 (DENV-3) and Dengue virus serotype-4 (DENV-4). Dengue virus is supposed to be evolved from the forests of Africa where all the four serotype were found and are evident on the basis of phylogenetic study. Dengue is an enveloped single stranded RNA virus which belongs to *flaviviridae* family and genus *flavivirus*. The viral RNA is surrounded by C protein and forms the inner core. [8,78]

The virus spherical particle is surrounded by the capsid and the envelope with and Membrane protein bond.

The virus measures 50 nm in size and the genome of dengue virus is 11000 base pair long which consists of 3,400 amino acid residues. The genome consists 3 structural protein genes which code for Core protein (C), membrane associated protein (M) an envelope Protein (E). Viral genome codes for seven non structural protein genes (NS). The NS proteins are nominated as NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. [79,80.]

- C protein: This is a basic protein of virus with 11kd. It is a compact dimmer with each monomer of four alpha helices. This is a group specific protein and can be detected by complement fixation test. [81]
- E protein: This is a group specific protein found in Flavivirus group. E protein is a glycoprotein made up of 500 amino acids with molecular weight of 50 kd and is synthesized by membrane protein containing 12 conserved cysteines to form disulphide bonds [53]. This protein helps in attachment of the virion to the

host cell receptors, act as a mediator for membrane fusion. E protein can be identified by Complement fixation test Haemagglutination Inhibition assay; neutralizing antibodies are found which play an important role in immunity. CMI has not yet been clearly understood in Dengue infection.[82]

- prM (precursor of membrane protein): E protein is stabilized and polymerized by prM protein.pr and M fragment fuse in Golgi complex and act as precursor of the M protein and is present on the surface of the virus, and is useful in differentiating in group of flavivirus.[82]

Non structural glycoprotein's: There are seven nonstructural proteins (NS-1,NS-2a,NS-2b,NS3,NS4a,NS4b and NS5)NS-1 glycoprotein is 46- 48 kd in size. The function of this protein is of diagnostic importance. It is one of the highly conserved glycoprotein. It can also initiate the infected cell lyses by activation of the C. Its role in assembly and maturation is not yet clear. [79]

NS 2A- This is one of the hydrophobic protein which smaller in size in comparison to NS1. It is a part formed from the cleavage of N- terminus of host singnalase and C terminus of viral protease. [80,83]

NS 2B – This is a still smaller component of the Non structural antigens and it co-ordinate with NS3 for insertion. [84]

NS3 – This protein is seen in *Flaviviridae* group it has an important role in apoptosis, while NS4 A interact with NS3 for various function it is also a hydrophobic protein while NS5 is one of the highly conserved multifunctional protein which is 105 kDa. This protein is also known to be inhibiting the role of IL-8, i.e inhibition of viral growth. [85,86].

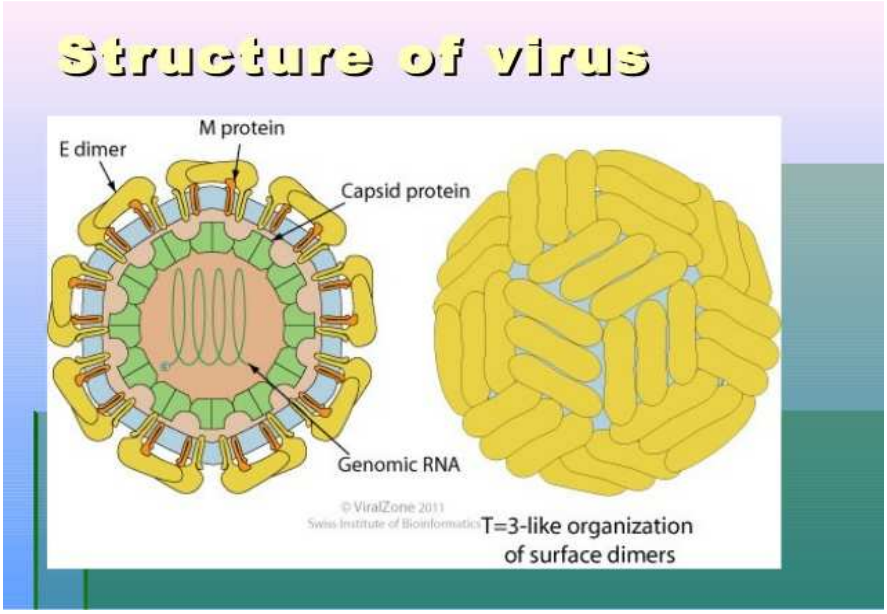
The virus genome is surrounded by capsid and then envelop with E and M proteins, Nucleo-capsid is surrounded by belayed M and E glycoprotein's. In the infected cell the M protein acts as precursor for prM which help in folding and assembly of E protein [87].

Dengue NS-1 glycoprotein is of 46kd, it is important in viral replication, assembly and maturation of the virus particle but not yet proved.

Mutational analysis show that NS4b is responsible for the maintenance of balance in replication in mosquito and human host [88]

NS5 is well conserved protein and is involved in replication of Dengue virus RNA, the NS5 contain the N terminal RNA cap processing activity and c terminal RNA dependent RNA polymerase activity. It also induce IL-8 secretion in late infection which may modulate the transcription of cytokines and affect the innate immunity.[88]

Figure no 3: Structure of Dengue Virus



2.4 THE VECTOR:

Dengue is an Arthropod born diseases; mosquitoes are the major vectors for transmission of Dengue virus from one person to the other. *Aedes aegypti* and rarely *Aedes albopictus* play an important role in transmission. *Aedes aegypti* is a small black mosquito with white stripes around 5 mm in size. *Aedes aegypti* is found in Tropical and Subtropical regions. It is the one of the primary vector for viral diseases like Chikunguniya, Yellow fever and Dengue.[89]

These mosquitoes breed in freshwater and stagnant water stored in proximity to human residents eg. Water coolers, water pots, flower pots, buckets, drums, unused or discarded rubber tiers of the vehicles, fountains and nearby open gutters draining rain water and other.[90]

Mosquitoes harboring the viruses usually bite the host during day time either in early morning or prior sunset but are active though out day time. *Aedes albopticus* can bite in day time and especially in late afternoon. *Aedes albopictus* is also small dark mosquito with white dorsal stripe and banded legs. It lays egg in the water logged container in urban, suburban and rural areas. [91]

Aedes aegypti and *Aedes albopictus* are sensitive to environmental changes such as temperature, precipitation and humidity. They do not survive in cold conditions; especially the activity of this vector is lowered in winter season. *Aedes* species lay the eggs in artificial water logged container in proximity to the residents and the eggs are resistant to environmental conditions, like drying so they remain for long time in the environment and can easily spread to new locations.[91,92]

2.5 Transmission of Dengue virus:

Life cycle of Dengue virus is carried out in humans and vector mosquito. Life cycle begins with the bite of the infected female *Aedes* species to human. The female *Aedes* mosquitoes harbor the virus in salivary gland and remain infective for the entire life which leads to spread of the disease.[89] Mosquitoes gain infection from the infected human in acute phase i.e during 2- 5 days of infection. Once the mosquitoes take up the virus during blood meal they need 8-15 days to become infective, so that the virus infect the salivary gland of the mosquito and starts transmitting the virus to the other vertebrates.[89] Virus is present in the entire tissues and organs of the mosquitoes involving the ovaries, midgut, central nervous cord, brain etc. The virus is present in the ovaries and multiplies in the female genital tract, while fertilization they enter the ovum and leads to infectious progeny. The vectors remain infectious for its lifetime and transmit the DEN virus.[93] No visible pathological changes are seen in the mosquito.[93]

Once the infected mosquito bites the vertebrates the visible signs and symptoms starts only after 2 to 14 days of incubation, which varies from human to human.[9] Onset of symptoms starts, fever usually is biphasic, muscle pain, Joint pain, rashes on the trunk and headache. This illness may last for 5 to 7 days as the cycle completes. Convalescence may last for long time.[94]

There are various factors which affect the transmission of dengue virus infection to human. Eg. Age, climate, sex, racial and genetic factors play important in transmission and pathogenesis of Dengue virus.

Climate plays an important role in growth and multiplication of the mosquito vector.[95] As Dengue is vector born disease, breeding of mosquito is an important

factor for transmission and multiplication. Many studies have stated that post monsoon season i.e from October to December is best for breeding of mosquito and this is the season were large number of dengue cases are seen. Increase in the temperature from 26^0 c to 30^0 c decreases the extrinsic incubation period of the dengue virus, which may facilitate the spread of the dengue virus especially the virulent genotypes.[74] Which results in increased immune response and disease.[95,96]

Age also plays an important role in disease, as many of the workers have seen the most commonly affected age group was between 15 to 45 years [97,98]. In many studies Dengue has been reported in pediatric age group. Problem related to its severity as more severe complications are seen in this group. In many studies it has been noted that all age groups are prone to infection with adult group predominance.[99] In few cases they have also stated the predominance of DENV-2 in children and DENV-3 in adults.[100]

Many of the studies have distributed the dengue cases in relation to sex ratio, male to female and most of them have coated, males are more commonly affected than female [97,101.] This may be due to the exposure of male to the environment than female. Few studies have stated the equal distribution of DENV infection in male and female. Serotype prevalence has been stated in many studies in relation with the male to female ratio, Dengue serotype-3 and serotype-2 was more common in male [100,102].

Genetic predisposition is also important, especially it is observed that Africans have deficiency of Glucose 6 phosphate dehydrogenase , polymorphism in the MBL-2 gene was shown to be associated with thrombocytopenia and an increase risk for dengue hemorrhagic fever.[8] HLA class 1 and II alleles are associated with the

development to DHF. Polymorphism in TNF-alpha , Fc receptors, Cytotoxic T cell associated antigen (CLA-4) cases.[8]

During 1981 and 1997 the hospitalization of blacks was less compared to whites, observed by Halstead et al stating the DENV resistance gene in the blacks, this study states racial difference seen in DENV infection. (CLA-4)[103]

Cases of vertical transmission have not been confirmed till now, vertical transmission of dengue can't be ruled out, and it needs further study. Only one case was reported in Thai women with febrile illness, who delivered healthy baby, which developed dengue fever after 6 days but was not confirmed weather it was mosquito born or vertical transmission.[104]

Age wise if we see, most of the researchers have seen Dengue virus infection is common in age group 15 to 45 years. Many studies have also stated about serious disease in small children. Infection in all age group was seen by Matlini et al[100] and other worker with more prominence in adult Male. DENV-2 was reported to be more common infection in children and DENV-3 in adult patients. [105,106].

Difference in sex ratio is seen in Dengue virus infection. Male patients are more commonly affected then female.[101,102] One of the study showed female were affected more, in another study in long duration of 8 years, male predominance was observed. DENV-3 and DENV-2 were the common serotype of Dengue virus isolated from both gender.[8,103]

2.6 PATHOGENESIS

Dengue virus replication in human

As the infected mosquito takes the blood meal, Inoculate the dengue virus in cutaneous Langerhans denderitic cells, where they multiply, triggering the cytokines and chemokine mediated infiltration of immune cells, they migrate through the lymphatics. They can also infect skin and intestinal denderitic cells. Skin and intestinal cells are the primary target site for virus attachment. Especially the denderitc cell adhesion molecule-3 and non- interim is essential for the productive Dengue virus infection.[107]

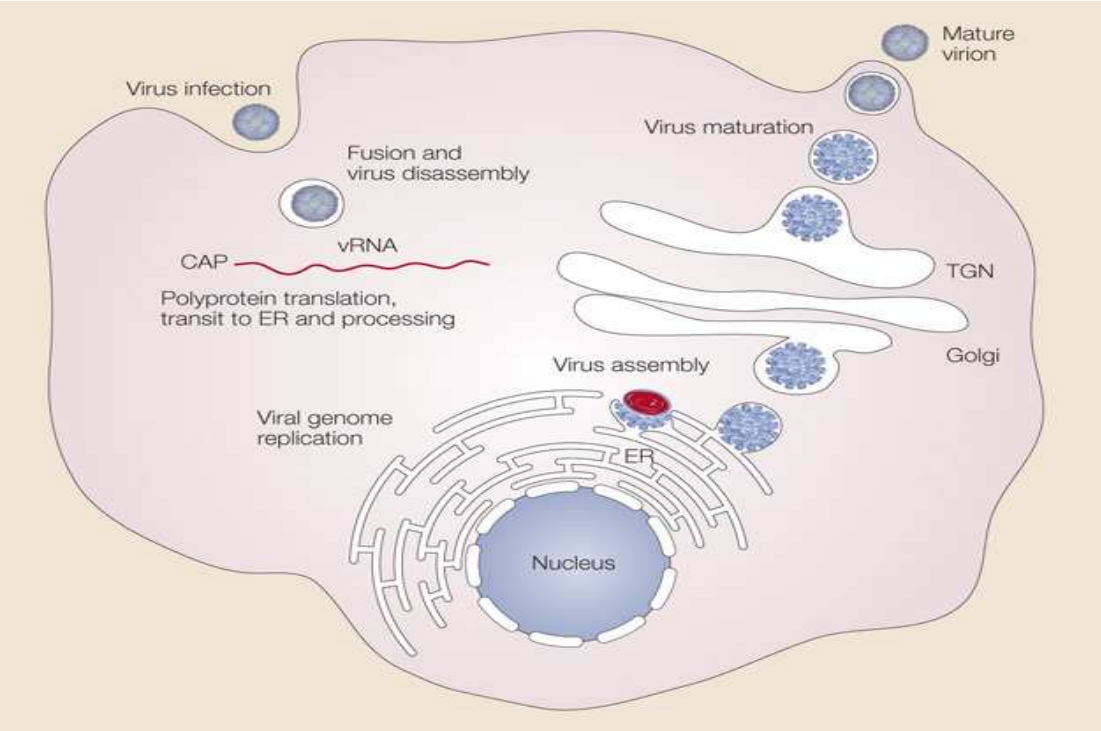
Intestinal Antigen presenting cells express the DC- sign or N- acetylegalactosamine specific C type lectin which helps in viral dissemination and antiviral immune response. After the virion are endocytosed in endosomic vesicles they are exposed to acidic pH, which trigger the modification of structural protein of viral envelop protein. Which in turn leads to changes in exposure of fusion peptide, and binds the edosomic membrane towards the viral membrane inducing the membrane fusion.[108]

The proteases present in the cells within endosomic vesicle un-coat the virion by digesting the envelope, releasing the genome in the endoplasmic reticulum. NS-3 and NS-4 help in invagination of ER, where RNA of the virus replicates. Further RNA synthesis and viral assembly takes place in the cell compartments. With the help of C protein nucleocapsid complex and viral RNA, capsid is developed.[109, 110]

After development of virus in host cell, it is released by exocytosis. Golgi complex helps in post translational modifications in addition to a sugar residue. The complex come out of the ER with a prM –E- lipid envelop. Where the virus is expelled via

exoveytic vesicles, mature M protein form furin mediated cleavage of prM and causes E protein to change to homodimer form.[16,111,112.]

Figure no 4: Dengue virus replication in Human cells.



Clinical manifestations and virus behavior

Virus is transmitted to vertebrate by the bite of infected mosquito during blood meal, the virus replicate in the regional lymph nodes and disseminates in blood after 2-3 days to different organs. Dengue virus can also replicate in macrophages, lymphoid cell and dendritic cells. The viremia is set up including involvement of monocytes, macrophages and even T and B cells in different organs.[113]

In viremia, fever is the major symptom; it is because of the release of interleukins followed by Rash which is due to lymphocytic dermal vasculitis. Release of cytokine in the circulation after viral infection leads to other symptoms.[114,115.]

Severity of the disease in dengue is major cause and may lead to high rate of mortality. Immune cells, liver and endothelial linings of blood vessels play an important role in Dengue shock syndrome and Dengue haemorrhagic fever.[114]

Role of cytokines in Dengue infection:

TNF alpha, interleukin (IL)-2, IL-8, IL-10, IL-12 and IFN gamma play an important role in pathogenesis of DENV infection. IL-8 is elevated in DHF where as plasma leakage is caused by TNF alpha.[82]

Complement in DENV:

Activation of complement factor plays an important role in inflammation which leads to plasma leakage to weakening the capillaries. The C3a and C5a complement factors reach the highest level at the defervescence.[82]

Cell response to DENV infection

CD 4 and CD 8 cells and Natural killer cells decrease usually in DSS or DHF and in most cases seen at the convalescence. Reversal of CD-4 : CD-8 ratio takes place after 10 days of onset of fever and noted with the patients with DHF, generalized BM suppression may occur in dengue infection which leads to Lymphopenia.[29]

Changes in liver enzyme in dengue infection

Liver enzymes are elevated and bleeding tendencies is seen in Dengue infection, which suggests that liver is involved very commonly. Cases of hepatic involvement in dengue have been reported with moderate necrosis of hepatocytes,

micro vesicular steatosis and councilman bodies, which is also seen in Yellow fever and Rift valley fever which belong to the *flaviviriadeae.* [116,117]

Role of endothelial cells in DENV infection:[118]

They play an important role in severe systemic inflammation, as EC are the physiological regulatory factor and the role in tropism of DENV is controversial. The Non structural proteins of Dengue virus show tropism to EC of Liver and Lung tissue. NS-1 and anti- NS-1 antibodies contribute to selective pulmonary vascular leakage.

Platelet count and DENV infection:

In Dengue virus infection suppression of erythroid, thromboid and myeloid precursors occur. Associated with peripheral cytopenias, especially thrombocytopenia, thrombocytopenia is because of IgM antibodies of anti-platelet, DENV specific antibodies, bone marrow which leads to increase in defective megakaryocytes or destruction of platelets in spleen and liver. Anti platelet antibodies cause lyses of platelet in association with complement. DENV serotype 2 is more associated with this, which binds to human platelets only in the presence of virus specific Abs, also known as immune mediated platelet clearance.[29,119]

Cell tropism has been seen in experimental animal and non human primates, which shows that virus was isolated in high concentration from skin and gastrointestinal tract and low level of viral load was seen in liver, lung, peripheral lymph node and spleen. Dengue virus can bind the mononuclear cells in the circulation, spleen, lymph nodes and bone marrow in AG 129 mice. Leucocytes are also infected with DENV. Dengue virus is isolated from the spleen, peripheral lymph

node and liver and CNS in IFN- alpha and beta deficient mice. Neural tropism is seen in mouse model, but not much in humans.[8,119]

Pathogenesis of DHF:[82]

Different theories have been put to explain the pathogenesis of dengue hemorrhagic fever, one is immune mediated, in which cross reacting antibodies develop, which augment the infection. The other one is related to the stain virulence, virulent dengue type cause DHF and less virulent strain of Dengue virus causes mild type or dengue fever.

Factors contributing the mechanism of pathogenesis

1. Dengue virus – virulent strain may cause severe infection
2. Anti – dengue antibodies- they activate complement and enhance dengue virus infection by virus – antibody immune complex serotype cross reactivity.
3. Complement – activation product that produce plasma leakage
4. Platelets – thrombocytopenia
5. T lymphocytes- produce lymhokines and lyses dengue virus infected cells
6. Endothelial cells- play an important role in plasma leakage and produce cytokines.

Hemorrhagic manifestations in DHF: [121]

Hemorrhagic manifestations are suppose to be one of the autoimmune manifestation, may be due to molecular mimicry. Few workers also state the role of cytokine, viral antibodies binding the platelets, vascular injury or with plasminogen or other clotting factors reduce platelet survival and consumptive coagulopathy. It is

caused by dysfunction of vascular endothelial cells rather than destruction of small blood vessels.

Shock in DENV infection:[121]

Due to massive extravasations of plasma to extra vascular sites, like pleural and abdominal cavity, person may go in shock. This is due to increased vascular permeability due to immune activation. Increased level of TNF receptor, Interferon gamma, Interleukin -8 and apoptotic endothelial cell death manifest immune activation. Complement is also activated in this sequence as a part of inflammation.

Antibody dependent enhancement:

Lack of neutralization of cross reacting antibodies of primary infection can cause severe manifestations in secondary infection, dengue virus and non neutralizing antibodies from complex. These complexes bind to Fcy receptor of the target cells and leads to the enhancement of DENV infection. This is called as ADE.[82]

Antibody dependent enhancement is based on, infection with two different serotypes of dengue virus. Manifestations of Dengue hemorrhagic fever is due to the immune response in the form of antibody formation to one serotype and if the another subtype of dengue virus infects the same individual, the virus will activate the immune system to attack it as if it was the first serotype. Immune complex suppresses the cellular immune response, increasing the intracellular infection and exaggerated inflammation. Chemokines and cytokines together contribute in the severity of the infection. Increased number of virion leads to high infection due to accelerated internalization and cell infection by immune complexes. Antibodies to prM are major

component of high cross reactivity in all dengue serotypes and these antibodies have ADE and low neutralization capacity.[105]

2.7 Clinical presentation:

Dengue may be present in various clinical forms, which vary for asymptomatic to sever dengue infection. Dengue may remain asymptomatic, symptomatic, undifferentiated fever, classical dengue with hemorrhage, with unusual hemorrhage; sever forms like DHF without shock or Dengue shock syndrome. DHF is also graded depending on the severity by World Health Organization, as grade I to IV. Dengue hemorrhagic fever III and IV is classified as Dengue shock syndrome.[122,123]

Dengue fever probable diagnosis:

An acute febrile illness with two or more of the following symptoms like headache, Retro-orbital pain, Myalgia, Arthralgia, Rash, Haemorrhagic manifestations, leucopenia and supportive serology i.e. IgG by ELISA or Positive IgM on a late acute or convalescent phase serum specimen or confirmed case of dengue fever in same location.[123]

Confirmed dengue fever diagnosis:

Isolation of dengue virus from serum or autopsy samples or demonstration of fourfold rise in IgG or IgM antibody titer of dengue virus antigen or Antigen demonstration in autopsy tissue serum or CSF by immunohisochemistry, immunofluroscence or ELISA or detection of DENV genome in autopsy or CSF by PCR.

Dengue hemorrhagic fever a case definition:[123]

If only all the below mentioned criteria are fulfilled it is defined as DHF.

Fever or history of acute fever since 2-7 days occasionally biphasic, hemorrhagic tendency, evident at least one e.g. positive tourniquet test, petechiae, ecchymoses or purpura, bleeding from the mucosa, GIT ,haematemesis or melaena. Thrombocytopenia (100000 cells per mm3 or less), evident plasma leakage.

Dengue syndrome case definition:[123] all the four criteria for DHF plus evidence of circulatory failure manifested by rapid and weak pulse, narrow pulse pressure, hypotension for age and cold , clammy skin and restlessness.

Dengue fever and dengue haemorrhagic fever grades:[123]

DF- fever with two or more of the flowing signs, Headache, rero-orbital pain, myalgia, arthralgia.

Grade 1 DHF- above signs plus positive tourniquet test with thrombocytopenia <_100000/mm3, haematrocrit>_20%.

Grade II DHF- above all signs plus spontaneous bleeding thrombocytopenia <_100000/mm3, haematrocrit>_20%.

Grade III DHF- above all signs plus circulatory failure, weak pulse, hypotension, restlessness thrombocytopenia <_100000/mm3, haematrocrit>_20%.

Grade IV DHF- profound shock with undetectable blood pressure and thrombocytopenia <_100000/mm3, haematrocrit>_20%.

Due to unclear picture of clinical symptoms and difficulties in reporting the dengue cases by the above criteria WHO in 2009 proposed a new classification according to the degree of severity it is as follows.

Dengue without warning signs (probable dengue):[122]

1. Live in / Travel to Dengue endemic area.
2. Fever and two or the following criteria.
    a. Nausea/ vomiting
    b. Rash
    c. Body ache and pain
    d. Leucopenia
    e. Tourniquet test positive
    f. Any warning sign
3. Laboratory confirmed dengue when no sign or plasma leakage.

Dengue with warning signs:[122]

Presence of any of below signs

- Abdominal pain
- Clinical fluid accumulation
- Persistent vomiting
- Mucosal bleeding
- Lethargy, restlessness
- Liver enlargement more than 2cm
- Laboratory diagnosis included decrease in platelet count and haematocrit

Severe Dengue:[122]

Patients from endemic area with fever of 2-7 days plus

Severe plasma leakage; leading to shock, fluid accumulation with respiratory distress

Severe bleeding as evaluated by clinician

Severe organ impairment; liver enzyme aspirate transaminase or alanine transaminase ALT >_ 1000 unites, with loss of consciousness, heart and other organs.

2.8 LABORATORY DIAGNOSIS OF DENGUE VIRUS INFECTION

There are various diagnostic methods used for detection of Dengue virus antigen or antibodies, which help in early diagnosis of the dengue infection, helpful for proper management of the patients in clinical care, surveillance of serotypes and to know the outbreak. Clinical profile may be similar with the other viral infections, so it is important to diagnose the disease in laboratory. [124]

Importance of the laboratory test lies in sensitivity and specificity. At present various test are done to detect the antibodies or antigen, rapid test card test, ELISA are one of them. Virus isolation and RNA detection is very much specific and specific antibody detection from patients serum.[125]

Laboratory test may be classified / divided into different types: a. Virus isolation from blood or serum samples, b. Serological test for detection of Antigen or Antibodies, C. Rapid methods and d. Newer methods with advance technology.[125]

a. **Isolation of Dengue virus from blood or serum samples** [125]: Isolation of virus can be done from serum, blood, plasma, Buffy coat, autopsy specimen (liver, spleen, lymph node etc), helpful in early diagnosis when antibodies are not developed. IgM is the antibody developed only after 5 to 7 days of infection, so virus isolation becomes important in the initial phase.

Isolation of Dengue virus can be done on various cell lines in tissue culture, animal inoculation and mosquito inoculation.

Tissue culture/ cell culture: Various mosquito cell lines are used for cultivation of dengue virus eg. C6/36 of *A. albopictus*, AP61 of *A pseudoscutellaris* are used, as well as animal cell lines Tra-284, CLA-1 and LLCMK2 are used for primary isolation of virus from plasma & serum of the patients. Detection of the virus in the cell culture is done by florescence, which is one of the time consuming methods replaced by RT- PCR, which is more sensitive and specific and less time consuming.[125,126,127.]

Animals used for isolation of dengue virus:

Mice with severe combined immunodeficiency, suckling Mice and Monkeys are used for isolation of Dengue virus but found to be less sensitive and the virus grows slow in the Mice and is more cost effective.[128] Serial inoculation may increase the sensitivity. Paralysis of the limbs is seen and extraction of dengue virus can be done from brain. Various experimentation are been done to isolate the virus in animal models but have very less infectivity and low viral growth.[129,130]

Inoculation of dengue virus in mosquitoes:

A.aegypti, *Toxorhychtiessplendens*, *A.albopictus* are the mosquitoes used for inoculation and isolation of dengue virus. Intra-thoracic inoculation is done and virus is extracted by squashing the head of the mosquitoes and detecting it by immunofluroscence or by flow cytometry, comparison have been done by few of the authors.[129,130,131] Neutralization test can be done for quantation of the dengue virus in culture.[132]

b. Serology for diagnosis of dengue virus:

Complement fixation test (CFT)[10]: one of the test very useful in identification of primary infection but not the secondary infection. This test is based on the Ag- Ab complex is formed and the C is utilized by the reaction, to perform this test highly trained person is needed. This is why test is not used now a day for detection of dengue.[10]

Complement fixation test is seen second to the HI test as it is short lived and can detect the initial phase infection. In some patients low level antibodies may be present for long time. The antibodies to CF appear late and remain unstable. This test is useful in primary infection rather than secondary infection.

Partiv k et al have used Complement fixation test for the detection of isolated virus and confirmation of the isolate from the human serum, and mosquito.

Hemagglutination test[10]: This was one of the commonly used test for diagnosis of primary and secondary dengue infection in the last few decades, major drawback of this test is it needs paired sera and cross reaction is seen it means this is one of the less specific test and may cross react with other viral infection. As this test needs minimal equipments and is easy to perform.[10]

The Haemagglutination test is based on the principle that the dengue virus can agglutinate the goose RBC at specific temperature and PH, and elution can be carried out by addition of specific antibodies.

The antigen is derived from the brain tissue of suckling mice or from the infected mosquito and then the serum is tested for antibodies. Antibody titer more than 10 can be detectable by this test and by 5 to 6 days after infection.

In primary infection the titer remain below 1:640 and then on progress usually the titer increases rapidly in the initial phase of infection. But lack of specificity of the Haemagglutination test is the major problem.

ELISA (IgM, IgG &IgA): Detection of IgM from blood, serum and plasma is routinely done in the laboratory for detection of Dengue virus infection.[94,131] As IgM is an antibody which only appear after 4 to 7 days of infection in the patients serum, detection is only possible after 5 days of infection so not helpful in early diagnosis. Many studies have showed the appearance of IgM in 3 days and rise in titer up to 2 weeks.[10]

IgM ELISA is one of the capture ELISA in which IgM antibodies are detected in patients serum by using a solid phase bound Antihuman antibodies and substrate enzyme conjugate is used to detect a colorimetric reaction. Various formats of this test are available e.g. Ultra micro ELISA, Au Bio Dot IgM capture ELISA, dipstick method Pan bio and J Mita co. kits are available for detection of IgM in serum and Plasma.[131]

These are very simple and easy to do test on routine bases and helpful in detection of primary and secondary infection, by calculation the OD and cut of value. 90 to 98 % sensitivity is seen and specificity is less as it can cross reacts with the other flaviviral group virus.[131]

IgG ELISA for detection of Dengue Virus infection: detection of IgG will help us to rule out the primary and secondary infection and four fold rise in the antibody titer in paired sera will let us know the stage of the disease. This test is also based on enzyme conjugate and substrate with chromomeric results based on solid phase. This is one of the non specific tests as it can cross react and mimic the hemagglutianation

test. These test are not routinely used and now they are not done to differentiate primary and secondary infection.[94,133]

As per the WHO guidelines 2009 the OD of IgG : IgM if less the 1: 2 is termed as primary infection and if OD greater than 1:2 is called as secondary infection. The ratio may vary depending on the stage of infection and clinical status and the serotype infected. Initial classification of Primary and secondary dengue infection was dependent on IgM to IgG unites.[122]

The ratio of IgM/ IgG ratio were more in Primary compared to secondary infection. Pan Bio kits showed the best result in classification of primary and secondary infection in Dengue, which was confirmed by Laxmi et al in their study.[(134)] Advantage found by using this kit was single sample is sufficient, there is no need of paired sera. And the disadvantage is cutoff value is variable and virus serotype cannot be determined.[94]

IgA can only be detected after one day of IgM appearance, as the half life of IgA is less it remain undetectable after the appearance.[27]

ELISA (NS-1):

NS-1 ELISA: this is one of the nonstructural protein found in initial phase of infection and can be detected in the initial phase of the diseases and can be detected upt0 8 days, as the half life of NS1 is more, it can be detected. Instead of going for PCR and isolation of virus in the acute phase of the Dengue viral infection we can do NS-1 ELISA for early diagnosis of the Disease.[133]

NS-1 is a solid phase Enzyme linked immunosorbent assay which is based on direct sandwich principle, the polystyrene micro wells are coated with the Anti

dengue NS-1 antibodies, which are specific and sensitive to detect even minute amount of NS-1 antigen. Patients sample containing the Ag is added to the wells and to which enzyme conjugate is added along with which contain monoclonal anti dengue NS-1 antibodies attached with horse radish peroxidase (detecting system). Those serum sample having antigen will bind to the antibodies in the well and forms a sandwiched structure only positive sample remain attached to the well while those serum sample doesn't have the antigen will get washed off by buffer wash. The intact system in the micro titer can be detected by addition of the substrate and after addition will show the chromogenic reaction. The optical density is read the intensity of the colour present in the well represent the amount of NS-1 antigen present in the serum.[135]

Datta S et al[135] studied the NS-1 assay and its efficacy on 600 patients sample and they found NS-1 assay to be more help full in detection of the dengue viral infection in early phase of the disease and when the serum sample are negative in the acute phase for IgM and IgG antibodies the positivity increased to 93.10% with Mac Elisa when compared with rapid card method.

Kassim et al[136] have compared both ELISA and PCR for the detection of the Dengue virus infection in the acute phase where he found NS-1 antigen detection was equally good in comparison with RT- PCR and showed 100% result. So he concluded that in many peripheral laboratories where IgM is done as a sole diagnostic method for the detection of the dengue virus infection, NS-1 also can be done for the detection so that it can improve the positivity.

Pei Yun et al[137] used NS-1 rapid kit for the detection of dengue virus infection among the air travelers and found to useful in detection of the acute infection and

found 17 cases positive by rapid test which were confirmed by PCR and found to be correct.

Singh MP et al[138] compared NS-1 by ELISA with RT PCR and IgM antibody detection in the patients with dengue. Where NS-1 detection was found to be the early marker, which they could detect up to 4th day of infection while IgM was only positive after 4 days and 75% to NS-1 positive cases were positive by RT PCR, stating that accurate and ease detection of the Dengue viral infection in the acute phase.

There are many other methods used for the detection of dengue virus in early and late phase, as well as for detection of Antigen and antibodies. Like Luminescence based optical fiber biosensor, monoclonal antibody technique using mAB4E11, natural cytotoxic receptor Ig, Microsphere based immune assay and Biosensor technology using mass spectrometry.[139]

Neutralization test for Dengue Virus:

This test was developed by Halstead and Nislak by using suckling mice. The major advantage of this test is that it is a specific test and very sensitive, used for identification of Primary Infection, but its cost and expertise needed, become the major disadvantage.[94,139]

Plaque reduction Neutralization test for dengue:

This test was first used by Nislak and Haslsted for virus neutralization by its antibodies showing plaque formation. This test was done to detect the serotype of dengue virus and was considered as gold standard test by WHO. The major disadvantage of this test is cross reaction with other virus. Also needs separate

dilution tubes, time consuming, separate staining procedures for counting of plaques. Also needs laboratory expertise and standardization for procedures.[140,141]

This test is based on the principal of interaction of the dengue virus and its antibodies and as a result there is inactivation of the virus, so that it is no more infective and do not replicate in culture.[139]

Difference in the results of the virus isolation and neutralization test is seen, as it basically depends on the virus stain and the cell lines used for cultivation of the virus.

Western blotting assay:

This test is based on prM protein detection present as the outer most layers of the Dengue virus and detection is based on substrate enzyme procedures where the sensitivity and specificity was very low. The sensitivity was 24% while specificity was 98% in Dengue virus detection.[142]

Loop mediated isothermal amplification method for dengue diagnosis:

This is one of new method which didn't need any high end instrumentation, but based on Reverse transcriptase –LAMP. Where C-prM gene or serotype specific 3' un-translated region is used for amplification by using a water bath with temperature between 60 to 65^0C for amplification, where turbidity is detected with the help of dye. Major advantages of this method is no cross reactivity is seen. It is sensitive as RT- PCR. Reaction time is less i.e. 1 hour and serotype can be identified as well as viral load. It is a simple and easy method. [7,143]

RT-PCR:

Reverse transcriptase Polymerase chain reaction: this is one of the most sensitive and specific method for detection virus in circulation during the acute phases of the disease where antibodies are not detectable and where virus isolation becomes cost effective and needs the high grade laboratories.[144]This test includes three steps i.e RNA extraction and purification, Amplification of nucleic acid and detection of the amplified product. This needs expertise and specialized equipments and trained staff technical. Chances of contamination are more at every step and needs sterile zone to perform the test.[94,144]

Nested PCR [94]:

Serotype specific primers are used in this where it can detect multiple serotype in the single reaction. prM / C region of viral gene is used for Rt and amplification .

Multiplex PCR[94]:

This is used instead of Nested PCR for detection of multiple serotypes, on gel documentation detection system and can be preserved and stained with ethidium bromide dye.

Real Time Polymerase reaction [10]:

One of the methods now a day's used for the detection of viral RNA. This is one of the quick, specific and quantitative methods. DNA Fluorophores, hairpin ologonucleotide probes and amplicons are most widely used in this test. Taqman assay i.e 533 Ologonucleotide probes are used and is used universally for detection of all four dengue serotypes. These techniques are based on nucleic acid detection which is specific but chances of false positivity lies in same group of organisms.

Nucleic acid sequence base amplification:[145,146] This is one of the methods which detect the Target RNA and runs at 41 degree centigrade and is more useful in detection of Dengue virus RNA from the tissue. The amplification of the nucleic acid is done at 41 degree centigrade and amplicon is detected in Agarose gel, other than this we can use electro chemilunesecne, enzyme linked gel assay. Advantage of this assay is it can amplify and can be used for sequencing.

Recently developed kits based on immunochromatographic principal and have short duration of detection with 20 min results can be obtained and also can detect both antigen and antibodies. Specificity of the kits is good but sensitivity varies from kit to kit. [147,148]

Yee ling lai[149] have studied the dengue virus by detecting the Dengue virus RNA by RT-PCR in early phase of the disease targeting the cDNA derived from the dengue RNA. Which forms the basis of the molecular diagnosis of the dengue viral infection, validation of the test was done by local sample collection during 2004 to 2006 and 87% of positivity was observed.

One of the study carried out by Abdul Rehman et al[150], developed the PCR kit which was able to detect the all four serotype in two step RT PCR. They used whole blood for detection of RNA. They have used all the NS-1 positive samples. 100% positivity was seen by this which detected serotype 1, serotypes -2 and also detected the mix or multiple virus infection with serotype 2 and serotype-3.

Rapid diagnostic kits and dengue positivity:

There are many rapid diagnostic kits available for detection of IgG , IgM antibodies and NS-1 antigen. They don't need skilled person; they are rapid showing

results in 20 minutes and can be performed easily, which can even be performed at the PHC level where laboratory facilities are not available.[151]

These kits are easy to handle but stored at request temperature and in outbreaks and epidemics as well as different areas they can be carried to get the test done on the spot.

These kits are based on the principal that the antibody from the patient's serum when incorporated in the test kit will attach the recombinant dengue virus particle conjugated with colloidal gold and forms a complex of Antigen and antibody. This Ag- Ab complex is captured by relevant anti human immunoglobulin IgG/ IgM immobilized on the test kit and develop s a colour line when migrate along with the by capillary action.[152]

Seok wang et al[152]evaluated the commercial kit with ELISA, RT PCR and HI and made a brief comparison between these kits. The study comprised of 420 suspected patients with dengue viral infection. 320 patient serums were positive by RT-PCR, while 300 serum samples were positive by ELISA and the commercial Dengue Duo kit showed 298 serums positive for IgM, NS-1 and IgG. The positivity noted for IgM was 98.75% while NS-1 positivity was 88.65%. He concluded that the Dengue duo test is highly specific and sensitive compared to RT-PCR and ELISA.

A study was conducted in Delhi in 2010 by Subhash et al.[153] where he use the rapid single step immunochromatigraphic test for detection of IgG, IgM and NS-1 in dengue suspected cases. A total of 1800 patients were tested 1208 were positive for NS-1 in different combination. The sensitivity and specificity was found to be good and concluded as a useful tool for tackling the rise in the suspected cases.

Sensitivity and specificity of the rapid test, ELISA and its correlation with clinical cases as per the WHO definition was done by Pramila devi R et al[154], where they found rapid test are less sensitive but are more specific then the ELISA. This study was conducted in Dengue suspected case, but less number of cases was positive, only 16 cases were positive by rapid test and 14 out of the same gave positive results by ELISA.

Jayasmiha et al[(155)] did a study on comparison of ELISA and rapid kits for detection of dengue viral infection where they tested 226 samples of which 150 were positive by ELISA. Positivity rate by rapid test was 66% with 80% sensitivity and 87.16% efficiency, and concluded that rapid test can be used for screening of dengue infection.

Hematological parameter:

As dengue patho- physiology includes the platelet and other immune cells so , a complete blood count, TC/ DC, clotting time bleeding time, platelet count and total haematocrit become important for progression of the disease.

Platelet count blow 100000/µl is called as thrombocytopenia with is seen in dengue viral infection and estimation of platelet count is helpful to manage the patients usually plate count drops in DHF and there is a general drop in Platelet below average in 3 to 8 days of infection and further recovery is seen[155].

A study conducted by Francisca et al, they compared the clinical condition in dengue viral infection and different stages of the disease, and correlated with the

hematological parameter and found that platelet count depletion one seen in DHF and DSS. Also noticed high heamatocrit with haemoconcentration.[156,157]

In a study by Aggrawal S et al[158] who studied in comparison the clinical and haematological profile of the dengue viral outbreak. Where 56 patients were studied and he found the major association of high hematocrit and low platelet count in dengue infection.

Most of the laboratory studies on dengue infection and laboratory parameter comparison it shows that the high prevalence of Hamatocrit and low platelet count is seen in babies and young adults aged up to 16 years.

Dot blot assay:

Commercially available dengue antibody dot blot is available in market with can detect the antibodies and a study conducted on this technique Cuzzub et al compared the dot blot technique with IgM and IgG ELISA, he found the dot blot technique to be more sensitive as compared to ELISA, where as IgM ELISA was more specific for JE. In detection of Dengue viral infection Dot blot and ELISA showed 100% results but ELISA was the more specific and sensitive.[159]

Sahrruachaman et al[160] study on semi-quantative dot assay for IgM antibody detection against dengue in human sera used biotinylated dengue antigen which is simple method wise and also rapid in performance. In this study he reported 23 cases of primary infection, 48 case of acute secondary infection and 33 recent dengue infections stating that this methodology was more sensitive in detecting secondary infection when compared with HI.

2.9 Dengue and Differential diagnosis: (WHO dengue guidelines for diagnosis treatment and prevention. 2009)

Dengue infection common signs and symptoms may resemble the other clinical conditions like: Flu like illness may be similar which is seen in Influenza, Chikunguniya, HIV, Measles, infectious mononucleosis etc. Rash may be seen in scarlet fever, Chikunguniya, Measles, Rubella etc. Abdominal symptoms may resemble Rotavirus diarrhea and other enteric infection, like bacterial, parasitic or fungal.

Critical conditions in dengue may also mimic with Malaria, Leptospirosis, Typhus, Typhoid, HIV, septic shock, malignancies like acute leukemia and other malignancy.

2.10 Management of Dengue fever[161]: (WHO dengue guidelines for diagnosis treatment and prevention. 2009)

As there is no treatment for the dengue viral infection, management of the patient by supportive therapy becomes the priority and fluid supplement in case of critical cases becomes must, as it can led to complications. So appropriate management, depending on the phase of the disease is must.

Therefore few guidelines have been made by WHO for the treatment of the dengue patients depending to which group they belong to the patients can be categorized in 3 different groups[162]

Group A: comprises of the patients with no complications who can remain in home and take treatment.

Group B: patients who needs to be managed in the hospital.

Group C: comprises the patients who need emergency treatment.

Dengue fever management is symptomatic by giving antipyretic; Aspirin is contraindicated as it may cause vomiting acidosis and gastritis. Sponging can be done to keep the body cool with bed rest. WHO in 2009 has made 3 categories for management of dengue patients and are divided as A, B &C.

Group A comprises of patients who can take in oral rehydration and can pass urine every six hourly and warning signs are not present. They should take oral fluids, juice and other oral rehydration for maintenance of sugar level, antipyretic can be given and instruct patients about warning signs and tell them to come to hospital if warning signs are observed at home. Full blood count and Haematocrit should be suggested.

Group B comprises of patients with warning signs. In this case first take the hematocrit of the patient and then start rehydration. Ringer lactate or Hartmann sol is given 5-7 ml/ hour reduced to 2-3 ml/kg/h according to the clinical status of the patient. Blood count and haematocrit should be suggested.

Re-assess the hematocrit and clinical feature if there is same status of hematocrit, continue with above rehydration. Glucose level and LFT, KFT and coagulation profile should be assessed. Depending on the patient condition oral or IV rehydration should be continued even in patients without warning signs.

Group C includes the patients who need emergency treatment and referral because of the sever dengue. The patients with severe dengue should be admitted in hospital under observation, where Intensive care and blood transfusion facilities are available. In cases with hypotensive shock colloidal sol are given IV.

Hematocrit should be observed if patients are not stable and bolus fluid administration or increase fluid. Shock management depends on type of shock separate guidelines are there for DHF, DSS and DF.

DHF average grade management: which is febrile phase of the disease, can be managed by antipyretics and oral or IV rehydration until the patients tolerate. Instead of water, fruit juice should be given.[165]

Management of DHF in febrile phase can be done by observation of hematocrit, if not HB should be checked.

DHF grade I and II [162]: Dengue fever patient with thrombocytopenia should be hospitalized and observed for signs of shock. Urine output should be checked and other wise Grade III and IV management regime should be done.

Management of DHF grade III and IV; Patients with grade III and IV should be given Oxygen. Examination of patient and hematocrit should be done and immediate rehydration should be done. Patients should be preferably treated with colloidal solution like Dextran. If continuously there is decrease in platelet count and hematotric, transfusion should be made available. Usually fresh whole blood is given 10ml/kg for patients with shock as a precautionary measure. Steroids don't have any role in recovery of Dengue infection. IV antibodies are of no use. There is no specific treatment for Dengue.

2.11 Antiviral drugs[162,163,164,165]

Direct antiviral administration can be preferred to kill the virus, but at present no specific antiviral drug is available, many attempts are been made to develop the antiviral against Dengue virus, but the drug should be equally effective to all the

serotypes of the virus. Seaweeds, polysaccharide extracts are studied and are under research to know its antiviral activity. Ravbvirin, 6 Azauridine are the cytostatic and control dengue virus replication.

There are many challenges to develop the antiviral drug which includes, these drugs should be active against all serotype and kill all serotypes, must be easily taken orally should be heat and moisture stable, should have long shelf life with low production cost.

The drug should be good, safe to handle and should work in both gender and all age group, must be active against all serotype and genotype present. Also must be effective in all stages of disease like primary and secondary dengue infection.

2.12 PREVENTION & CONTROL OF DENGUE VIRUS INFECTION [166]

No specific treatment against dengue virus is available, whatever so treatment is done are symptomatic and vaccines are also not available, they too are under trial phase. Mosquito control is one of the method by which dengue virus transmission can be stopped.

How to reduce breeding of *Aedes* mosquito: This can be done by improvement in the water storage and supply. Prolong storage of water is the major cause for the multiplication of the mosquito. Timely changing of water from the stored container may break the cycle.[167]

Proofing of over head water tanks, underground water storage or containers should be proofed [167]

Empting the container regularly and filling fresh water after washing will reduce the multiplying mosquitoes.

Personal prevention:[168]

Cloths made from thick fabrics should be worn especially with long sleeves and trousers and stockings [166].

Mosquito repellent mats, coils and house hold insecticides should be sprayed to reduce the load, electric vaporizers are available can be used. Natural or chemical repellents can be used for the killing.[169,170]

Travelers especially should take care while travelling to the endemic areas as they may themselves carry the virus with them, to avoid infection, screened stain or air condition room should be preferred, use of mosquito repellent cream and coils in stay places and protective clothing.

There are many other methods to destroy larval form of mosquitoes so that trans-ovarian transmission can be broken, like biological methods to control larvae by using microbes like *Bacillus thuringiensis* H14 & *Bacillus sphaericus* which have been found to effective in destroying the larval form in the water.[171]

Nematodes have been used to destroy the larval forms. *Gambusia affinis*, *Poecilia reticulate* fish are also used for control of *Aedes* larva. [171]

Copepods (small fish) are used to eliminate larval form from the storage containers.

Adult mosquito can be killed by insecticides and other pesticides also. [167]

Chemical controls can also be used to eliminate the mosquito; drinking water storage container can also be treated with the chemical agents to avoid the growth of larval form in water. They are mainly of three types [166]

Bacillus thuringiensis H-14 can be used as a lavicidal and is safe for humans.

Temephos sand granules: one percent temephos can be used in the 1ppm dosage is effective in 8-12 weeks, especially in earthen pots, jars. [172]

Insect growth regulators can be used to control the growth of mosquito where in it interferes with the chitin synthesis during the process in larval molting and cause disruption of the pupa. [176] Thermal fog and cold fogs can also be used to destroy the mosquito by spraying in the space where in the droplet of this space spray can kill the *Ae. Aegypti*. These thermal droplet fogs are of 15 micron when condensed at high temperature.[173,174]

2.13 Dengue Vaccines:[175]

As there are no models to detect the dengue vaccine efficacy and antibody dependent pathogenesis become the important challenge for development of dengue virus vaccine.

Vaccine if developed should be tetravalent as dengue has four serotypes and all should be included.

Tissue culture vaccine and recombinant vaccine are under trial since 2003 and other than this 6 various workers are still working towards viral vaccine for dengue.

Live attenuated vaccine for dengue:

Many efforts have been made to develop the dengue vaccine, first such initiative was done in Hawaii by using non human model for serial passage of dengue virus for production of vaccine and further development was done by Mahidol University Bangkok,.[176]

Walter reed Army Institute and research center then started development of tissue culture vaccine at the same time. Apart from this a vaccine with recombinant, live, attenuated, tetravalent vaccine was under trial. Trials were imitated in Asia and Latin America to check the efficacy of the vaccine dose given 0,6 and 12 months schedules, which involved children of age group 2-14 years. After the third dose efficacy increased from 56% to 80% and overall efficacy taken by the committee was 60%. But the Antibody response increased in the second and third dose.[177,178]

This vaccine reduced the burden of hospital, the hospitalization rate was reduced in the age group 2 to 16 years, but in Asian phase trial 3 the 3rd dose efficacy was low. In Thailand the vaccine was safe but failed to protect against DENV-2 infection and there was large variation in protective immunity in various serotypes.[178]

An inactivated dengue viral vaccine has got many advantages; they are safe and won't revert as like there are chances of live vaccine to get converted into virulent strain. Dengue virus -2 attenuated vaccines combined with adjuvant is used for trial in monkeys and mice, where high level of antibody development and protection against virus is observed.

A live attenuated tetravalent vaccine is been developed by Sanfi Pasture Chimeri vax dengue vaccine. Where in prM and E genes of all the four serotypes are stable and are less neurovirulent, the YFV 17D and immunogenic in monkeys. Phase I study showed better results but now phase II showed 30% effect and efficacy.

Recombinant vaccine for Dengue:[178,179]

Envelope gene (E) & prM genes are used for the production of recombinant vaccine, as these are the portions of the virus surface and help in attachment of the virus to the host cells. Chemeric vaccine is developed in such a way that includes the genes of all four serotypes. CDC has developed such vaccine including E and prM gene of all dengue virus serotypes. The vaccine has been licensed In Viragen and Takeda respectively has undergone successful clinical trial.

USA has developed the vaccine derived from the recombination using Yellow fever vector, technology known as chimerl Vax technology for dengue fever. Envelop and prM gene of Dengue is combined with the yellow fever 17 D full length genome which is replaced by Dengue virus genes. Immune response trialed in monkey showed good results with the mono valent vaccine of individual serotype vaccine of Dengue. But DENV-2 response was good as compared to 1, 3 and 4 serotype, balanced formulation is needed to develop good immune response in monkeys and then human trials should be done.[178]

Inactivated Dengue Virus vaccine is under trial, since many years as they have various advantages over live vaccine e.g. these strains do not show reversion and cannot show change in phenotypic character and they can easily be combined with other serotype and chances of mixing within serotype is less. Also good humoral and cell mediated immune response is there. One of the disadvantages of this vaccine is that, there may be immune enhanced pathogenesis and breakdown into wild type. AS03 and AS04 are the two adjutants now a day's used for vaccines licensed for human use. [179]

Subunit vaccines are tried and first phase trial is over in USA against DENV-1 and its safety and efficacy is been checked.[179]

DNA vaccine are made up of plasmids containing dengue virus gene, trial taken in monkeys using multivalent vaccine showed raised antibody response. In recent studies on rhesus monkey model by DNA vaccine showed good results. But the major disadvantage is that there is risk of gene integration in host chromosome and further which may lead to autoimmune antibodies.[179]

Vectored vaccines recombined with poxvirus and adenovirus is used to demonstrate strong response in human against various pathogens. Alpha virus, vaccine virus, adenovirus are use to design and engineer the dengue virus envelop vaccine. Moreover E protein expressed in insects, yeast and virus like particles dengue virus vaccine have been under test and are under pre clinical trial studies.[179]

Even though the knowledge about dengue virus pathogenesis and virulence is increased and lot of research is ongoing, still up today we don't have a licensed vaccine against dengue virus.

2.13 Changing pattern of dengue virus:

Dengue virus is emerging and prevalent in India, almost in all states, all the four serotypes are present in India. Chances of mono infection and multiple infection is common and the severity of the disease is in the areas where multiple serotype are co circulating at a given time. Also changing serotype circulation during the years is observed in many places.

Ekta G et al[64] in the year 2003 to 2005 in Delhi have seen the changing pattern of the serotype with predominance of serotype 3 in 2005. She has reported circulation

of all the four serotypes in the year 2003, in the year 2004 only dengue serotype -1 was isolated, while in the year 2005 the predominant serotype seen was DENV-3.

Since 1997 dengue serotype-1 was observed, in following years, 2004 multiple serotypes were isolated from North India. Also it is observed that last 2 decades DENV-3 was responsible for epidemic in Srilanka, Latin America and East Africa.[15,180]

Aim and Objectives of the study

Aim: To evaluate the utility of Serodiagnosis of dengue virus infection and correlate results with demographic, clinical and laboratory profile in patients clinically suspected of dengue infection.

Objectives:

1. To evaluate the performance of rapid immunochromatographic test in detecting antibodies to dengue virus and its comparison with reference antibody capture assay (MAC - ELISA)
2. To evaluate the usefulness of NS-1 antigen for early diagnosis of Dengue Infection.
3. To detect dengue viral RNA by PCR using whole blood or serum from patients in acute phase and know the possibility of multiple serotype infection among dengue patients.

4.1Material and Methods:

4.1.A Type of study and period: This is a cross sectional study done from the year 2012 to 2016.

B. Study population:

- Patients attending with symptoms of dengue viral infection from rural and urban area attending OPD's of Medicine and Pediatric department of Bidar Institute of Medical Sciences teaching hospital, Bidar.
- The study protocol was approved by Institutional Ethics Committee and informed consent was taken from the patients before collecting the samples.

C. Sample size: The sample size (n=1000) was estimated with an expected prevalence of Dengue as 15% with 4% absolute precision and 95% confidence interval. An interim analysis was carried out and the estimate from the interim analysis was used to modify the sample size.

D. Inclusion criteria: cases of both sex and all age group with Fever, Joint pain, Myalgia, Arthralgia. Two or more signs as per WHO criteria were included in study.

E. Exclusion criteria: cases of known etiology eg. Malaria, Typhoid, Chickunguniya etc were excluded.

4.2. A Sample collection:

- 3 to 5 ml blood sample was collected under aseptic conditions from all patents suspected of Dengue infection and was divided in two vials, plain and vial containing EDTA.

B. Sample processing:

- **Hematological analysis:** Blood in EDTA vial was subjected after homogenization to fully automated cell counter to detect the Platelet count, haematocrit, TC and DC.
- **Serodiagnosis:** Blood collected in plain vial was allowed to clot and serum was separated for detection of NS-1, IgM and IgG antibodies by rapid card method and ELISA.
- After rapid test was done, the serum samples were preserved at -80^{0}C until ELISA and PCR was done.

C. RAPID TEST

- Collected serum samples from suspected cases were subjected for detection of NS-1, IgM and IgG by using (J. Mitra. Co. New Delhi, India kits). Manufacturer's instructions were followed to carry out the test.
- Dengue NS-1: Three drops of serum was added to the antigen well and results were observed within 20 minutes.
- Dengue IgM and IgG: 10 µl of serum sample was added to the antibody well with help of dropper provided with proper precaution, then 2 drops of Dengue Antibody buffer provided was added to the well(buffer well) of the antibody card. Results were observed at 20 minutes.

Interpretation of results of rapid test: the results were interpreted as follows

For NS-1 Ag:

- Non-Reactive : Development of pink color line only in control (C) region
- Reactive: Development of pink color line in Test (T) and control (C) region.
- Invalid: No color development in C (control) region on the kit membrane.

Interpretation of IgG and IgM rapid test:

- If IgM and IgG reactive: Pink color band in "M" , "G" & C region
- If only IgG positive : Pink color band in "G" & C region
- If only IgM positive: Pink color band in "M" & C region
- If non reactive: Pink band in C region only
- Invalid test: if no band in C region.

D. MAC-ELISA

- All serum samples from suspected cases positive or negative by rapid test were subjected to ELISA for NS-1, IgM and IgG (Pan Bio), manufacturer's instructions were followed to carry out the test.

Procedure for NS-1 ELISA

- Micro-titer plates were brought to room temperature
- Wells were labeled, first well were left blank, and in second well negative control were added.
- Calibrator was added to 3rd and 4th well and to the fifth well positive control was added, from 6th well samples was added.
- 100µl diluents were put into all well except the blank well and wells were covered and incubated at 37^{0}c for one hour.
- Wells were washed with wash buffer for 5 times in automated washer
- Then 50µl of working conjugate was added to each well except the blank and incubated at 37^{0}c for 1 hour, then washed with buffer again.
- 100 µl of TMB substrate was put into each well and incubated for 30 min at room temperature
- 100µl of stop solution was added to each well containing TMB.
- Absorbance was calculated in ELISA reader by using 450nm filter.

Calculation and interpretation of NS-1 ELISA results:

- Cut of value was calculated, taking the mean of absorbance of calibrator.
- Index value taken by dividing the sample absorbance by cut of value (index value = sample absorbance/ cut-off value)

- Calculation of Pan bio units was done by multiplying index value by 10.(Pan bio unit= Index value x 10)

Index	**Pan bio unit**	**Result**
<0.9	<9	Negative
0.9- 1	10-11	Equivocal
>1.1	>11	Positive

Test procedure for IgM and IgG ELISA :

- All the wells were brought at Room Temperature and the wells were labeled.
- Antigen was diluted to 1/250 with antigen diluent provided (10ul of Ag in 2.5 ml of diluent was added)
- Solution became pale blue in colour in IgM ELISA and red in IgG when Antigen was added to Antigen diluent.
- As per the requirement and number of test, antigen was mixed with equal volume of MAb tracer in a sterile clean vial and preserved until test at Room temperature.
- Control and patents serum sample were added to each wells, covered and incubated at 37^0c for one hour. Wells were washed for six times with wash buffer.
- 100µl of Ag monoclonal antibody tracer was added to each well
- Plate was covered and incubated at 37^0c for one hour
- Again the plate was washed with wash buffer for 6 times

- 100µl of TMB substrate was added to each well and incubated at Room temperature for 10 minutes
- 100µl of stop solution was added to each well and reading was taken by using 450nm filter in ELISA reader.

Calculation and interpretation of IgM & IgG ELISA results:

- Cut-off value was detected by taking the mean of absorbance of three calibrator and multiplied with calibrator factor.
- Index value taken by dividing the sample absorbance by cut of value (index value = sample absorbance/ cut-off value)
- Pan bio units were calculated by multiplying index value by 10.(Pan bio unit= Index value x 10)

Index (IgM)	**Pan bio unit (IgM)**	**Result (IgM)**
<0.9	<9	Negative
0.9- 1.1	0-11	Equivocal
>1.1	>11	Positive
Results of IgG		
<1.8	<18	Negative
1.8-2.2	18-22	Equivocal
>2.2	>22	Positive

Calculation of OD to differentiate between Primary and secondary dengue

- Samples positive for either IgM antibodies and /or IgG antibodies by ELISA were used for differentiation of primary and secondary dengue viral infection.

- OD of IgM antibodies / OD of IgG antibodies, which was calculated as per WHO guidelines
- IgM : IgG ratio greater than 1.2 were considered as Primary Dengue Infection (PDI)
- IgM : IgG ratio less than 1.2 were considered as secondary dengue infection (SDI)

E. Molecular diagnosis:

- Serum samples positive for NS-1 Antigen by ELISA were subjected for detection of viral RNA by RT- PCR.
- Serotype specific primers were used for identification of dengue virus serotype.
- Dengue RNA was identified in 119 NS-1 positive samples

Dengue virus RNA extraction:

- QIAmp RNA mini kit (Qiagen Valencia, CA, USA) was used for RNA extraction.
- For conventional PCR, serotype specific E-NS1 region primers were used; viral RNA was then reverse transcribed to cDNA. Enzymatic DNA amplification was done by use of MoMLV reverse transcriptase. For RT of viral RNA to cDNA before Taq polymerase amplification, corresponding reverse primers of the particular set was used.

Primer and Probe used for Molecular study:

- Initial viral quantitation was done using real-time PCR to see that there were enough viral loads to carry out amplification and sequencing reactions. The primers (Eurofins genomics) were designed to target the 3 prime un-translated regions on the dengue viral genome. The primers were combined in the required ratio with the corresponding Taqman probe (Eurofins Genomics) to make a common screening primer- probe mixture (ppm). The primer probe mix also contained primers and probes for a housekeeping gene (internal control region). VLC dye channel were used to see the internal control probe, Viral C-DNA amplification signal were seen in the FAM channel on real-time machine.
- Viral load for all serum samples was detected.
- Serotyping by serotype specific primer-probe mix (Eurofins Genomics) was done to separate serotype.

Procedure:

- 150 µl of serum sample was taken in a microc tube
- 560ul AVL buffer was added to the sample and incubated at 25^0c for 10 min
- Sample were transferred to the high pure filter tube and centrifuged at 8000rpm for 15 seconds
- 560 ethanol was added to the tube and pulse vortexed for 15 seconds

- RNA precipitation was obtained which was transferred to mini column QIAmp.
- 50µl of elution buffer was added to filter tube and incubated at RT for 1 min
- Centrifugation of the tube was done at 8000 rpm for 1 min
- Which yielded the final RNA extract

Viral RNA extracted from the serum was converted to cDNA for RT- PCR detection

Procedure:

Viral RNA was reverse transcribed to c DNA by use of Reverse transcriptation kit (Applied Biosystem fosta city CA, USA)

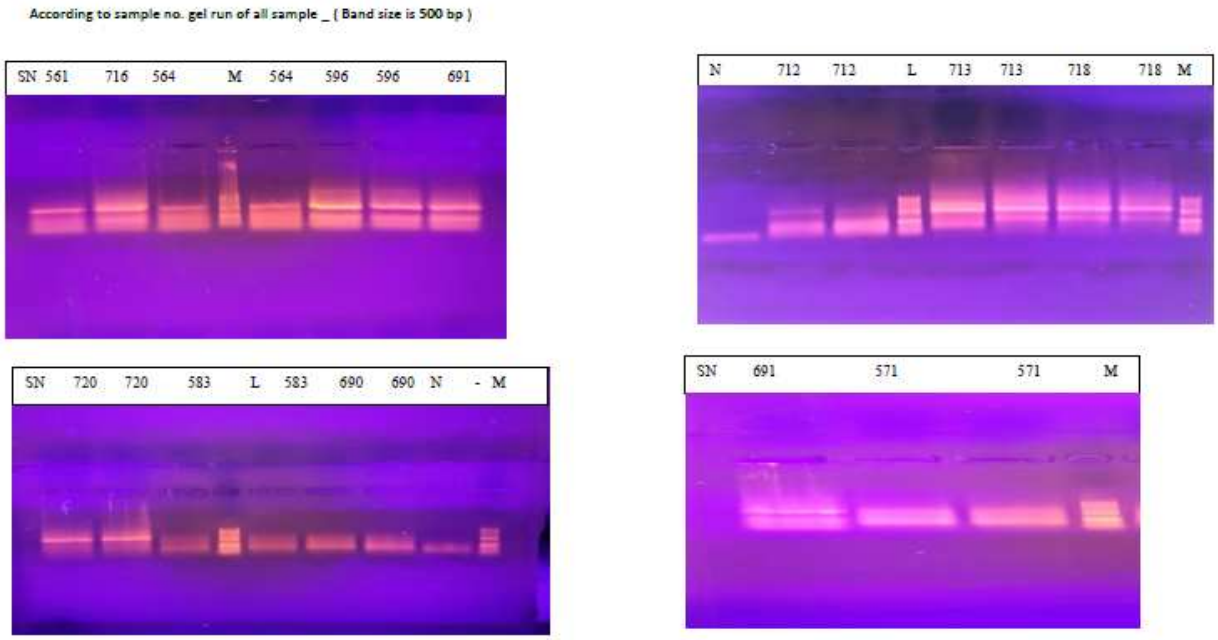
SS DNA was prepared by using dengue specific primes

5'-TCAATATGCTGAAACGCGCGAGAAACCG-3(511) and

5'-TTGCACCAACAACAGTCAATGTCTTCAGGTTC-3 (511)

- 10µl of RNA and RT master mix was used
- Reaction was set on thermo cycler using serotype specific primers and temperature at 72^0c for 10 min
- Visualized on 2% agarose gel, images of gel run below. (Fig 4.)

Figure no 5: Gel run of Dengue virus RNA

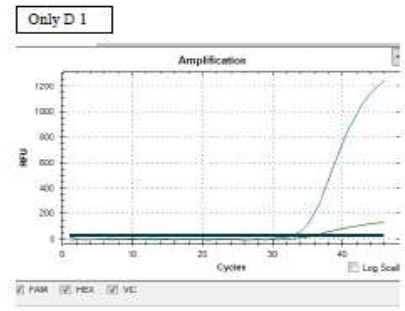


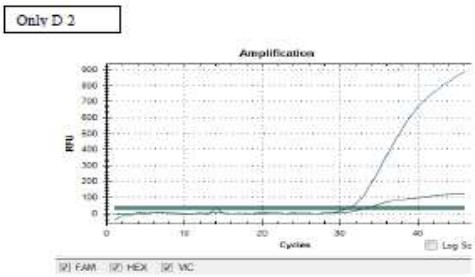
Dengue virus serotyping :

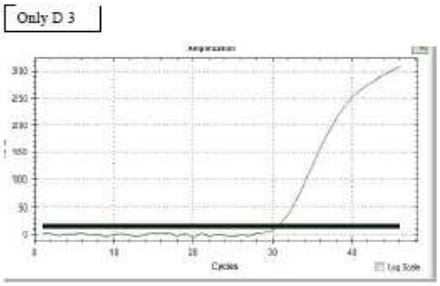
- Dengue virus serotyping was done using cDNA synthesized
- 1:10 diluted cDNA was used as template
- Second round PCR was set using Dengue serotype specific primers and temperature chart below.

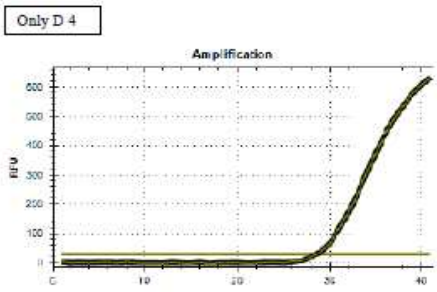
Table no: 3 Dengue serotype specific primers and temperature chart

Serotype	Primers	PCR program	Product size
DEN -1	D1-1229F	95^0c – 5min x 1 95^0c-30s x35	482 BP
	D1-1710R	57^0c – 30s 72^0c -1 in 72^0c -5min x1	
DEN -2	ENVF-PO1	95^0c – 2min x 1 95^0c-30s x30	455 BP
	D2R-PO1	57^0c – 30s 72^0c -2 in 72^0c -7min x1	
DEN -3	D3-1307F	95^0c – 5min x 1 95^0c-30s x35	881 BP
	D3-1867R	57^0c – 30s 72^0c -1min 72^0c -5min x1	
DEN -4	D4-1954R	95^0c – 5min x 1 95^0c-30s x35	195 BP
	D4-1760F	57^0c – 30s 72^0c -1min 72^0c -5min x1	









Phylogeny, sequencing and Restriction analysis

- The phylogenetic relation between the isolates was generated by using NCBI blast tree view software and is based on neighbor joining tree type. Consensus sequences were generated for each sample and were checked for molecular confirmation in the Pub Med using NCBI-BLAST tool (https://blast.ncbi.nlm.nih.gov).
- Sequence alignment : the obtained sequence data was subjected to multiple sequencing alignment using Clustal W software (to check the mutations in the envelop and prM gene)
- Insilco restriction digestion: sequences were subjected to restriction analysis using Vector NTI software. The unique sites Bam H1 and Nco1 sites were selected to generate restriction map.

Analysis of data and statistics

- One way Annova test was done, to analysis the variance followed by post hoc "t" test was done to determine the significance difference between the group
- SPSS software version 22.0 (IBM Corp. Released 2013 was used for data analysis. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) was used to analyse the data.
- Chi-square test was applied wherever necessary and p value was calculated.
- *P*-value <0.05 was statistically significant.

Results: A total of 1000 Dengue suspected cases based on WHO dengue case definition, fever plus one or more other manifestations were included in the study, during the period 2012 to 2016.

DEMOGRAPHIC PICTURE: AGE, SEX, AREA, SEASON, CLINICAL PICTURE

Table no 4. Age and Sex wise distribution of Total suspected cases.

Age in years	Male	Female	Total
0 to 15	91 (9.1%)	79 (7.9%)	170 (17.0%)
16 to 30	271 (27.1%)	220 (22.0%)	491 (49.1%)
31 to 45	89 (8.9%)	102 (10.2%)	191 (19.1%)
46 and above	77 (7.7%)	71 (7.1%)	148 (14.8%)
Total	528 (52.8%)	472 (47.2%)	1000 (100%)

Chi-square = 4.149 DF (degree of freedom)=3 P = 0.330 not significant

Male to female ratio of Dengue suspected cases was 1.2:1, a total of 528 (52.8%) Male patients and 472(47.2%) female patients were included, male were more affected then female, but significant difference was not seen. (Table no 4)

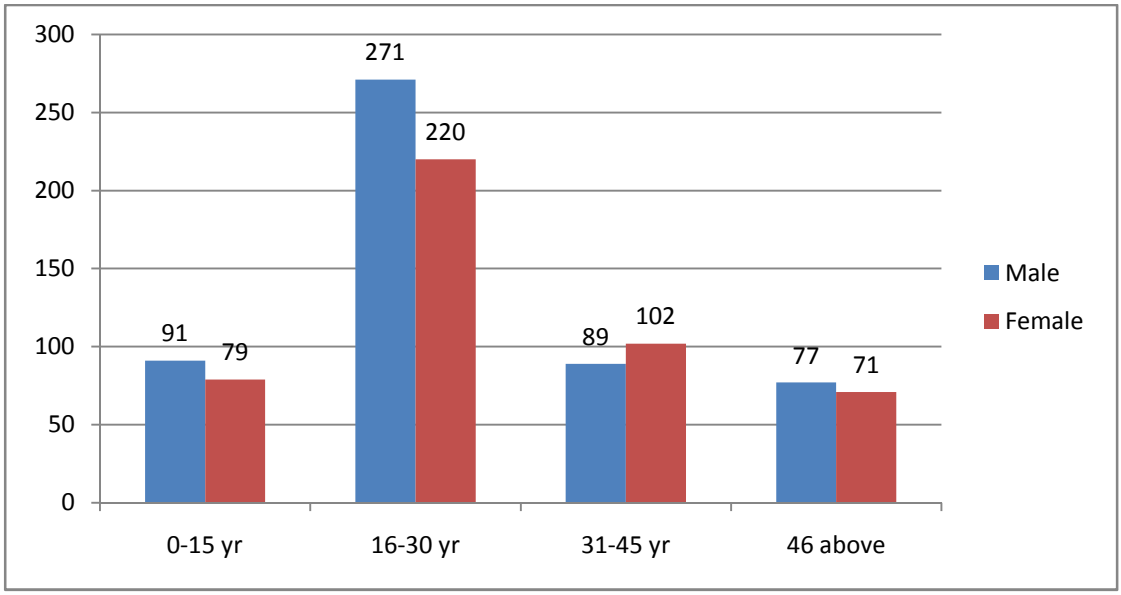


Fig no 6: Year wise distribution of Dengue infection suspected cases and number of cases positive for Dengue serology (NS-1, IgM &IgG).

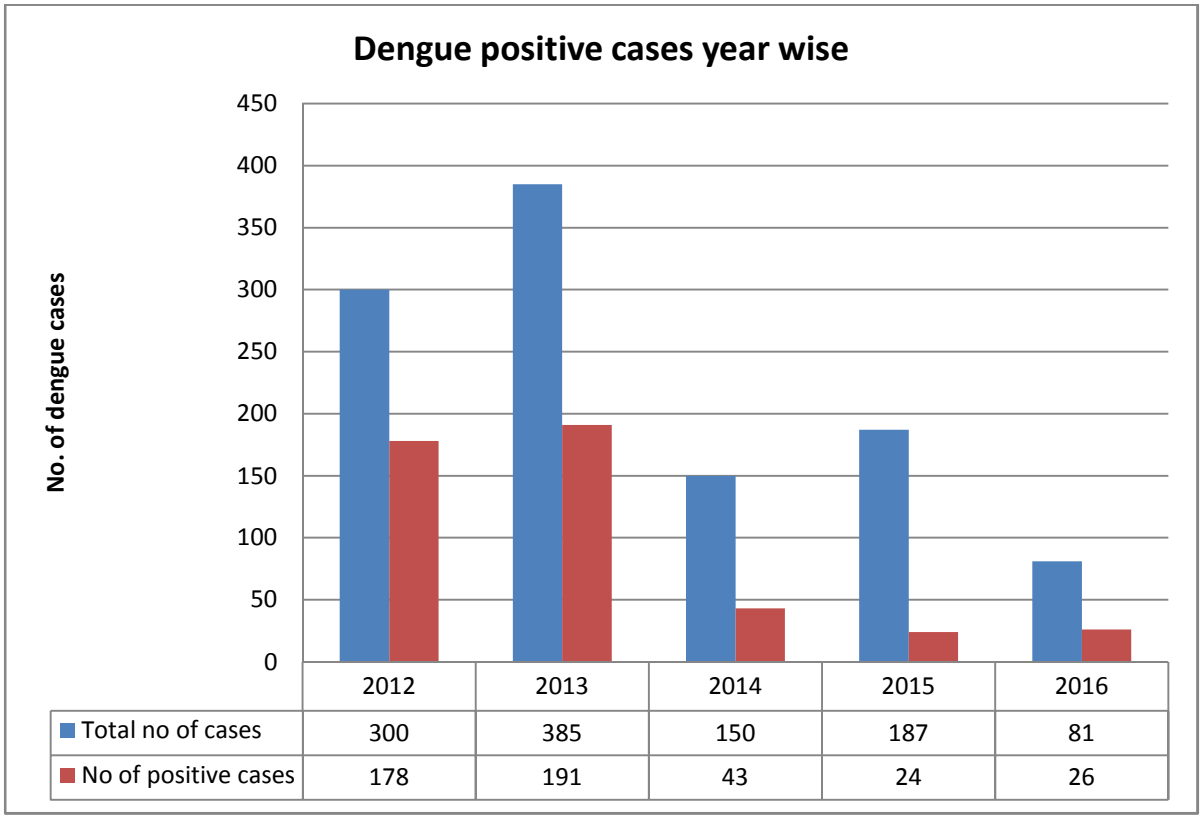


Fig 6 shows the total number of samples tested each year and number of positive cases every year, more number of cases were seen in the year 2013(56.33%), followed by 2012(49.61%), 2014 (28.66%), 2016(32.09%) & 2015(12.83%), as the number of suspected cases were also respectively more in those years.

Age & sex wise distribution: Out of 1000 suspected cases, 462 samples were positive for Antigen, Antibodies or positive for both NS-1 Antigen, IgM, IgG antibodies, either by Rapid or ELISA. Most of the cases were from the age group 16 to 30 (51.1%) followed by 31-45 year (23.1%), 46 and above (13.2%) and 0-15 year (12.1%). No significant difference was seen between male to female infection but male predominance was there with (53.03%) infection and female (46.96%)

Table 5:Age & sex wise distribution of Dengue seropositive cases.

Age in years	Male (%)	Female (%)	Total (%)
0-15	31 (6.70)	25(5.41)	56(12.1)
16-30	121(26.19)	117(25.32)	238(51.01)
31-45	65(14.06)	42(9.09)	107(23.16)
45& above	28 (6.06)	33(7.14)	61(13.20)
Total	245 (53.03)	217(46.96)	462(100)

Fig no 7: Age and sex wise distribution of seropositive cases.

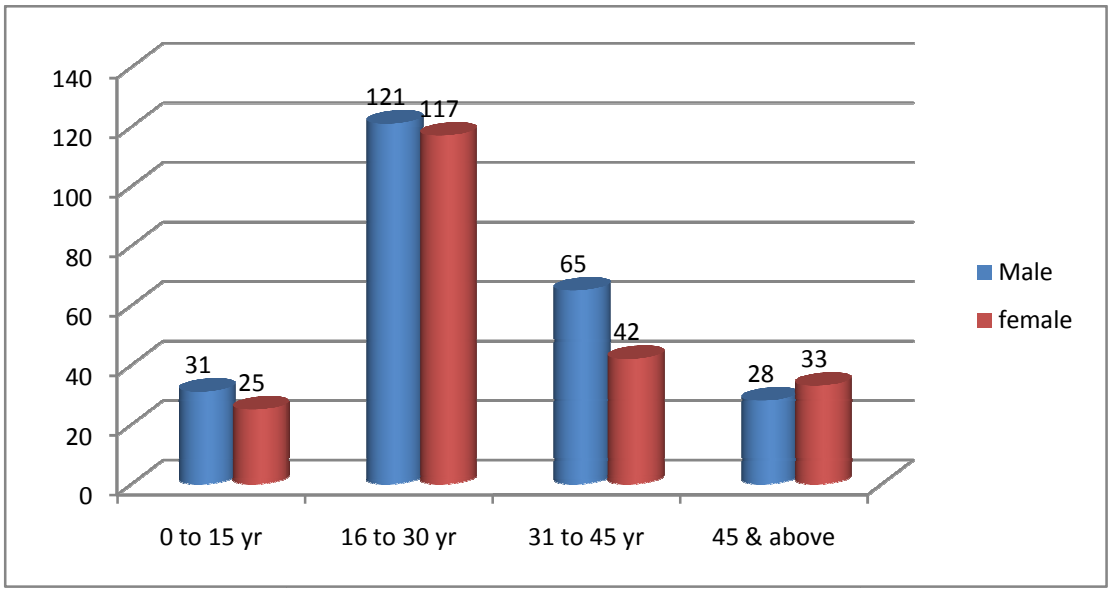
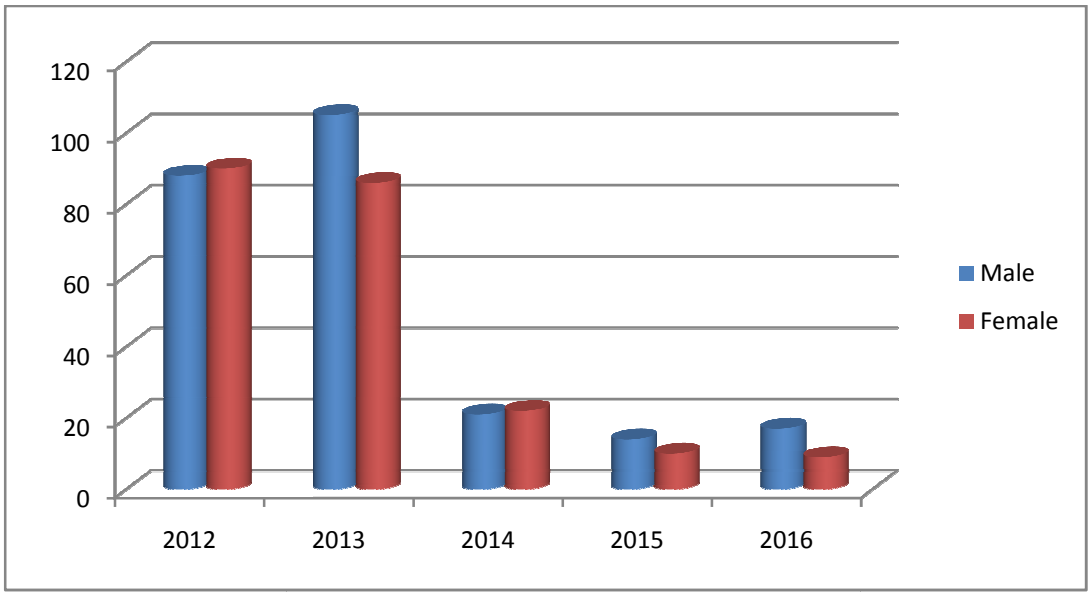


Table no 6: Age, sex and year wise distribution of Dengue Seropositive cases.

Age in years	2012 (n=178)		2013(n=191)		2014 (n=43)		2015(n=24)		2016 (n=26)		Total
	M	F	M	F	M	F	M	F	M	F	
0 to 15	08	06	16	12	03	05	01	01	03	01	**56**
16 to 30	49	55	52	46	08	08	05	03	07	05	**238**
31 to 45	22	17	26	17	08	04	05	03	04	01	**107**
45and above	09	12	11	11	02	05	03	03	03	02	**61**
Total	**88**	**90**	**105**	**86**	**21**	**22**	**14**	**10**	**17**	**09**	**462**

Year wise distribution of seropositivity: Dengue seropositive cases were significantly more in the year 2013, 191(41.34%) followed by 2012, 178(38.52%), 2014, 43(9.30%), 2016, 26 (5.62%) & 2015, 24(5.19%). Female patients were more in the year 2012 while in rest of the years male predominance was seen, significant difference was not observed. Significant decrease in incidence of Dengue virus infection was seen from 2012 to 2016($p<0.05$).

Fig no 8: Age, sex and year wise distribution of Dengue seropositive cases.

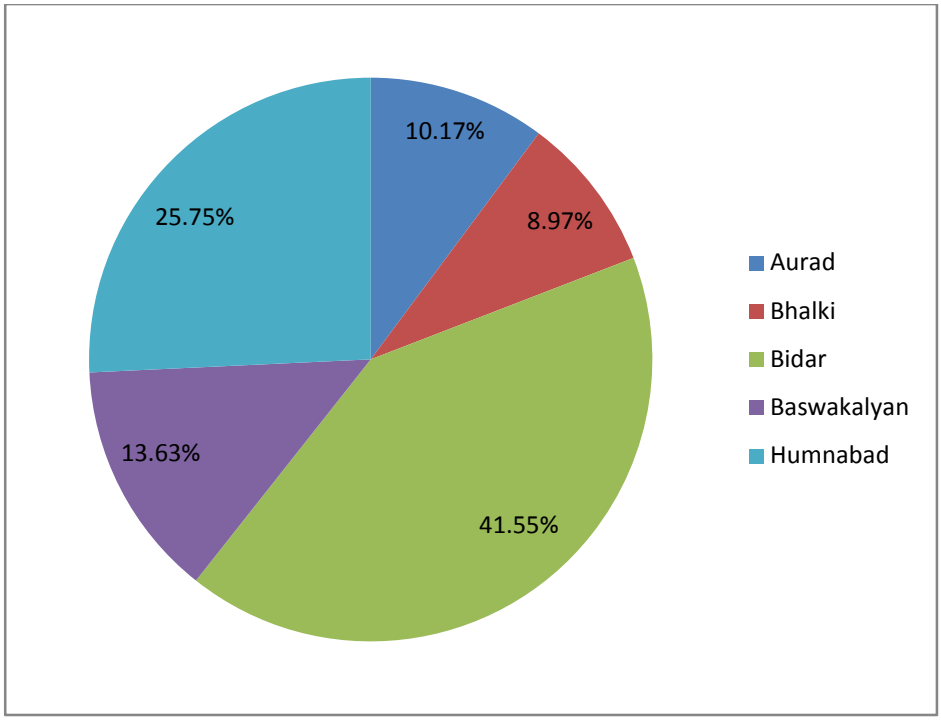


Taluka wise distribution of dengue case: More number of dengue positive cases were from Bidar city (41.55%) followed by Humnabad (25.75%), Baswakalyan (13.63), Aurad (10.17%) and Bhalki (8.97%).

Table no 7: Taluka wise distribution of dengue seropositive case

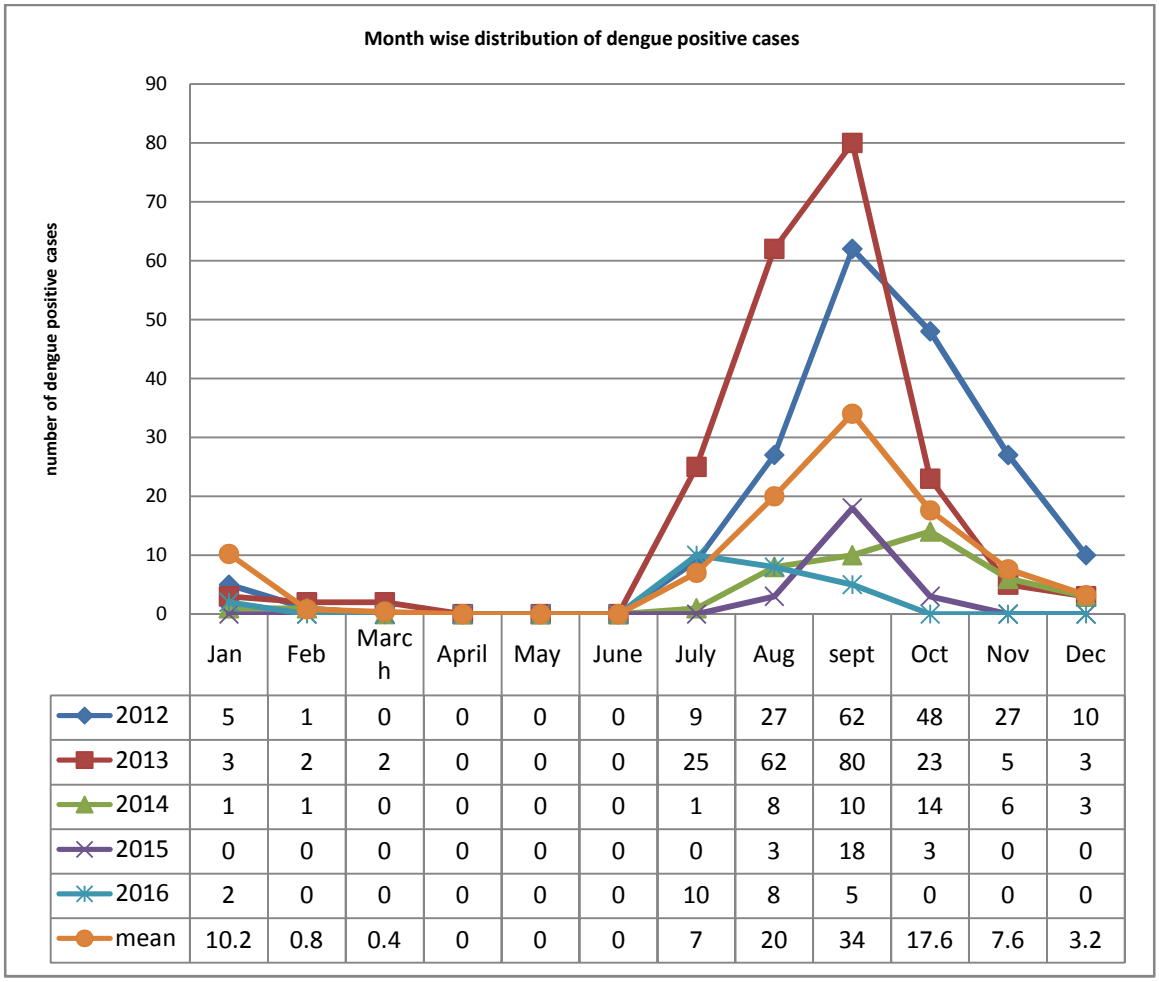
Taluka	Aurad	Bhalki	Bidar	Baswakalyan	Humnabad
No of cases n= 462 (%)	47(10.17%)	41 (8.97%)	192 (41.55%)	63 (13.63%)	119 (25.75%)

Fig no 9: Showing Taluka wise distribution of Dengue Seropositive cases.



Seasonal distribution of dengue cases: significantly more number of cases were seen in post monsoon season, with highest number in the month of September with a mean of 34 cases, followed by August (mean 20 cases) and October (mean 17.6) which was significant (p value <0.05). According to the number of cases suspected and tested more number of cases was positive in September 2013, followed by September 2012, 2015, 2014 &2016 with a mean of 34. No cases of Dengue were identified in the month of April, May and June in either of the year.

Fig no 10: Month wise distribution of Dengue positive cases.



	Jan	Feb	March	April	May	June	July	Aug	sept	Oct	Nov	Dec
2012	5	1	0	0	0	0	9	27	62	48	27	10
2013	3	2	2	0	0	0	25	62	80	23	5	3
2014	1	1	0	0	0	0	1	8	10	14	6	3
2015	0	0	0	0	0	0	0	3	18	3	0	0
2016	2	0	0	0	0	0	10	8	5	0	0	0
mean	10.2	0.8	0.4	0	0	0	7	20	34	17.6	7.6	3.2

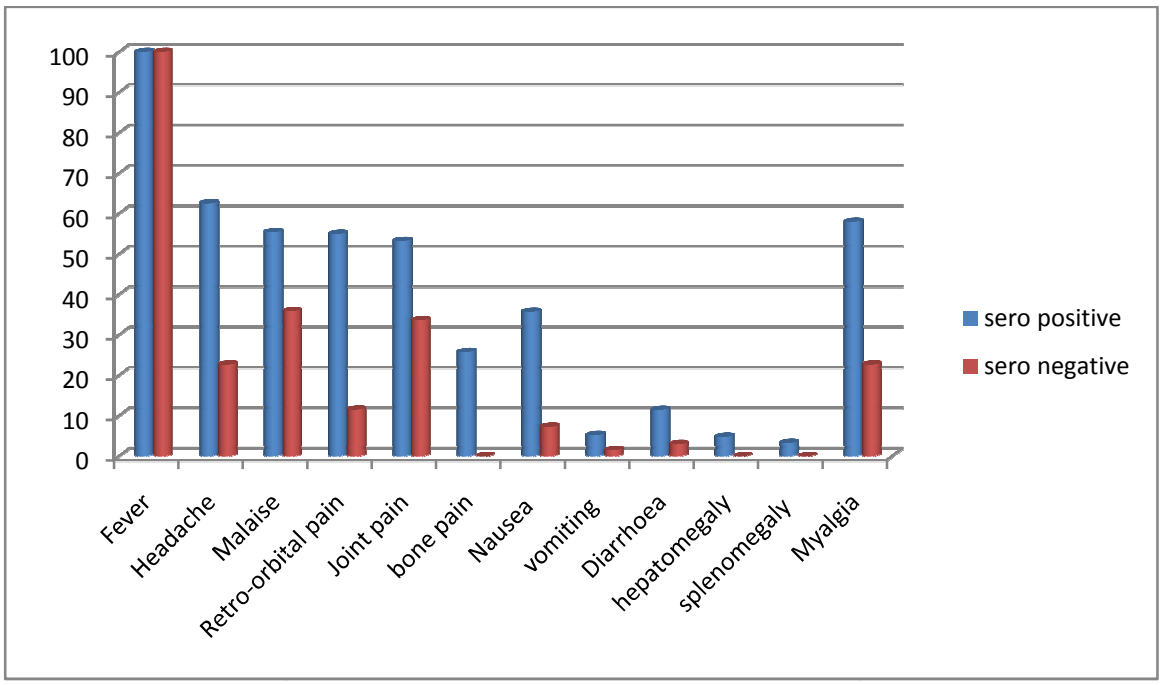
Clinical presentation: Seropositivity and classification according to 2009 WHO: Maximum, number of cases were Dengue with warning signs 255 (55.19%), 195(42.20%) belong to Dengue without warning signs and 12 (2.59%) showed sever dengue infection.

Table 8: showing classification of seropositive cases percentage.

Categories of Dengue	**Dengue seropositive cases (%)**
Dengue without warning signs	195 (42.20%)
Dengue with warning signs	255 (55.19%)
Severe dengue	12 (2.59%)
Total	462 (100%)

Table no 9: Clinical manifestations of total seropositive cases and seronegative cases.

Signs and symptoms	**Seropositive (n=462)**	**Seronegative (n=538) (%)**	**Total (n=1000) %**	**p- value**
Fever	462 (100)	538(100)	1000 (100)	0.96
Malaise	256(55.41)	193(35.87)	449(44.9)	0.00
Headache	289(62.55)	122(22.67)	411(41.1)	0.00
Retro-orbital pain	254(54.97)	62(11.52)	316(31.6)	0.00
Nausea	165(35.71)	39(7.24)	204(20.4)	0.00
Vomiting	24(5.19)	08(1.48)	32(3.2)	0.02
Abdominal pain	174(37.66)	28(5.20)	202(20.2)	0.00
Diarrhea	53(11.47)	16(2.97)	69(6.9)	0.00
Spleenomegaly	15(3.24)	00(00)	15(1.5)	0.00
Hepatomegaly	22(3.24)	00(00)	22(2.2)	0.00
Cough	32(6.92)	10(1.85)	42(4.2)	0.00
Dyspnea	41(8.87)	06(1.11)	47(4.7)	0.00
Rhinorrhea	38(8.22)	02(0.37)	40(4.0)	0.00
Congestion	16(3.46)	00(00)	16(1.6)	0.00
Myalgia	267(57.97)	122(22.67)	389(38.9)	0.00
Joint pain	246(53.24)	181(33.64)	427(42.7)	0.00
Bone pain	119(25.75)	00(00)	119(11.9)	0.00
Maculopapular rash	15(3.24)	00(00)	15(1.5)	0.00



All the seropositive patients were suffering from fever 100% ,62.55% had headache, 55.41% malaise, 54.97% retro- orbital pain, 37.66% abdominal pain, 11.47% diarrhea, 35.71% nausea, 57.79% myalgia,53.24% joint pain, 25.75% bone pain, 3.24% splenomegaly, 4.76% hepatomegaly.

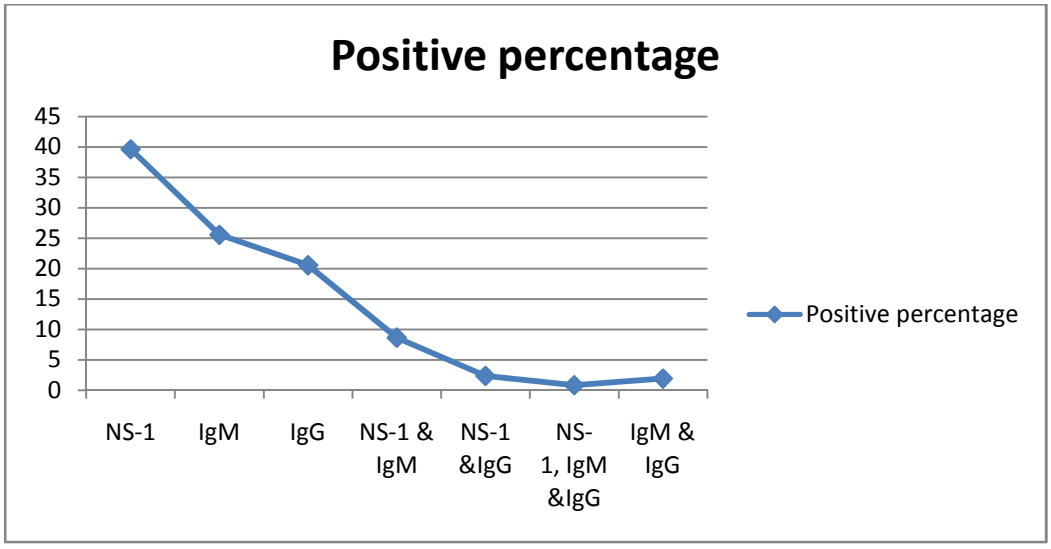
Fever was most common symptom followed by headache, myalgia, retro-orbital pain nausea and vomiting.

Serology: NS-1, IgM & IgG positive cases alone or in combination

39.61% cases were alone NS-1 positive, 25.56% were IgM poisitve, 20.56% cases were IgG positive, 8.56% were NS-1 & IgM positive, 2.38% were NS-1 and IgG positive, 1.94% were IgM & IgG positive and 0.86% were positive for NS-1, IgM and IgG.

Table no 10: Total number of cases positive for Antigen and Antibodies & in combination

Sr. no	Test name	Number of positive cases	percentage
1.	NS-1 Antigen	183	39.61 %
2.	IgM Antibodies	120	25.56%
3.	IgG antibodies	95	20.56 %
4.	NS-1 and IgM	40	8.65 %
5.	NS-1 and IgG	11	2.38 %
6.	NS-1, IgM and IgG	04	0.86 %
7.	IgM and IgG	09	1.94%



Comparison of Rapid card test with ELISA:

100% positivity was seen by ELISA method for NS-1, IgM and IgG detection, rapid card method showed 98.90%, 98.33%, 97.89%, 95%,75% positivity for NS-1, IgM, IgG, NS-1&IgM, IgM & IgG respectively. ELISA method was specific and sensitive compared to rapid card method which was specific but less sensitive. Significant difference was not seen.

Table no 11: Cases positive by Rapid method and ELISA

Sr. no	Test name	Number of positive cases		Percentage	
		Rapid card test	ELISA	Rapid	ELISA
1.	NS-1 Antigen	181	183	98.90 %	100 %
2.	IgM Antibodies	118	120	98.33 %	100 %
3.	IgG antibodies	93	95	97.89 %	100 %
4.	NS-1 and IgM	60	64	97.82 %	100 %
5.	NS-1 and IgG	11	11	100 %	100 %
6.	NS-1, IgM and IgG	04	04	100 %	100 %
7.	IgM & IgG	09	09	100%	100%

T = -0.038 DF =10 p value = 0.971 not significant.

Fig no 11: showing no of seropositive cases by Rapid method and ELISA.

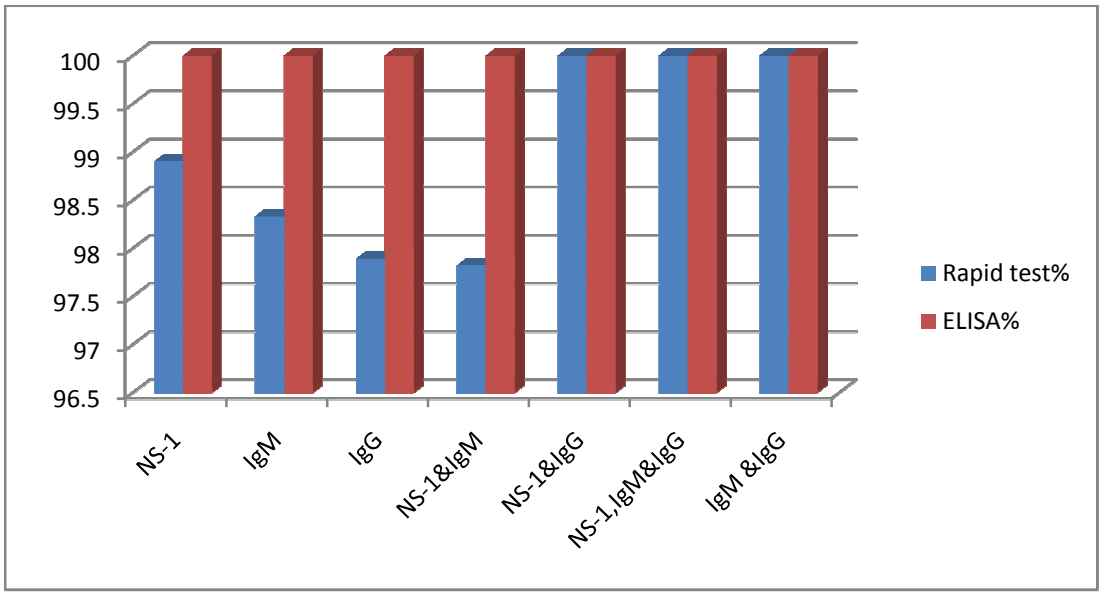


Table no 12: Comparison of sensitivity, specificity, PPV and NPV of ELISA with Rapid Immunochromatographic test.

Test	Result by rapid	Positive by ELISA	Negative by ELISA	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
NS-1	Reactive	234	0	234	76.64	100	100	99.48
	Non reactive	04	762	766				
	Total	238	762	1000				
IgM positive	Reactive	169	0	169	82.90	100	100	99.62
	Non reactive	04	827	831				
	Total	173	827	1000				
IgG positive	Reactive	117	0	117	88.20	100	100	99.88
	Non reactive	02	881	883				
	Total	119	881	1000				

NPV: Negative predictive value PPV: positive predictive value.

All samples positive by rapid test were also positive by ELISA, no false positive results were shown by Rapid card method (means rapid test positive and ELISA negative). If ELISA is taken as standard, the specificity and PPV of rapid test was found to be 100%.

Sensitivity of NS-1, IgM and IgG by Rapid card method was 76.64%, 82.90% and 88.20% respectively when compared with ELISA. The NPV was 99.48%, 99.62% and 99.88% for NS-1, IgM and IgG respectively.

Early and late Dengue:

NS-1 positivity was more in cases with onset of illness in 1 to 7 days, which was significant in comparison with IgM detection, with p value <0.05. While the rate of detection of IgM increased after 8 days up to 14 days of illness.

Table no 13: showing NS1 and IgM positivity in early and late stages of laboratory Diagnosed dengue cases.

Day of post onset of illness	NS1 positive	IgM positive	Chi square	DF	p-value
1 to 7 days	241/247(97.57%)	10/184 (5.43%)	364.27	01	<0.05
8 to 14 days	06/24 (2.42%)	174/184 (94.56%)	82.325	01	<0.05

Hematological parameter:

Platelet count was significantly decreased in dengue seropositive case with a mean of 75200 cell/mm^3. TLC was significantly decreased with a mean of 6520 cells/mm^3. While the sero negative cases showed the mean platelet count of 1,75,000c/mm^3

Table no 14: Showing hematological picture in Dengue seropositve and seronegative cases.

Hematological parameter	Dengue Seropositive (n=462)	Dengue seronegative (n=538)	P value
Hb (mmHg)	11.5	12.3	<0.05
TLC (cells/mm^3)	6520	10456	<0.01
Hematocrit	40.1	43.2	<0.05
Platelet (cells/mm^3)	75200	1,75,000	<0.01

Comparison of hematological parameter with NS-1 IgM and IgG positivity:

Significant decrease in mean platelet count (42000 cell/mm^3) was seen in patients with NS-1, IgM & IgG all positive. Followed by cases positive for IgM and IgG both with a mean of (55000 cells/mm^3). Patients with only IgG positive showed a mean platelet count of 1,25,000 cells/mm^3.

Table no 15: Showing the various seropositive cases and its comparison with hematological parameter

Hematological parameter	**Ns-1 positive**	**IgM positive**	**IgG positive**	**NS-1 & IgM positive**	**IgM & IgG positive**	**NS-1& IgG positive**	**NS-1, IgM & IgG positive**	**P value**
HB	12.2	11.5	12.0	12.05	11.2	11.45	12.1	<0.05
TLC	6121	6520	6852	6441	6752	6985	6258	0.6
Hematocrit	39.1	37.57	39.05	38.10	36.12	37.15	38.15	<0.05
Platelet count	75000	67150	125000	71800	55000	62000	42000	<0.01

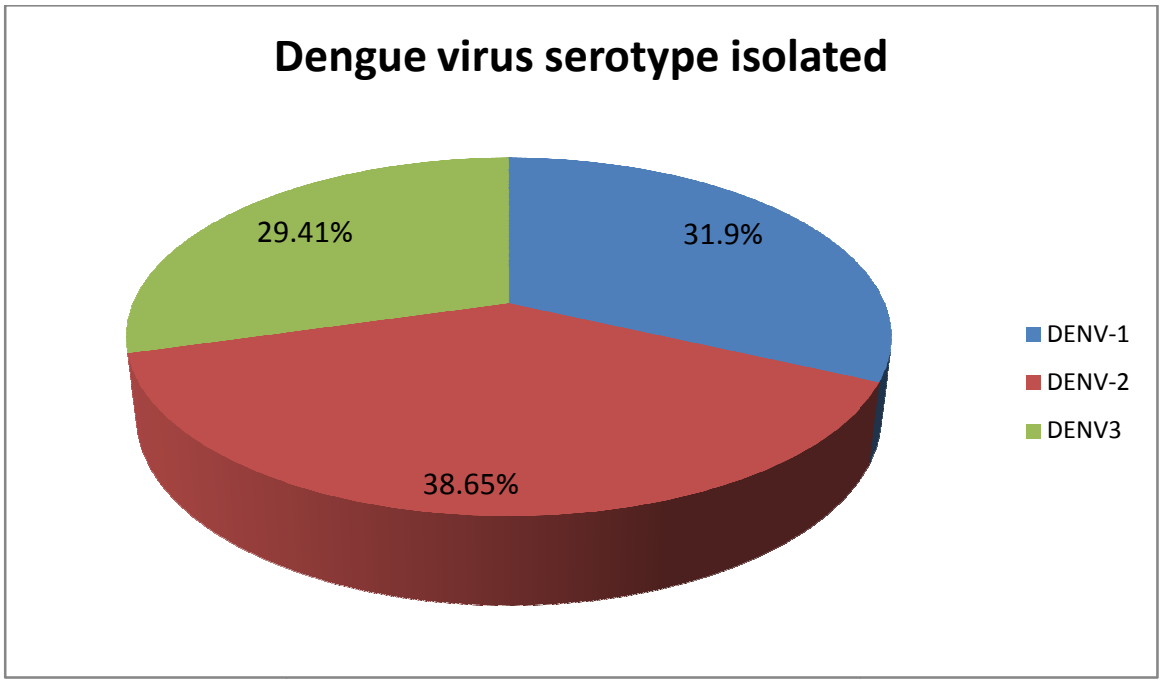
Results of Molecular study:

Only NS-1 positive samples were subjected for RNA detection, of which 119 samples showed different serotype RNA. Of the total 119 Dengue RNA detected 38(31.93%) belonged to Dengue serotype -1, 46(38.65%) belonged to serotype-2 and 35(29.41%) were serotype-3. Dengue serotype -4 was not identified from our samples.

Table no 16: Showing different serotypes isolated from tested samples.

Total	DENV-1	DENV-2	DENV-3
119	38 (31.93%)	46 (38.65%)	35 (29.41%)

Fig no 12: Dengue serotype isolated percentage wise.



Demographic Picture of Dengue virus serotype1, 2&3:

Age,sex and Taluka wise distribution of Dengue cases. (Fig no 14)

Maximum number of cases were from the age group 15-30 years, 30-45 years & 45 and above with DENV-2, DENV-3 & DENV-1. Age group 0-15 years were affected with DENV-1, DENV-3 &DENV-2.There was no significant difference in Male to female ratio of DENV serotype infection.

Table no 17: Showing details of Demographic study and Dengue serotype.

Parameters	Total case (n=119)	DEN-1 (n=38)	DEN-2 (n=47)	DEN-3 (n=35)	Chi-square	DF	p-value
Age wise							
0-15	19 (15.96%)	08	05	06	3.125	02	0.210
15-30	62 (52.10%)	17	26	19	1.096	02	0.578
30-45	28 (23.52%)	09	11	09	1.289	02	0.525
45 and above	10 (8.40%)	04	05	01	0.092	02	0.955
Sex wise							
Male	71 (59.66%)	22	29	20	0.483	02	0.786
Female	48 (40.33%)	16	17	15			
Taluka wise							
Aurad	04 (3.36%)	03	01	00	3.319	02	0.192
Bidar	80 (67.22%)	20	35	25	4.516	02	0.105
Humnabad	22 (18.48%)	06	06	10	3.118	02	0.210
Bhalki	06 (5.04%)	03	03	00	0.607	02	0.738
Baswakalyan	07 (5.88%)	06	01	00	7.748	02	0.021

Taluka wise serotype detection:

More number of cases of Dengue serotype -1 were observed in Baswakalyan and Bidar. All three serotypes were isolated from Bidar and Humnabad, serotype 1 and 2 was isolated from Baswakalyan, Bhalki and Aurad and serotype -3 was seen in Bidar and Humnabad.

Year wise distribution of serotype: (Fig 13)

Changing trend of serotype was seen from 2012 to 2016. Dengue serotype2 was seen throughout the years but serotype-1 was seen in 2012 to 2014 and serotype-3 emerged in 2014 and was seen in 2015 and 2016.

Fig no 13: Showing year wise Dengue serotype prevalence

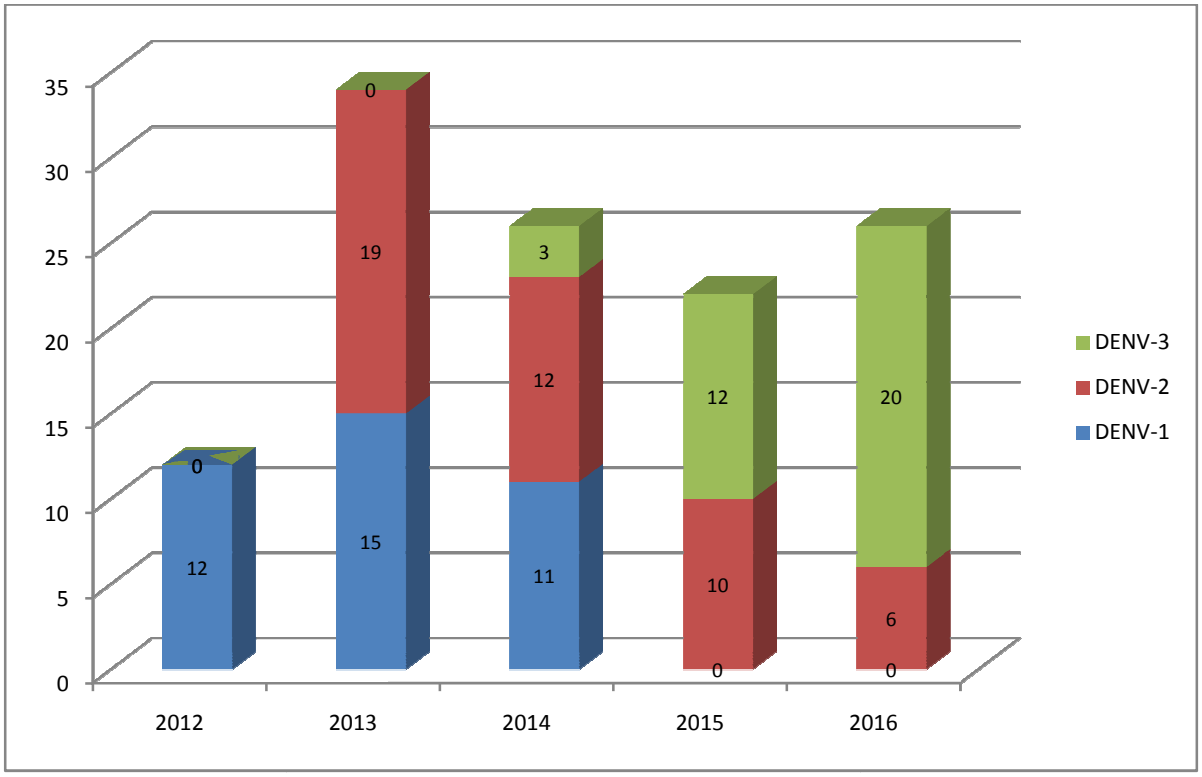


Table no 18: Showing year wise Dengue serotype prevalence

Year	Total case (n=119)	DEN-1 (n=38)	DEN-2 (n=47)	DEN-3 (n=35)	Chi-square	DF	p-value
2012	12	12	00	00	19.611	02	0.000
2013	33	15	18	00	4.450	02	0.108
2014	26	11	12	03	1.032	02	0.597
2015	22	00	10	12	17.694	02	0.000
2016	26	00	06	20	0.193	02	0.908

Serotype and hematological picture:

Hematological assessment revealed that the mean platelet count was less in DENV-2 (67150 cells/mm3), followed by DENV-3(71800 cells/mm3) and DENV-1(75200 cells/mm3) ($p<0.05$). Hematocrit was 37.57 in DENV-2, 38.10 in DENV-3 and 39.01 in DENV-1 ($p<0.05$)

Table no 19: Correlation of dengue serotypes with hematological parameters

Hematological parameter	DENV-1	DENV-2	DENV-3	P -value
Hb (mmHg)	12.2	11.5	12.5	<0.05
TLC (cells/ mm^3)	6121	6520	6441	0.6
Hematocrit	39.01	37.57	38.10	<0.05
Platelet count (cells/ mm^3)	75200	67150	71800	<0.01

Clinical picture and dengue serotype 1,2 &3: (Table 19)

Clinical manifestations of patients were compared with the dengue serotype identified from 119 samples, which was statistically analyzed by Chi-square test. Malaise was a predominant symptom in DENV-3 ($p<0.05$), while headache ($p<0.001$) and retro-orbital pain ($p<0.05$) were predominant symptoms in DENV-2. GI symptoms (nausea, abdominal pain, and diarrhoea) were also significantly observed in DENV-2(17.02%),($p<0.05$). Respiratory symptoms (cough, dyspnea, rhinorrhea and congestion) were more commonly seen in DENV-3 infection. Masculoskelatal symptoms (myalgia, joint pain and born pain) commonly observed ($p<0.05$) in DENV-2 with 89%, 78.72% and 53.19% incidence, respectively. Maculopapular rash was frequently ($p<0.05$) seen in DENV-2 (12.07%).

Table no 20: Serotypes isolated and its comparison with clinical manifestations.

Signs and symptoms	**Total cases (n=119)**	**DENV-1** (n=38)31.93%		**DENV-2** (46) 38.65%		**DENV-3** (35)29.41 %	
		No.	**%**	**No**	**%**	**No.**	**%**
Fever	**119 (100)**	**38**	**100**	**46**	**100**	**35**	**100**
Malaise	**63(52.94)**	**10**	**26.31**	**23**	**50**	**30**	**85.71**
Headache	**78(65.54)**	**11**	**28.94**	**44**	**95.65**	**23**	**65.71**
Retro-orbital pain	**64(53.78)**	**17**	**44.73**	**31**	**65.95**	**16**	**45.71**
Nausea	**44(36.97)**	**07**	**18.42**	**37**	**78.72**	**00**	**00**
Vomiting	04(3.36)	02	5.2	60	24.25	00	00
Abdominal pain	**42(35.29)**	**08**	**21.05**	**32**	**68.08**	**02**	**5.71**
Diarrhoea	**09(7.56)**	**00**	**00**	**09**	**19.14**	**00**	**00**
Spelnomegaly	15(12.60)	05	13.15	10	21.27	00	00
Hepatomegaly	**12(10.08)**	**03**	**7.89**	**08**	**17.0**	**20**	**12.85**
Cough	**08(6.72)**	**01**	**2.6**	**30**	**24.25**	**05**	**14.28**
Dyspnea	**08(6.72)**	**00**	**00**	**06**	**12.76**	**02**	**5.71**
Rhinorrhoea	**06(5.04)**	**01**	**2.6**	**30**	**24.25**	**03**	**8.57**
Congestion	**04(3.36)**	**00**	**00**	**01**	**2.1**	**20**	**38.57**
Myalgia	**62(52.10)**	**18**	**47.36**	**42**	**89.36**	**02**	**5.71**
Joint pain	**53(44.53)**	**12**	**31.57**	**37**	**78.72**	**04**	**11.42**
Bone pain	**34(28.57)**	**09**	**23.63**	**25**	**53.19**	**00**	**00**
Maculopapular rash	**06(5.04)**	**00**	**00**	**06**	**12.70**	**00**	**00**

Molecular analysis: Results of Sequencing of DENV-2 and DENV-1

- DENV-2 detected from North Karnataka resembles the strains isolated from Singapore in the year 2013.
- DENV-1 detected in the year 2014 and 2015 had sequence variations in a couple of isolates which indicates existence of novel variant with the genotype
- DENV-3 could not be sequenced due to insufficient genome sequence.

Fig no 14: Dengue virus isolate D2/SG/CT36/2013 polyprotein gene, compete cdsSequence ID: KX380829, length:10722. Range 1:2025 to2436 GenBankGraphics.

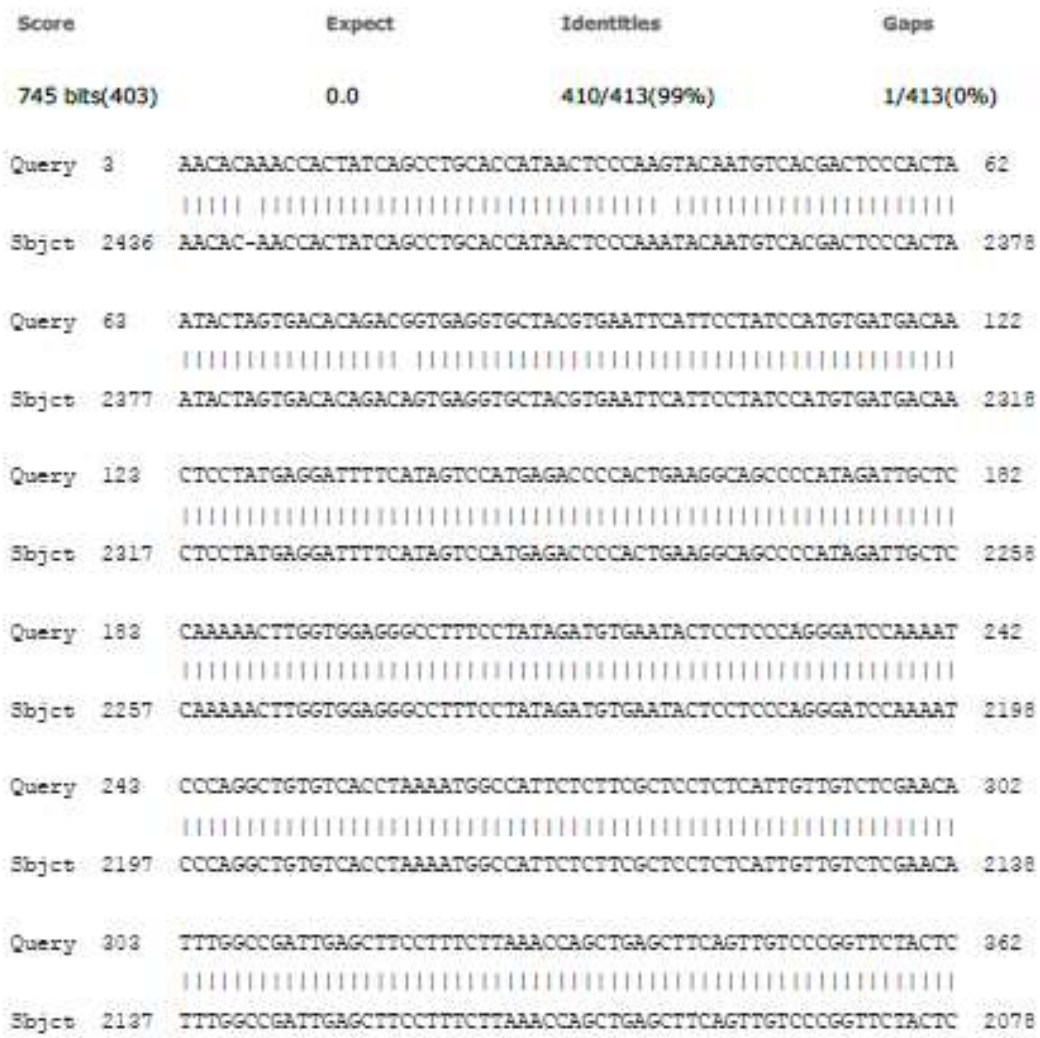


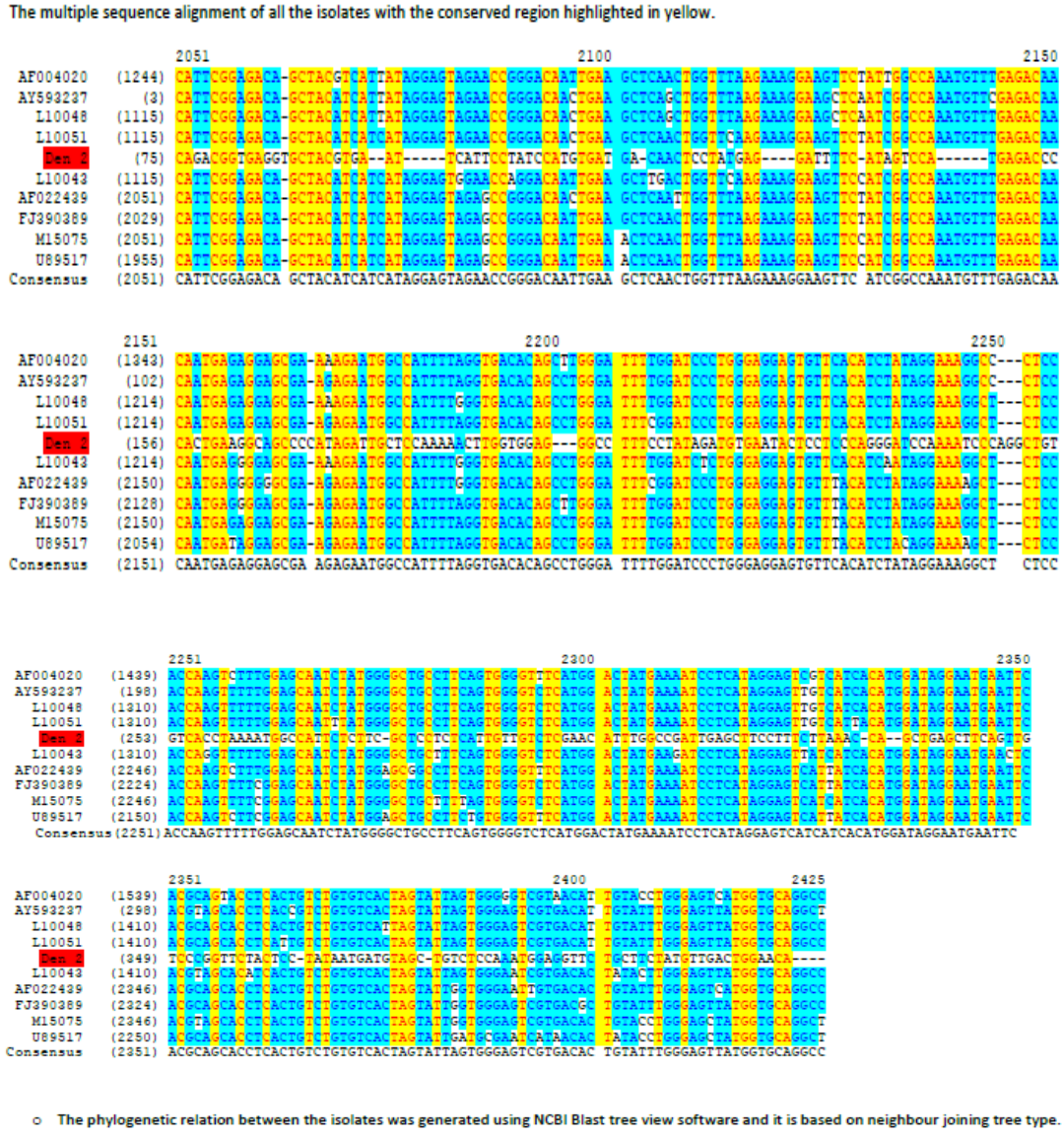
Fig 14 represents pair wise nucleotide sequence alignment of current strain of DENV-2 was analyzed using nucleotide blast software available on NCBI website. It shows 99% match with the GenBank: KX380829.1, Dengue virus 2 isolated D2/SG/CT36/2013 poly protein genes, complete cds isolated in Singapore. The sequence generated was aligned.

Dengue 2 isolate was also compared with the few important isolates worldwide using multiple sequence alignment on the vector NTI software. The data is below.

Table no 21: List of important isolates worldwide used for multiple sequence alignment.

Virus isolated ID no.	**Gen Bank Accession no**	**Country**	**year**
NGC (prototype)	AF 038403	New Guinea	1944
P9-122	L 10043	India	1957
S-4452	L 10048	Seychelles	1977
ARAC-8110827	M 15075	Jamaica	1982
S-10	L 10051	Somolia	1984
ThNH- P14/93	AF 022439	Thailand	1993
838/96	AY 593237.1	Delhi	1996
Cook Island-1	AF 004020	Cook Islands	1997
P7-863	U 89517	Malaysia	Unknown
D2/SG/CT36	**KX380829.1**	**Singapore**	**2013**

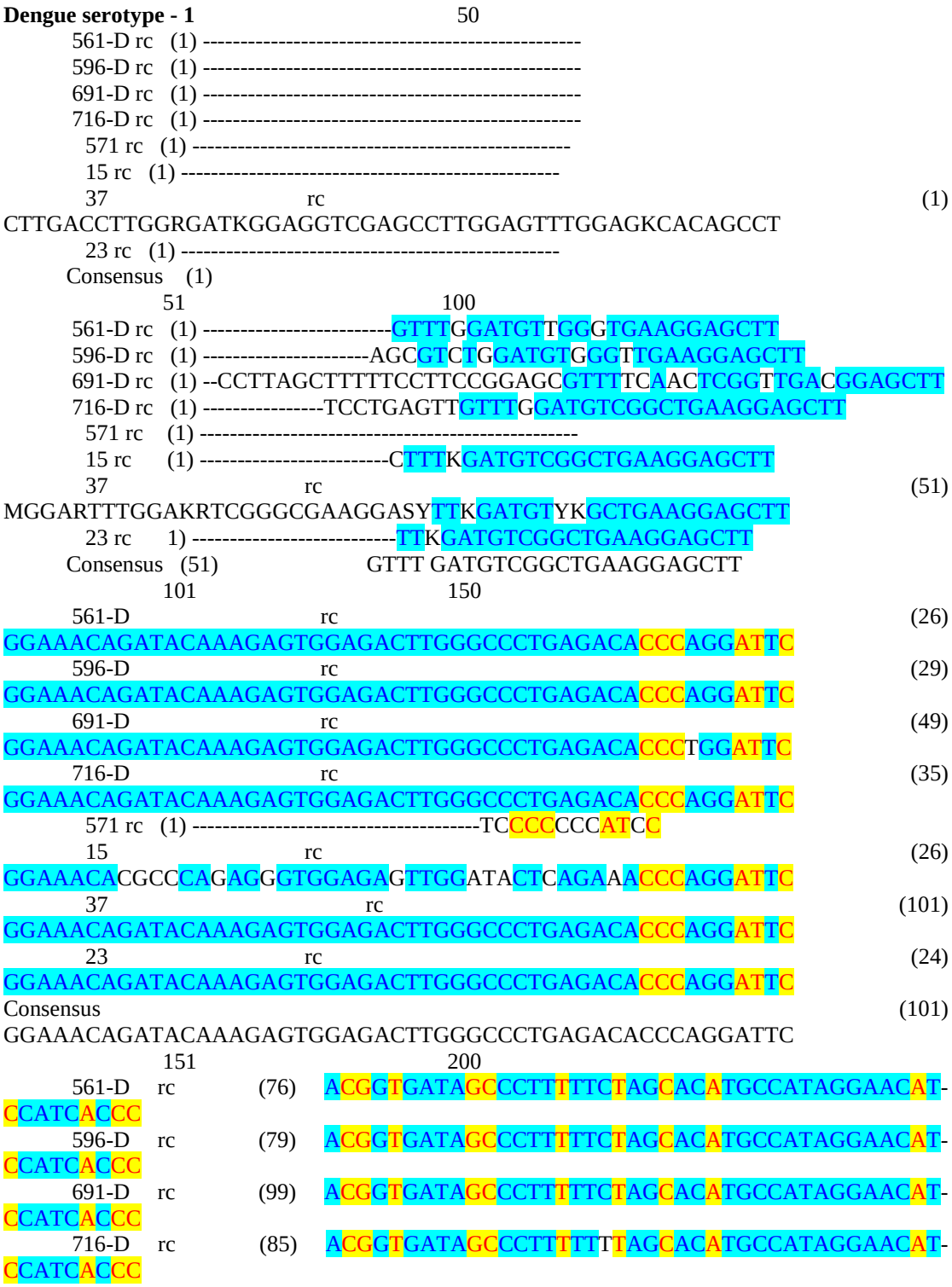
Fig no 15: Multiple sequence alignment of DENV 2 isolates with the conserved region highlighted in yellow.



As observed on the multiple sequencing alignments, the query sequence blast result and the phylogenetic tree, it can be assumed that the E_NS1 region of the query D2 isolated is very much similar to the isolate form Singapore identified in 2013. However the close proximity of the query DEN 2 isolate on to the

Delhi 1996 isolate (which was earlier classified by the authors as genotype IV) phylogeny shows that it may have evolved very recently and is a genotype IV.

Fig no 16: Showing Results of Multiple Sequence Alignment of Amplified regions of DENV-1, conserved region highlighted in yellow.



571 rc (13)
GCGCTCCCCGCCCGCTCTCTCCCTTACACCCCCCCCCCAAACTCGGATCC
15 rc (76) GCGCTCTTGGCAGGATTTATGGCTTATATGATTGGGCAAA-
CAGGGATCC
37 rc (151) ACGGTGATAGCCCTTTTCCTAGCACATGCCATAGGAGCAT-
CCATCACCC
23 rc (74) ACGGTGATAGCCCTTTTTCTAGCACATGCCATAGGAACAT-
CCATCACCC
Consensus (151) ACGGTGATAGCCCTTTTTCTAGCACATGCCATAGGAACAT CCATCACCC
201 250
561-D rc (125)
AGAAAGGGATTATTTTCATTTTGTTAATGCTGGTAACACCATCCATGGCC
596-D rc (128)
AGAAAGGGATTATTTTCATTTTGTTAATGCTGGTAACACCATCCATGGCC
691-D rc (148)
AGAAAGGGATTATTTTCATTTTGTTAATGCTGGTAACACCATCCATGGCC
716-D rc (134)
AGAAAGGGATTATTTTCATTTTGTTAATGCTGGTAACACCATCCATGGCC
571 rc (63)
AGCGGACGGTCTTCTCTCTTTCCATACCGCCCGTCCCCTCATCCCACGGA
15 rc (125)
AGCGGACGGTCTTCTTTATTTTAATAATGCTGGTTGCCCATCCTACGGA
37 rc (200)
AGAAAGGGATTATTTTYATCTTGTTAATGCTGGTAACACCATCCATGGCC
23 rc (123)
AGAAAGGGATTATTTTCATTTTGTTAATGCTGGTAACACCATCCATGGCC
Consensus (201) AGAAAGGGATTATTTTCATTTTGTTAATGCTGGTAACACCATCCATGGCC
251 300
561-D rc (175)
ATGCGATGCGTGGGAATAGGCAGCAGGGACTTTGTGGAAGGACTGTCAGG
596-D rc (178)
ATGCGATGCGTGGGAATAGGCAGCAGGGACTTTGTGGAAGGACTGTCAGG
691-D rc (198)
ATGCGATGCGTGGGAATAGGCAGCAGGGACTTTGTGGAAGGACTGTCAGG
716-D rc (184)
ATGCGATGCGTGGGAATAGGCAGCAGGGACTTTGTGGAAGGACTGTCAGG
571 rc (113) CTCCGATGCGTTGGAGTGGCGAACAGAGACTC-
GTAGAAGGAGTCTCAGC
15 rc (175)
ATGCGATGCGTAGGAGTGGGGAACAGAGACTTTGTGGAAGGAGTCTCAGG
37 rc (250)
ATGCGATGCGTGGGAATAGGCAGCAGGGACTTTGTGGAAGGACTGTCAGG
23 rc (173)
ATGCGATGCGTGGGAATAGGCAGCAGGGACTTTGTGGAAGGACTGTCAGG
Consensus (251)
ATGCGATGCGTGGGAATAGGCAGCAGGGACTTTGTGGAAGGACTGTCAGG
301 350
561-D rc (225)
AGCAACTTGGGTAGATGTGGTACTGGAACATGGAAGTTGCGTCACCACCA
596-D rc (228)
AGCAACTTGGGTAGATGTGGTACTGGAACATGGATGTTGCGTCACTACCA
691-D rc (248)
AGCAACTTGGGTAGATGTGGTACTAGAACATGGAATTGGTGTCAGCACCA
716-D rc (234)
AGCAACTTGGGTAGATGTGGTACTGGAACATGGAAGTTGCGTCACTACCA
571 rc (162) -GGAGCATGGGTCGAT-TGCTGCC-GAACATGGAGGATGT-
TCACAACCA
15 rc (225)
TGGAGCATGGGTCGATCTGGTGCTAGAACATGGAGGATGTGTCACAACCA

```
        37                                  rc                                  (300)
AGCAACTTGGGTAGATGTGGTACTGGAACATGGAAGTTGTGTCACTACCA
        23                                  rc                                  (223)
AGCAACTTGGGTAGATGTGGTACTGGAACATGGAAGTTGTGTCACTACCA
Consensus  (301) AGCAACTTGGGTAGATGTGGTACTGGAACATGGAAGTTGTGTCACTACCA
                 351                                 400
      561-D                                 rc                                  (275)
TGGCTAAAGACAAACCAACACTGGACATTGAACTCTTGAAGACGGAAGTC
      596-D                                 rc                                  (278)
TGGCTAAAGACAAACCAACACTGGACATTGAACTCTTGAAGACGGAAGTC
      691-D                                 rc                                  (298)
GGGGTAAGGACAAACCAACACTGGACATTGAACTCTTGAAGACGGAAGTC
      716-D                                 rc                                  (284)
TGGCTAAAGACAAACCAACACTGGACATTGAACTCTTGAAGACGGAAGTC
      571          rc              (208)          TGGCCCAGGGAAAACCAACCTTGGAT-
TTGAACCCATCAAAACTTCGGCG
      15                                    rc                                  (275)
TGGCCCAGGGAAAACCAACCTTGGATTTTGAACTGATCAAGACAACAGCC
      37   rc     (350)   TGGCCAAAGACAAACCAACACTGGACATTGAACTCTTGAAGACG-
AAGTC
      23                                    rc                                  (273)
TGGCCAAAGACAAACCAACACTGGACATTGAACTCTTGAAGACGGAAGTC
Consensus                                                                       (351)
TGGCTAAAGACAAACCAACACTGGACATTGAACTCTTGAAGACGGAAGTC
                 401                                 450

      561-D        rc              (325)        ACAAACCCTGCCGTCTTGCGCAAACTGTGCAT-
TGAAGCCAAAATATCAA
      596-D        rc              (328)        ACAAACCCTGCCGTCTTGCGCAAACTGTGCAT-
TGAAGCCAAAATATCAA
      691-D        rc              (348)        ACAAACCCTGCCGTCTTGCGCAAACTGTGCAT-
AGAAGCCAAAATATCAA
      716-D        rc              (334)        ACAAACCCTGCCGTCTTGCGCAAACTGTGCAT-
TGAAGCCAAAATATCAA
      571                rc                          (257)          TCCGAA---GTGGTCTTG-
GCCAACGGAACACCACAAACCTCCATATCAA
      15          rc              (325)        AAGGAAGTGGCTCTGTTAAGAACCTATTGCAT-
TGAAGCCTCCATATCAA
      37          rc              (399)        ACAAACCCTGCCGTCTTGCGCAAACTGTGCAT-
TGAAGCCAAAATATCAA
      23          rc              (323)        ACAAACCCTGCCGTCTTGCGCAAACTGTGCAT-
TGAAGCCAAAATATCAA
Consensus  (401) ACAAACCCTGCCGTCTTGCGCAAACTGTGCAT TGAAGCCAAAATATCAA
                 451                                 500
      561-D rc  (374) ACACCACTACTGACTCAAGATGTCCAACACAAGGAGAAGCCACAC-
TGGT
      596-D rc  (377) ACACCACTACTGACTCAAGATGTCCAACACAAGGAGAAGCCACAC-
TGGT
      691-D rc  (397) ACACCACTACTGACTCAAGATGTCCAACACAAGGAGAAGCCACAC-
TGGT
      716-D rc  (383) ACACCACTACTGACTCAAGATGTCCAACACAAGGAGAAGCCACAC-
TGGT
      571       rc          (303)      ATATAACCACACAA-CACGATGTCCAACGCAAGGAGA-
GCCATACCTCAA
      15 rc  (374) ATATAACCACGGCAACARGATGTCCAACGCAAGGAGR-GCS---------
      37   rc     (448)  ACACCACTACTGACTCAAGATKTCCAACACAAGGAGAAGCCACRS-
TGGT
      23   rc     (372)  ACACCACTACTGACTCAAGATGTCCAACACAAGGAGAAGCCACAC-
TGGT
Consensus  (451) ACACCACTACTGACTCAAGATGTCCAACACAAGGAGAAGCCACAC TGGT
```

```
                501                     537
561-D rc  (423) GGAAGAACAGGACGCGAACTTTGGGTCGACAATCTT-
596-D rc  (426) GGAAGAACAGGACGCGAACTTTGGGTCGACGAACTT-
691-D rc  (446) GGAAGAACAGGACGCGAACTTGTGG-CTACTAACTT-
716-D rc  (432) GGAAGAACAGGACGCGAACTTGTGGTCGACAAACGTT
                571 rc  (351) AGAGGAACAAGTCAACCGACATCGC-CGAAAACCCTT
 15 rc  (414) ------------------------------------
 37 rc  (497) GGAAGAACAGGACGCGRASTTKGGGACGAACA-----
 23 rc  (421) GGAAGAACAGGACGCGAACTTTGTGKCRCAT------
Consensus  (501) GGAAGAACAGGACGCGAACTTTGGG CGAC A C T
```

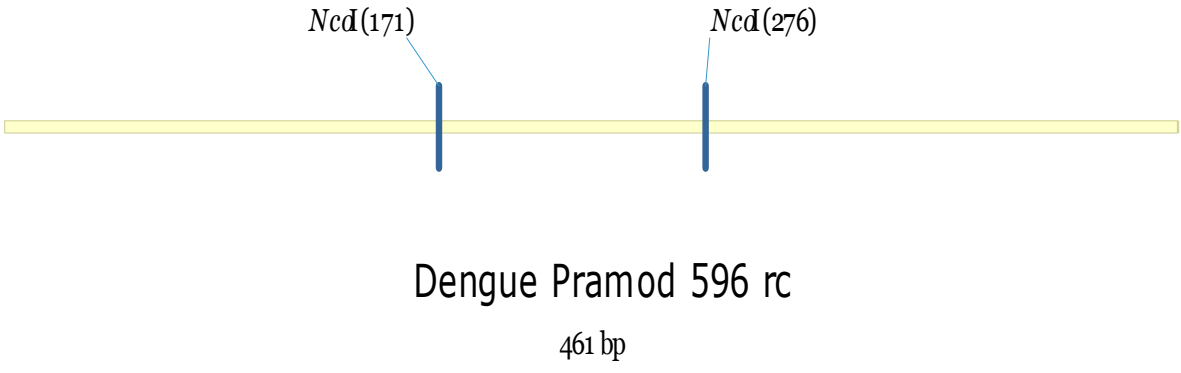
Insilico Restriction Digestion:

- The sequences were subjected to restriction analysis using Vector NTI software. The unique sites BamHI and Ncol sites were selected to generate restriction map.
- Ncol region was conserved in serotype-1 from our isolates which can be used for RFLP.

Insilico **Restriction Digestion**

- Dengue serotype -1, 2, E-NS-1 genes were subjected to Restriction digestion , where Ncol region was found conserved in Serotype-1 when compared with the other strains of Dengue serotype -1, 2,3 & 4.
- The sequences were subjected to restriction analysis using Vector NTI software. The unique sites BamHI and NcoI sites were selected to generate restriction map.
- Ncol region was conserved site in serotype I from our isolate which can be used for RFLP.

Fig no 17: Representative Restriction Map of Serotype 1sequence



Analysis and Interpretation of Data:

Dengue is an important arthropod borne disease in Tropical and sub Tropical region of the world, and is endemic in many countries. High incidence of Dengue infection in recent year is seen in south East Asian regions. Depending on various factors the south Asian regions are divided into three categories. [180] India is in category 'A' based on the severity of dengue as health problem, Leading cause of hospitalization and deaths in children, All four serotypes are found in India hence hyper endemic, Rural areas are more affected. The first epidemic of dengue like illness was found in Chennai in the year 1780, but only in 1963-64 DENV etiology was proved in Calcutta and eastern coast of India [180,181]. The largest DF outbreak was seen in Delhi and surrounding areas in 1996. This outbreak of DENV was of DENV-2, which was followed by epidemic of Dengue serotype-1 in 1997. Subsequent outbreak was reported of co-circulation of two different serotype-1 and serotype -3 in the year 2003 [45]. Karnataka has experienced in recent years epidemic with all serotypes in Bangalore, Mysore, Vellore etc[75].

As no licensed vaccine is available and treatment is only symptomatic, early detection and specific diagnosis of Dengue cases is very important. This study was carried out to assess the dengue seropositivity and to know which serotype is prevalent. Here by in the present study we report complete demographic, serological and molecular study, also the co-circulation of serotype-1 and 2 and replacement of serotype 2 and 3 during the year 2012 to 2016.

Dengue incidence:

Incidence of Dengue varies in different parts of the country, in the present study we report 46.2% seropositivity, our findings are similar to Gupta et al [64]who reported 44.56% of seropositivity in Delhi, while Laksmi et al[182] reported 34.17%, Kalyanarooj et al[183] reported 34.88%, Saini et al[184] showed the incidence of 35.64%. Increase in Dengue cases is because of various factors which include vector transmission, sanitation conditions (poor sanitation condition may lead to increase incidence), rapid urbanization, and presence of stagnant water reservoirs. Subsequently from 2012 to 2016 we report decrease in the number of seropositive cases, decrease in the seropositive cases may be explained, due to improved vector control, sanitation and early diagnosis.

Age and Sex wise seropositivity:

Predominance of the disease among the male patients is observed in many studies, Kamal et al[185] reported the male to female ratio of 0.72:1 in this case females were affected more than male, while Dash et al[44] in 2003 reported 1.28:1 and Raju et al[186] in 2003 from Tirupati reported 1.5:1 while Neerja et al[187] reported the male to female ratio of 2:1, our study shows 1.2:1 male to female ratio which is in concurrence to previous reports of Pathankar et al[188] and Mehta et al[189]. Majority of the cases were observed in the age group 16-30 years, this is comparable to the previous studies from other regions by Gupta et al[64], Kaup S et al[190], Saini et al[184], Raju et al[186], Neeraja et al[187]. This age group is constantly exposed to the outer environment and mosquito vector, at work place or travel. Infection in young adults result in non productivity and large

number of DF cases in young age group indicates the disease is prevalent in the region.

Seasonal variation in Dengue seropositivity:

We observed dengue virus incidence rate of 46.2% in our study population. Dengue cases started in monsoon season and reached the peak in September, which is post monsoon period. We have observed the most dengue sero positive cases with a mean number of 34 cases every September, followed by August, October, November with a mean of 20, 17.6, 7.6 cases respectively every year from 2012 to 2016. We could not find a single case of Dengue in the month of April, May and June which is significant ($p<0.05$). Which is similar to the study done by Gupta et al[64], Lakmi et al [134],Ukey et al.[191]. A study conducted by Amin et al [96] showed more number of cases in the month of October to December i.e post monsoon season. Pathankar et al[188] study shows high incidence of DF in October followed by September and November. In a study by Sarkar et al[192] in 2012 dengue cases started in July and maximum number of positivity was seen in November. This is because of the fact that stagnant fresh water from August to October (rainy season) favors the breeding of vector mosquitoes. Hence, these months must be effectively targeted to initiate preventive measures to control the breeding of mosquitoes.

Clinical presentation of Dengue seropositive cases:

In our study, fever was seen in 100% cases followed by Headache (62%), malaise (55.41%) Myalgia (57.79%), Nausea (35.71%), abdominal pain

(37.66%), retro- orbital pain (54.97%). Our study showed 100% fever which is in correlation with the study by Singh et al[49], Bansal et al[193], Dash et al[44] and Kale et al[194]. In our study Headache was the next prominent symptom which was found in 62.55% cases which is in correlation with Deora et al[195] and Lakshmi V et al[134] where they found 67.79% and 74.2% respectively. Myalgia was 57.79% which is similar to the study done by Dora et al and Sharma et al[167] showing 54.23% and 45.91% of cases respectively. In our study Arthralgia was seen in 53.24% which is in correlation with the study of Deor et al and Fazal et al[196]. Nausea and vomiting reported by us is similar with the reports shown by Dash et al, Kumiria et al[69]and Fazal et al[196]. In present study abdominal pain was major warning sign which is comparable with Aggrawal et al[58]. In our study hepatomegaly and splenomegaly was 4.76% and 3.24% respectively which is in contrast with Kumar MP et al[19] where he showed 33.1% cases and 27.13% cases respectively, our study correlates with Bansal et al[193] and Sharma et al who showed 8.0% and 5.0% cases.

Platelet count and Dengue seropositivity:

Our study showed that 72.2% patient had platelet count less than one lakh, which is similar to the study done by Fazal et al[196] who reported 73% of cases. While kulkarni et al reported 68.75% , Bansal et al[193] in 2014 reported 80.11% of cases, Tathe et al[197] in 2013 reported 81.72% of cases having platelet count below one lakh. Significant decrease in platelet count was seen in seropositve patients with a mean platelet count of 75200 c/mm^3 when compared with seronegative who had a mean platelet count of 1,75,000 c/mm^3.

we also compared the platelet count with NS-1, IgM and IgG positive cases alone or in combination where we found the patients with NS-1, IgM and IgG all positive had very low platelet count of i.e. 42000/mm^3 with a p value of less than 0.05 which was significant, whereas IgM & IgG positive cases had a mean platelet count of 55000 c/mm^3, NS-1 &IgG positive had mean platelet count of 62000 c/mm^3, only IgM positive had mean platelet count of 67150 c/mm^3, NS-1 & IgM positive cases had mean platelet count of 71800 c/mm^3, only NS-1 positive had 75000 c/mm^3 and IgG alone positive had high Platelet count compared to other i.e. mean 125000 c/mm^3 It proves the association of thrombocytopenia with Dengue virus infection.

Rapid test vs ELISA:

When rapid Dengue card method was compared with ELISA for dengue, positivity of Dengue Ag or Ab was more by ELISA, compared to the rapid test, out of the 462 sample positive for Dengue NS-1, IgM or IgG or in combination, all the test which were positive by ELISA were positive by rapid test. Significant difference was not seen, only when compared rapid test gave 98.90%, 98.33%, 97.89%, 95% and 75% with NS-1, IgM IgG NS-1&IgM , IgM and IgG. Shrivastav et al[198] observed 26% positivity by ELISA and 16.56% by rapid card method, while Lakshmi P et al[182] found 48% positivity by ELISA and 32% positivity by rapid method. All samples positive by ELISA were positive by Rapid card method.

Considering ELISA as standard test according to the literature, when specificity, sensitivity and positive predictive value and negative predictive value was estimated, ELISA was found to be more sensitive and more cases were detected when compared with rapid test. But rapid test did not show false positive reaction i.e positive by rapid and negative by ELISA. 100% sensitivity and PPV was determined by rapid card method, this study is in correlation with Laxmi P et al[182] and Chakraverti et al[199].

Chakraveti V et al[199] reported rapid card test to be less sensitive than ELISA. Jyasimah et al[155] reported 80.7% sensitivity and 100% PPV and 72.14%NPv of rapid over ELISA. Shrivastav et al[198] showed ELISA as best method for identification with62.55% sensitivity,100% specificity ,100%PPV, 88.9%NPV which is in correlation with our study. This variation of the results in rapid card method may be because of different company kits used and nature of sample and duration of test and also cross reacting other flavivral diseases in endemic areas. IgM to IgG ratio was used to determine the primary and secondary dengue infection, the Optical density ratio greater than 1:2 was considered as primary infection and IgM to IgG ratio less then 1:2 was considered as secondary infection as per the WHO protocol 2009. Parng et al[200] suggest this as the best method for detection of primary and secondary dengue, but Pe Yen Shu et al[10] suggested that cutoff value calculation to be best for distinguishing primary and secondary dengue.

Early diagnosis and NS-1/ IgM which is better:

IgM capture ELISA is commonly used test in India due to its cost affectivity and ease of use. But here it is important to understand that, NS-1 antigen detection assay has an advantage of detecting infection very early, however it disappears early also and is of little use in the early convalescence phase when IgM is useful [201]. Seroconversion should be shown in patients to confirm a case of acute dengue infection by serology. In this study, NS-1 antigen alone was positive in 183 samples and IgM positive in 120 but both tests done on a single sample statistically increased the positivity rate to 431. Rapid test kits commercially available can detect Ns-1 from first 7 to 9 days of infection, but IgM antibodies can be detected only after 8 to 10 days of infection, this is why NS-1 antigen capture ELISA could detect high number of cases compared to IgM capture ELISA alone.[201,202,203.] Ns-1 Ag detection decreased from 97.57% in acute phase to 2.42% in early convalescent sera and detection rate of IgM increased from 5.43% in acute phase sera to 94.56% in convalescence sera. Wattal and Gupta et al have seen the increase in sensitivity of detection when two assays were used on a single sample. [200,204]

Clinical manifestations and Dengue serotype:

Although the symptoms correlated with serotype observed in our study is similar to Dash et al[46]and Kumaria R et al [69]. However, our findings are in contrast with the studies reporting hemorrhagic manifestations associated with dengue serotype -4. In our study we could not isolate dengue serotype-4 from

serum samples. In the Present study we saw high incidence of Anorexia in serotype 2 when compared to serotype 1 and 4. Hepatomegaly was seen in DENV serotype-2 , followed by 1,3 and 4. In this study, hepatomegaly was more in dengue serotype2 followed by 3 and 1. Out of 119 serum samples positive for dengue RNA, 38 belonged to DENV-1 infection, 46 DENV-2 monotype infections and 35 DENV-3 monotype infections, only four dengue monotypic infected patients (age group 16-30 years) suffered from DHF which can be explained by the absence of immunity against all the four serotype of dengue virus in these patients.

As Dengue virus is emerging and prevalent in India almost in all states, all the four serotypes are present in India. Chances of mono infection and multiple infections are common and the severity of the disease is in the areas where multiple serotypes are co circulating at a given time is high. Also changing serotype circulation during the years is observed in many places. Ekta G et al[64] in the year 2003 to 2005 in Delhi have seen the changing pattern of the serotype with predominance of serotype 3 in 2005. She has reported circulation of all the four serotypes in the year 2003, in the year 2004 only dengue serotype -1 was isolated, while in the year 2005 the predominant serotype seen was DENV-3. Mehta T K et al[189] has reported in his study about co-circulation of Dengue serotype -2 and serotype -3. Since 1997 dengue serotype one was observed in the following years 2004 multiple serotypes were isolated from North India. Also it is observed that in the last two decades DENV-3 was responsible for epidemic in Sri lanka, Latin America and East Africa.

Parida et al[7] have reported the changing trend of dengue virus circulation in the year 2004 to 2010, replacement of DENV-1 isolated in 2004 with DENV-4 in 2007 was observed and further in 2008 recurrence of DENV-1 was seen, in 2010 it was replaced by DENV-3. Infection with more than one serotype of dengue leads to severe infection. Incidence of multiple serotype infection has been reported from India. May be individual serotype may have different genetic features associated with infectivity and virulence, but not yet proved. Partial protection with one serotype may lead to Antibody dependent enhancement (ADE). Predominance of the serotype may vary according to the herd immunity and competition in serotype infecting.

During the years 2012 and 2013, the serotype prevalent was DENV-1 and DENV-2. In the year 2013, 2014 and 2015 displacement of DENV-1 was seen. The present study shows the change in the serotype from one to another. Our study is similar with the previous reports which showed DENV-1 replaced by DENV-4.[191] As the samples in our study were taken from hospitalized patients, the results may not actually tell the predominant serotype in the population in a given year. However, as currently there are not any approved vaccines or therapeutics for dengue the treatment in mainly supportive. Hence it is important to detect and know the spread of the DF and its vector. [49,192] Detailed study of circulating serotype will help to develop, improved, proactive, laboratory –based surveillance systems that can predict and impending dengue outbreak which will help to timely initiate preventive and control measures.

Rocha Hesse[206] states that comparative analysis of E gene, the E-NS-1 junction sequence using 6% divergence as cutoff value , genotype within each serotype have been linked with varying epidemic potentials. As DENV-2 and DENV-3 originate from Southeast Asia, which correlate with increased severe dengue infection i.e. DHF and DSS in Latin America. So we need the information of molecular epidemiology and phylogeny.

As DENV-2 showed more severe clinical manifestations of the disease, we tried to find out where it has come from, for which Phylogenitic analysis was done for DENV-2. phylogenetic relation between the isolates was generated by using NCBI blast tree view software and is based on neighbor joining tree type, showed its close proximity to the strain isolated from Singapore. DENV-2 and DENV-1 E- gene could be sequenced and due to insufficient genome of DENV-3 sequencing was not possible. DENV-2 E-gene showed 16 base substitutions but it did not affect the phenotypic or genotypic character, as all of them were silent mutations, this is in similarity with the other authors who have observed mutations in more than 56 sites in E gene and 120 mutations in prM gene from DENV-3[204,205]. DENV-1 did not show significant changes in E- gene, but when compared with few important isolates worldwide using multiple sequence alignment on the Vector NTI software we found the Ncol region to be conserved in our strains. Which can be used for (RFLP), and many such conserved sites in different strains will help us in developing vaccine and future regime for dengue virus Infection.

Summary and conclusion:

- The present study showed incidence of 46.2% dengue virus infection in North Karnataka.
- Significant decrease in incidence of Dengue virus infection was seen from 2012 to 2016.
- Male patients were more affected than female patients with a ratio of 1.2:1, 16-30 year age group were more affected followed by 31-45 years.
- More number of Dengue cases was from Bidar city and Humnabad.
- Dengue cases were significantly high in post monsoon season between June to October with peak number of cases in the month of September.
- Fever, headache, myalgia and arthrelgia were major symptoms in Dengue fever patients.
- Platelet count was depleted in cases positive for Ns-1, IgM and IgG (all three)
- ELISA was more satisfactory than rapid, NS-1 and IgM if performed both together showed increased sensitivity.
- NS-1 was highly positive in acute phase, IgM was positive in early convalescence.
- Molecular typing showed prevalence of DENV-1,2&3 in North Karnataka.
- Monotypic infections were seen, even though multiple serotypes were circulating.

- Change in serotype circulation was seen during 2012 to 2016.
- Shift of serotype 1 to serotype 3 was seen with Constant presence of DENV-2.
- Severe clinical manifestations were seen in DENV-2 infection.
- Platelet count was depleted in DENV-2 followed by DENV-3 and DENV-1.
- DENV-2 was closely associated with Singapore strain.
- Ncol region was conserved site in serotype 1 from our isolate which can be used for RFLP.

Conclusion:

- Detection of Antigen and Antibody when compared to Immunochromatographic test with ELISA, ELISA was found to be more sensitive.
- NS-1 Antigen detection is useful for early diagnosis of Dengue when antibodies are not detectable.
- When multiple serotypes were circulating, monotypic infection were seen in North Karnataka with severe clinical presentation by DENV-2 and 3.
- Ncol site in DENV-1 was conserved, which is a unique finding from the strains of North Karnataka.

Final conclusion of our study:

Dengue virus serotype 1, 2 and 3 were prevalent in our study population and severe clinical manifestations were observed in patients suffering from Dengue Virus serotype-2 infection, Ncol region was conserved in Dengue serotype-1.

Limitations of the study:

- Stage of the disease is important for isolation of RNA from serum, RNA isolation is only possible in the initial phase of the disease when high amount of circulating viruses are present, and hence we have chosen NS-1 positive samples for isolation. As antibodies develop, RNA load is lowered in circulation, which may result in less isolation rate, which may be the reason for less isolation of the serotypes from our study samples.

Future direction

- Large number of samples should be processed.
- Entire genome sequencing is needed for detail study.
- Detection of mutations constantly needed to be checked for the transformation.
- Find out more such conserved sites, which may in turn help in vaccine production and antiviral agents.

BIBLIOGRAPHY

1. World health organization. Key issues in Dengue Vector Control towards the operationalization of global strategy. Report of consultation (CTD/FIL(Den)/IC.96.1) Geneva: WHO;1995.
2. World Health Organization (WHO). Global Strategy of Dengue Prevention and Control, 2012-2020. Geneva: WHO Press;2012.p1-43.
3. Lin C, Haung Y, Shu P, Wu H, Lin Y, Yeh T et al. Characteristic of Dengue Disease in Taiwan: 2002-2007. Am J Trop Med Hyg.2010;82(4):731-739.
4. Gubler D, Clark G. Dengue/Dengue Hemorrhagic Fever: The Emergence of a Global Health Problem. Emerg. Infect Dis.1995 Apr-Jun:1(2):55-57.
5. Bhatia R, Sash AP, Sunyoto T. Chanign epidemiology of dengue in South-East Asia. WHO Southeast Asia J Public Health 2013 Jan-Mar;2(1):23.-7.
6. Lindenbach BD, Thiel HJ, Charles CM. Flaviviridae: The Viruses and Their Replication in : Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B et al, editors. Fields virology. Fifth edition. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins;2007.
7. Parida M. Horioke K, Ishida H, Dash P, Saxena P, Jana A et al. Rapid Detection and Differentiation of Dengue Virus Serotypes by a Real-Time Reverse Transcription-Loop-Mediated Isothermal Amplification Assay. J Clin Microbiol. 2005 Jun;43(6):2895-2903
8. Martina B, Koraka P. Ossterhaus A. Dengue Virus Pathogenesis; an Integrated View. Clin Microbiol Rev. 2009 Oct;22(4):564-581.
9. Gubler DJ, Kuno G, Markoff L. Flaviviruses. In:Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA, Roizman B et al, editors. Fields virology. Fifth edition. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins;2007.

10. Shu P, Hung J. Current Advances in Dengue Diagnosis. Clin Diagn Lab Immunol. 2004 ul;11(4):642-650.

11. Wattal C, Data S. Dengue NS1 antigen detection: A useful tool in early diagnosis of dengue virus infection. Indian J Med Microbiol. 2010 Apr-Jun:28(2):107-110.

12. Hermann L, Thaisomboonsuk B, Poolpanichupatam Y, Jarman R, Kalayanarooj S, Nisalk A et al. Evaluation of a Dengue NS1 Antigen Detection Assay Sensitivity and Specificity for the diagnosis of Acute Dengue Virus Infection. PLoSNegl Trop Dis.2014 Oct;8(10)e3193.

13. Subedi D, Taylor-Robinson A.W. Laboratory Diagnosis of Dengue Infection: Current Techniques and future Strategies. Open Journal of Clinical Diagnostics. 2014;4:63-70.

14. Shekharan SD, Kanthesh BM, subramaniam. Sensitivity of dengue virus NS1 detection in Primary and secondary infection. African J Micro Res 2009;3(3):105-110.

15. Gupta N, Srinivastava S, Jain A, Chaturvedi U. Dengue in India. Indian J Med. Res. 2012 Sep;136(3):373-390.

16. Ananthnarayan R, Panikar CKJ, Arboviruses. In Ananthanarayan and Panikar Textbook of Microbiology, 7th edition Hydrabad: Orient Longman; 2005.

17. Murray N, Quam M, Smith A. Epidemiology of dengue: past, present and future prospects. Clin Epidemiol. 2013;5:299-309.

18. Gublar DJ. Dengue and Dengue Haemorrhagic fever. Clin Microbiol Rev 1998;11(3):480-496.

19. Bancroft WH , Scott RM, Eckels KH, Hoke CH, Simms TE, Jesrani KDT, et al. Dengue virus type 2 vaccine: reactogenicity and immunogenicity in soldiers. J Infect Dis1984;149 (6):1005–1010.

20. Craiz DE. Chikungunya and dengue: a case of mistaken identity? J Hist Med Allied Sci 1971;26(3):243–262.

21. Hotti AK. Dengue serosurveillance in Kolkata, facing an epidemic in West Bengal, India. J Vector Borne Dis.2009 Sep;46:197-204.

22. Sabin AB. Research on dengue during World War II. *Am J Trop Med Hyg*1952; 1.1:30-50.

23. Gupta BP, Manandar KD, Malla R, Mishra SK. Emergence of Dengue Virus in Nepal. Int J Appl Sci Biotechnol. 2013;1(3):79-84.

24. Malavige GN, Fernando S, Fernando DJ, Seneviratne SL. Dengue viral infections. Postgrad Med J 2004;80(948):588-601.

25. En.wikipedia.org/wiki/dengue-fever;dated August 2011.

26. Sarkar JK, Chatterjee SN, Chakravarty SK. Haemorrhagic fever in Calcutta: some epidemiological observations. Indian J Med Res 1964;52: 651-659.

27. Teo D, Neg L C, Lam S. Is Dengue a threat to a blood supply.Tranfus med 2009 April;19(2):66-77.

28. Chaturvedi U, Nagar R. Dengue and dengue haemorrhagic fever: Indian perspective. J Biosci. 2008 Nov:33(4)429-441.

29. Sirisena P, Noordeen F. Evolution of dengue in Sri Lanka- changes in the virus, vector, and climate. IJID. 2014;19:6-12.

30. Wasay M, Channa R, Jumani M, Zafar A. Changing patterns and outcome of Dengue infection; report form a tertiary care hospital in Pakistan. J Pak Med Assoc.2008 Sep;58(9):488-489.

31. Khan J, Khan A. Incidence of dengue in 2013: Dengue outbreak in District Swat, Kyber Pakhtunkhwa, Pakistan. IJFBS. 2015;2(1):01-07.

32. Chatterjee SN, Chakravati SK, Mitra AC, Sarkar JK. Virology investigation of cases with neurological complications during the outbreak of haemorrhagic fever in Calcutta. J Indian Med Assoc.1965;45:314-6.

33. Balaya S, Paul SD, D'Lima LV, Parvi KM. investigations on an outbreak of dengue in Delhi in 1967. Indian J Med Res 1969;57:767-74.

34. Chatruvedi UC, Mathur A, Kapoor AK, Mehrotra NK, Mehrotra RML. Virological study of an epidemic of febrile illness with haemorrhagic manifestations at Kanpur, India, during 1968.Bull world health organ 1970;43:289-93.

35. Chatruvedi UC, Kapoor AK, Mathur A, Chandra D, Khan AM,Mehrotra RML. A clinical and epidemiological study of an epidemic of febrile illness with haemorrhagic manifestations which occurred at Kanpur, India in 1968.Bull world Health Organ 1970;43:281-7.

36. Myer RM, Varkey MJ, Reuben R, Jesudass ES. Dengue outbreak in Vellore, South India, in 1968, with isolation of four dengue types from man and mosquitoes. Indian J of Med Res 1970;58:24-30.

37. Mahadev PV, Kollali VV, Rawal ML, Pujara PK, Shaikh BH, IIKal MA. et al. Dengue in Gujarat state, India during 1988 & 1989. Indian J Med Res 1993;97:135-44.

38. Ghosh SN, Pavri KM, Singh KR, Sheikh BH, D'lima LV, Mahadev PV, et al. Investigation of the outbreak of dengue fever in Ajmer city, Rajasthan state in 1969. Part 1. Epidemiological, clinical and virological study of the epidemic. Indian J Med Res 1974;62:511-22.

39. Chouhan GS, Rodrigues FM, Shaikh BH, IIKal MA, Khangaro SS, Mathur KN, et al. Clinical &virological study of dengue fever outbreak in Jalore city, Rajasthan 1985. Indian J Med Res 1990; 91: 414-8.

40. Rodrigues FM, Pavri KM, Dandawate CN, Banerjee K, Bhatt PN. An investigation of the aetiology of the 1966 outbreak of febrile illness in Jabalpur, Madhya Pradesh. Indian J Med Res 1973; 61: 1462-70.

41. Kumar A, Sharma SK, Padbidri Vs, Thakare JP, Jain DC, Datta KK. An outbreak of dengue fever in rural area of northen India. J Commun Dis 2001; 33:274-81.

42. Parida. MM, Dash PK, Upadhyay C. Saxena P, Jana AM. Serological L&virological investigation of an outbreak of dengue fever in Gwalior, India. Indian J Med Rs. 2002;116:248-54Kumaria R. correlation of disease spectrum among four dengue serotypes: A five years hospital based study from India. Braz.J Infect Dis 2010;14:141-6.

43. Singh UB, Maritra A, Baroor S, Rai A, Pasha ST, Seth P. Partial nucleotide sequencing and molecular evolution of epidemic causing dengue 2 strain. J Infect Dis 1999;180: 959-65.

44. Dash PK, Saxena P. Abhayankar A. Bhargava R and Jana AM. Emergence of dengue virus type-3 in northern India. Southeast Asian J Trop Med Public Health. 2005 Mar;36(2):370-77.

45. Dash PK, Saxena P. Abhayankar A. Bhargava R and Jana AM. Reemergence of dengue virus type-3(subtype III) in India: implications of increased incidence of DHF and DSS. Virol J 2006 ;3: 55-65.

46. Dash PK, Parida MM, Sexena P, kumar M, Rai A, Pasha ST, et al. Emergence and continued circulation of dengue-2 (genotype IV) virus strain in India: J Med Virol 2004;74: 314-22.

47. Padbidri VS, Adhikar P, Thakare JP, IIkal MA, Joshi GD, Pereira P et al. The 1993 epidemic of dengue fever in Mangalore, Karnataka State, India. Southeast Asian J Trop Med and Public Health . 1995 Dec;26(4):699-704

48. Ratho RK, Mishra B, Kaur J, Kakkar N, Sharma K. An outbreak of dengue fever in periurban slums of Chandigarh, India with special reference to entomological and climatic factors. Indian J Med Sci.2005 Dec; 59(12):518-526.

49. Singh NP, Jhamb R, Agarwal SK, Gaiha M, Dewan R, Daga MK et al. The 2003 outbreak of Dengue in Delhi, India. Southeast Asian J Trop Med Public Health.2005 Sep;36(5):1174-78.

50. Myer RM, Cary DE. Concurrent isolation from patient of two arboviruses. Chikungunya and dengue type -2. Science 1967;157: 1307-8.

51. Myer RM, Cary DE, Benerjee K, Ruben R, Ramamurti DV. Recovery of dengue virus from human serum and Aedes aegypti in south India. Indian J Med Res 1968;56: 781-7.

52. Chatruvedi UC, Mathur A ,Kapoor AK. Clinocopathological study of recurrence of dengue epidemic with haemorrhagic manifestation at Kanpur, during 1969. Indian J Med Res1972;60:329-33.

53. Chaturvedi UC, Mathur A, Mehrotra RM. Experimentally produced cardiac injury following dengue virus infection. Indian J PatholBacteriol1974;17:218-20.

54. Mukherjee KK, Chakarverti SK, Dey PN. Outbreak of febrile illness due to dengue virus type-3 in Culcutta during 1983. Trans R Soc Trop Med Hyg1987;81:1008-10.

55. Kabra SK, Verma IC, Arora NK, Jain Y, Kalra V. Dengue haemorrhagic fever in children in Delhi. Bull world Health Organ 1992;70: 105-8.

56. Kaur H, Prabhakar H, Mathew P, Marshalla R, Arya M. Dengue haemorrhagic fever ougbreak in October- November 1996 in Ludhiana, Punjab, India. Indian J Med Res 1997;106:1-3.

57. Agarwal R, Kapour S, Nagar R, Misra A, Tandon R, Mathur A et al. A clinical study of the patients with dengue hemorrhagic fever during the epidemic of 1996 at LUcknow, India. Southeast Asian J Trop Med Public Health. 1999 Dec;30(4):735-40.

58. Agrawal A, Chandra J, Aneja S, Patwari A K, and Dutta A K. An epidemic of dengue haemorrhagic fever and dengue shock syndrome in children in Delhi. Indian Pediatr. 1998 Aug;35(8):727-32.

59. Dar L, Broor S. Sengupta S, Xess I, Seth P. The First Major Outbreak of Dengue Hemorrhagic Fever in Delhi, India. Emerg Infect Dis. 1999 Jul-Aug;5(4):589-590.

60. Vajpayee M, Manmohan K, Wali JP, Dar L, Seth P ,Broor S. Dengue virus infection during post-epidemic period in Delhi, India. Southeast Asian J Trop Med Public Health 1999;30: 507-10.

61. Victor TJ, Malathi M, Gurusamy D, Desai A, Ravi V, Narayanaswami G et al. Dengue fever outbreak in two villages of Dharampuri district in Tamil Nadu. Indian J Med Res 2002;116: 133-9.

62. Kabilan L, Velayutham T, Sundaram B , Tewari SC, Natarajan A, Rathnaswamy R, et al. Field- and laboratory- based active dengue survillence in Chenni, Tamil nadu, India: observations before and during the 2001 dengue epidemic. Am J Infect Control 2004; 32: 391-6.

63. Thenmozi V, Paramasuvabet R, Hiriyan J, Dhananjeyan K, Tyagi B, Dash AP. Serological and entomological investigations of an outbreak of dengue fever in certain rural areas of kanyakumari district, Tamil Nadu. Indian J Med Res 2006; 123:697-701.

64. Gupta E, Dar L, Kapoor G. Broor S. The changing epidemiology of Dengue in Delhi, India. Virol J. 2006 Nov;3:92-6.

65. BharajP ,Charaha HS, Pandey A, Diddi K, Dar L, Guleria R, et al. Concurrent infection by all four serotypes during an outbreak of dengue I 2006 in Delhi, Inida. Virol J 2008;5:1.

66. Hati Ak. Studies on dengue and dengue haemorrhagic fever (DHF) in West Bengal State, India J Commun Dis 2006; 38: 124-129.

67. Kumar SR, Patil JA, Cecilia D, Cherian SS, Barde PV, Walimbe AM, et al. Evolution, dispersal and replacement of American genotype dengue-2 in India(1956- 2005): selection pressure and molecular clock analyses. J Gen Virol 2010; 91:707-720.

68. Chahar HS, Bharaj P Dar L, Guleria R, Kabra SK, Broor S. Co-infections with dengue and chikunguniya virus in Delhi, India. Emerg Infect Dis 2009;15:1077-80.

69. Kumaria R. Correlation of disease spectrum among four Dengue serotypes: a five year hospital based study from India. Braz J Infect Dis 2010; 14: 141-146.

70. Paramasivan R, Leo SV, Muniraj M. Dengue fever caused by dengue virus serotype-3 in rural area of Madhuraidist TN. Indian J Med Res 2010;132:339-342.

71. Dash Pk, Sharma S, Santhosh SR, Parida MM Neerja M et al. Emergence of dengue virus type-4(genotype-1) in India. Epidemiol Infect 2011; 139: 857-859.

72. Kukrei H, Dash PK, ParidaM,Chaudhary A, Saxena P, Rautela RS, et al. Phylogenetic studies reveal existence of multiple lineages of single genotype of DENV-1 genotype III in India during 1956-2007. Virol J 2009;6: 1-9.

73. Matlani M, Chakravarti A. Changing trends of dengue diseases: a brief report from a tertiary care hospital in New Delhi. Braz J Infect Dis 2011;15 : 184-185.

74. Anoop M, Issac A, Mathew T, Philip S, Kareem NA, Unnikrishnan R, et al. Genetic characterization of dengue virus serotypes causing concurrent infection in

an outbreak in Ernakulam, Kerala, South India. Indian J Exp Biol 2010;48:849-857.

75. Sharma S, Dash PK, Agrawal S, Shukla J, Parida MM, Rao PV. Comparative complete genome analysis of dengue virus type-3 circulating India between 2003-2008. J Gen Virol 2011; 92: 1595-1600.

76. Dayaraj C, kakade MB, Bhagat AB, Vallentyne J, Singh A, Patil JA, et al. Detection of dengue-4 virus in Pune, western India after an absence of 30 years-its association with two severe cases. Virol J 2011;8: 46-49.

77. Balmaseda A, Hammond SN, Tellez Y, Imhoff L, Rodriguez Y, Saborio SL, et al. High seroprevalence of antibodies against dengue virus in a prospective study of school children in Managua, Nicaragua.Trop Med Int Health 2006;11(6):935-942.

78. Fry SR, Meyer M, Semple MG, Simmons CP, Sekaran SD, Huang JX, et al. The Diagnostic Sensitivity of Dengue Rapid Test Assays is significantly enhanced by using a combined antigen and antibody testing approach. PLoS Negl Trop Dis 2011;5(6):e1199.

79. Sankar SG, Dhanajeyan KJ, Paramasivan R, Thenmozhi V, Tyagi BK, Vennison SJ. High-Level Expression of Functionally Active Dengue-2 Non-Structural Antigen 1 Production in Escherichia coli. BioMed Res Int 2013;2013:343195.

80. Falgout B, MarkoffL. Evidence that flavivirus NS1-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. J Virol 1995; 69(11):7232-7243.

81. Lindenbach BD, Thiel HJ, Charles CM. Flaviviridae: The Viruses and Their Replication in : Knipe DM, Howley PM, Griffin DE, Martin MA, Lamb RA,

Roizman B et al, editors. Fields virology. Fifth edition. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins;2007.

82. Kurane I. Dengue hemorrhagic fever with special emphasis on immunopathogenesis. Comp Immun Microbiol Infect Dis. 2007; 30(5-6):329-340.

83. Chambers TJ, McCourt DW, Rice CM. Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. Virology 1990;177(1):159-174.

84. Yon C Teramoto T, Mueller N , Phelan J, Ganesh VK, Murthy KH, et al. Modulation of the nucleotide triphosphatase/ RNA helicase and 5" –RNA triphosphastase activities of Dengue virus type-2 nonstructural protein 3 (NS3) by interaction with NS5, the RNA-dependent RNA polymerase. J Biol Chem 2005;280 (29): 27412-27419.

85. Zhang L, Mohan P M, Padmanabhan R. Processing and localization of Dengue virus type 2 polyprotein precursor NS3-NS4A-NS4B-NS5. J Virol 1992;66(12):7549-7554.

86. Jones M, Davidson A, Hibbert L, Gruenwald P, Schlaak J, Ball S, et al. Dengue Virus Inhibits Alpha Interferon Signaling by Reducing STAT2 Expression.J Virol 2005;79(9):5414-5420.

87. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M. Enzyme linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary and secondary infections. J. Clin. Microbiol 2002; 40(2):376.

88. Medin CL, Fitzgerald KA, Rothman AL. Dengue virus nonstructural protein

NS5 induces interleukin-8 transcription and secretion. J Virol 2005;79(17): 11053-61.

89. Quintero J, Brochero H, Saide PM, Perez MB, Basso C, Romero S, et al.Ecological, biological and social dimensions of dengue vector breeding in five urban settings of Latin America: a multi-country study BMC Inf Dis 2014; 14:38.

90. www.cdc.gov/dengue/resources/30jan2012/aegyptifactsheet.pdf

91. www.cdc.gov/dengue/resources/30jan2012/albopictusfactsheet.pdf

92. Campbell KM, Lin CD, Iamsirithaworn S, Scott TW. The Complex Relationship between Weather and Dengue Virus Transmission in Thailand. Am J Trop Med Hyg 2013; 89(6):1066–1080.

93. Park K. Park's Textbook of preventive and social medicine, 23rd ed. Jabalpur,India: M/s BanarasidasBhanot publishers;2015 February.p246-255.

94. World Health Organization (WHO). Regional office for Southeast Asia. Comprehensive Guidelines for Prevention and Control of Dengue and Dengue Haemorrrhagic Fever, Revised and Expanded Edition. New Delhi. WHO;2011.p1-212.

95. Md. Nazmul Karim, Mushi SU, Anwar N, Md Shah Alam. Climatic factors influencing dengue cases in Dhaka city: a model for dengue prediction. Indian J Med Res. 2012;136:32-39.

96. Amin MM, Hussain Azam, Murshed M. Chowdhury I A, MannanS, Chowdhuri SA et al. Sero-Diagnosis of Dengue Infections by Haemagglutination Inhibition test (HI) in Suspected Cases in Chitagong, Bangladesh. Dengue Bulletin. 1999;23:34-38.

97. Laxmi V. Teja V. mabala P, Subbalakshmi M, Neeraja M. Serodiagnosis of dengue virus infection in patients presenting to a tertiary care hospital. Indian J Med Microbiol. 2006 Oct:24(4):280-282.

98. Shah GS, Islam S, Ds BK. Clinical and Laboratory profile of Dengue infection in children Kathmandu Univ Journal. 2006 Jan-Mar;4(1):40-43.

99. Wilder-Smith A, Chen L, Massad E, Wilson M. Threat of Dengue to Blood Safety in Dengue-Endemic Countries. Emerg Infect Dis. 2009 Jan; 15(1):8-11.

100. Matlani M, Kumar A, Chakravarti A, Kashyap B. Awareness of changing trends in epidemiology of dengue fever is essential for epidemiological surveillance. Indian J Med Microbiol. 2012 Apr-Jun;30(2):222-226.

101. KumarMP, .Rao C, Pandit V, Shetty S, Bammigatti C, Samarasinghe C. Clinical manifestations and trend of dengue cases admitted in tertiary care hospital, Udapi District, Karnataka. Indian J Community Med.2010 Jul;35(3):386-390.

102. Khan SA, Dutta P, Topno R. Soni M. Mahanta J. Dengue outbreak in a Hilly State of Arunachal Pradesh in Northeast India. The Scientific World Journal. 2014;2014:1-6.

103. Halstead SB, Streit TG, Lafontant JG, Putvatana R, Russell K, Sun W et al. Haiti: absence of dengue hemorrhagic fever despite hyperendemic dengue virus transmission. Am J Trop Med Hyg. 2001 Sep;65(3):180-3.

104. Enders G. Viral infections of the fetus and neonate, other than rubella. In: Collier L, Balows A, Sussman M, Mahy BWJ, editors. Topley and Wilson's Microbiology and Microbial infections. 9th edition. Great Britain - London: Arnold, a member of the Hodder Headline Group:1998.

105. Guzman M, Vasquez S. The Complexity of Antibody-Dependent Enhancement of Dengue Virus Infection. Viruses. 2010 Dec;2(12):2649-2662.

106. Singhi S. Kisson N, Bansal A. Dengue and dengue hemorrhagic fever: management issues in an intensive care unit. J Pediatr (Rio J) 2007;83(2)S22-35.

107. Pham AM, Langlois RA, Tenoever BR. Replication in cells of hematopoieticorigin is necessary for Dengue virus dissemination. PLoS pathog 2012;8(1):e 1002465.

108. Luplertlop N, Misse D, Bray D, Deleuze V, Gonzalez JP, Leardkamolkarn V, et al. Dengue-virus-infected dendritic cells trigger vascular leakage through metalloproteinase overproduction. EMBO Rep 2006 ;7(11):1176-1181.

109. GollinsSW,PorterfieldJS. Flavivirus infection enhancement in macrophages : radioactive and biological studies on the effect of antibody onviral fate. J Gen Virol 1984; 65(8): 1261-1272.

110. Heinz FX, Auer G, Stiasny K, Holzmann H, Mandl C, Guirakhoo F, et al. . The interactions of the flavivirus envelope proteins: implications for virus entry and release. Arch Virol Suppl 1994;9: 339-348.

111. Kwan WH, Helt AM, Maranon C, Barbaroux JB, Hosmalin A, Harris E, et al. Dendritic Cell Precursors Are Permissive to Dengue Virus and Human Immunodeficiency Virus Infection. J Virol 2005; 79(12): 7291-7299.

112. Wang S, He R, Anderson R. PrM- and cell-binding domains of the dengue virus E protein. J Virol 1999;73(3): 2547-2551.

113. Wu SJ, Vogel GG, Sun W, Mascola JR, Brachtel E, Putvatana R, et al. Human skin Langerhans cells are targets of dengue virus infection. Nat Med 2000;6(7): 816-820.

114. Malheiros SM, Oliveira AS, Schmidt B, Lima JG, Gabbai AA. Dengue. Muscle biopsy findings in 15 patients. Arq Neuropsiquiatr 1993;51(2):159-164.

115. Mandell GL, Bennett JE, Dolin R. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 7th Edition. Elsevier Churchill Livingstone Publisher, 2009.

116. Paes MV , Pinhao AT, Barreto DF, Costa SM, Oliveira MP, Noqueira AC, et al. Liver injury and viremia in mice infected with dengue-2 virus. Virol 2005; 338(2):236-246.

117. Kyle, JL, Beatty PR, Harris E. Dengue virus infects macrophages anddendriti ccells inamousemodel of infection. JInfectDis2007; 195(12):1808-1817.

118. Sahaphong, S, Riengrojpitak S, Bhamarapravati N, Chirachariyavej T. Electron microscopic study of the vascular endothelial cell in dengue Hemorrhagicfever. SoutheastAsianJ. Trop.Med.PublicHealth1980; 11(2):194-204.

119. Funahara Y, Ogawa K, Fujita N, Okuno Y. Three possible triggers to induce thrombocytopenia in dengue virus infection. Southeast Asian J Trop Med Public Health 1987 ;18(3):351-355.

120. Lee MS, Hwang KP, Chen TC, Lu PL, Chen TP. Clinical characteristics of dengue and dengue haemorrhagic fever in a medical centre of southern Taiwan during the 2002 epidemic. J Microbiol Immunol Infect 2006; 39(2): 121-129.

121. National Rural Health Mission, National Vector Borne disease control programme, Government of Karnataka. Guidelines for treatment of dengue fever/dengue hemorrhagic fever in small hospitals.

122. WHO. Dengue: Guidelines for diagnosis, treatment, prevention and control. New edition. Geneva: World Health Organization; 2009.p1-160.

123. World Health Organization. Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control. 2nd edition. Geneva, World Health Organization;1997. p1-92.

124. http://www.who.int/dengue dated 2012.

125. Philip SP, Tyagi BK. Diagnostic methods for detection and isolation ofdengue viruses from vector mosquitoes. Ind J Med Res 2006;123(5):615-628.

126. Pant U, Ilkal MA, Soman RS, Shetty PS, Kanojia PC, Kaul HN. First isolation of Japanese encephalitis virus from the mosquito Culex triateniorhynchus Giles 1901 (Diptera:Culicidae) in Gorakhpur district, Uttar Pradesh. Indian J Med Res 1994; 99 : 149-51.

127. Morens MD, Halstead SB, Repik MP. Simplified plaque reduction neutralization assay for dengue viruses by semimicro methods in BHK-21 cells. Comparison of BHK suspension tests with standard plaque reduction neutralization. J Clin Microbiol 1985; 22 : 205-14.

128. Singhraj P, Simasathien P, Sukhavachana P, Halstead SB, Scanlon JE. Recovery of dengue and other viruses in mice and tissue culture from Thai mosquitoes. Bull World Health Organ 1966; 35 : 67-68.

129. Sudeep AB, Mourya DT, Mishra AC. Insect cell culture in research: Indian scenario. Indian J Med Res 2005;121(6) : 725-38.

130. Subedi D, Taylor-Robinson A.W. Laboratory Diagnosis of Dengue Infection: Current Techniques and future Strategies. Open Journal of Clinical Diagnostics. 2014;4:63-70.

131. Buchy P, Yoksan S. Peeling RW, Hunsperger E. Laboratory Tests for The Diagnosis of Dengue Virus Infection. Scientific Working Group, Report on Dengue, Geneva,Switzerland.2006Oct.1-5.

132. Samuel PP, Tyagi BK. Diagnostic methods for detection and isolation of dengue viruses from vector mosquitoes. Indian J Med Res.2006 May;123(5):615-28.

133. Matheus S. Deparis X, Labeau B, Lelarge J, Morvan J, Dussari P. Discrimination between Primary and Secondary Dengue Virus Infection by an Immunoglobulin G

Avidity Test Using a Single Acute-phase Serum Sample. J Clin Microbiol.2005 Jun;43(6):2793-2797.

134. Laxmi V. Teja V. mabala P, Subbalakshmi M, Neeraja M. Serodiagnosis of dengue virus infection in patients presenting to a tertiary care hospital. Indian J Med Microbiol. 2006 Oct:24(4):280-282.

135. Datta S, Wattal C. Dengue NS1 antigen detection: A useful tool in early Diagnosisofdengue virus infection. Indian J Med Microbiol 2010;28(2):107-110.

136. Kassim FM, Izati MN, TgRogayah T, Apandi YM, Saat Z. Use of dengue NS1 antigen for early diagnosis of dengue virus infection. Southeast Asian J Trop Med Public Health. 2011;42(3):562-9.

137. Shu PY, Yang CF, Kao JF, Su CL, Chang SF, Lin CC, et al. Application of the Dengue Virus NS1 Antigen Rapid Test for On-Site Detection of Imported Dengue Cases at Airports. Clin Vaccine Immunol 2009; 16(4): 589–591.

138. Singh MP, Majumdar M, Singh G, Goyal K, Preet K, Sarwal A, et al. NS1 antigen as an early diagnostic marker in dengue: report from India. Diag Microbiol Infect Dis 2010;68(1):50-54.

139. Thomas S. Nisalak A, Andrson K, Librraty D, Kalayanarooj S, Vaughn D et al. Dengue Plaque Reduction Neutralization Test (PRNT) in Primary and Secondary Dengue Virus Infection. How Alterations in Assay Conditions Impact Performance. Am J Trop Med Hyg. 2009 Nov;81(5):825-833.

140. Vorndam V, Beltra M. Enzyme-linked immunosorbent assay-format microneutralization test for dengue viruses. Am J Trop Med Hyg. 2002;66(2):208-212.

141. Timiryasova T. Bonaparte M, Luo P, Zedar R, Hu B, Hildreth S. Optimization and Validation of Plaque Reduction Neutralization Test for the Detection of Neutralizing Antibodies to Four Serotypes of Dengue Virus Used in Support of Dengue Vaccine DeveloW;pment. Am J Trop Med Hyg. 2013 May;88(5)962-970

142. Wichit S. Yongpradoem H, Surasombatpoattana P, Laungwuttwong P, Kalambahet T, Jampangem W et al. Cloning and application of recominant Dengue virus prM-M protein for serodiagnosis of Dengue Viral infection. Southeast Asian J trop med public health.2013 Mr;44(2)218-225.

143. Teoh B, Sam S, Tan K, Johari J, Danlami M, Hool P et al. Detection of dengue viruses using reverse transcription -loop mediated isothermal amplification. BMC Infect Dis.2013;13:387.

144. Lolekha R. Chokephaibulkt K, Yoksan S. Vanpraper N, Phongsmart W, Chearskul S. Diagnosis of dengue infection using various diagnostic tests in the early stage of illness. Southeast Asian J trop Med public health. 2004;35(2):391-395.

145. Usawattanakul W. Jittmittraphap A. Tapchaisri P, Siripanichgon K, Buchachart K, Hong-ngarm A et al. Detection of Dengue Viral RNA in Patient's Sera by Nucleic Acid Sequence -Based Amplification (NASBA) and Plymerase Chain Reaction (PCR). Dengue Bulleting. 2002;26:131-139

146. Fakruddin MD, Muzumdar RM, Chowdhury A, Mannan K. Nucleic acid sequences based amplifications (NASBA). Int J life Sci pharma Res. 2012 Jan-Mar;2(1):106-121.

147. Tee H, How S, Jamalludin A, FarizSafhan M, MohdSapain M, Kaun Y et al. Risk Factors Associated with development of Dengue Haemorrhagic Fever or Dengue Shock Syndrome in Adults in Hospital TengkuAmpuanAfzan Kuantan. Med J Malaysia. 2009 Dec;64(4):316-320.

148. Chen RF, Yang KD, Wang L, Liu JW, Chiu CC, Chang JT. Different clinical and laboratory manifestations between dengue hemorrhagic fever and dengue fever with bleeding tendency. Trans R Soc Trop Med Hyg 2007; 101(11): 1106-13.

149. Lai YL, Chung YK, Tan HC, Yap HF, Yap G, Ooi EE. Cost-Effective Real-Time Reverse Transcriptase PCR (RT-PCR) To Screen for Dengue Virus followed by Rapid Single-Tube Multiplex RT-PCR for Serotyping of the Virus. J Clin Microbiol 2007;45(3):935-941.

150. Siregar AR, Wibawa T, Wijayanti N. Early Detection and Serotyping of Dengue Viruses by Using Reverse Transcription Polymerase Chain Reaction (RT-PCR) 2 Primers. I J Biotech 2011;16(2):71-75.

151. Gubler DJ. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century.Trends Microbiol 2002 ;10(2):100-103.

152. Wang SM, Sekaran SD. Early Diagnosis of Dengue Infection Using aCommercial Dengue Duo Rapid Test Kit for the Detection of NS1, IGM, and IGG. Am J Trop Med Hyg 2010; 83(3): 690–695.

153. SubhashAC, Agarwal N, Parikh SC, Agarwal S. Simultaneous Detection ofDengue NS1 Antigen, IgM plus IgG and Platelet Enumeration during an Outbreak. Sultan Qaboos Univ Med J 2011; 11(4): 470–476.

154. Pramiladevi R, Kaivalya, Kora S. Study of Rapid Serological Tests for

Diagnosis of Dengue. Sch J App Med Sci 2013; 1(5):548-551.

155. Jayasimha VL , Thippeswamy MTR , Yogesh Babu KV , Vinodkumar CS,Niranjan HP , Raghukumar KG , et al. Dengue: seroprevalence, comparisonof rapid test with ELISA. Nat J Basic Med Sci 2014;3(1):57-60

156. http://www.who.int/dengue dated 2012.

157. Raimunda F F. Azin G, Gonçalve RP, Pitombeira MH, Lima DM, BrancoICDeng ue: profile of hematological and biochemical dynamics. Rev BrasHematol Hemoter 2012; 34(1): 36–41.

158. Agarwal S, Rukadikar A, Laghawe A, Tripathi A. Importance of platelet count and serological markers in diagnosing Dengue infection with special reference to NS1 antigen. J of Evolution of Med and Dent Sci. 2014 Mar;3(10)2543-2547.

159. Cuzzubbo AJ, Vaughn DW, Nisalak A, Solomon T, Kalayanarooj S, AaskovJ, et al. Comparison of PanBio Dengue Duo IgM and IgG capture ELISA and venture technology dengue IgM and IgG dot blot. J Clin. Virol 2000;16(2):135-44.

160. Sjahrurachman A , Ernawati B, Ibrahim F, Mardiastuti , Sudiro TM, Sudarmono P. Semiquantitative dot immunoassay for detection of IgM antidengue antibodies in human sera. Med J Indones 2000;9(1):9-12.

161. Rajapakse S, Rodrigo C, Rajapakse A.Treatment of dengue fever. Infec Drug Res 2012;5 :103–112.

162. Government of India. Guidelines for clinical management of Dengue , Dengue Haemorrhagic Fever and Dengue Shock Syndrome. Directorate of National Vector Borne Dis Control Programm, Delhi. 2008;p1-39 (*www.nvbdcp.gov.in/Doc/Clinical%20guidelines*)

163. DamonteEB, MatulewiczMC, CerezoAS.Sulfatedseaweedpolysaccharides as antiviral agents. Curr Med Chem 2004 ;11(18):2399-2419.

164. Crance JM, Scaramozzino N, Jouan A, Garin D. Interferon, ribavirin, 6-azauridine and glycyrrhizin: antiviral compounds active against pathogenic flaviviruses. Antiviral Res 2003;58(1):73-79.

165. Yina Z, Chena YL, Schula W, Wanga QY, Gua F, Duraiswamy J, et al. An adenosine nucleoside inhibitor of dengue virus. Proc Natl Acad Sci U S A 2009 ;106(48):20435–20439.

166. WHO (2011), Comprehensive Guidelines for Prevention & Control of Dengue and Dengue Haemorrhagic fever, Revised and expanded edition Regional office of SEAR.

167. Sharma RS, Sharma GK, Dhillon GPS. Epidemiology and control of malaria in India. New Delhi: National Malaria Control Programme, 1996.

168. Kittayapong P, Strickman D. Three simple devices for preventingdevelop ment of Aedes aegypti (larvae in water). Am J Trop Med Hyg 1993;49(2):158–65.

169. Katz TM, Miller JH, Hebert AA. Insect repellents: historical perspectives and new developments. J Am Acad Dermatol 2008; 58(5): 865–71.

170. Kroeger A, Lenhart A, Ochoa M, Villegas E, Levy M, Alexander N,et al.Effectiv e control of dengue vectors with curtains and water container coverstreated with inse cticide in Mexico and Venezuela:Cluster randomised trials.BMJ 2006 ;7: 332(7552): 1247–1252.

171. Rozendaal JA, ed. Vector control: Methods for use by individual andcommu nities. Geneva: World Health Organization, 1997.

172. Seng CM, Setha T, Nealon J, Socheat D, Chantha N, Nathan MB. Community-base use of the larvivorous fish Poecilia reticulata to control the dengue vector Aedes aegypti in domestic water storage containers in rural Cambodia. Journal of Vector Ecology 2008; 33(1): 139–144.

173. Tikar SN, Mendki MJ, Chandek K, Parashar BD, Prakash S. Susceptibility of immature stages of aedes(stegomyia) aegypti; vector of dengue and chikungunya to insecticides from India. Parasitol Res 2008;102(5):907-913.

174. Mani TR, Arunachalam N, Rajendran R, Satyanarayana K, Dash AP. Efficacy of thermal fog application of deltacide, a synergized mixture of pyrethroids, against Aedes aegypti, the vector of dengue. Trop Med Int Health 2005;10(12):1298-304.

175. Murrell S, Wu SC, Butler M. Review of dengue virus and the development of a vaccine .Biotechnol Adv 2011;29(2):239-47.

176. Murphy BR, Whitehead SS. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol 2011;29:587-619.

177. Villar L. Dalyan G Arrendondo-Garcia J. Rivera D, Cunha R Deseds C. et al . Efficacy of Tetravalent Dengue Vaccine In Children in Latin America. N Engl J Med. 2015Jan;3372:113-123.

178. Swaminathan S. Khanna N. Viral Vaccines for Dengue: The Present and the Future .Dengue Bulleting. 2003;27:181-191.

179. Thisyakorn U, Thisyakorn C. Latest development and future directions in dengue vaccines. Ther Adv Vaccines. 2014 Jan;2(1):3-9.

180. ChakravertiA.Matlani M. Awerness of changing trends in epidemiology of dengue fever is essential for epidemiological surveillance. Indian J of Med Microbiol 2012;30:222-30.

181. Gunasekaran P ,Kaveri K, Mohana S, Arunagri K, Babu BV, Priya PP et al. Dengue disease status in Channai(2006-8). Indian J of Med Res 2011;133:322-325.

182. Lakshmi P, Nainar P. Challenges in the early diagnosis of dengue: A practical approach. J Sci Soc. 2014;41(2):85-88.

183. Kalayanarooj S, Vaughn D. Nimmannitya S, Green S, Suntayakorn S, Kunentrasai N et al. Early Clinical and Laboratory Indicators of Acute Dengue Illness. JID. 1997 Aug;176(2):313-321.

184. Saini S, Kinikar A, Deorukhakar S, Bhalerao D. Roushani S. Epidemiology and seropositivity of dengue fever cases in a rural tertiary care hospital of western Maharashtra, India. Int J of Biomed & Adv Res. 2013;4(7):473-477.

185. Kamal S, Jain SK, Patnaik SK, Lal S. An outbreak of dengue fever inVeerran napet village, Cherial Mandal of Warangal district, Andhra Pradesh.
J Commun Dis 2005; 37 (4): 301-306.

186. Raju BJ, Rajaram G. Prevalence of dengue fever and dengue hemorrhagicfever i n government general hospital tirupati. Int J Res Health Sci 2013; 1(1):23-27.

187. Neeraja M, Lakshmi V, TejaVD, Umabala P, Subbalakshmi MV. Serodiagnosis of dengue virus infection in patients presenting to a tertiary care hospital. Indian J Med Microbiol 2006;24(4): 280-282.

188. Pathankar M, Patel B, Gandhi V, Sahah P, Vegad M. Seroprevalence of dengue in Gujarat, western India. Virol J 2006;3:92.

189. Mehta TK, Shah PD. Identification of prevalent dengue serotypes by reverse transcriptase polymerase chain reaction and correlation with severity of dengue fever as per the recent WHO classification(2009). Indian J of Med Microbiol 2018;36:273-278.

190. Kaup S, Sankiarankutty J. Seroprevalence and Seasonal Trend of Dengue Virus Infection at a Teaching Hospital in Tumkur, Indi. Sch J App Med Sci. 2014;2(3A):922-926.

191. Ukey P, Bondade S, Paunipagar P, Powar R, Akulwar S. Study of seroprevalence of dengue fever in central India. Indian J Community Med.2010 Oct-Dec;35(4):517-519.

192. Sarkar A, Taraphdar D, Chatterjee S. Molecular Typing of Dengue Virus Circulating in Kolkata. India in 2010. Journal of Tropical Medicine. 2012;2012:1-5.

193. Bansal N, Uniyal N, Khrime D, Verma A. Clinical Profile and outcome of Dengue fever in Tertiary level Hospital in Uttrakhand , India. Transworld Medical Journal. 2014;2(3):165-169.

194. Kale AV, Haseeb M, Sandeep Reddy C, Khan S. Golwalkar A, Bedaam KM, ClinicalProfile and Outcome of Dengue Fever from a Tertiary Care Centre at Aurangaba Maharashtra India: An Observational Study. IOSR-JDMS. 2014 Sep;13(9):14-19.

195. Deora (Kaswa)P, Bhalchanda M, Mulay V. Early diagnosis of Dengue-Need of the hour. National Journal of Medical Sciences. 2015 Jan;4(1):60-65.

196. Fazal F, Biradar S. Clinical and Laboratory Profile of Dengue Fever. J of Evidence Based Med & Healthcare. 2015;2(9):1136-1147.

197. Tathe SS, Chincholkar VV, Kulkarni DM, Nilekar SL, Ovhal RS, Halagarkar et al. Astudy of NS1 antigen and platelet count for early diagnosis of dengue infection. Int. J. Curr. Microbiol. App. Sci. 2013;2(12):40-44.

198. Shrivastava A, Dsh P. Tripathi N, Sahni A. Gopalan N, Lakshaman Rao P. Evluationof a commercial Dengue NS1 enzyme-linked immunosrobent assay for early diagnosis of dengue infection. Indian J Med Microbiol. 2011 Jan-Mar;29(1):51-55.

199. Chakraverti T, Malkappa S, Saileea. K. Comparison of Diagnostic efficacy on NS1 antigen based Immunochromatographic test with Immuno-sorbent assay as its role in detection of early Dengue infection. J of Evolution of Med and Dent Sci. 2012 Dec;1(6):1066-1070.

200. Parng G, Chokephaibulkit K. Immunologic hypo- or no-responder in natural dengue virus infection. J Biomed Sci. 2013;20(1):34-39.

201. Ampaiwan C, Wathane C, Viroj P, Kanchana T, Sarapee L, Sutee Y. The use of Non structural antigen for the early diagnosis during the febrile stage in patients with dengue infection.JPaed infect Dis.2008;27:43-48.

202. Tricou V, Vu HT, Quynh NV,Nguyen C HT, Farrar J et al. Comparison of two dengueNS1 rapid tests for sensitivity, specificity and relationship to viremia and antibody responses. BMC Infect Dis 2010;10:142.

203. Duong V, Ly S, Lom Try P, Tuiskunen A, Ong S, Chroeung N et al. Clinical and virological factors influencing the performance of a NS-1 antigen capture assay and potential use as a marker of dengue diseases severity. PLoSnegl Trop Dis 2011;5(7):e 1244.

204. Fry SR , Meyer M, Semple G, SimmonsCP, Sekharan SD, Huang et al. The diagnostic sensitivity of dengue rapid test assay in significantly enhanced by using a combined antigen and antibody testing approach. PLosNegl Trop Dis 2011;5(6):1244.

205. Schiling S Ludolfs D, Le VA. SchmizH . Laboratory diagnosis of primary and secondary dengue infections. J Clin Virol2004;31:179-184.

206. Rocha BA, GuilardeAo, Argolo AF, Tassara MP, da Silveria LA, Junqueria IC et al. Dengue specific serotype related to clinical severity during the 2012/2013 epidemic in central of Brazil. Infect Dis Poverty 2017;6: 116.

Annexure -I

List of Publications

1. Pramod SM, Peerapur BV. Utility of NS1 antigen for diagnosis of Dengue virus infection JKIMSU,Vol. 6, No.1, Jan-March 2017. **(Indexed in *Scopus*)**

2. Pramod SM, Basavaraj PV. Demographic and clinical profile of patients infected with dengue virus serotype 1,2 and 3 in North Karnataka . Journal of Natural Science, Biology and Medicine. 2019 Jul 1;10 **(Indexed in Pub Med)**

ANNEXURE - II

CASE PROFORMA

Performafor Dengue

1. Name of the patient :
2. Age :
3. Sex :
4. Address :
5. IPD No./MRD No.

   Identification No. :
6. Ward and Unit :
7. Date of onset of symptom :
8. Day of sample collection after onset of symptoms:

Clinical findings

1. Fever (Since how many days):
2. Nausea and Vomiting :
3. Headache :
4. Bodyache (myalgia) :
5. Joint pain (Arthralgia) :
6. Rash :
7. Retro-orbital pain :

Other Sings: (warning signs and signs of shock)

1. Abdominal pain :
2. Bleeding manifestations :

3. Restlessness :
4. Hepatomegly :
5. Spleenomegaly :
6. Altered sensorium :
7. Shock :
8. Ascitis :
9. Pleural effusion :

History of Dengue in the past:

Laboratory Investigations:

a) Platelet count :

b) WBC count :

Serological results

1. NS1 Rapid :
2. IgM Rapid :
3. IgG Rapid :
4. NS1 ELISA :
5. IgM ELISA :
6. IgG ELISA :
7. Serotype detected :

ANNEXURE III

LIST OF ABBREVIATIONS

Ab	:	Antibody
ADE	:	Antibody Dependent Enhancement
Ag	:	Antigen
C	:	Core protein
CDC	:	Centers for Disease Control and prevention
CF	:	Complement Fixation
CFT	:	Complement Fixation Test
CTLA4	:	Cytotoxic T Lymphocyte Associated antigen4
DC-SIGN	:	Dendritic Cells Specific Intercellular Molecule-3-Grabbing Non -integrin
DEN	:	Dengue
DENV	:	Dengue Virus
DF	:	Dengue Fever
DHF	:	Dengue Haemorrhagic Fever
DSS	:	Dengue Shock Syndrome
E	:	Envelope protein
EC	:	Endothelial Cells

ECL	:	Electro - chemiluminescence
ELGA	:	Enzyme Linked Immuno Sorbent Assay
G6PD	:	Glucose 6 phosphate Dehydrogease
HI	:	Haemagglutination inhibition
HLA	:	Human Leucocyte Antigen
HRP	:	Horseradish Peroxidase
ICT	:	Immunochromatographic test
IFN	:	Interferon
IgA	:	Immunoglobulin A
IgM	:	Immunoglobulin M
IgG	:	Immunoglobulin G
IL	:	Interleukin
ITMN	:	Insecticides reated Mosquito Nets
i.e.	:	That is
JE	:	Japanese Encephalitis
LAMP	:	Loop Mediated Isothermal Amplification
M	:	Membrane protein
MAbs	:	Monoclonal Antibodies

MAC ELISA	:	IgM Antibody Capture Enzyme Linked Immunosorbent Assay.
MBL 2	:	Mannose Binding Lectin 2
MIA	:	Microsphere based Immunoassays
NASBA	:	Nucleic Acid Sequence Based Amplification
NC	:	Negative Control
NCR Igs	:	Natural CytoxicityRecepotor Immunoglobulins
NCR	:	Natural Cytotoxicity Receptors
NS	:	Non-Structural
NT	:	Neutralization Test
OD	:	Optical Density
ORS	:	Oral Rehydration solution
PC	:	Positive Control.
PCR	:	Polymerase Chain Reaction
prM	:	Precursor of Membrane protein
PRNT	:	Plaque Reduction Neutralization Test
RDT	:	Rapid Diagnostic Test
RT-LAMP	:	Reverse Transcriptase Loop mediated Isothermal Amplification

RT-PCR	:	Reverse transcriptase Polymerase Chain Reaction
qRT-PCR	:	Real Time Polymerase Chain Reaction
SOP's	:	Standard Operative Procedure.
SPCEs	:	Screen Printed Carbon Electrodes
TMB	:	Trimethyle benzidine
TNF α	:	Tumor Necrotic Factor α
VCD	:	Virologically Confirmed Dengue
WRAIR	:	Walter Reed Army Institute of Research
YF	:	Yellow Fever
WB	:	Western Blot
WHO	:	World Health Organization

Original Article

Demographic and Clinical Profile of Patients Infected with Dengue Virus Serotypes 1, 2, and 3 in North Karnataka

Pramod Sidram Manthalkar, Basawaraj V. Peerapur[1]
Department of Microbiology, BLDEU'S Shri B M Patil Medical College, Bijapur, [1]Department of Microbiology, RIMS, Raichur, Karnataka, India

Abstract

Introduction: Dengue fever is a mosquito-borne disease caused by flavivirus and has clinical presentation varying from being asymptomatic to severe complications (dengue shock syndrome and dengue hemorrhagic fever) depending on the serotype of the virus involved. Cross-protective immunity between the serotypes is lacking, hence the severity of the disease is more if multiple infections occur with two different serotypes. Hence, data on the demographic-specific prevalence of virus serotypes are vital to optimal clinical measures. **Aim:** The present study aimed to identify the dengue virus serotypes prevalent in the North Karnataka region of India in correlation to the clinical presentation of the disease. **Materials and Methods:** A prospective study was carried out in a Teaching hospital of North Karnataka, India, from June 2012 to March 2016. One thousand serum samples were tested for NS 1 antigen aIgM and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) method. Samples positive for NS-1 was subjected to reverse transcription polymerase chain reaction (RT-PCR) for the detection of serotypes. **Results:** Of the 1000 serum sample test 462 serum samples were positive for dengue virus antigen or antibodies. Two hundred and forty-five patients (53.03%) were male and 217 patients (46.96%) were female. Age group of16-–30 years was more affected followed by 31–45 years, over 45 years, and 0–15 years of age group. Maximum number of cases were observed in Bidar city followed by Humnabad, Aurad, Bhalki, and Basavakalyan regions. Malaise was a predominant symptom in dengue virus serotype-3 (DENV-3) ($P < 0.05$), while headache ($P < 0.001$), and retro-orbital pain (<0.05) were predominant symptoms in DENV-2. GI symptoms (nausea, abdominal pain, and diarrhea) were significantly common in DENV-2 ($P < 0.001$). Hepatomegaly was frequently observed in DENV-2 (17.02%), ($P < 0.05$). A total of 462 samples were positive for either NS-1, IgM, or IgG or in combination. Viral RNA was extracted from 119 samples positive for NS-1 antigen by ELISA. Of the 119 samples tested for serotyping by RT-PCR, 38 belonged to dengue serotype-1 (DENV-1), 46 were of dengue serotype 2 (DENV-2) and 35 belonged to dengue serotype 3 (DENV-3). A change in the earlier serotype 1 and 2 from 2011 to 2013 to the present serotype DENV-2 and DENV-3 was observed and constant presence of DENV-2 in circulation was recorded. **Conclusion:** Dengue virus serotype 1, 2, and 3 were prevalent in our study population, and severe clinical manifestations were observed in patients suffering from dengue virus serotype 2 and 3.

Keywords: Dengue, North Karnataka, serotype

Introduction

Dengue fever is one of the mosquitos borne diseases caused by flavivirus. Dengue infection is reported in 50–100 million individuals from developing world and is responsible for over 500,000 hospitalizations.[1] The severity of the disease varies from acute infection to fatal consequences such as dengue shock syndrome and dengue hemorrhagic fever (DHF).[2] Dengue virus has four different serotypes, DENV-1, DENV-2, DENV-3, and DENV-4, which are further subclassified based on their individual genotype.[3]

In India, a major outbreak of dengue serotype 2 was reported in the year 1996.[4] This was later displaced by dengue serotype 3, although many outbreaks with mixed serotypes are also reported subsequently. The first isolated dengue viruses belonged to serotype 3 and 4 in the year 1964 and 1965, respectively. Dengue virus type 3 has been very rarely reported in North Karnataka.[4-6] There are various studies from the Indian subcontinent investigating DHF in various parts of the country. However, there are no studies investigating the overall prevalence of the dengue serotypes

Address for correspondence: Mr. Pramod Sidram Manthalkar, Department of Microbiology, BLDEU'S Shri B M Patil Medical College, Bijapur, Karnataka, India.
E-mail: pramodmanthalkar@gmail.com

Access this article online

Quick Response Code:

Website: www.jnsbm.org

DOI: 10.4103/jnsbm.JNSBM_207_18

How to cite this article: Manthalkar PS, Peerapur BV. Demographic and clinical profile of patients infected with dengue virus serotypes 1, 2, and 3 in North Karnataka. J Nat Sc Biol Med 2019;10:144-8.

circulating in the endemic zone, apart from the epidemic outbreak regions.

The purpose of our study was to identify the circulating serotypes of dengue virus in North Karnataka from suspected cases of dengue presenting to our hospital during the years 2012–2016 and correlate it with the clinical presentation.

Materials and Methods

This is a prospective study, which was carried out in Bidar Institute of Medical Sciences Teaching Hospital of North Karnataka, India during the year 2012–2016. Ethical clearance was obtained from the Institutional Ethical Committee. Patients attending the hospital with a history of fever, headache, retro-orbital pain, nausea/vomiting, joint pain, malaise, and generalized skin rashes were initially considered for the diagnosis of dengue fever.[7-11] Patients presenting with two or more of these symptoms were included and were classified according to the WHO criteria[12] (dengue with or without warning signs). Patients of both gender and all age groups were included in the study.

A volume of 2–5 ml peripheral venous blood was collected in plain vial and blood was allowed to clot at room temperature and then centrifuged. Serum was separated for detection of NS-1antigen, IgM, and IgG antibodies by enzyme-linked immunosorbent assay (ELISA). In case of delay, test serum samples were preserved at −70°C. Positive samples were stored at −80°C until they were processed for serotyping by reverse transcription polymerase chain reaction (RT-PCR).[13]

Blood samples were collected in ethylenediaminetetraacetic acid vial from the patients for complete blood count (i.e., Hb estimation [Hb], total leukocyte count, differential count, hematocrit, and platelet count) using an automated cell counter.

All the 1000 serum samples were tested for the presence of NS-1 antigen (Pan-bio) and IgM and IgG antibodies by capture ELISA[12,13] by using the kit prepared by the National Institute of Virology, Pune, India. Following the prescribed protocol, optical density was measured at 450 nm using ELISA reader.

RNA extraction: Samples which were positive for NS-1 antigen by ELISA were only subjected for extraction of viral RNA by using the QiagenQIAmp RNA Mini Kit according to the manufacturer's protocol.

For the conventional PCR using serotype-specific E-NS1 region primers, viral RNA was reverse transcribed to cDNA before enzymatic DNA amplification by use of MoMLV reverse transcriptase. For RT of viral RNA to cDNA before Taq polymerase amplification, corresponding reverse primer of the particular set was used.

Primer and probe details

The initial viral quantitation of the samples was done using real-time PCR to establish that there was enough viral load for carrying out amplification and sequencing reactions. The primers (Eurofins genomics) were designed to target the 3 prime untranslated region on the dengue viral genome. The primers were combined in the required ratio with the corresponding Taqman probe (Eurofins Genomics) to make a common screening primer-probe mixture (ppm). The primer probe mix also contained primers and probe for a housekeeping gene (internal control region). The internal control probe signal was read in the VIC dye channel whereas the viral C-DNA amplification signal was read in the FAM channel on the real-time machine.

Table 1: List of primes and programs for E-NS-1 region amplification

Serotype	Primers	PCR program		Product size
DEN 1	D1-1229F	95°C- 5 min	X 1	482 BP
		95°C - 30 s	X 35	
	D1-1710R	57°C - 30 s		
		72°C - 1 min		
		72°C - 5 min	X 1	
DEN 2	ENVF-PO1	95°C - 2 min	X1	445 BP
		95°C - 30 s	X 30	
	D2R-PO1	52°C - 30 s		
		72°C - 2 min		
		72°C - 7 min	X1	
DEN 3	D3-1307F	95°C - 5 min	X 1	881 BP
		95°C -30 s	X35	
	D3-1867R	57°C - 30 s		
		72°C - 1 min		
		72°C - 5 min	X 1	
DEN 4	D4-1954R	95°C - 5 min	X 1	195 BP
		95°C - 30 s	X 35	
	D4-1760F	57°C -30 s		
		72°C -1 min		
		72°C - 5 min	X 1	

PCR: Polymerase chain reaction

After the viral load for all the serum samples was carried out, serotyping by serotype-specific primer-probe mix (Eurofins Genomics) was done to categorize the samples into their particular serotypes.

Primers were designed in-house at Huwel Lifesciences Pvt. Ltd [Table 1].[14]

Statistical analysis

Statistical analysis was done by using SPSS version 22 software (IBM corporation., Armonk, NY, USA). Chi-square test was applied. $P < 0.05$ was considered statistically significant.

Results

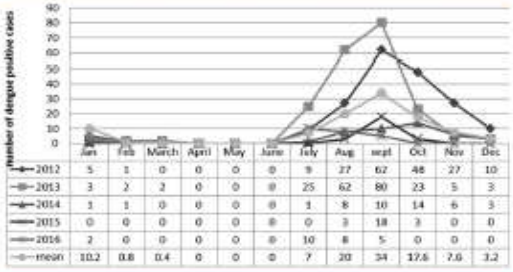
Of the 1000 clinically suspected patients, only 462 serum samples were positive for dengue antigen or antibodies, hence achieving an incidence rate of 46.2% in our patient population evaluated. Age group wise more number of positive case were observed in the age group 16–30 years, i.e., 238 patients (51.1%) followed by 31–45 years (107 patients; 23.16%), 46 years and above (61 patients; 13.20%), and

0–15 years (56 patients; 12.1%). Male patients (245 patients; 53.03%) were relatively more affected then female patients (217 patients; 46.96%) [Table 2]. Sporadic cases occurred throughout the year, more number of dengue cases were observed in monsoon and postmonsoon months, i.e., in July and attained maximum number of cases in the month of September, with a mean number of seven cases in July, 20 cases in August, 34 cases in September, 17 cases in October, 8 cases in November, and 3 cases in December [Table 3].

Table 2: Age and sex wise distribution of seropositive samples for dengue antigen and antibodies

Age (years)	Male (%)	Female (%)	Total (%)
0-15	31 (6.70)	25 (5.41)	56 (12.1)
16-30	121 (26.19)	117 (25.32)	238 (51.1)
31-45	65 (14.06)	42 (9.09)	107 (23.16)
45 and above	28 (6.06)	33 (7.14)	61 (13.20)
Total	245 (53.03)	217 (46.96)	462 (100)

Table 3: Month wise distribution of dengue positive cases



More cases of dengue DENV-1 (7) serotype were observed in Basavakalyan. All the three serotypes were isolated from Bidar and Humnabad, while serotypes 1 and 2 were isolated from Basavakalyan, Bhalki, and Aurad regions.

Of the 462 positive samples, 183 (39.61%) were only NS-1 positive, 120 (25.97%) were IgM positive and 247 (53.46%) were both NS-1 and IgMpositve by ELISA method. The positivity of NS-1 was 241 (97.57%) in acute phase (1–7 days), and IgM positivity (174 cases; 94.56%) was more in convalescence (6–14 days) phase.

Clinical manifestations of patients were compared with the dengue serotypes identified from 119 samples, which was statistically analyzed by Chi-square test. Malaise was a predominant symptom in DENV-3 ($P < 0.05$), while headache ($P < 0.001$) and retro-orbital pain (<0.05) were predominant symptoms in DENV-2. GI symptoms (nausea, abdominal pain, and diarrhea) were also significantly observed in DENV-2 ($P < 0.001$). Hepatomegaly was commonly observed in DENV-2 (17.02%), ($P < 0.05$). Respiratory symptoms (cough, dyspnea, rhinorrhea, and congestion) were more commonly observed in DENV-3 infection. Musculoskeletal symptoms (myalgia, joint pain, and bone pain) commonly observed ($P < 0.001$) in DENV-2 with 89.36%, 78.72%, and 53.19% incidence, respectively. Cutaneous signs (maculopapular rash) was frequently ($P < 0.05$) seen in DENV-2 (12.07%) [Table 4].

Hematological assessment revealed that the mean platelet count was less in serotype-2 (67150 c/mm^3), followed by serotype-3 (71800 c/mm^3) and 75200 in DENV-1 ($P < 0.01$). Hematocrit was 37.57 in DENV-2, 38.10 in DENV-3, and 39.01 in DENV-1 ($P < 0.05$) [Table 5].

PCR Result: NS-1 ELISA positive samples were subjected for dengue virus RNA isolation. We could identify dengue virus

Table 4: Serotypes isolated and its comparison with clinical manifestations

Signs and symptoms	Total cases (n=119)	DENV-1 (n=38; 31.93%), n (%)	DENV-2 (n=46; 38.65%), n (%)	DENV-3 (n=35; 29.41%), n (%)
Fever	119 (100)	38 (100)	46 (100)	35 (100)
Malaise	63 (52.94)	10 (26.31)	23 (50)	30 (85.71)
Headache	78 (65.54)	11 (28.94)	44 (95.65)	23 (65.71)
Retro-orbital pain	64 (53.78)	17 (44.73)	31 (65.95)	16 (45.71)
Nausea	44 (36.97)	7 (18.42)	37 (78.72)	0 (00)
Vomiting	4 (3.36)	2 (5.2)	60 (24.25)	0 (00)
Abdominal pain	42 (35.29)	8 (21.05)	32 (68.08)	2 (5.71)
Diarrhea	09 (7.56)	0 (00)	9 (19.14)	0 (00)
Splenomegaly	15 (12.60)	05 (13.15)	10 (21.27)	0 (00)
Hepatomegaly	12 (10.08)	03 (7.89)	8 (17.0)	20 (12.85)
Cough	8 (6.72)	1 (2.6)	30 (24.25)	5 (14.28)
Dyspnea	8 (6.72)	0 (00)	6 (12.76)	2 (5.71)
Rhinorrhea	6 (5.04)	1 (2.6)	30 (24.25)	3 (8.57)
Congestion	4 (3.36)	0 (00)	1 (2.1)	20 (38.57)
Myalgia	62 (52.10)	18 (47.36)	42 (89.36)	2 (5.71)
Joint pain	53 (44.53)	12 (31.57)	37 (78.72)	4 (11.42)
Bone pain	34 (28.57)	9 (23.63)	25 (53.19)	0 (00)
Maculopapular rash	6 (5.04)	0 (00)	6 (12.70)	0 (00)

RNA from 119 (24.68%) samples by Multiplex RT-PCR. Out of 119 samples positive by RT-PCR, 38 samples had monotypic infection with DENV-1 (31.93%), 46 samples (38.65%) had monotypic infection with DENV-2 and 35 samples (29.41%) had monotypic infection with DENV-3. No DENV-4 serotype was detected among the samples [Table 6].

Serotype 1 was identified from the samples collected in 2012, 2013, and 2014 while Serotype 2 was identified from the samples collected in 2012, 2013, 2014, and 2015. Serotype 3 was identified from samples collected in the year 2013, 2014, and 2015.

Out of the 38 Serotype 1, we could identify 12 (31.57%), 15 (39.47%), 11 (28.94%) in 2012, 2013 and 2014 respectively and out of 47 DENV-2 19 (40.42%), 12 (25.53%), 10 (21.27%), and 05 (12.7%) were identified in the year 2013, 2014, 2015, and 2016, respectively and DENV-3 was isolated 03 (8.57%), 12 (34.28%), 20 (57.14%) in the year 2014, 2015, and 2016, respectively [Table 7].

Discussion

Dengue is the major public health problem in endemic regions of India.[15] The first epidemic of clinical dengue-like illness was recorded in Chennai in the year 1780 but proven virological epidemic of Dengue fever in India occurred in Calcutta and Eastern coast of India in 1963–1964.[16] The largest outbreak occurred in Delhi and surrounding areas in the year 1996. This outbreak was due to dengue serotype 2, which was followed by epidemic in 1997 due to dengue serotype 1. Subsequent epidemics were reported to be due to co-circulation of both serotype-1 and serotype 3 in the year 2003.[17] In this study, we are reporting the co-circulation and replacement of serotype-1 and 2 by serotype 2 and 3 during the years 2012–2016.

The relative predominance in the prevalence of disease among male patients observed in our study is in concurrence to previous reports.[18,19] Majority of cases were observed in the age group of 16–30 years, this is comparable to the studies previously reported from other regions.[18,19] This age group is highly exposed to the external environment and mosquito vector. Infection in young patients results in nonproductivity and high number of cases in young age group indicates the disease is endemic to the region. We observed dengue virus incidence rate of 46.2% in our study population. Dengue cases started during July and reached the peak in September, which is the post-monsoon period. It may be explained by the fact that stagnant fresh water from August to October (Rainy season) favored the breeding of vector mosquitoes. Hence, these months must be effectively targeted to initiate preventive measures to control the breeding of mosquitoes.

Although the symptoms correlated with serotypes observed in our study is consistent with previous reports;[19-21] however, our finding is in contrast to the studies reporting hemorrhagic manifestations associated with dengue serotype-4.[20] In our study, we could not isolate dengue serotype 4 from any of the blood samples. A previous study reported from India[20] evaluated 80 serum samples and isolated all four serotypes of DENV. This study reported a high prevalence of Anorexia in serotype 2 as compared to serotype 1 and 4. Hepatomegaly was a prominent feature in serotype-2 followed by 1, 3, and 4. In our study, hepatomegaly was seen more in dengue serotype 2 followed by dengue serotype 3 and 1. In our study, out of the total 119 serum samples positive, 38 suffered from DENV-1 monotype infection, 46 DENV-2 monotype infection and 35 DENV-3 monotype infection, only four dengue monotypic-infected patients (age group 16–30 years) had suffered from DHF which may be explained by the absence of immunity against all-serotype of dengue virus in these patients.

In the year 2012 and 2013, the main serotype prevalent was DENV-1 and DENV-2. However, in the following years 2013, 2014, and 2015 displacement of DENV-1 was observed. The study shows that the serotype predominance can shift from one to another. Our study is in agreement with previous reports demonstrating DENV-1 was replaced by DENV-4.[18,19] As the majority of the samples in our study were obtained from hospitalized patients, the results may not actually reflect the predominant serotype in the general population in a given year. However, as currently there are not any approved vaccines or therapeutics for dengue the treatment is mainly supportive. Hence the containment of the spread of the vector and the disease is important.[22,23] Data on serotype will help develop improved, proactive, laboratory-based surveillance systems that can predict an impending dengue outbreak which will help to timely initiate preventive and control measures.

Table 5: Correlation of dengue serotypes with hematological parameters

Hematological parameter	DENV-1	DENV-2	DENV-3	*P*
Hb (mmHg)	12.2	11.5	12.5	<0.05
TLC (cells/mm³)	6121	6520	6441	0.6
Hematocrit	39.01	37.57	38.10	<0.05
Platelet count (cells/mm³)	75,200	67,150	71,800	<0.01

TLC: Total leukocyte count, Hb: Hemoglobin

Table 6: Different serotypes isolated from tested samples

Total	DENV-1	DENV-2	DENV-3
119	38 (31.93%)	46 (38.65%)	35 (29.41%)

Table 7: Year-wise distribution of dengue serotypes isolated

Serotype	2012	2013	2014	2015	2016	Total (%)
DENV-1	12	15	11	00	00	38 (31.93)
DENV-2	00	19	12	10	5	46 (38.65)
DENV-3	00	00	3	12	20	35 (29.42)
Total (%)	12 (10.08)	34 (28.5)	26 (21.8)	22 (18.4)	25 (21.0)	119

Conclusion

We observed circulation of dengue serotype-2 and serotype-1 and also a rise in number of dengue serotype-3. Co-circulations of these mixed serotypes may lead to severe clinical manifestations. Hence, detection of dengue serotypes helps achieve proper clinical management and probably develop an effective vaccine.

Limitations

Stage of the disease is important for isolation of RNA from serum. RNA isolation is only possible in the initial phase of the disease when high amount of circulating viruses are present, hence we have chosen only NS-1 positive samples for isolation. As the antibody develop, RNA load is lowered in circulation, which may result in less isolation rate, which may be the reason for less isolation of serotypes from our study samples.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Gubler DJ, Meltzer M. Impact of dengue/dengue hemorrhagic fever on the developing world. Adv Virus Res 1999;53:35-70.
2. World Health Organization. Key Issues in Dengue Vector Control Towards the Operationalization of a Global Strategy: Report of Consultation. (CTD/FIL (Den)/IC.96.1). Geneva: WHO; 1995.
3. Clyde K, Kyle JL, Harris E. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol 2006;80:11418-31.
4. Shu PY, Huang JH. Current advances in dengue diagnosis. Clin Diagn Lab Immunol 2004;11:642-50.
5. Mackenzie JM, Jones MK, Young PR. Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology 1996;220:232-40.
6. Alcon S, Talarmin A, Debruyne M, Falconar A, Deubel V, Flamand M, *et al.* Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J Clin Microbiol 2002;40:376-81.
7. Pongsiri P, Thamboonlers A, Poovorawan Y. Changing pattern of dengue virus serotypes in Thailand between 2004 and 2010. J Health Popul Nutr 2012;30:366-70.
8. Sarkar A, Taraphdar D, Chatterjee S. Molecular typing of dengue virus circulating in Kolkata, India in 2010. J Trop Med 2012;2012:960329.
9. Guzmán MG, Kouri G. Dengue: An update. Lancet Infect Dis 2002;2:33-42.
10. Kumarasamy V, Wahab AH, Chua SK, Hassan Z, Chem YK, Mohamad M, *et al.* Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection. J Virol Methods 2007;140:75-9.
11. Bessoff K, Delorey M, Sun W, Hunsperger E. Comparison of two commercially available dengue virus (DENV) NS1 capture enzyme-linked immunosorbent assays using a single clinical sample for diagnosis of acute DENV infection. Clin Vaccine Immunol 2008;15:1513-8.
12. Chaterji S, Allen JC Jr., Chow A, Leo YS, Ooi EE. Evaluation of the NS1 rapid test and the WHO dengue classification schemes for use as bedside diagnosis of acute dengue fever in adults. Am J Trop Med Hyg 2011;84:224-8.
13. Valdés K, Alvarez M, Pupo M, Vázquez S, Rodríguez R, Guzmán MG, *et al.* Human dengue antibodies against structural and nonstructural proteins. Clin Diagn Lab Immunol 2000;7:856-7.
14. Singh UB, Maitra A, Broor S, Rai A, Pasha ST, Seth P, *et al.* Partial nucleotide sequencing and molecular evolution of epidemic causing dengue 2 strains. J Infect Dis 1999;180:959-65.
15. Chakravarti A, Matlani M, Kashyap B, Kumar A. Awareness of changing trends in epidemiology of dengue fever is essential for epidemiological surveillance. Indian J Med Microbiol 2012;30:222-6.
16. Gunasekaran P, Kaveri K, Mohana S, Arunagiri K, Babu BV, Priya PP, *et al.* Dengue disease status in Chennai (2006-2008): A retrospective analysis. Indian J Med Res 2011;133:322-5.
17. Gupta E, Dar L, Kapoor G, Broor S. The changing epidemiology of dengue in Delhi, India. Virol J 2006;3:92.
18. Pathankar M, Patel B, Gandhi V, Shah P, Vegad M. Seroprevalence of dengue in Gujarat, Western India: A study at Tertiary care hospital. Int J Med Sci Public Health 2014;3:16-8.
19. Mehta TK, Shah PD. Identification of prevalent dengue serotypes by reverse transcriptase polymerase chain reaction and correlation with severity of dengue as per the recent World Health Organization classification (2009). Indian J Med Microbiol 2018;36:273-8.
20. Kumaria R. Correlation of disease spectrum among four dengue serotypes: A five years hospital based study from India. Braz J Infect Dis 2010;14:141-6.
21. Rocha BA, Guilarde AO, Argolo AF, Tassara MP, da Silveira LA, Junqueira IC, *et al.* Dengue-specific serotype related to clinical severity during the 2012/2013 epidemic in centre of Brazil. Infect Dis Poverty 2017;6:116.
22. Li DS, Liu W, Guigon A, Mostyn C, Grant R, Aaskov J, *et al.* Rapid displacement of dengue virus type 1 by type 4, pacific region, 2007-2009. Emerg Infect Dis 2010;16:123-5.
23. Whitehead SS, Blaney JE, Durbin AP, Murphy BR. Prospects for a dengue virus vaccine. Nat Rev Microbiol 2007;5:518-28.

JKIMSU, Vol. 6, No. 1, January-March 2017 ISSN 2231-4261

ORIGINAL ARTICLE

Utility of NS1 Antigen for Diagnosis of Dengue Virus Infection

Pramod S. Manthalkar[1], B.V. Peerapur[2]*

[1]Department of Microbiology, BLDE University's, Shri B.M Patil Medical college Vijayapura-(Karnataka) India, [2]Department of Microbiology, RIMS, Raichur-(Karnataka) India,

Abstract:

Background: Dengue has become a major global public health problem in the developing countries. *Aim and Objectives:* This study was carried out to evaluate the utility of NS1 antigen assay in early diagnosis of dengue infection. *Material and Methods:* The performance, detection rate of NS1 antigen assay in comparison to IgM Antibody Capture Enzyme Linked Immunosorbent Assay (MAC-ELISA) was evaluated in a single sample constituted Group 1. One thousand acute/ early convalescent sera were screened by both the assays. NS1 antigen assay was used to evaluate the efficacy of single assay in 30 acute phase sera of paediatric OPD patients constituted Group II. Specificity of NS1 assay in comparison to MAC-ELISA was evaluated on 40 samples used as controls constituted group III. *Results:* Out of 1000 samples in Group I, 247 (24.7%) and 184 (18.4%) samples were positive by NS1 assay and MAC –ELISA respectively. Increase in the detection rate to 431 (43.1%) was seen when both the assays were used together on a single sample. NS 1 Ag positivity varied from 97.57% to 2.42% in acute and early convalescent sera, conversely IgM detection rate was 94.56% and 5.43% in early convalescent and acute phase sera respectively (P<0.0001). Twenty (66.66%) samples were positive by NS 1 assay in Group II. All 40 samples in Group III were negative showing 100% specificity of both the assays. *Conclusion*: NS-1 Ag assay is a useful tool for early diagnosis of dengue virus infection. When used in combination with MAC-ELISA on a single sample it significantly improves the diagnosis algorithm without the requirement of paired sera.

Keywords: Dengue diagnosis, comparison, NS1 antigen assay, MAC- ELISA.

Introduction:

Dengue has become a major global public health problem in the developing countries. The estimated risk of acquiring Dengue Virus (DV) infection is approximately 2.5 billion people living mainly in urban areas [1]. DV causes various clinical symptoms ranging from asymptomatic or undifferentiated fever, known as Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF), Dengue Shock Syndrome (DSS), leading to death, especially among the children [2].

Viral isolation by culture or viral RNA detection by Polymerase Chain Reaction (PCR) helps in the diagnosis of recent dengue infection. But this viral isolation is very time consuming and requires specialized laboratory equipment [3]. Newer PCR types like nested PCR and real time PCR have significantly reduced processing time but are expensive and technically exacting [4]. As a result, dengue culture and PCR have limited utility in routine clinical use.

Rapid newer test for presumptive diagnosis of dengue is the detection of Non Structural Protein Antigen (NS1). This antigen is a highly conserved glycoprotein that is essential for the viability of dengue virus [5]. The first immunoglobulin isotype to appear is IgM antibody, suggesting recent infection. One of the most recent advances for routine dengue diagnosis is IgM Antibody Capture Enzyme Linked Immunosorbent Assay (MAC-ELISA) [6]. To confirm dengue during both early and late infection, combined usage of

NS1 antigen and IgM antibody ELISA are promising [7].

In this study, we have performed both types of immunoassays; NS1 and IgM, on the samples received in our laboratory and the results of the combined tests have been compared individually with each test separately.

Materials and Methods:

One thousand samples were collected from the patients suspected of DV infection, attending the Bidar Institute of Medical Sciences and Teaching Hospital, Bidar. Demographic details of the patients were collected. Depending on the reporting time of the patients, sera comprised of both acute and early convalescent phases. Samples were divided into three groups I, II, III.

Group I: consisted of 1000 samples obtained from suspected cases of DF, classified as undifferentiated fever/ Dengue fever (DF)/ DHF (WHO classification). Adult samples from Out-Patients Department (OPD) and In-Patients Department (IPD) and paediatric IPD patients were included in this group. Single blood sample was screened by NS1 Ag assay and MAC ELISA and then compared.

Group II: consisted of 30 samples obtained from paediatric OPD patients classified as undifferentiated fever/ Dengue fever (DF). Acute phase sera (presenting within 7 days of fever) from this group were screened only by NS1 Ag assay, to evaluate the cost effectiveness of this assay in the acute phase. Since, this group patient attended OPD at regular intervals; paired samples of the patients who were negative by NS1 Ag assay were screened for antibodies in the early convalescent phase.

Group III: consisted of 40 samples, 25 were obtained from patients with fever due to known etiology other than dengue (enteric fever [18], bacterial meningitis [3], UTI [4]) and 15 were from healthy blood donors. All these 40 were screened by both the assays.

The samples were screened for the presence of dengue specific IgM antibodies by Mac ELISA, using a kit prepared by National Institute of Virology, Pune, India, (as an integral part of National Vector Borne Disease Control Programme), strictly following the manufacturer's protocol [8]. NS1 Ag was detected in the sera by Pan Bio NS1 Ag ELISA kit. Haematological parameters (leukocyte count, Hb and platelet count) of dengue positive patients were also observed and correlated. Fisher's exact test was used to find out the P values.

Results:

In Group I, of the 1000 samples, 462 (46.2%) were positive either for NS1 or IgM antibody or both. 247 (24.7%) samples were positive for NS1 Ag and 184 (18.4%) were positive for IgM antibody including those that were positive by both (Table 1). When single sample was tested by both the assays, the detection rate increased to 431.

Out of 247 NS1 Ag positive samples, 97.57% (241) were from acute phase sera and 2.42% (6) were from early convalescent phase. NS1 Ag detection rate decreased from 97.57% in acute phase sera to 2.42% in early convalescent sera ($P<0.0001$). All the 183 samples that were positive for NS1 Ag alone in this study, Group I belonged to acute phase sera (Table 2).

Table 1: Detection rate of NS1 Ag Assay and MAC-ELISA in Dengue Positive Samples of Group 1 (n=462)

NS 1 Ag	IgM Antibody		Total
	Negative	Positive	
Negative	95 (9.5%)*	120 (12%)	215 (21.5%)
Positive	183 (18.3%)	64 (6.4%)	247 (24.7%)
Total	278 (27.8%)	184 (18.4%)	462 (46.2%)

*(*95 samples were negative by both NS1 Ag assay and MAC ELISA but positive by IgG ELISA)*

Table 2: Positivity of NS1 and IgM in (1-7 days) and (8-14 Days)

Day post onset of illness	NS1	IgM
1 to 7 days	241/247(97.57%)	10/184 (5.43%)
8 to 14 days	06/24 (2.42%)	174/184 (94.56%)

IgM antibodies were positive in 10 (5.43%) cases during 1-7 days period of illness and 174 (94.56%) were positive during 8-14 days of illness. All the 120 samples that were only IgM positive belonged to convalescent phase (Table 2). IgM antibody detection rate increased from 5.43% to 94.56% (P<0.0001). NS1 Ag was positive in 241(97.57%) samples during 1-7 days period and 06(2.42%) in 8-14 days period

All the 30 samples from study Group II were screened only for NS1 Ag and 20 (66.66%) were positive. All these 20 patients were classified as DF. All had normal platelet count. The 10 NS1 Ag negative samples from patients with undifferentiated fever remained seronegative on subsequent screening at early convalescent stage. All the 40 samples from Group III were negative by both the assays.

Discussion:

The IgM capture ELISA is most commonly used in India due to its low cost and ease of handling. But here it is important to understand that, NS1 antigen detection assay has an advantage of detetecting infection very early, however it disappears early also and is of little use in the early convalescence phase when IgM is useful [7]. To confirm a case of acute dengue infection by serology, IgM sero-conversion or a fourfold increase of IgG antibody titer in paired sera must be demonstrated [9].

In this study, NS1 Ag was only positive in 183 (18.3%) samples and IgM was positive in 120 (12%) but both the assays performed on a single sample could statistically increase the number of positive to 431. NS1 antigen is detectable by most of the commercial kits in first 7 to 9 days of infection while IgM antibodies are detectable only after 8 to 14 days of infection, the reason why NS1 antigen capture ELISA could detect more cases

compared to IgM capture ELISA alone [9-11]. NS1 Ag detection decreased from 97.57% in acute phase sera to 2.42% in early convalescent sera and detection rate of IgM increased from 5.43% in acute phase sera to 94.56% in convalescent sera. Similar findings were seen in other studies along with an increase in sensitivity of detection when both the assays were used together in a single sample [12, 13].

Since it is difficult to judge accurately which post infection day the sample is being tested, it is ideal to use both assays, not to miss the diagnosis.

Conclusion:

Although, there is no specific treatment available for dengue, early diagnosis has a role in individual case management as well as planning and implementing control strategies. Of the two different tests used to diagnose dengue, NS1 antigen detection had the highest sensitivity in the early stages while IgM detection was more sensitive in the latter half of the illness. Because of these characteristics of the assays, it is recommended to use both assays simultaneously to ensure that the diagnosis is not missed.

References

1. Halstead SB. Dengue. *Lancet* 2007; 370:1644-52.
2. Gubler DJ. Melzer M. Impact of dengue/ dengue hemorrhagic fever on the developing world. *Adv virus Res* 1999; 53:35-70.
3. Satish N, Vijayakumar TS, Abraham P, Sridharan G. Dengue fever: its laboratory diagnosis, with special emphasis on IgM detection. *Dengue Bull* 2003; 116-21.
4. Bessof K, Delorey M, Sun W, Hunsperger E. Comparison of two commercially available dengue Virus (DENV) NS1 capture ELISA using a single clinical sample for diagnosis of acute DENV infection. *Clin Vaccine Immunol* 2008; 15:1513-8.
5. Dussart P, Labeau B, Lagathu G, Louis P, Nunes MRT, Rodrigues SG, *et al*. Evaluation of Enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. *Clin Vaccine Immunol* 2006; 13: 1185-90.
6. Vaughn DW, S Green, S Kalayanarooj *et al*. Dengue viremiaiter, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 2000; 181(1): 2-9.
7. Andries AC, Duong V, Nagan C, Ong S, Huy R, Sroin KK *et al*. Field evaluation and impact on clinical management of a rapid diagnostic kit that detects dengue NS1, IgM and IgG. *PLoS Negl Trop Dis* 2012; 6(12): e1993.
8. Cecilia D, Kakade MB, Bhagat AB *et al*. Detection of dengue-4 virus in pune, western India after an absence of 30 years- its association with two severe cases, *Virol J* 2011; 8, article 46.
9. Tricou V, Vu HT, Quynh NV Nguyen C, Tran HT, Farrar J *et al*. comparison of two dengue NS1 rapid tests for sensitivity, specificity and relationship to viraemia and antibody responses. *BMC Infect Dis* 2010; 10:142.
10. Duong V. Ly S. Lom Try P, Tuiskunen A, Ong S, Chroeung N *et al*. clinical and virological factors influencing the performance of a NS1 antigne capture assay and potential use as a marker of dengue diasease severity. *PLoS Negl Trop Dis* 2011; 5(7): e1244.
11. Fry SR, Meyer M, Semple MG, Simmons CP, Sekaran SD, Huang JX *et al*. The diagnositic sensitivity of dengue rapid test assays in significantly enhanced by using a combined antigen and antibody testing approach. *PLoS Negl Trop Dis* 2011; 5(6): e1244.
12. Schiliing S, Ludolfs D, Le VA, Schmiz H. Laboratory diagnosis of primary and secondary dengue infections, *J Clin Virol* 2004; 31:179-84.
13. Ampaiwan C, Wathanee C, Viroj P, Kanchana T, Sarapee L, Sutee Y. The use of Nonstructural protein 1 antigen for the early diagnosis during the febrile stage in patients with dengue infection. *J Paed infect Dis*: 2008; 27: 43-8.

*Author for Correspondence: Mr. Pramod S. Manthalkar, Ph. D Scholar, BLDE University, Vijayapura, Karnataka
Email: pramodmanthalkar@gmail.com Cell: 09880032382

Annexure -V

Consent form

Title of Project: Serological and Molecular Characterization of Dengue virus in North Karnataka.

Name of the Participant:

Address:

The details of the study has been explained to me in my language, and I have understood the study and given chance to ask the questions. My participation in this study is voluntary and free to withdrawn any time, without giving any reason. Without affecting the medical care being given by the hospital. I fully consent to participate in the above study.

Signature/ thumb impression: Date:

In Minor patients

Parents/ patients assistant signature/ Thumb impression: Date:

Name of the child: Date:

Signature of Investigator: Date:

IAMM-KC's

MICRO - KAR 2019

XXIII Annual Conference
8th & 9th Feb 2019

A Commitment to

Organized by
Department of Microbiology

Sri Dharmasthala
Manjunatheswara University

Certificate of Participation

This is to certify that

Mr./Ms. Pramod S. Mattikalkar

has participated as a Delegate in the Conference held on 8th & 9th Feb 2019.

~~He/She has Chaired a Session / Judged an Academic~~

~~Presentation Session~~ / Presented a Paper (Oral / ~~Poster~~).

Dr Praveen C Shetty
Organising Secretary
MICRO-KAR 2019

Dr Ajantha G. S.
President
IAMM-KC 2018-19

Dr S. K. Joshi
Principal
SDMCMSH, Dharwad

MANIPAL ACADEMY of HIGHER EDUCATION

McGill | GLOBAL HEALTH PROGRAMS | PROGRAMMES DE SANTÉ MONDIALE

3rd Manipal International Infectious Diseases Conference 2019

Theme: Tropical Infections and Global Health

CERTIFICATE

This is to certify that

Dr/Mr/Ms Pramod S M

has presented ~~PAPER/POSTER~~ titled

Demographic and Clinical Profile of Patients Infected with Dengue Virus serotypes 1, 2 and 3 in North Karnataka.

in 3rd Manipal International Infectious Diseases Conference held on 10th & 11th August, 2019 in Manipal

organized by

Manipal Center for Infectious Diseases (MAC ID), PSPH, MAHE, Manipal

in association with

McGill Global Health Programs, Canada & Department of Medicine, Kasturba Medical College, MAHE, Manipal

Prof Kavitha Saravu	Prof Helmut Brand	Prof Raviraja V Acharya	Prof Madhukar Pai
Joint Coordinator MAC ID Professor of Medicine Kasturba Medical College, MAHE, Manipal	Director Prasanna School of Public Health MAHE, Manipal	HOD, Department of Medicine Kasturba Medical College MAHE, Manipal	Joint Coordinator MAC ID Director, McGill Global Health Programs Director, McGill International TB Center McGill University, Canada