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MOLECULAR CHARACTERIZATION OF CHIKUNGUNYA AND DENGUE VIRUS AND THEIR ASSOCIATION WITH LIVER AND RENAL PROFILES

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SHRI GURU RAM RAI INSTITUTE OF MEDICAL AND HEALTH SCIENCES

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DOCTOR OF MEDICINE

(BIOCHEMISTRY)

BY

DR. ABHINAV MANISH

Under The Guidance of

Dr Tariq Masood M.D.,

Prof. & Head of Deptt. of Biochemistry

S.G.R.R. UNIVERSITY, DEHRADUN

UTTARAKHAND

DEPARTMENT OF BIOCHEMISTRY

SHRI GURU RAM RAI INSTITUTE OF MEDICAL & HEALTH SCIENCES

CERTIFICATE

This is to certify that the dissertation entitled "**Molecular characterization of chikungunya and dengue virus and their association with liver and renal profiles**" by **Dr. Abhinav Manish**, for the award of **Doctor Of Medicine (Biochemistry)** from S.G.R.R University Dehradun (Uttarakhand) is a piece of original work carried out under my supervision and guidance. The matter embodied in this dissertation has not been submitted for the award of degree in any other university. His work of conduct during the period was very good.

Dr. Tariq Masood
Professor & Head
Department of Biochemistry
SGRRIM&HS, Patel Nagar,
Dehradun.

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RECOMMENDATION OF CHIEF SUPERVISOR

Dr. TARIQ MASOOD

M.D. (Biochemistry)
Professor & Head
Shri Guru Ram Rai Institute of Medical & Health Sciences
Patel Nagar, Dehradun.

RECOMMENDATION OF CO SUPERVISORS

Dr Amit Varma

Professor & Head,

Department of Medicine,

SGRRIMHS, Dehradun.

Dr. Narotam Sharma

Biotechnology Scientist,

Central Molecular Research Laboratory,

Department of Biochemistry,

SGRRIHMS, Dehradun.

Dr. Rajeev S. Kushwaha

Associate professor,

Department of Biochemistry,

SGRRIHMS, Dehradun.

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ABBREVIATION

LFT	Liver function test
KFT	Kidney function test
DENV	Dengue virus
CHIKV	Chikungunya virus
PCR	Polymerase chain reaction
RT-PCR	Real time polymerase chain reaction
AGE	Agarose gel electrophoresis
PP	Primers and probes
cT-value	Thermocycler value
DF	Dengue fever
CHIK	chikungunya
ALT	Alanine Transaminase
AST	Aspartate Transaminase
ALP	Alkaline phosphatase
GGT	Gamma glutamyl transpeptidase
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
WHO	World Health Organization
NVBDCP	National Vector Borne Disease Control Programme
CDC	Centre for Disease Control
IL	interleukins
TNF	Tumor necrosis factors
RNA	Ribonucleic Acid
c-DNA	Complementary Deoxyribonucleic Acid
BSC	Biosafety Cabinet
FDA	Food and drug administration
BMV	Brome Mosaic Virus
ELISA	Enzyme linked immunosorbent assay

INTRODUCTION

Dengue is a mosquito borne arbovirus infection endemic to most tropical and subtropical countries (1). Elevation of liver enzymes Aspartate Transaminase (AST) and Alanine Transaminase (ALT) is common in acute Dengue illness, occurring in 65-97% (2,3,4,5) of Dengue patients peaking during the convalescent period of illness (days7-10) (2,4,6). In Dengue endemic countries Dengue fever (DF) is important cause of the acute viral hepatitis (7). Dengue fever represents a severe flu like symptomatology which includes severe headache (retro-orbital), nausea, vomiting, muscle and joint pain (bone-break fever) and skin rash. According to WHO, the incidence of DF has increased 30 folds over last 50 years with approximately 50-100 million infections occurring annually in over 100 endemic countries, placing almost half of the world population at risk (1, 8).

Dengue virus comprises of four distinct serotypes of Dengue Virus DENV 1, DENV 2, DENV 3, DENV 4, belonging to genus Flavivirus, family Flaviviridae. Among them, Asian genotype of DENV 2 and DENV 3 are frequently associated with severe Dengue disease (9). The etiology of elevated aminotransferases level during Acute Dengue is unclear since, AST is expressed in heart, skeletal muscle, red blood cells, kidney, brain, and liver while ALT secreted primarily by liver (10, 11) so raised Aminotransferases are not entirely due to liver.

Chikungunya virus (CHIKV) infection is a self limiting illness characterized by fever, headache, weakness, rash and arthralgia. Some patients have prolonged weakness or arthralgia lasting several months (12). The causative agent is an Arbovirus, CHIKV a member of Alphavirus genus from the Togaviridae family (13). Maternal fetal transmission has been confirmed if the mother is acutely positive during childbirth (14). Mild to moderate elevation of the liver aminotransferases are also seen in Chikungunya infection (15). In Jan 2006 there was a very large epidemic in Reunion Island followed quickly by the one in India (16). Almost 1.3 million suspected cases were reported in India (17).

Both Dengue and Chikungunya spreads via same vector Aedes mosquito. Both infections have almost the same clinical picture so a chance of misdiagnosis is very frequent. It is important to diagnose the causative agent early because misdiagnosis of Dengue as Chikungunya risks delaying or disrupting Dengue specific intensive supportive treatment which can have a tenfold impact on likelihood of progression from Dengue Fever to severe disease (18). It also risks inappropriate prescription of arthralgia alleviating non steroidal anti inflammatory agents which could lead to severe bleeding in patients with thrombocytopenia (19). This study aims to determine the serological and molecular profiling of the DENV and molecular characterization of CHIKV along with a comparison of the Liver function tests and Renal function tests in Dengue and Chikungunya positive patients.

DENGUE DISEASE

"The pains which accompanied this fever were exquisitely severe in the head, back, and limbs. The pain is retro-orbital in nature, and many times it confined to eyeballs only. In some people, the pains were so acute in their backs and hips that they could not lie in bed. Some complains pain on little pressure to any art of body. From these circumstances, the disease was sometimes believed to be a rheumatism. But its more general name among all classes of people was Break-bone fever" (20).

DENGUE VIRUS

The etiologic agent of "break-bone fever" was found to be dengue virus (DENV). DENV is a flavivirus of the family Flaviviridae. Other flavi-viruses in the same genus include Japanese encephalitis, yellow fever, West Nile, and tick borne encephalitis viruses. Dengue viruses are single-stranded positive-sense RNA viruses. The DENV genome is 11kb in length and encodes three structural and seven nonstructural proteins (21). DENV has four different serotypes: DENV1, DENV2, DENV3, and DENV4. Infection with one serotype provides lifelong immunity to the infecting serotype only but has been associated with increased risk of severe dengue illness upon secondary infection with a different serotype.

It is debatable if one serotype is more infectious or causes a more severe infection compared to another. Some studies have suggested there are differences in the pathophysiology of the different dengue serotypes, but currently no one serotype is considered more dangerous than another (22,23,24).

DENGUE ILLNESS

Dengue viruses are transmitted through the bite of an infected mosquito, usually Aedes aegypti or Aedes albopictus (25).

Once a susceptible host is infected, symptoms of dengue infection may occur and usually appear after an incubation period typically between 4 and 7 days, with a range from 3 to 14 days (26). Dengue illness can range from an uncomplicated febrile illness, as seen in most dengue fever (DF) cases, to a more severe illness with bleeding tendency, thrombocytopenia, and plasma leakage as seen in dengue hemorrhagic fever (DHF). DF and DHF are emerging infectious diseases that are endemic in tropical and subtropical areas (27, 28, 29).

Patients with confirmed dengue are classified as having DF if fever and any two of the following are present: headache, myalgia, arthralgia, rash, hemorrhagic manifestations, and leucopenia (30). Patients are classified as having DHF according to World Health Organization (WHO) guidelines based on the presence

of all four of the following four signs: fever, thrombocytopenia (platelet count <100,000/µL), bleeding tendency (positive tourniquet test or spontaneous bleeding), and evidence of plasma leakage (evidence of pleural effusion, ascites or ≥20% haemoconcentration) (29); however, these findings may not appear until patients are already critically ill.

DHF is categorized by severity into four grades (29). A diagnosis of DHF grades 3 and 4, termed dengue shock syndrome (DSS) includes all DHF criteria with the addition of circulatory failure. There is not a reliable definition of what constitutes a severe dengue illness and much controversy surrounds the WHO definition of DHF. This classification system is often impractical in the clinical setting, which leads to inconsistency of scientific data, such as under- or over reporting of severe dengue cases. Studies have shown that the WHO classification of DHF doesn't account for all severe dengue illnesses (31, 32, 33, 34, 35).

The term 'hemorrhagic' in DHF can lead to the false assumption that suspected dengue cases must have hemorrhage before being classified as: 1) dengue can be severe even without significant hemorrhage, 2) hemorrhage is not the sole criterion for DHF and 3) dengue can be severe without meeting all the criteria for DHF.

Dengue vector

The Aedes aegypti mosquito, the most important vector for transmission of DENV, is known to be a nervous feeder and will disrupt a feeding at the slightest movement and return later to continue feeding on the same individual or a different individual. Due to this type of feeding, the female Aedes aegypti can infect numerous individuals in a single blood meal spreading the virus to each person it feeds on. They are indoor mosquitoes and prefer to feed inside a residence, making control efforts more cumbersome due to the inability to effectively reach breeding sites with spraying of insecticides. Despite major efforts from the Pan American Health Organization and the CDC to prevent and control dengue, such strategies have proven to be poorly implemented and mostly ineffective (36). Community-wide participation and active involvement in prevention is needed to sustain any mosquito control effort. In a survey of knowledge, attitudes, and practices in rural Thailand, a negative association was observed between respondent's knowledge of mosquito development sites and the number of unprotected containers; however, preventative practices were only carried out after already having mosquito infestation (37).

HISTORY OF DENGUE AND CURRENT MAGNITUDE

The first known report of symptoms similar to dengue-like illness was in China 265 to 420 AD; however, the first dengue virus was not isolated until the 1940s

during World War II (38). There has been an increased occurrence of dengue illness in the 20th century which has been attributed to poor vector control, rapid urbanization, and increased globalization (39).

DHF first appeared in Asia in the 1950s and the first major epidemic of DHF/DSS in the America was caused by the introduction of DENV2 shortly after a large DENV1 outbreak in Cuba in 1981. The first cases of DHF in the Americas were due to secondary DENV2 infections that followed a DENV1 outbreak in 1977 (40, 41).

Dengue infection continues into the 21st century; recent estimates are that 3.6 billion people (55% of the global population) are at risk for dengue infection and 500 million dengue virus (DENV) infections occur annually, 2.1 million of which are severe dengue illnesses with ~21,000 deaths (42).

Moreover, industrialized nations, such as the United States and European countries, are at risk of dengue outbreaks, because it is the most common systemic febrile illness among American and European travelers returning from Southeast Asia, the Caribbean, and South America (43). Additionally, recent outbreaks of dengue fever have occurred in south Texas and Hawaii (44, 45).

ECONOMIC IMPACT OF DENGUE

The economic impact of dengue in developing countries is substantial and has been associated with higher costs and longer disease duration when compared to non-dengue febrile illnesses (46, 47, 48).

As with most severe illnesses, individuals and families are impacted by lost wages from missed work, costs of seeking care, costs of treatment, missed school, and extended effects of recovery. For families who are already in the low socioeconomic bracket, a severe infectious illness could be detrimental both physically and economically. Clark et al estimated that an amount equal to approximately 82% of the average Thai family's monthly household income is lost for each household member with dengue infection (47). Although healthcare treatment for children in Thailand is paid for by the government under the "30-baht to cure every disease project" (Dr. Praon Sup radish, personal communication), Clark et al also found that most families first sought care at a private clinic that was not government funded (47).

Transportation costs for seeking care have also been shown to substantially impact the economic burden of disease in Thailand (46).

TREATMENT OF DENGUE ILLNESS

Recently in May 2019 FDA approved a live, attenuated vaccine for Dengue illness namely DENGVAXIA. This vaccine prevents against all four dengue serotypes in people ages 9 through 16 who has laboratory confirmed previous dengue infection and who live in endemic areas. It is licensed to use in 16 countries among European and Asian countries.

The standard treatment for patients with suspected dengue is supportive care consisting of oral rehydration therapy, bed rest, paracetamol (to reduce fever), and avoidance of aspirin.

In practice, patients suspected to have DF or DHF are usually treated the same up to defervescence (initial febrile phase is subsiding); they are sometimes hospitalized and their condition is closely monitored with routine laboratory tests and maintenance of fluid intake. Around the time of defervescence, usually within 24 hours after defervescence, patients with DHF will develop severe symptoms and may become severely ill often with decreases in platelet count, hemorrhage, and signs of plasma leakage. However, many DF cases are considered mild and may not require hospitalization but are often hospitalized until 24 hours after defervescence to ensure that the characteristics of DHF do not manifest. Currently, there are no early diagnostic/prognostic tools available to distinguish dengue from

OFI or DF from DHF or severe dengue from non-severe dengue. If such tools were developed, they could potentially impact clinical practice in many ways, including:

1) decreasing the number of un-necessary hospitalizations, 2) improving utilization of limited hospital resources to treat more severely ill patients, 3) improving outcomes of severely ill patients by getting them the care they need earlier, and 4) improving the capability of physicians in developing or rural areas to make a more accurate early diagnosis with limited resources.

DISTINGUISHING DENGUE FROM OTHER FEBRILE ILLNESSES

Differences in clinical and laboratory features between dengue and other febrile illnesses have been reported; however, published studies differ in terms of duration of symptoms, age of patients, and quality of the study, which could impact the clinical applicability of their findings (49).

CHIKUGUNYA DISEASE

Chikungunya is a debilitating non-fatal viral illness caused by Chikungunya virus. The disease reemerged in the country after a gap of three decades. In India a major epidemic of Chikungunya fever was reported during 60s & 70s. Symptoms of Chikungunya fever are most often clinically indistinguishable from those observed in dengue fever. It is characterized by fever with severe joint pain (arthralgia) and

rash. Chikungunya outbreaks typically result in large number of cases but deaths are rarely encountered. Joint pains sometimes persist for a long time even after the disease is cured. Resurgence of chikungunya has been attributed to various factors including globalization, increase in the mosquito population, loss of herd immunity and the mutation A226V in the E1 gene causing a significant increase in CHIKV infectivity for Ae. albopictus (50)

CHIKUNGUNYA ILLNESS

Symptoms generally start 4–7 days after the mosquito bite. The acute phase is characterized by painful polyarthralgia, high fever, asthenia, headache, vomiting, rash and myalgia. In the chronic phase, incapacitating arthralgia persists for months. Neurological syndromes included encephalitis, encephalopathy and myelopathy or myeloneuropathy. Non-neurological systemic syndromes included renal, hepatic, respiratory, cardiac and hematological manifestations together with atypical manifestations including lymphadenopathy, oral ulcers and encephalitis (51, 52). Optical abnormalities have also been associated with CHIKV infections (53, 54). Vertical transmission of CHIKV from mother to child has been documented (55).

CHIKUNGUNYA VECTOR

This disease is transmitted by Aedes mosquito. Both Ae. aegypti and Ae. albopictus can transmit the disease. Humans are considered to be the major source or reservoir of Chikungunya virus. Therefore, the mosquitoes usually transmit the disease by biting infected persons and then biting others. The infected person cannot spread the infection directly to other person (i.e. it is not contagious disease).

HISTORY OF CHIKUNGUNYA AND CURRENT MAGNITUDE

The first recorded chikungunya outbreak was in Kolkata in 1963. This was followed by epidemics in Tamil Nadu, Andhra Pradesh and Maharashtra in 1964–65 and in Barsi in 1973 (56). CHIKV then seems to have disappeared from India. The virus re-emerged in 2006 after a gap of 32 years and caused an explosive outbreak affecting 13 states. The states first affected were Andhra Pradesh, Karnataka, Maharashtra, Madhya Pradesh, Tamil Nadu, Gujarat and Kerala. All ages and both sexes were affected. The virus isolates belonged to the African genotype different from the viruses circulating in 1963–1973, which belonged to the Asian genotype.12,23 The A226V shift in the E1 protein that was detected with progression of the epidemic in Reunion Island was absent in all of the Indian isolates. The A226V mutation was found to occur only in the 2007 isolate from India. (57)

TREATMENT

There is neither any vaccine nor drugs available to cure the Chikungunya infection. Supportive therapy that helps to ease symptoms, such as administration of non-steroidal anti-inflammatory drugs and getting plenty of rest are found to be beneficial.

CASE DEFINITIONS

Dengue and chikungunya cases were classified according to WHO (58, 59). In brief, probable dengue was defined as participants with fever and any two of the following criteria: nausea, vomiting, rash, pains, tourniquet test positive, leucopenia and/or a positive IgM ELISA test. Probable chikungunya: a patient meeting both the clinical (a characteristic triad of fever, rash and joint manifestations plus the following: nausea, vomiting, headache and/or an IgM positive test by ELISA) and epidemiological criteria (residing or having visited epidemic areas). Confirmed acute dengue or chikungunya was defined as a positive PCR result. Prior exposure was defined as presence of anti-dengue or anti-chikungunya IgG or IgM in serum.

AIMS AND OBJECTIVES

Serological and molecular profiling of Dengue and molecular characterization of Chikungunya virus in clinical isolates.

OBJECTIVES

1. To determine the various serotypes of the Dengue virus. DENV1 DENV2 DENV3 DENV4.

2. To perform molecular characterization of Chikungunya virus.

3. To establish a correlation of Dengue and Chikungunya viral diseases with respect to liver enzymes.

4. To establish a correlation of Dengue and Chikungunya viral diseases with respect to kidney function.

REVIEW OF LITERATUFRE

DENGUE & CHIKUNGUNYA

Mosquitoes have survived million years or more. Mosquitoes in addition to being a buzzing and biting annoyance are the vectors of life threatening zoonotic diseases. A total of 34 genera and 3500 species of mosquitoes are known out of which three genera Anopheles, Aedes and Culex, are the primary vector of important viral pathogens. (60)

Table (1) Vectors and Diseases associated.

Mosquitoes: (vectors)	**Diseases**
Aedes aegypti	Dengue, yellow fever, chikungunya, Zika virus
Aedes albopictus	Chikungunya, dengue, West Nile virus
Culex quinquefasciatus	Lymphatic filariasis
Anopheles (more than 60 known species can transmit diseases)	Malaria, lymphatic filariasis (in Africa)
Haemagogus	Yellow fever

Mosquito-borne diseases cause significant human health problems, and their incidence has increased significantly within last two decades. Estimates from the World Health Organization (WHO) indicates that, around the developing nations three mosquito born diseases Malaria, Filaria and Dengue are leading cause of morbidity and mortality. Malaria, Filaria, Dengue fever, Chikungunya, Yellow fever, and various forms of viral encephalitis transmitted by mosquitoes, share a major fraction of the global infectious disease burden. (61)

Dengue- an Arbovirus and Chikungunya- an Alphavirus, transmitted by Aedes mosquitoes (figure.1), are a cause of great concern to public health. Both viruses are known to cause acute febrile illness with almost identical symptoms in the early phase of infection, although the clinical profiles differ as the infection progresses. Every year, thousands of individuals are affected and contribute to the burden of health care. Presently, about 50% of the world's population is at risk and there are 50–100 million cases every year. An estimated 500,000 people with severe dengue require hospitalization each year and about 2.5% of those affected die (World Health Organization). (61, 62)

Dengue virus belongs to the family Flaviviridae and genus flavivirus. It is spherical in shape and size of about 48 to 50nm in diameter surrounded by lipid envelope contains electron dense core of about 30nm. Envelope (E) and membrane (M)

proteins are present on the surface of the viral particles. The nucleocapsid contains the capsid (C) protein and genomic RNA. (63)

Genome is a single stranded positive-sense RNA and 10.7 kb in size. It has genes which encodes for ten proteins. Three structural proteins: the capsid (C), envelope (E), and membrane (M) proteins and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. (63)

In India, dengue is widespread and endemic in most major cities. Dengue outbreaks have continued since the 1950s but severity of disease has increased in the last two decades. According to National Vector Borne Disease Control Programme (NVBDCP) recently millions dengue cases were reported from all over India. Almost 31 states of India including Union Territories are found to be affected with dengue virus. (64)

Figure (1)- Aedes Mosquitoes

There are four serotypes of dengue virus such as DENV-1, DENV-2, DENV-3 and DENV-4. DENV-1 formed into five clades designated as genotype I (Southeast Asia, China and East Africa), genotype II (Thailand), genotype III (Malaysia), genotype IV (South Pacific) and genotype V (America, Africa)(65).

Analyses of E gene sequences have revealed that DENV -1 and DENV-2 can be divided into five and six genotypes, respectively, and DENV-3 and DENV-4 into four genotypes, including the sylvatic lineages found in non-human primates (66). Emergence of DENV-5 was announced in 2013 which was isolated from a 37-year-old patient in Malaysia. Phylogenetic analysis showed it was distinct from DENV-4 and some similarity with DENV-2 and the disease caused by DENV-5 is mild (67).

All the Indian DENV-1 isolates belong to the American African genotype and distributed into four lineages, India I, II, III and the Africa lineage. Of these, India III is the oldest and extinct lineage; the Afro-India is a transient lineage while India I am imported from Singapore and India II, evolving in situ, are the circulating lineages. The American genotype of DENV-2 which circulated predominantly in India during early-1971 subsequently replaced by the Cosmopolitan genotype (68).

PATHOGENESIS OF DENGUE

Dengue viruses can cause nonspecific febrile illness (pantomorphic) and hemorrhagic fever (visceromorphic) in human host. Clinical spectrum of dengue virus includes dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). When pre-existing dengue antibodies present in the host due to primary (first) DENV infection, antibody dependent enhancement of infection occurs by binding of pre-existing antibody binding to an infecting DENV particle during subsequent infection with different dengue serotype. Partial neutralization of DENV by antibodies during subsequent infection with a different serotype of dengue enhances the attachment of Ab-virus complex to receptors called Fcγ receptors (FcγR) on circulating monocytes. This complex helps the virus to infect monocyte more efficiently and it leads to rapid replication of the virus and development of severe dengue (69).

DENGUE FEVER

The disease begins abruptly, after a 2 - to 14-day incubation period, with high fever, headache, retroocular pain, lumbosacral pain, conjunctival congestion, and/or facial flushing with biphasic fever. Generalized myalgia and arthralgia are reported. Bradycardia associated with fever is seen. Maculopapular rash appears first on the trunk and then spreads centripetally to the face and limbs. A second

phase of fever called saddleback fever is seen in some patients. Generalized lymphodenopathy, cutaneous hyperesthesia, and altered (metallic) taste sensation may accompany this stage of the disease. The peripheral white blood cell count is depressed with an absolute granulocytopenia, and the platelet count may fall to less than 100,000 per mm3. Haemorrhagic phenomena may include petechiae, epistaxis, intestinal bleeding, menorrhagia, and a positive tourniquet test. Unusually myocarditis, various neurologic disorders, including encephalopathy and peripheral mononeuropathy, polyneuritis, and Bell's palsy are associated with Dengue fever (70).

DENGUE HEMORRHAGIC FEVER AND DENGUE SHOCK SYNDROME

DENVs being a viscerotropic virus can cause a range of severe disease, including fulminant hepatitis with massive hemorrhage, vascular leak with hypovolemia, organ failure, cardiomyopathy and encephalopathy. The severe form of dengue is a vascular leak syndrome known as DHF. Onset is generally sudden, with fever of 2 to 7 days duration and a variety of nonspecific signs and symptoms. Hemorrhagic manifestations are common, and the tourniquet test may be diagnostically helpful though false positivity is high. Scattered petechiae, gastrointestinal hemorrhage, hematemesis and melena usually occur after prolonged shock (70).

DHF is classified into four grades of illness based on severity, Grade I is mild, scattered petechiae or a positive tourniquet test. Grade II is more severe with one or more hemorrhagic manifestations. Grade III is characterized by mild shock with signs of circulatory failure; the patient may be lethargic or restless and have cold extremities, clammy skin, a rapid but weak pulse and Grade IV is the most severe form of DSS and characterized by profound shock with undetectable pulse and blood pressure (WHO, 1997). Thrombocytopenia and haemoconcentration indicating plasma leakage are constant findings in DHF and DSS. A platelet count of less than 100,000 per mm3 is usually found between the third and eighth day of illness. Elevated liver enzymes are common. Pleural effusion or ascites may be detected by physical examination or radiography (71).

DENGUE IMMUNOPATHOGENESIS

Human cytotoxic factor (hCF) stimulates certain pathways that can shift Th1 response to Th2 response. Th1 cells secrete IFN-γ, IL-2, TNF-β which involve in cell mediated immunity, delayed type hypersensitivity, tissue injury. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 induces antibody production and involve in humoral immunity. Cross regulation of Th1 and Th2 are mediated by IL-10 and IFN-γ. Th1 is linked to the disease recovery and Th2 is linked to severe disease progression. Th1 is raised in dengue fever and Th2 is elevated in severe dengue. Non -Th1, Th2

includes IL-1 and TNF-α, plays a role in the development of acute inflammatory response (72).

DENV infected endothelial cells produce IL-6, IL-8. IL-8 along with ICAM-1 enhances the attachment of polymorphonuclear cells lead to the release of thrombomodulin and vasopermeability. IL-8 was raised in patients with dengue infections (73).

IL-6 levels are elevated in DHF and less in DF. Levels of TNF-α are less in DF and DHF. Levels of IFN-γ, IL-6, IL-8 are elevated in dengue cases. IL-6/IL-8 is elevated in DHF than DF. The association of IFN-γ with ALT levels were significant than with thrombocytopenia. During the analysis of cytokine response, a time trend was noted in IFN-γ and IL-8 response. Decreasing trend was observed in IFN-γ and increasing trend in IL-8 in DHF cases (74).

CHIKUNGUNYA

Chikungunya Virus (CHIKV) a member of alpha virus genus belonged to Togaviridae family. The size of the virus is about 50 -70 nm and has an icosahedral like nucleocapsid surrounded by an envelope. The envelope is made up of lipid bi-layer which is derived from host plasma membrane. Envelope has two major virus encoded glycoproteins E1, E2 and a small peptide 6k1. Nucleocapsid consists of

single stranded positive sense RNA approximately 11.8 kb surrounded by multiple copies of capsid proteins with size of about 30 kDa (75).

Chikungunya outbreaks started in the 1960s and declined to sporadic cases until resurgence in 2006. Since 2003, there has been a resurgence of Chikungunya outbreaks in the islands of the Pacific Ocean, including Madagascar, The Comoros, Mauritius and Reunion Island (76).

Mosquitoes involved in the transmission of CHIKV vary based on the geographical regions and conditions of the ecosystem. Aedes Aegypti an urban vector is most common in Asia. Trend of resurgence of these diseases has been attributed to various factors including globalization, increase in the mosquito population and in case of Chikungunya particularly loss of herd immunity and the mutation A226V in the E1 gene causing a significant increase in CHIKV infectivity for Aedes albopictus (77).

CHIKV genome is positive-sense single-stranded RNA with 11805 nucleotides long. It has genes encoding for nonstructural proteins (NSP1, NSP2, NSP3, and NSP4) and structural proteins (C, E1, E2, E3; 6K). In CHIKV genome capping of 5' end is with a 7-methylguanosine and the 3'end is with poly A tail (78).

The non-structural protein (NSP) plays a role in transcription and replication of viral RNA. For viral RNA replication, NSP1 is a cytoplasmic capping enzyme.

The NSP2 proteins act as helicase and proteinase, and cleave NS to form individual proteins. The role of NSP3 in replication is not clear. NSP4 codes for RNA polymerase (78).

STRUCTURAL PROTEINS

Capsid, E3, E2, 6K and E1 are structural proteins which are synthesized as single polypeptide and further cleaved to individual proteins and these react with the host antibodies. Junction region promotes transcription of an intracellular sub-genomic 26s RNA. Untranslated region at the 5' end plays a key role in plus-strand RNA synthesis and the region at 3'end helps in proteins translation. The 6K is a constitutive membrane protein plays a key role in the processing of glycoprotein, permeabilization of membrane and budding of viral particles (79).

E2 PROTEIN

E2 protein involves in attachment by binding to the host cell receptor. The p62 precursor is processed by furin at the cell membrane prior to virion budding and it develops an E2 -E1 heterodimer. This heterodimer is unstable and dissociate at low pH. Apparently to avoid E1 fusion before its final export to cell surface, processing of p62 occurs at the last step. The C terminal region of E2 protein has ephemeral

transmembrane which upon n disruption results in change in orientation to the exterior cytoplasm. Critically 2 C-terminus is involved in budding by interacting with capsid proteins. The virus starts secreting the E2 protein and prevents the viral assembly that occurs in the membranes of the endoplasmic reticulum of the infected cell (80).

REPLICATION

Virus enters to the cell through receptor-mediated endocytosis and endosomal dependent fusion (81). Characteristic features of CHIKV supports it replication in both vertebrate host as well as in the invertebrate vector. Mainly, the virus replication is similar in both and little variation was observed in the release of virions in mosquitoes. Viruses were discharged from insect host cells via plasma membrane through exocytosis process (82).

The attachment of Alphavirus with different receptors of different types of cells in various host are not well established except for muscle cells where it is targeted to satellite cells. Autocatalytic cleavage of the budding structural proteins occurred due to protease activity of capsid proteins. After the self-cleavage, capsid associates with ribosomes instantly and the proteins binds to viral RNA within few minutes then assembles as icosahedric core particles rapidly. Transportation of virus into the cell is by endocytosis of clarithrin-coated vesicles. The reduction of

pH in the vesicle is essential for the E1 protein activation in the E1 -E2 complex. The complex is promoted by viral and endosomal membrane fusion, which results in the release of the nucleocapsid into the cytoplasm. The replication of CHIKV occurs in the cytoplasm (83).

The synthesised nucleocapsid eventually associates with cytoplasmic E2 at the cell membrane, leading to budding and formation of mature virions, which interacts with cellular receptors (84).

During infection, viral fusion process is initiated by E1 trimerization formed by involvement of E1 protein domain I and II. E1-E2 interactions are mediated by domain II during virus maturation and budding (85).

New virions attach to target cells, and after endocytosis their membrane fuses with the target cell membrane. This leads to the release of the nucleocapsid into the cytoplasm, followed by an uncoating event necessary for the genomic RNA to become accessible. The uncoating might be triggered by the interaction of capsid proteins with ribosomes. Binding of ribosomes would release the genomic RNA since the same region is genomic RNA-binding and ribosome-binding. E3 protein's function is unknown (86).

IMMUNOPATHOGENESIS

13 cytokines were studied in acute and convalescent sera obtained from CHIKV infected cases. Level of CXCL9/MIG, CCL2/MCP-1, IL-6 a CXCL-10/IP-10 were significantly elevated in acute phase when compared to the follow up samples. TNF-α, IL-1β, IL-5, IL-10, IL-12 and IFN-γ were low at initial acute phase and significantly elevated at later stage of the disease. Analysis based on the severity of the symptoms such as crippling joint pain mimicking rheumatoid arthritis associated with CXCL-9/MIG, CXCL10/IP-10 and IgG levels. Several studies have been carried out to better understand CHIKV disease, some studies targeted acute phase of illness, while others used both acute and convalescent sera (87).

Among rural population IFN-γ, IFN-β, IFN-α, CXCL10/IP10 and IL-1β was observed in early acute phase. Other cytokines such as TNF-α, MCP-1, IL-4, IL-6 and IL-10 was extreme in prolonged symptomatic phase and elevated levels were continued in recovered group. IL-4 a maker of Th2 response was highest in the sub-acute illness. Modest positive correlation was observed between myalgia and the pro inflammatory cytokine TNF - α. This study also reported that IFN-β and IFN-α were significantly raised in seronegative group whereas IL-4 and IL-10 elevated in seropositive group (88).

Diseases that mimic chikungunya Dengue is the principal mimic of CHIK fever West Nile and Zika present with similar clinical features of CHIKV. In India a major epidemic of Chikungunya fever was reported during the last millennium viz.; 1963 (Kolkata), 1965 (Pondicherry and Chennai in Tamil Nadu, Rajahmundry, Vishakhapatnam and Kakinada in Andhra Pradesh; Sagar in Madhya Pradesh; and Nagpur in Maharashtra and 1973, (Barsi in Maharashtra). Based on the data from NVBDCP, the number of cases reported in 2014 was about 16049 for Chikungunya. A226V mutation enhanced the virus to choose an alternate vector Aedes albopictus which had significance in the epidemiology (89).

Ae. Albopictus is a container breeder dark colored mosquito that can be identified by the white band on thoracic region and on its legs, and a silver-white pattern of scales on its body (Figure- Aedes mosquitoes). Due to this pattern it is also called “Asian tiger mosquito”. Ae. Albopictus is highly adapted for rural and suburban environment; dwell in tropical and subtropical regions all over the world, mainly between the latitudes of 35°N and 35°S where the winter temperature is no colder than 10°C. Widespread deforestation and uncontrolled urban development forced Ae. Albopictus to adapt to breed in manmade containers besides natural sites (90).

It breeds in both natural and manmade habitats including rock pools, leaf axils, tree holes, cut bamboo stumps, etc (Figure - breeding places). Ae. Albopictus have a complex life cycle that includes aquatic and terrestrial stages including egg, larvae,

pupae and adult stages (Figure - life cycle). Female mosquito ovipositor their eggs in the water holding substrates near the human settlements. Eggs hatched in to larvae, which are aquatic and feed on microorganisms found in the water. These larvae converted in to pupae and then adult mosquito emerged out from pupae. The adult mosquito is able to fly and is no longer aquatic. It has a terrestrial habitat and females take blood from human host. (90)

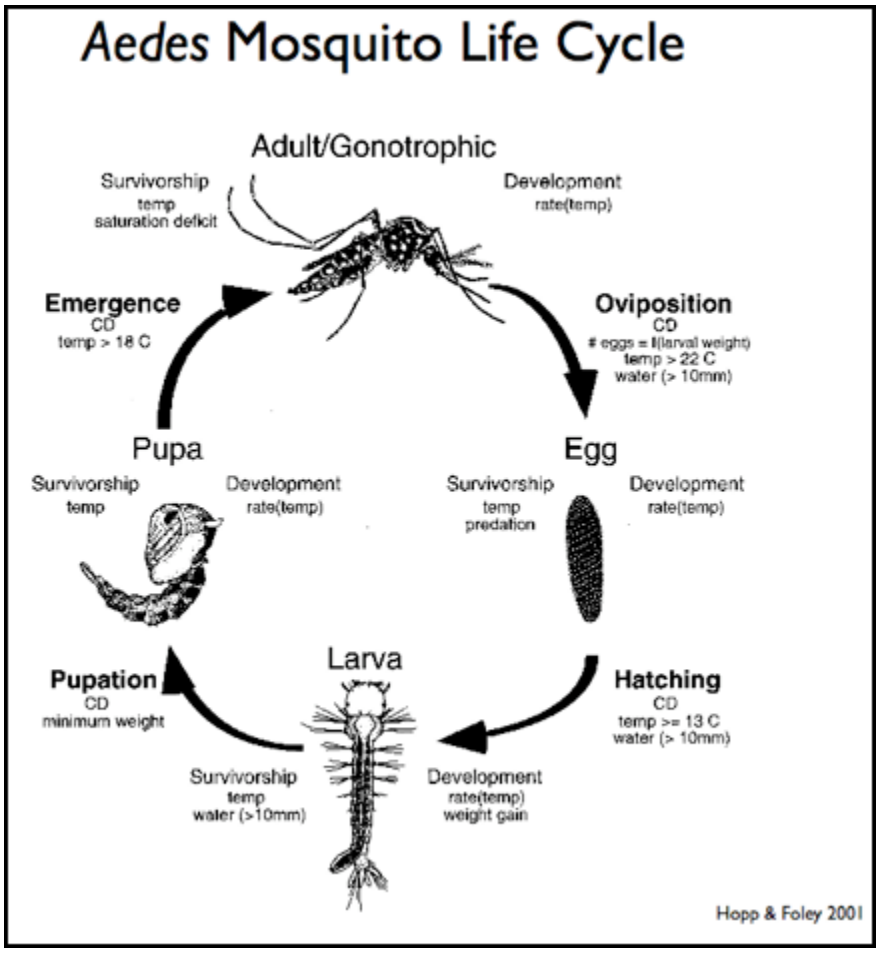


Figure (2) - Life cycle of Aedes

Figure (3)- Common Breeding sites of Aedes mosquito

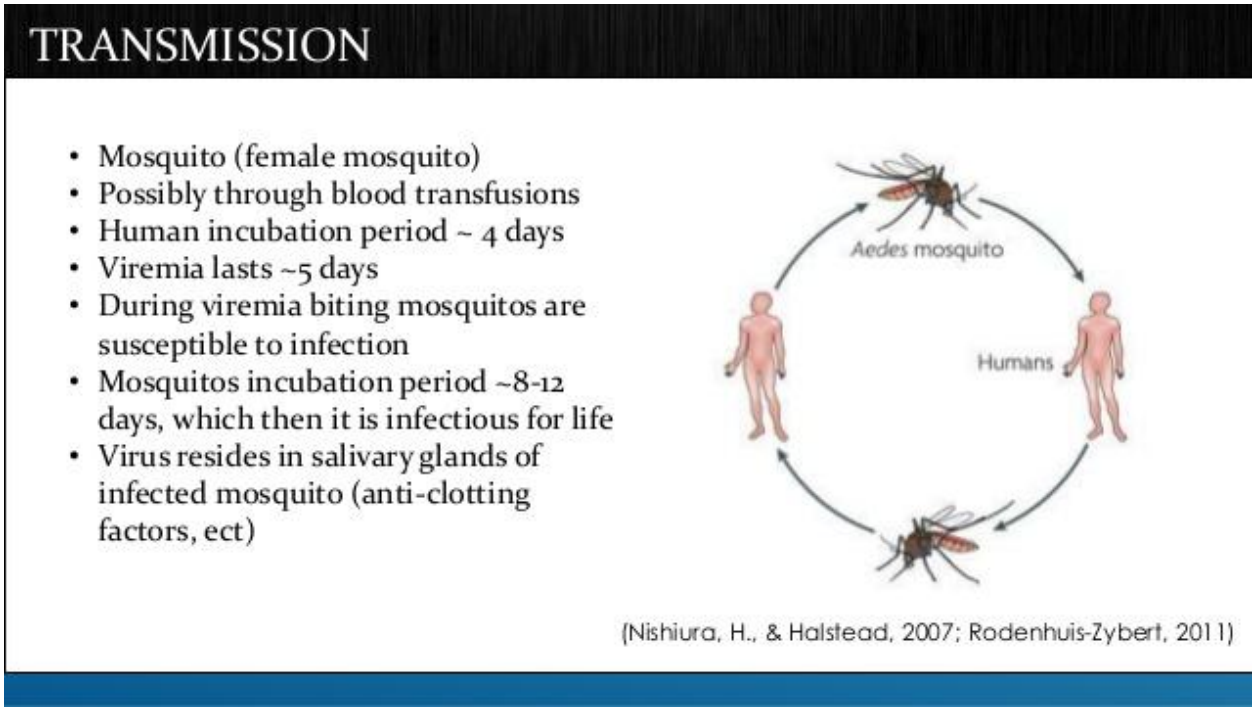


Figure (4)- Disease transmission (human - mosquito cycle)

DISEASE TRANSMISSION

Aedes is a daytime biting mosquito. Upon feeding on an infected host, the female mosquito becomes infected with the dengue/chikungunya virus. After certain time period the ingested virus enters the midgut epithelium, replicate in it and transferred to the salivary glands via the haemolymph. (Figure- disease transmission). An infected mosquito can later transmit that virus to healthy people by biting them. Following an incubation period in humans of 3-14 days (4-6 days average), there is often a sudden onset of the disease, with fever, headache, myalgia, loss of appetite, and a variety of nonspecific signs and symptoms, including nausea, vomiting and rash in case of dengue (Figures- sign and symptoms). This is the crucial period when the patient is most infective for the vector mosquito and contributes to maintaining the transmission cycle if the patient is not protected against vector mosquito bites. It has also been reported that Ae. Albopictus is capable of ovarian transmission of dengue virus from infected female mosquitoes to the next generation. (91)

FIGURE (5) Pathophysiology of Dengue infection endothelial cell activation & Coagulopathy

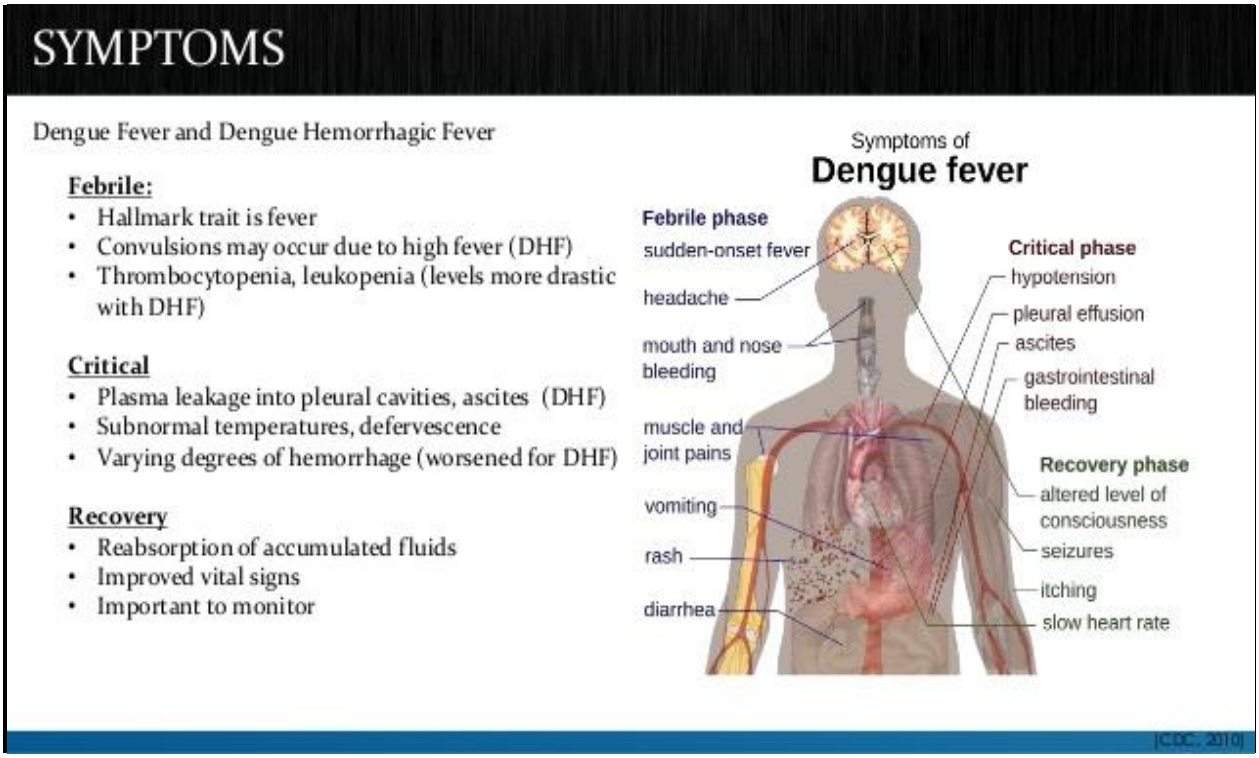


Figure (6)- Signs And Symptoms

Chikungunya (chik'-en-GUN-yah), also called chikungunya virus disease or chikungunya fever, is a viral illness that is spread by the bite of infected mosquitoes. The disease resembles dengue fever, and is characterized by severe, sometimes persistent, joint pain (arthiritis), as well as fever and rash. It is rarely life threatening. It usually starts suddenly with fever, chills, headache, nausea, vomiting, joint pain, and rash. (92)

In Swahili, "chikungunya" means "that which contorts or bends up". This refers to the contorted (or stooped) posture of patients who are afflicted with the severe joint pain (arthritis) which is the most common feature of the disease. Infection appears

to confer lasting immunity. The time between the bite of a mosquito carrying chikungunya virus and the start of symptoms ranges from 1 to 12 days. (92)

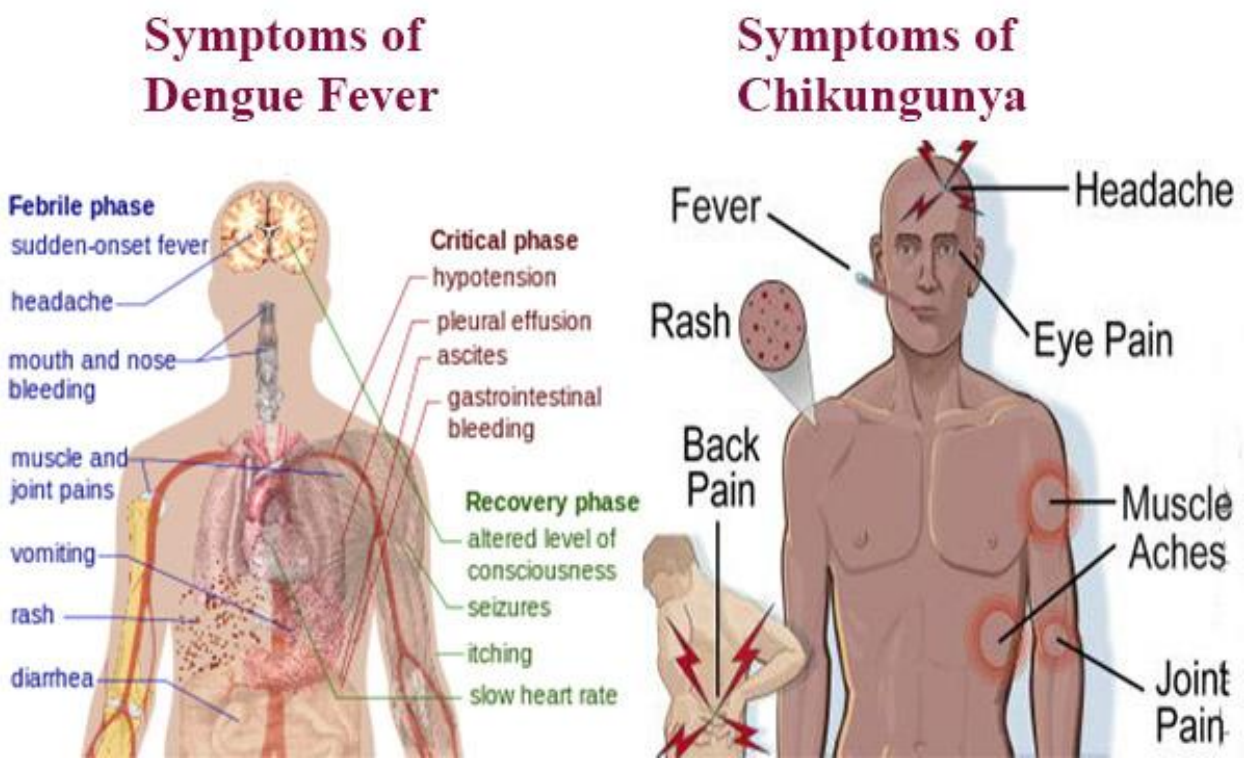


Figure (6) - Common Symptoms of dengue and chikungunya

DENGUE AND CHIKUNGUNYA CO-INFECTION

As dengue and chikungunya are transmitted by same species of Aedes mosquitoes, the frequency of confection is higher. The regions that are endemic for both the diseases will also increase the rate of co infection. (93)

The co-infection of DENV and CHIKV was reported from a patient who returned to Taiwan from Singapore. (94)

MOSQUITO CONTROL

Since no vaccine is available yet for the prevention of dengue/chikungunya infection and there are no specific drugs for the treatment. Hence the mosquito control is essential to prevent proliferation of these diseases. The major tool in mosquito control operation is the application of synthetic insecticides such as organochlorine and organophosphate compounds. But this has not been very successful due to human, technical, operational, ecological, and economic factors. The control of dengue/chikungunya and other vector-borne diseases in India is dependent mainly upon residual spraying of insecticides that interrupts their transmission. These include organochlorine (chlorinated hydrocarbon) insecticides such as DDT, hexachlorocyclohexane (HCH) benzene hexachloride (BHC), dieldrin, etc. Most of these insecticides have been banned because of their persistence in the environment and the vectors have developed resistance to them. The second category of insecticides used for the control of vector-borne diseases, the organophosphates (malathion, fenthion, pirimiphos-methyl, and temephos chlorpyrifos) do not persist in the environment but prolonged use has resulted in the development of resistance in some vector. Several other insecticides belong to different categories including propoxur, bendiocarb (carbamates), deltamethrin, cyfluthrin, and lambdacyhalothrin (pyrethroids) are used in the control of vector-borne diseases. But the continuous use of these chemicals has brought some

environmental threats including harmful effect on human health and other non-target populations, biological magnification through ecosystem, non biodegradable nature and increasing insecticide resistance on a global scale. Furthermore many mosquito species have developed resistance to various classes of insecticides. Ae. albopictus and Ae. aegypti have also been found resistant against malation, temephose, fenthion and other insecticides. (95)

MATERIAL AND METHODS

STUDY DESIGN

A prospective observational study will be conducted in the Department of Biochemistry in conjugation with Department of Medicine at Shri Mahant Indiresh (SMI) Hospital, Dehradun.

STUDY GROUP

It comprises of 100 confirmed cases of Dengue and 60 confirmed cases of Chikungunya.

SAMPLE COLLECTION

5 ml of blood will be collected in serum separation tubes (SST). Serum will be separated and tests will be performed according to WHO guidelines and as per manufacturer's instructions.

Various procedures and assays involved in the proposed study will be carried out in the Department of Biochemistry SGRR Institute of Medical and Health Science, Dehradun.

DENGUE

The blood samples of the patients suspected of Dengue will be collected from the Department of Medicine of SMI Hospital and received at the molecular lab in the Department of Biochemistry. Here we will perform Reverse Transcriptase method for preparation of complementary DNA (cDNA) from the viral RNA, further cDNA will be utilized by Real Time Polymerase Chain Reaction (RT-PCR) technique for qualitative determination of the DENV. By End Point PCR, positive cases of DENV will be further characterized for the detection of the different serotypes.

CHIKUNGUNYA

The blood samples of the patients suspected of Chikungunya will be collected from the Department of Medicine of SMI Hospital and received at the molecular lab in the Department of Biochemistry. Here we will perform Reverse Transcriptase method for preparation of cDNA from the viral RNA, further cDNA will be utilized by RT-PCR technique for qualitative determination of the CHIKV.

ESTIMATION OF LIVER FUNCTION TEST (LFT) AND RENAL FUNCTION TEST (RFT)

The biochemical markers of Liver Enzymes **AST** (96)**, ALT** (97)**, Alkaline Phosphatase (ALP)** (98)**, Gamma-GlutamylTtransferase (GGT)** (99) and of Renal profile **Blood urea** (100)**, Serum creatinine** (101)**, Uric acid** (102) will be

evaluated in the study group at Central Biochemistry lab of SMI Hospital using VITROS 5600 fully automated system.

STATISTICAL ANALYSIS

Data so obtained will be subjected to suitable statistical analysis using Microsoft Excel and SPSS software.

WORK AT CENTRAL MOLECULAR RESEARCH LAB

All the experiments were done at CMRL, Shri Guru Ram Rai Institute of Medical & Health Science (SGRRIM & HS), Patel Nagar Dehradun (U.K.). All the experimental work was performed in the Biosafety cabinet III [BSCIII] and all the procedures were done according to WHO and FDA guidelines.

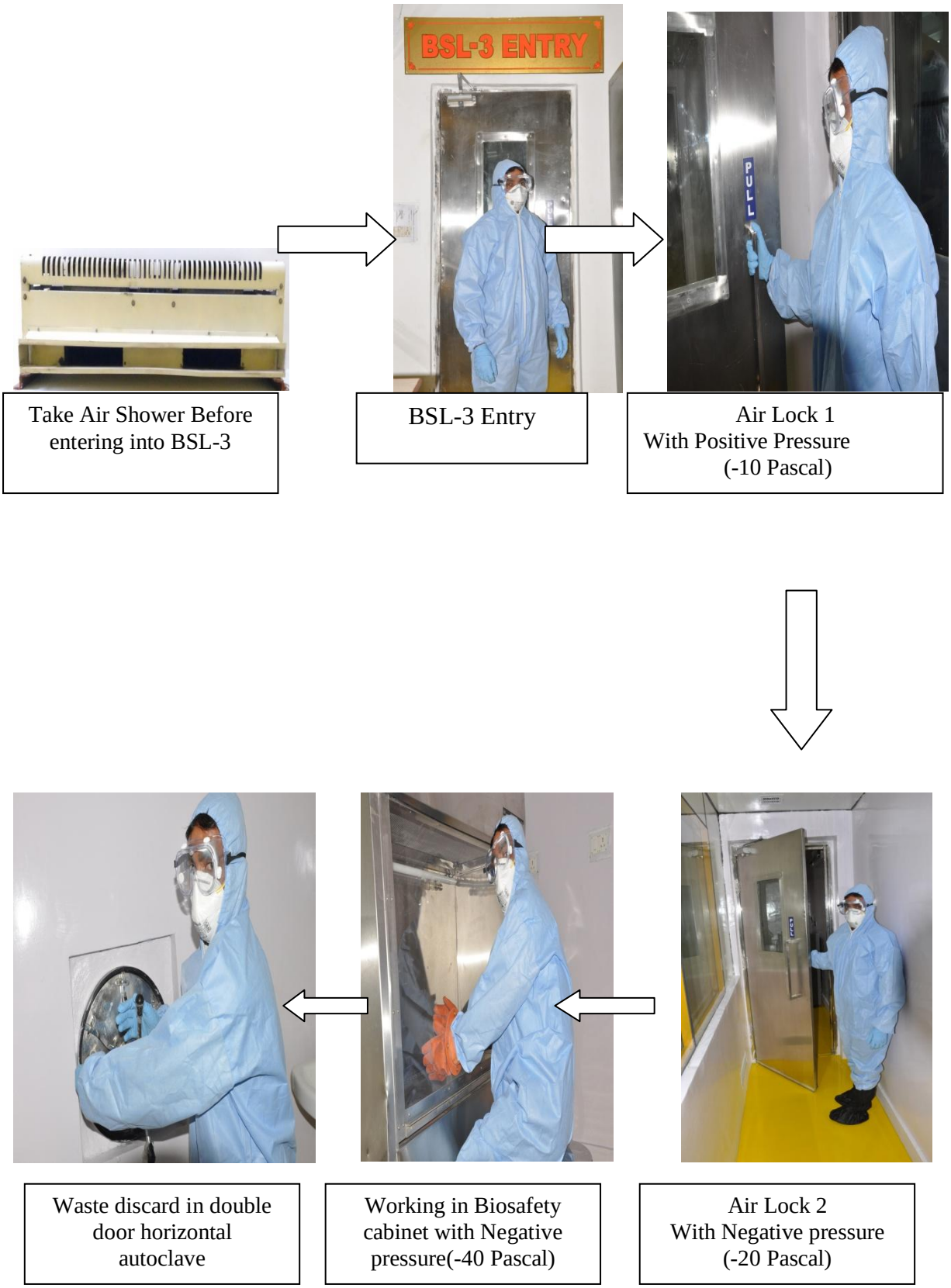


Figure (7) Entry of laboratory personal into Biosafety -3 laboratory.

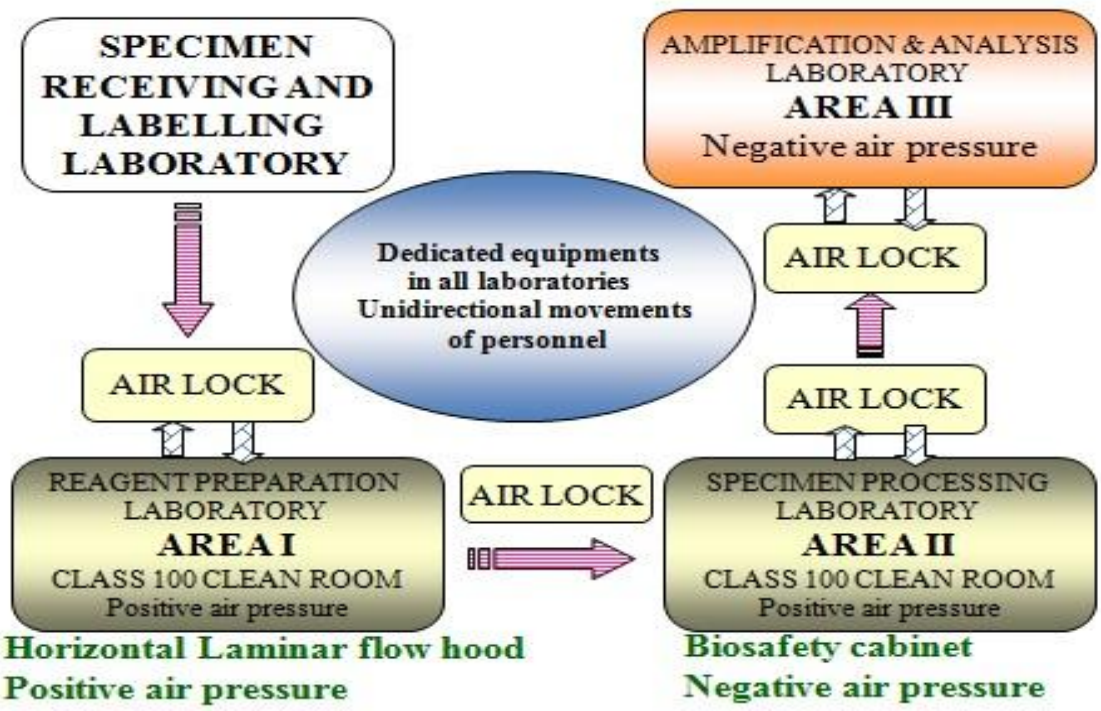


Figure (8) - A unidirectional workflow while performing pre-amplification, amplification and post amplification.

INSTRUMENTS UTILIZED

(a) Dry Bath

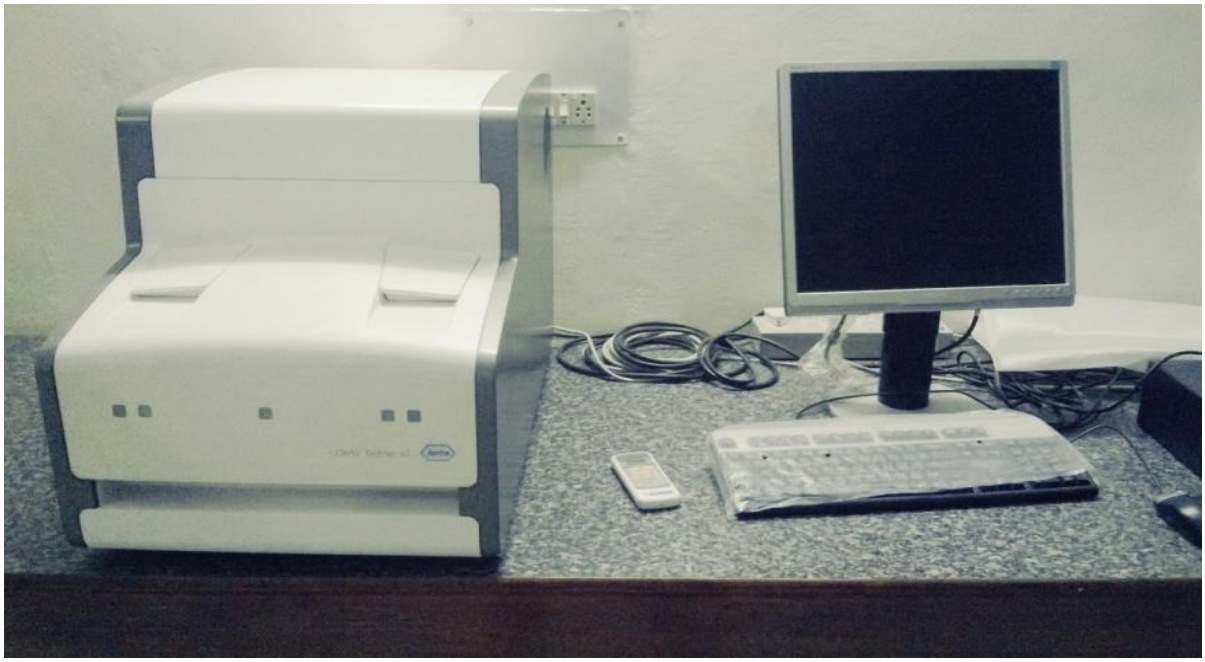
(b) Fig. Vortex

(c) PCR Workstation

(d) Cooling Centrifuge

(e) Gel Electrophoretic Unit

(f) Biosafety cabinet (class 2, A2)



(g) COBAS Taqman 48 Real Time PCR machine

(h) Rotor –Gene Q Real time PCR machine

(i) Spin win (j) Conventional PCR machine.

Figure (9)-Instruments used in CMRL

Table.2- Reagents required for the process:

1	LYS (Lysis buffer/Binding buffer)
2	CAR (Carrier RNA)
3	Absolute Ethanol
4	Washing Buffer 1
5	Washing Buffer 2
6	ELB (Elution Buffer)
7	gDNA removal mix
8	RNAse free water
9	Reverse Transcription Enzyme

10	Reverse Transcription Mix
11	Buffer
12	MgCl2
13	DNTPs
14	D1
15	D2
16	TS1- Primer for detection of dengue serotype
17	TS2-Primer for detection of dengue serotype
18	TS3- Primers for detection of different dengue serotype
19	TS4-Primer for detection of dengue serotype
20	Taq Polymerase
21	NFW

Table(3)- Reagents required for dengue profiling in RT-PCR.

	Contents	**FTD-43-32**	**FTD-43-64**
DG PP	Primer/Probe mix for Dengue virus and internal control	1 x 48 µl	2 x 48 µl
DG PC	Positive control containing plasmids for detection of	1 x 150µl	2 x 150 µl

	Dengue virus.		
NC	Negative control	1 x 2000µl	1 x 4000 µl
IC	Internal control	1 x 128 µl	2 x 128 µl
Enzyme	25x RT-PCR Enzyme mix (Fast-track master mix)	1 x 32 µl	2 x 32 µl
Buffer	2x RT-PCR Buffer(Fast-track master mix)	1 x 400 µl	2 x 400 µl

PP- primer and probe, IC- internal control, PC- positive control, NC- negative control.

COLLECTION OF SAMPLE FROM CONFIRMED DENGUE PATIENTS AND TRANSPORTATION TO CMRL LABORATORY

ELIZA confirmed blood samples of dengue patients were taken from SHI hospital and further processed at the CMRL, SGRRIM&HS for the Molecular and Serological profiling of Dengue virus.

Figure (10)- Shri Mahant Indiresh Hospital

Figure (11) Blood Samples from suspected cases of Dengue

SERUM/PLASMA SEPARATION FROM CLINICAL SAMPLE

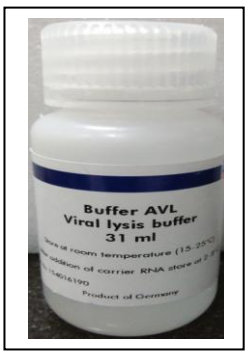
Whole blood samples were centrifuged at 10,000rpm for 10-15 minutes at 4°C. The serum was separated from blood & then subjected for nucleic acid extraction.

Figure(12)- Serum separation from blood samples

Molecular profiling of Dengue virus

(i)-Isolation of RNA by silica column method for Molecular detection of Dengue virus. by QIAGEN viral RNA mini kit.

RNA Isolation



560 µl LB + 5.4 µl carrier RNA + 140 µl serum sample

⇩

Vortex & Mixing

⇩

Hold it for 10 minutes at room temperature

⇩

Add 560 µl Absolute Ethanol

⇩

Vortex

⇩

Transfer 700 µl samples in silica column

⇩

Centrifuge the sample at 10,000rpm for 1 minute in cooling centrifuge (4ºC)

⇩

Add 500 µl Washing Buffer-1

⇩

Centrifuge the sample at 10,000rpm for 1 minute in cooling centrifuge (4ºC)

⇩

Decant the supernatant from the collection tube

⇩

Add 500 µl Washing Buffer-2

⇩

Centrifuge the sample at 10,000rpm for 1 minute in cooling centrifuge (4ºC)

⇩

Decant the supernatant from the collection tube

⇩

Dry washing at 10,000rpm for 1 minute

⇩

Centrifuge the sample at 13000rpm for 2 minutes at 4ºC

⇩

Transfer the silica column in fresh new labeled MCT

⇩

Add 60 µl Elution Buffer in mid of the silica column

⇩

Hold for 3-4 minutes at room temperature

⇩

Centrifuge the sample at 10,000rpm for 1 minute in cooling centrifuge (4 ºC)

⇩

RNA extracted and can be further used as template

Master mix preparation for Amplification and Detection of 3'untranslated region of Dengue virus by Rotor –Gene Q Real time PCR machine.

PRINCIPLE

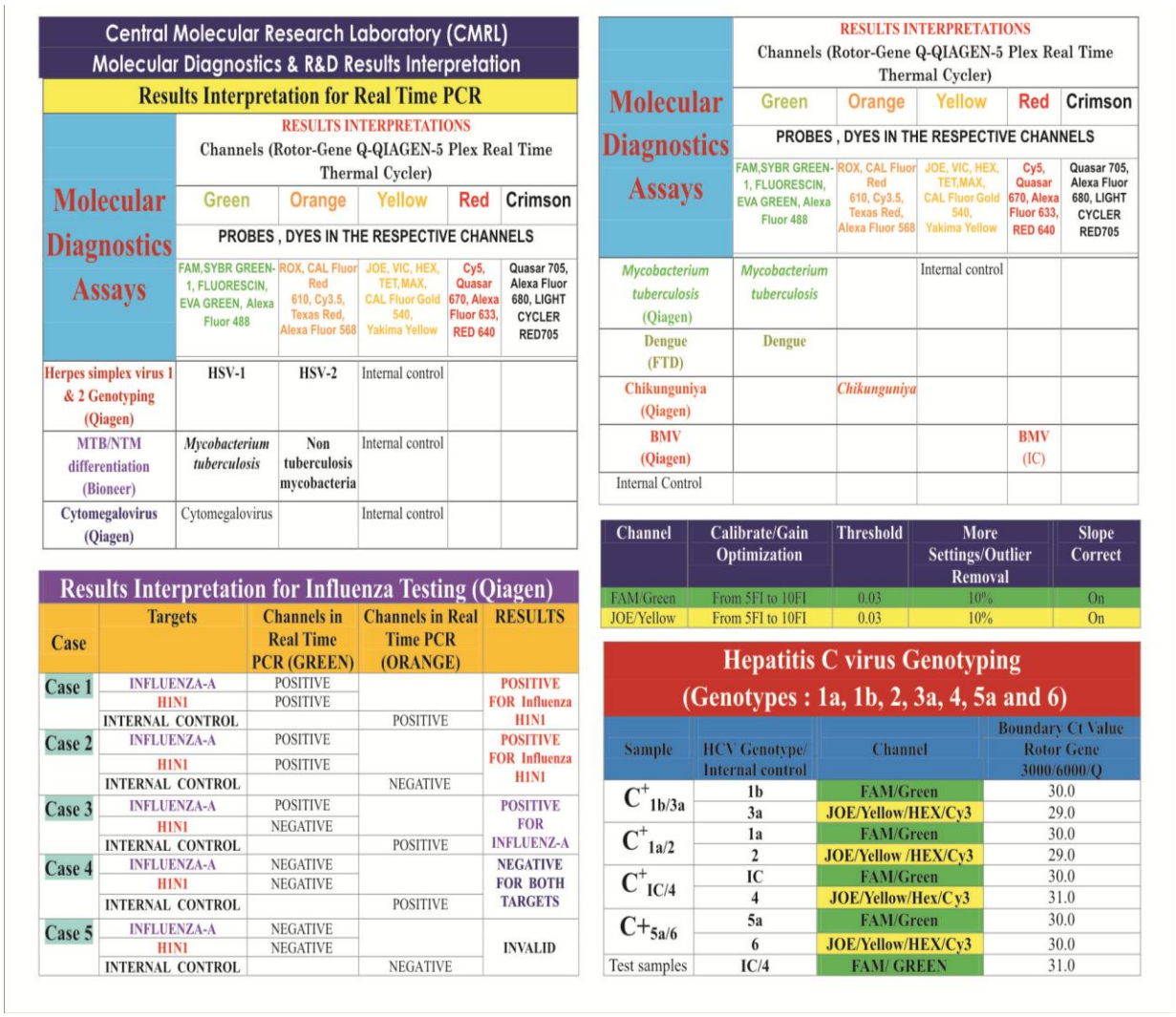
Pathogen detection by the polymerase chain reaction (PCR) is based on the amplification of specific regions of the pathogen genome. In real-time PCR the amplified product is detected via fluorescent dyes. These are usually linked to Oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e., in real-time) allows the detection and quantification of the accumulating product without having to re-open the reaction tubes after the PCR run.

(a) (b

Figure (14) Rotor-Gene Q Real Time PCR machine

(a)- Rotor of RT-PCR machine

(b)- 5 Plex RT-PCR machine



Central Molecular Research Laboratory (CMRL)
Molecular Diagnostics & R&D Results Interpretation
Results Interpretation for Real Time PCR

Molecular Diagnostics Assays	RESULTS INTERPRETATIONS Channels (Rotor-Gene Q-QIAGEN-5 Plex Real Time Thermal Cycler)				
	Green	Orange	Yellow	Red	Crimson
	PROBES , DYES IN THE RESPECTIVE CHANNELS				
	FAM,SYBR GREEN-1, FLUORESCIN, EVA GREEN, Alexa Fluor 488	ROX, CAL Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568	JOE, VIC, HEX, TET,MAX, CAL Fluor Gold 540, Yakima Yellow	Cy5, Quasar 670, Alexa Fluor 633, RED 640	Quasar 705, Alexa Fluor 680, LIGHT CYCLER RED705
Herpes simplex virus 1 & 2 Genotyping (Qiagen)	HSV-1	HSV-2	Internal control		
MTB/NTM differentiation (Bioneer)	*Mycobacterium tuberculosis*	Non tuberculosis mycobacteria	Internal control		
Cytomegalovirus (Qiagen)	Cytomegalovirus		Internal control		

Results Interpretation for Influenza Testing (Qiagen)

Case	Targets	Channels in Real Time PCR (GREEN)	Channels in Real Time PCR (ORANGE)	RESULTS
Case 1	INFLUENZA-A	POSITIVE		POSITIVE FOR Influenza H1N1
	H1N1	POSITIVE		
	INTERNAL CONTROL		POSITIVE	
Case 2	INFLUENZA-A	POSITIVE		POSITIVE FOR Influenza H1N1
	H1N1	POSITIVE		
	INTERNAL CONTROL		NEGATIVE	
Case 3	INFLUENZA-A	POSITIVE		POSITIVE FOR INFLUENZ-A
	H1N1	NEGATIVE		
	INTERNAL CONTROL		POSITIVE	
Case 4	INFLUENZA-A	NEGATIVE		NEGATIVE FOR BOTH TARGETS
	H1N1	NEGATIVE		
	INTERNAL CONTROL		POSITIVE	
Case 5	INFLUENZA-A	NEGATIVE		INVALID
	H1N1	NEGATIVE		
	INTERNAL CONTROL		NEGATIVE	

Molecular Diagnostics Assays	RESULTS INTERPRETATIONS Channels (Rotor-Gene Q-QIAGEN-5 Plex Real Time Thermal Cycler)				
	Green	Orange	Yellow	Red	Crimson
	PROBES , DYES IN THE RESPECTIVE CHANNELS				
	FAM,SYBR GREEN-1, FLUORESCIN, EVA GREEN, Alexa Fluor 488	ROX, CAL Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568	JOE, VIC, HEX, TET,MAX, CAL Fluor Gold 540, Yakima Yellow	Cy5, Quasar 670, Alexa Fluor 633, RED 640	Quasar 705, Alexa Fluor 680, LIGHT CYCLER RED705
Mycobacterium tuberculosis (Qiagen)	*Mycobacterium tuberculosis*		Internal control		
Dengue (FTD)	Dengue				
Chikunguniya (Qiagen)		*Chikunguniya*			
BMV (Qiagen)				BMV (IC)	
Internal Control					

Channel	Calibrate/Gain Optimization	Threshold	More Settings/Outlier Removal	Slope Correct
FAM/Green	From 5FI to 10FI	0.03	10%	On
JOE/Yellow	From 5FI to 10FI	0.03	10%	On

Hepatitis C virus Genotyping
(Genotypes : 1a, 1b, 2, 3a, 4, 5a and 6)

Sample	HCV Genotype/ Internal control	Channel	Boundary Ct Value Rotor Gene 3000/6000/Q
$C^{+}_{1b/3a}$	1b	FAM/Green	30.0
	3a	JOE/Yellow/HEX/Cy3	29.0
$C^{+}_{1a/2}$	1a	FAM/Green	30.0
	2	JOE/Yellow /HEX/Cy3	29.0
$C^{+}_{IC/4}$	IC	FAM/Green	30.0
	4	JOE/Yellow/Hex/Cy3	31.0
$C+_{5a/6}$	5a	FAM/Green	30.0
	6	JOE/Yellow/HEX/Cy3	30.0
Test samples	IC/4	FAM/ GREEN	31.0

Figure(15)- Result Interpretation of Rotor-Gene Q Real Time PCR machine.

Molecular Diagnostics Assays	RESULTS INTERPRETATIONS Channels (Rotor-Gene Q-QIAGEN-5 Plex Real Time Thermal Cycler)				
	Green	Orange	Yellow	Red	Crimson
	PROBES , DYES IN THE RESPECTIVE CHANNELS				
	FAM,SYBR GREEN-1, FLUORESCIN, EVA GREEN, Alexa Fluor 488	ROX, CAL Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568	JOE, VIC, HEX, TET,MAX, CAL Fluor Gold 540, Yakima Yellow	Cy5, Quasar 670, Alexa Fluor 633, RED 640	Quasar 705, Alexa Fluor 680, LIGHT CYCLER RED705
Mycobacterium tuberculosis (Qiagen)	*Mycobacterium tuberculosis*		Internal control		
Dengue (FTD)	Dengue				
Chikunguniya (Qiagen)		*Chikunguniya*			
BMV (Qiagen)				BMV (IC)	
Internal Control					

Figure (16)- Result interpretation for Dengue virus (Green channel)

Programming of the Thermocycler by Fast Track Diagnosis (FTD), 32 reactions (catalog No.FTD-43-32), 64 reactions (catalog No-43-64).

PRINCIPLE OF THE METHOD

The viral RNA is transcribed into cDNA using a specific primer mediated reverse transcription step followed immediately in the same tube by polymerase chain reaction. The presence of specific pathogen sequences in the reaction is detected by an increase in fluorescence observed from the relevant dual-labeled probe, and is reported as a cycle threshold value (Ct) by the Real-time thermo cycler. The assay uses Brome Mosaic Virus (BMV) as an extraction control- the internal control (IC) - which is introduced into each sample and the negative control at the Lysis buffer stage of the extraction process.

Table (4)- Settings for the detectors.

PP mix	**Pathogen**	**Dye**	**Detection wave length (nm)***
DG	Dengue virus	Green	520
	Chikungunya virus	Orange	610
	Brome Mosaic Virus (BMV) (internal control)	Red	670

PP- Primers and Probes

DG- Dengue

Table (5) Oligonucleotide primers used to amplify and type dengue viruses

Primer	Sequence	Genome positional	Size, in bp, of amplified DNA product (primers)b
Dl	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	134-161	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616-644	511
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	568-586	482(Dl and TS1)
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	232-252	119 (Dl and TS2)
TS3	5'-TAACATCATCATGAGACAGAGC-3'	400-421	290 (Dl and TS3)
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506-527	392 (Dl and TS4)

PCR Programme

42ºC for 15 minutes hold

- At this temperature the isolated template RNA get converted in cDNA, generated by reverse transcription.

94ºC for 3 minutes hold

40 cycles of: 94°C for 8 seconds

60°C for 34 seconds.

Table (6) Master mix preparation for Dengue virus detection by Real Time PCR (Catalog No. R0115308)

Sr.No.	Component	Quantity (for 1 rxn.)	Quantity (for 4 rxn.)
1.	Pre amplification mix	10 µl	40 µl
2.	Template RNA	10 µl	40µl
	TOTAL	20µl	80 µl

SEROTYPES DETECTION BY CONVENTIONAL PCR MACHINE

Principle

The method relies on **thermal cycling,** consisting of cycles of **repeated** heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

AGAROSE GEL ELECTROPHORESIS

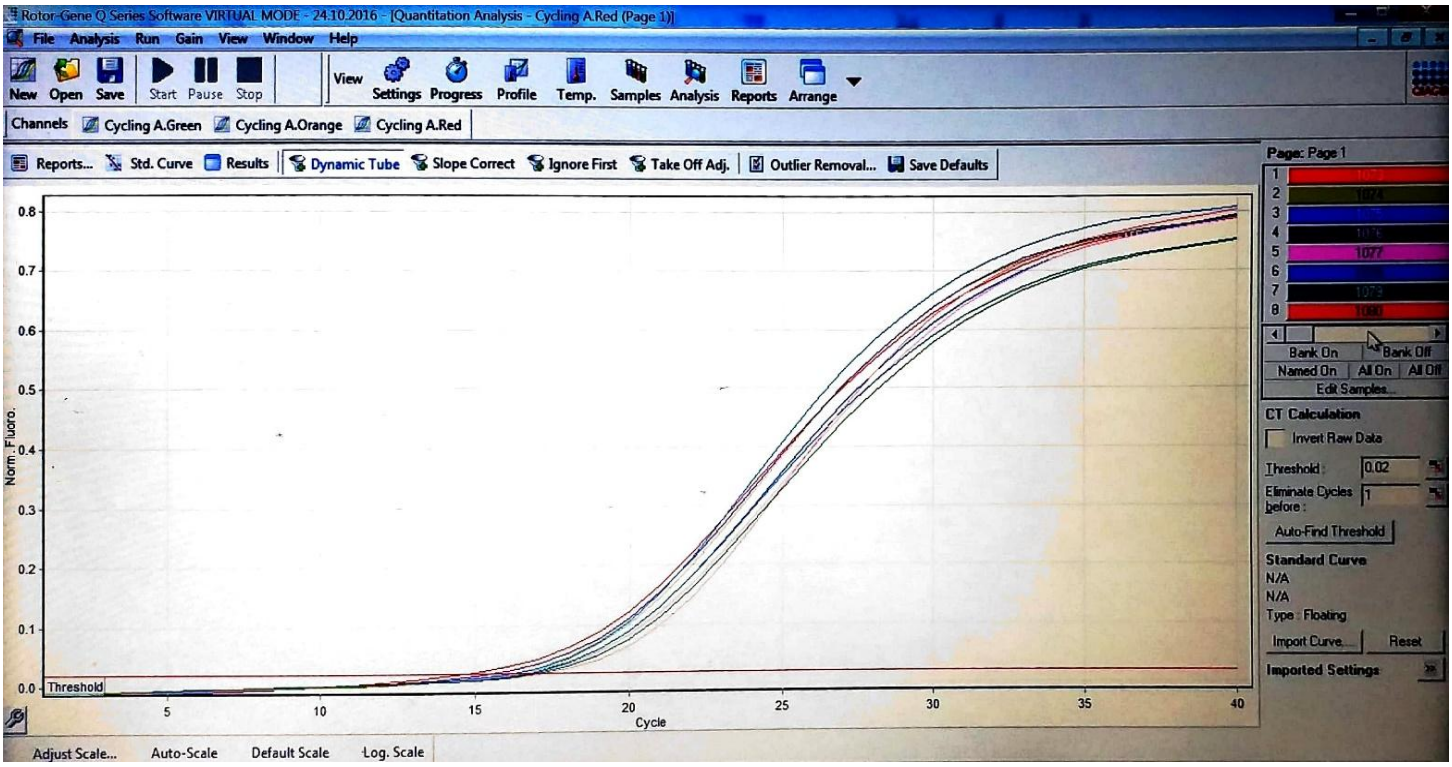
The Agarose gel electrophoresis is based on the principle of movement of charged Macromolecules of different or same sizes across a matrix of uniform pore size, under the influence of an applied electric field.

RESULT AND OBSERVATIONS

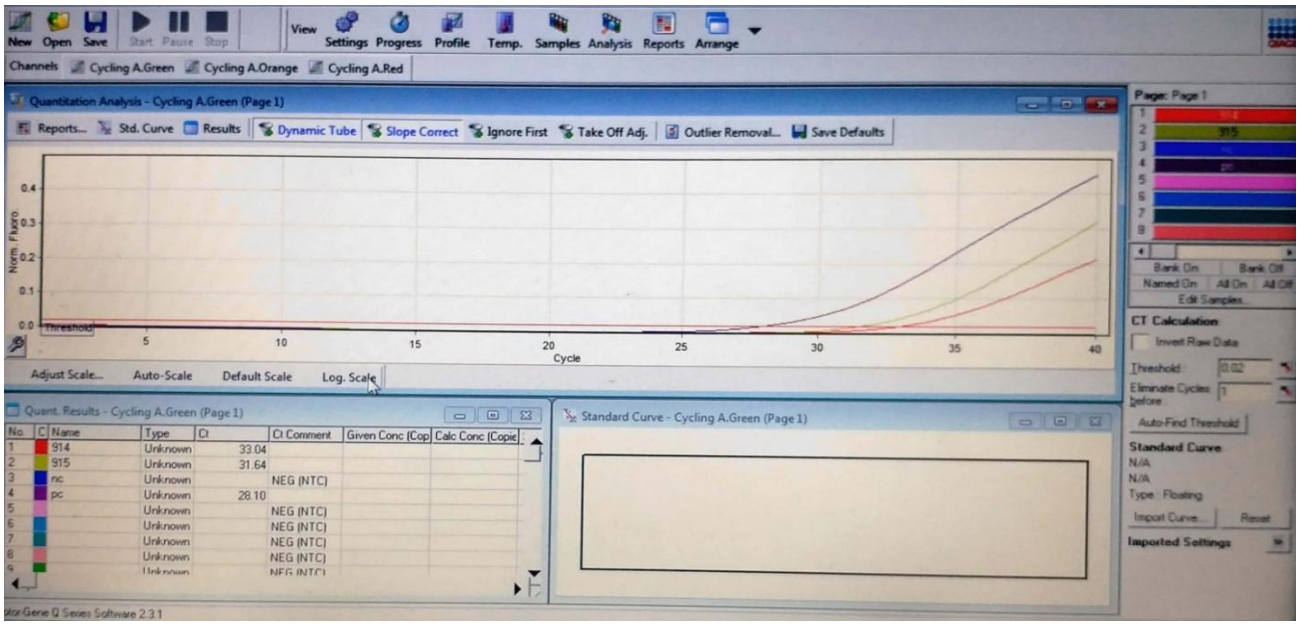
Out of 100 samples of dengue patients, 64 dengue positive were selected for the typing of Dengue virus among their serotypes because of degradation of the RNA and less viral load in remaining 36 samples. Detected Serotypes are tabulated in (**Table 7)**. The cut of value for PCR Thermocycler for the Dengue viral fever was 40. The lesser the value, the more was the viral load and early exponential amplification curve was detected in thermocycler, **(Graphs 1,2,3)** showing the exponential amplification due to high viral load. The result is presented as (Mean±SD±SE). The mean value for the thermocycler for dengue was found to be 21.82±5.17±1.34 for DENV-1, 22.79±5.99±1.06 for DENV-2, 20.39±5.40±1.31 for DENV-3, and is tabulated in **(Table 10)**, DENV-4 was not detected during the epidemic of 2017-18 in our study. **(Figures 17,18,19,20)** showing the detected serotypes on the Gel electrophoresis via ultraviolet gel image capture technique.

Out of 64 Dengue cases 26 (40%) were female and 38(60%) were males. DENV-2 was found most frequent among the all serotypes of DENV, Result is Graphically presented in **(Graph 5)**. Out of 15 DENV-1 serotype, 5 were female and 10 were male, for DENV-2 serotype 15 were female and 17 were male, for DENV-3 serotype 6 were female and 11 were male, Result is tabulated in **(Table 10).** The values of thermocycler for different serotypes, DENV-1 for male and female were found

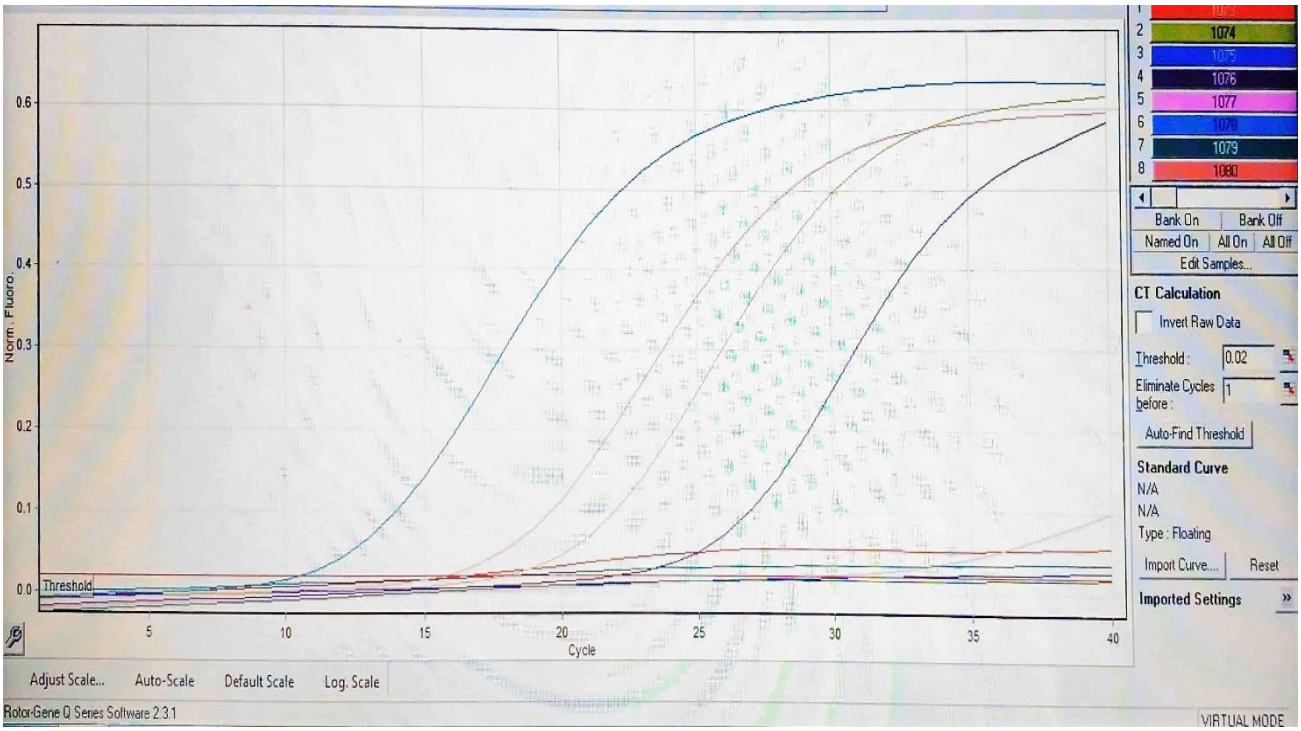
to be 22.06±6.22±1.9 and 21.3±2.50±1.12 respectively, DENV-2 for male and female were found to be 20.24±5.21±1.26 and 25.67±5.6±1.4 respectively, DENV-3 for male and female were found to be 19.92±6.42±1.94 and 21.22±3.11±1.27 respectively and result is tabulated in**(Table 11).**



Graph (1) Amplification curves for the Dengue sera samples including internal controls along with their ct values



Graph (2) Amplification curves for the Dengue sera samples including internal controls along with their ct values.



Graph (3) Amplification curves for the Dengue sera samples including internal controls along with their ct values

AGAROSE GEL PICTURE REPRESENTING PCR AMPLICON OF 290 BP AND 382 BP FOR DENV-3 AND DENV- 4 SEROTYPE RESPECTIVELY

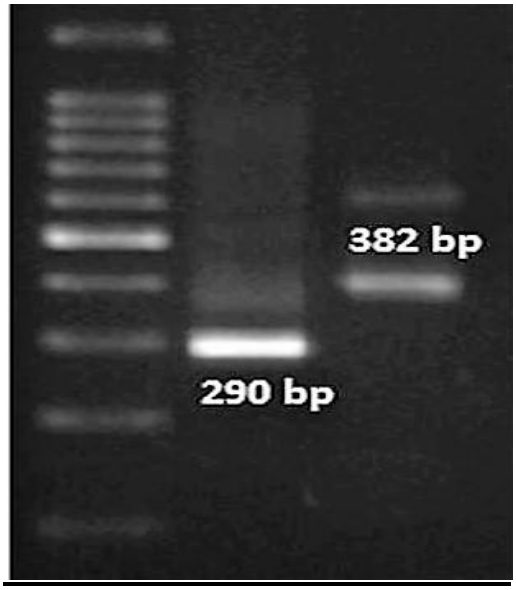


Figure (17) DENV-3 on 290bp and DENV-4 on 382bp on base pair ladder

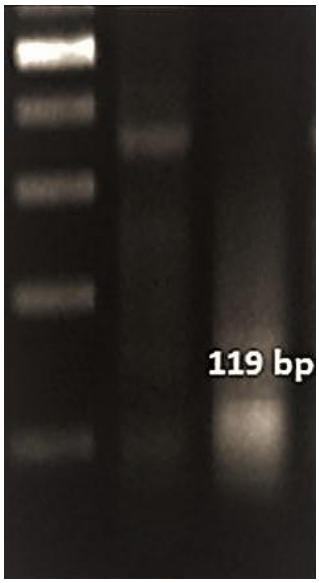


Figure (18) Agarose gel picture representing PCR amplicon of 119 bp for DENV-2 serotype

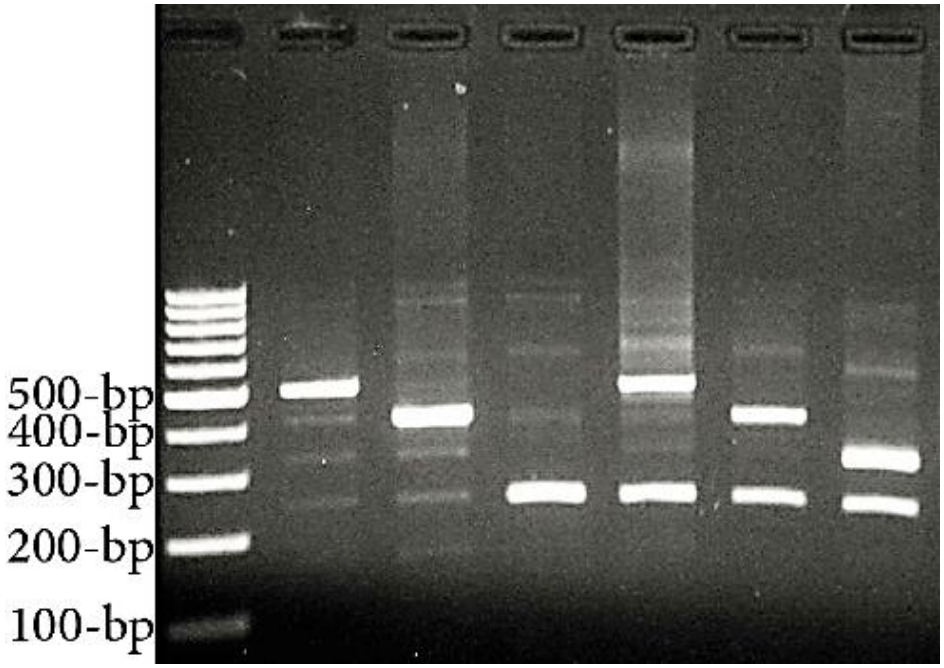


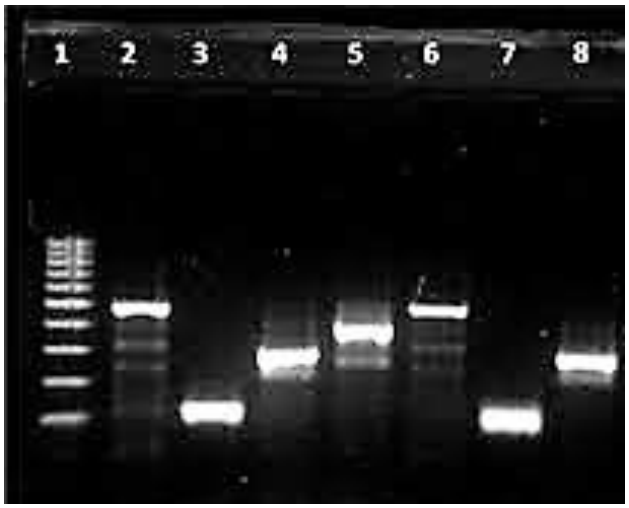
Figure (19) Agarose gel electrophoresis images for Dengue Serotyping.

Well 1: 100 bp DNA difference ladder

Well 2: PCR amplicon of 482 for DENV-1 serotype

Well 3: PCR amplicon of 392 for DENV-4 serotype

Well 4: PCR amplicon of 290 for DENV-3 serotype



Well 1: 100 bp DNA difference ladder
Well 2: PCR amplicon of 482 for DENV-1 serotype
Well 3: PCR amplicon of 119 for DENV-2 serotype
Well 4 and 5: PCR amplicon of 392 for DENV-4 serotype
Well 6: PCR amplicon of 482 for DENV-1 serotype
Well 7: PCR amplicon of 119 for DENV-2 serotype
Well 8: PCR amplicon of 290 for DENV-3 serotype

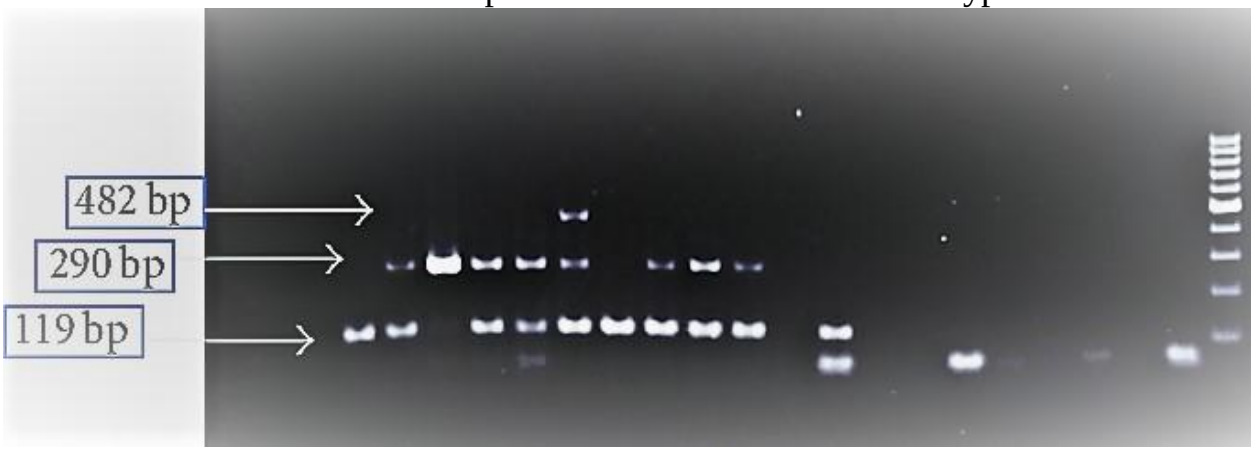


Figure (20) Agarose gel electrophoresis image for Dengue Serotyping, PCR products (bp) at 482, 119, 290 and 392 represent Dengue Serotyping, DENV-1, DENV-2, DENV-3 and DENV-4 respectively

Table (7) Serotypes detected from the amplified product of RT-PCR (amplicon) in Gel electrophoresis.

Sr.No	**AGE(yr.)**	**GENDER**	**Ct-value**	**SEROTYPE**
			(>40 = -ive)	**(1/2/3/4)**
1	48	male	23.3	DENV-2
2	23	female	25.8	DENV-2
3	28	female	26	DENV-2
4	22	female	29.1	DENV-2
5	64	female	18.1	DENV-1
6	75	male	16	DENV-3
7	46	male	27.4	DENV-1
8	75	male	24.1	DENV-1
9	52	male	29.5	DENV-2
10	18	male	15.9	DENV-1
11	48	male	28.6	DENV-2
12	31	male	28.2	DENV-2
13	30	female	24.2	DENV-3
14	48	male	27.2	DENV-2
15	21	male	20.9	DENV-1
16	38	male	24.2	DENV-3

17	39	male	19.7	DENV-3
18	60	male	22.2	DENV-3
19	18	male	21	DENV-3
20	37	male	30.2	DENV-2
21	28	female	23.3	DENV-2
22	19	female	28	DENV-2
23	44	female	29.1	DENV-2
24	27	female	7	DENV-2
25	45	female	21.4	DENV-3
26	49	male	25.5	DENV-2
27	30	female	24.3	DENV-2
28	45	female	28.6	DENV-2
29	28	male	23.8	DENV-2
30	30	female	19.8	DENV-1
31	25	male	28.1	DENV-3
32	61	male	8	DENV-1
33	21	female	21.7	DENV-1
34	22	male	29.4	DENV-2
35	55	male	24.1	DENV-1
36	32	female	25	DENV-2

37	56	male	18.7	DENV-2
38	60	female	28.8	DENV-3
39	60	male	22.5	DENV-2
40	28	male	23.8	DENV-1
41	63	male	15.1	DENV-3
42	26	male	23.7	DENV-3
43	65	female	22.5	DENV-1
44	28	male	24.17	DENV-3
45	40	male	15.5	DENV-2
46	35	male	15.1	DENV-2
47	62	female	17.4	DENV-2
48	35	female	15.2	DENV-2
49	50	female	28.4	DENV-2
50	38	male	20.3	DENV-2
51	30	male	23.8	DENV-1
52	33	male	7.8	DENV-3
53	13	female	14.6	DENV-3
54	35	male	17.7	DENV-2
55	4	male	14.8	DENV-2
56	49	female	24.6	DENV-1

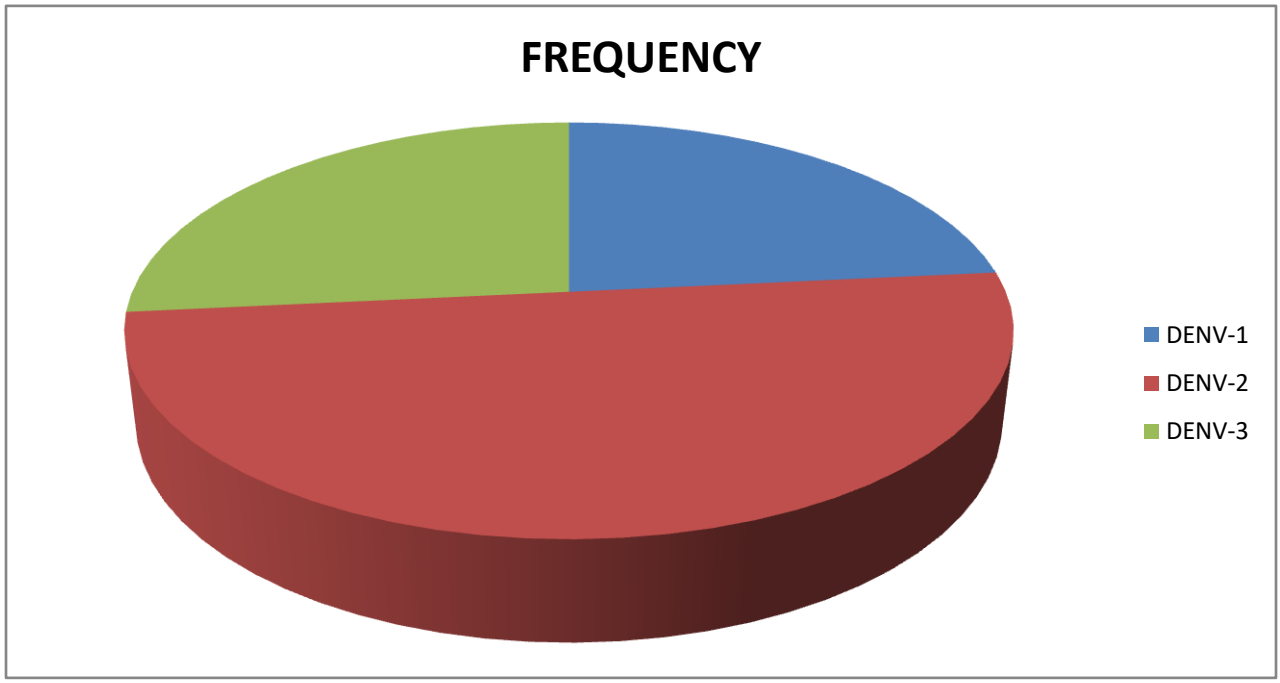
57	20	female	22.7	DENV-3
58	26	male	30.3	DENV-1
59	45	male	22.3	DENV-1
60	33	female	13.3	DENV-2
61	36	male	16.4	DENV-3
62	56	male	19.9	DENV-2
63	46	female	18.5	DENV-2
64	24	female	16.3	DENV-3

Out of 64 dengue RNA viruses the DENV-2 serotype was found to be more prevalent other serotypes DENV-3 and DENV-1 were also found in decreasing order. The results are tabulated below and represented graphically.

Table (8) Frequency of different Serotypes.

SEROTYPES	**FREQUENCY**
DENV-1	15
DENV-2	32
DENV-3	17

Graph (4) Pie Graph for frequency distribution of dengue serotypes.

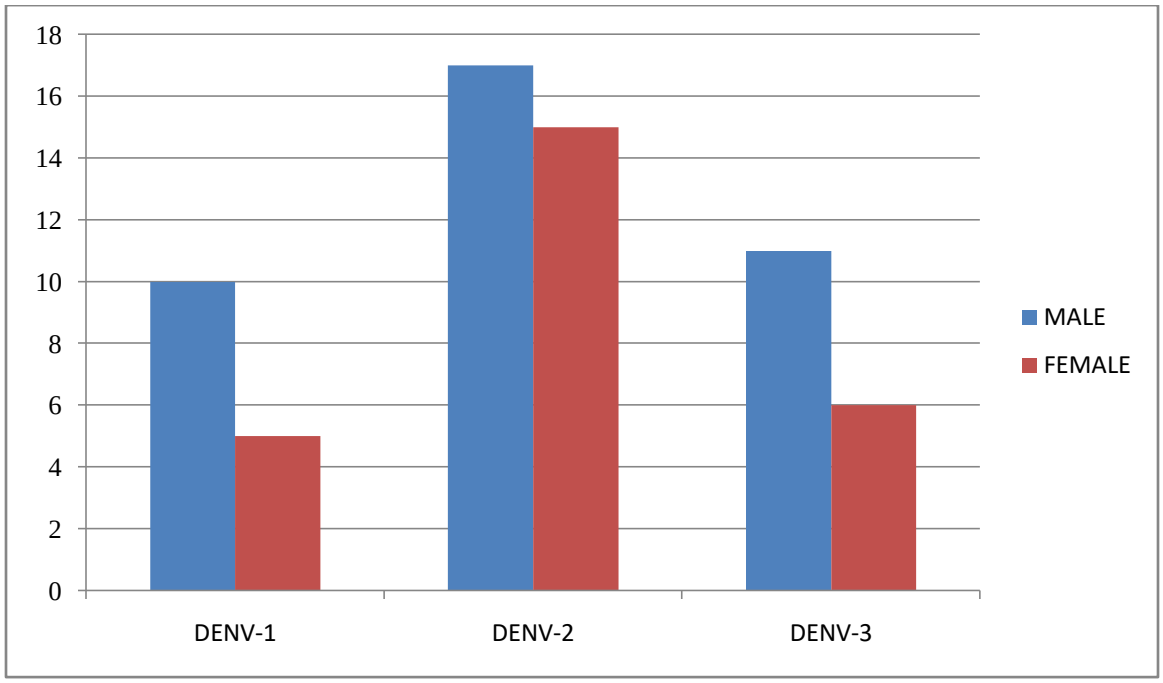


Out of 15 DENV-1 serotype 5 were female and 10 were male. For DENV-2, 15 were female and 17 were male. For DENV-3, 6 were female and 11 were male.

Table (9) gender wise frequency analysis of Dengue serotypes.

SEROTYPE	FEMALE	MALE
DENV-1	5	10
DENV-2	15	17
DENV-3	6	11

Graph (5) gender wise frequency analysis of Dengue serotypes.

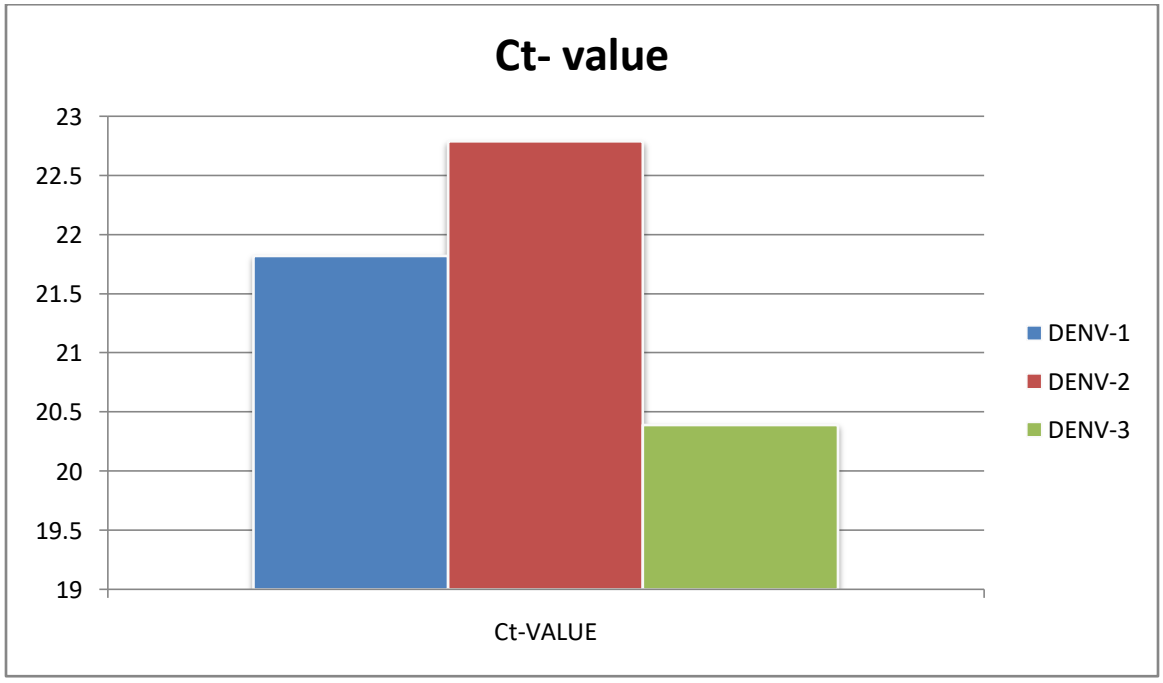


The viral load and Ct-value has the inverse relationship with each other hence the DENV-3 was found with more viral load. Although the DENV-2 was more prevalent but the infectivity of DENV-3 was found more due to more viral load of the DENV-3 serotype.

Table (10) thermocycler value for different serotypes.

SEROTYPES	Ct- VALUE (mean±SD±SE)
DENV-1	21.82±5.17±1.34
DENV-2	22.79±5.99±1.06
DENV-3	20.39±5.40±1.31

Graph (6) Thermocycler value for different serotypes.

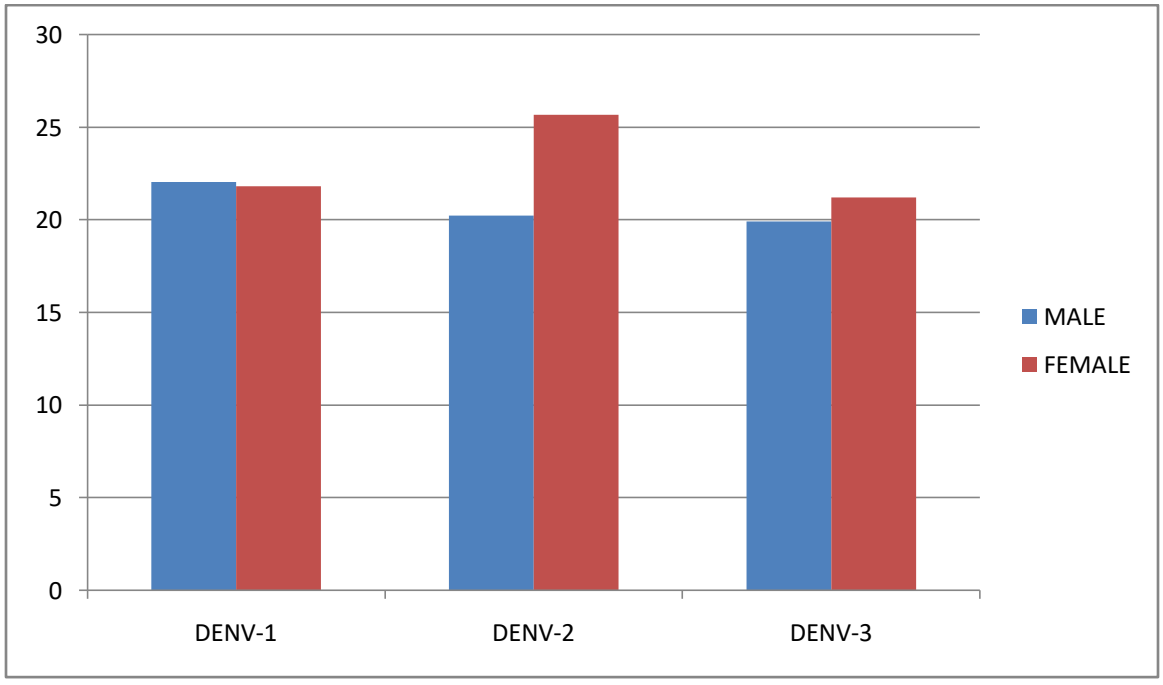


Males were found to be more affected by Dengue fever as compared to females. The infectivity of DENV-2 and DENV-3 was more in males as compared to females. The infectivity of DENV-1 was found more in females.

Table (11) Gender wise comparison of thermocycler value.

SEROTYPES	Ct- VALUE MALE (mean±SD±SE)	Ct- VALUE FEMALE (mean±SD±SE)	t-value	p-value	Sig.
DENV-1	22.06±6.22±1.9	21.3±2.50±1.12	-0.3	0.7	NS
DENV-2	20.24±5.21±1.26	25.67±5.6±1.4	2.84	0.008	ES
DENV-3	19.92±6.42±1.94	21.22±3.11±1.27	0.4	0.46	NS

Graph (7) gender wise comparison of thermocycler value.



Out of 60 confirmed cases of chikungunya 30 were males and 30 were females. Result is tabulated in **(Table 12)**. Females was found to be affected more with the Chikungunya viral infection the mean values for thermocycler was 18.89±6.31±1.15 for males and 16.89±7.10±1.3 for females. Result is tabulated in **(Table 13),** and is graphically represented in **(Graph 8).**

The cut-off value for PCR Thermocycler for the Chikungunya viral fever was 40. The lesser the value the more was the viral load and early exponential amplification curve was detected in thermocycler. **(Graph 9,10,11)** showed the exponential growth of the curve because of high viral load.

Table (12) Chikungunya RNA detected via RT-PCR.

S. NO	AGE	GENDER	C-t Value (>40 = -ive)
1	12	F	12.4
2	18	F	13.1
3	18	M	28.7
4	19	M	29.6
5	20	M	16.5
6	21	F	13.3

7	21	M	21.7
8	22	M	31.1
9	24	F	14.5
10	26	M	15.3
11	27	M	23.3
12	28	F	12.9
13	28	M	16.4
14	28	M	16.1
15	29	M	29.4
16	30	M	14.4
17	31	M	24.1
18	31	M	18.8
19	32	F	30.7
20	32	M	11.2
21	34	F	20.7
22	35	F	7.4
23	35	F	28.7
24	35	M	20.3
25	38	F	14.5
26	38	M	15.5

27	39	F	14.7
28	40	F	22.8
29	40	M	9.6
30	42	F	17.1
31	42	M	26.4
32	42	M	12.8
33	43	M	18.2
34	44	F	32.2
35	44	F	14.7
36	44	M	14.2
37	44	M	19.7
38	45	F	9.2
39	46	F	14.3
40	48	F	32.6
41	48	F	16
42	48	F	17.4
43	48	M	17.4
44	55	F	8
45	55	F	9.5
46	55	F	18.6

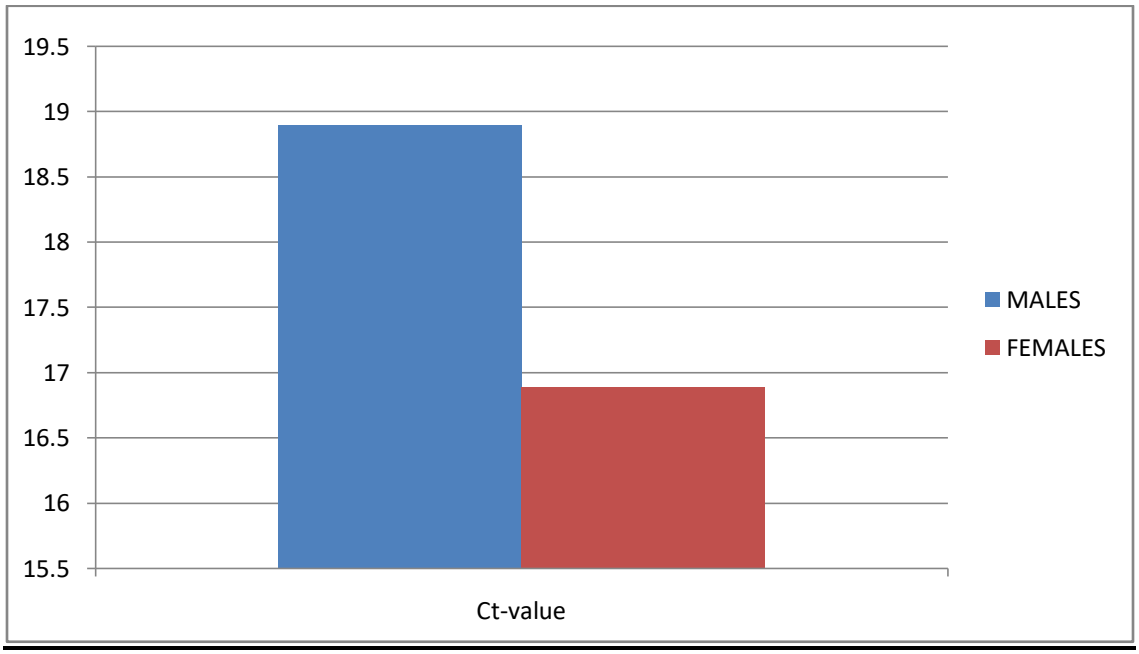
47	56	F	14.2
48	57	F	8.5
49	57	F	21.1
50	59	F	25
51	60	M	7.9
52	60	M	20.6
53	61	F	16.5
54	62	M	15.9
55	63	M	21.6
56	63	M	25.4
57	65	M	16.4
58	65	M	8.3
59	75	F	9.1
60	80	F	17.1

Out of 60 confirmed cases of chikungunya 30 were found to be males and 30 were found to be females.

Table (13) Gender wise thermocycler value of chikungunya virus.

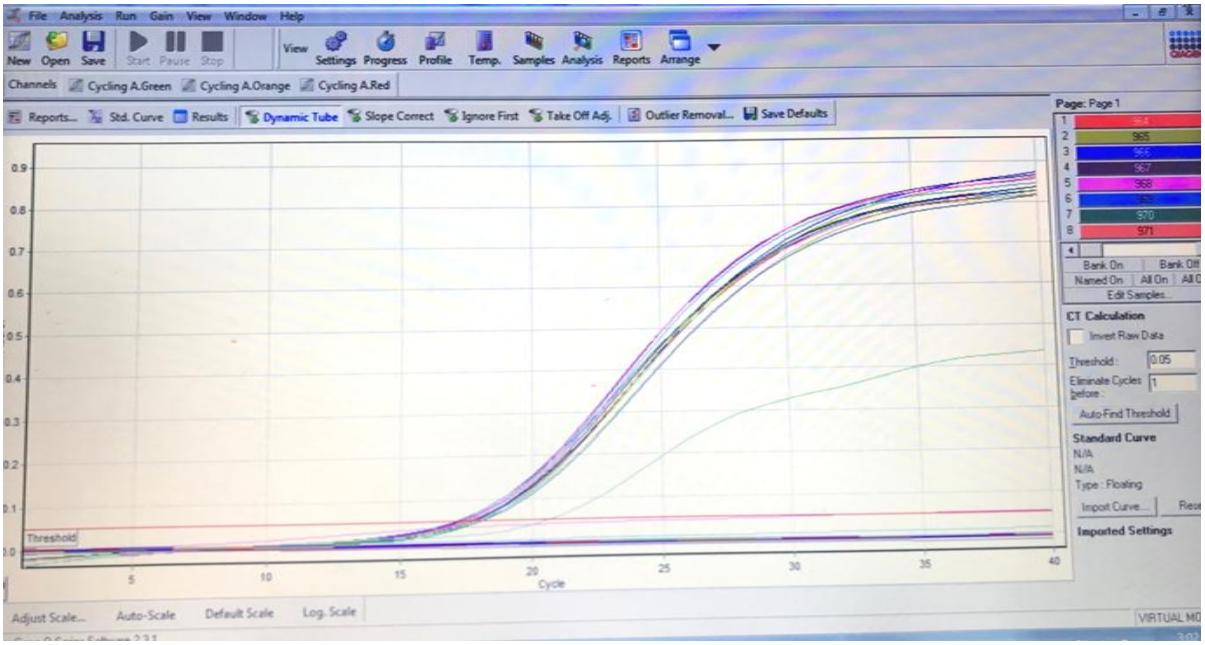
PARAMETER	MALES Mean±SD±SE	FEMALES Mean±SD±SE	t-value	p-value	Sig.
Ct-value	18.89±6.31±1.15	16.89±7.10±1.3	-1.1	=0.25	NS

Graph (8) Gender wise thermocycler value of chikungunya virus.

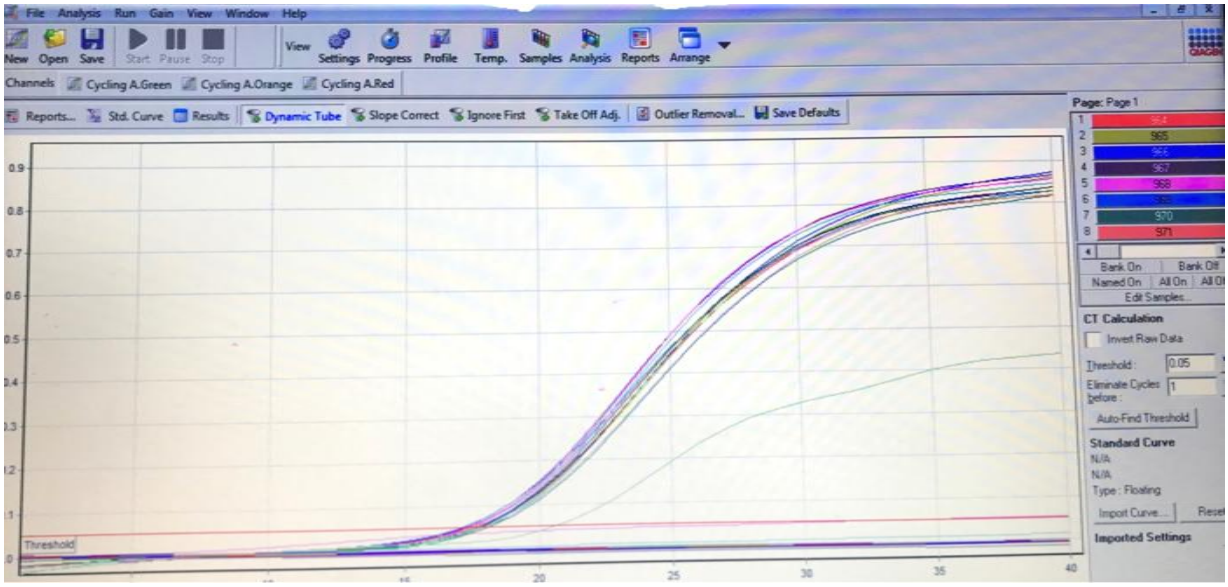


Chikungunya infectivity was found more in females as compared to the males.

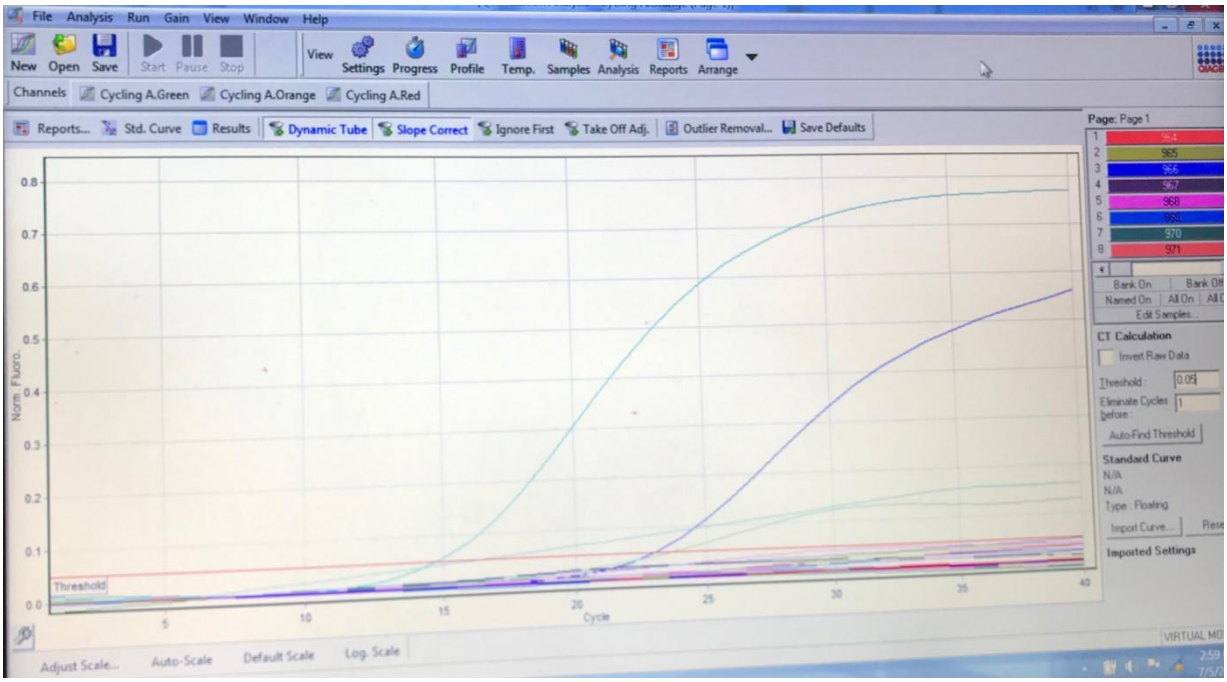
Graph (9) Amplification curves for the Chikungunya RNA with Ct- values



Graph (10) Amplification curves for the Chikungunya RNA with Ct- values



Graph (11) Amplification curves for the Chikungunya RNA with Ct- values



The negative correlation of Thermocycler values of dengue viral disease with the liver enzymes was detected. The ***'r'*** values for correlation between Ct-value and liner enzymes are tabulated below in **(Table 15)** and graphically presented by scatter plot **(Graph 12)** and correlation is evaluated. The correlation could be found as: '+' = indicates positive correlation, '-'= indicates negative correlation. The strength of correlation was tabulated in **(Table 14)**.

Result shows the negative correlation of dengue viral disease with the liver enzymes. The *'r'* values for correlation between Ct-value and liner enzymes are tabulated below and graphically presented by scatter plot and correlation is evaluated. The correlation could be found as: '+' = indicates positive correlation, '-'= indicates negative correlation.

Table (14) Strength of correlation.

correlation *'r'* value	Strength of correlation
0	No correlation
< -2	Weak correlation
-2 to -4	Moderate correlation
-4 to -6	Strong correlation
>-6	Very strong correlation

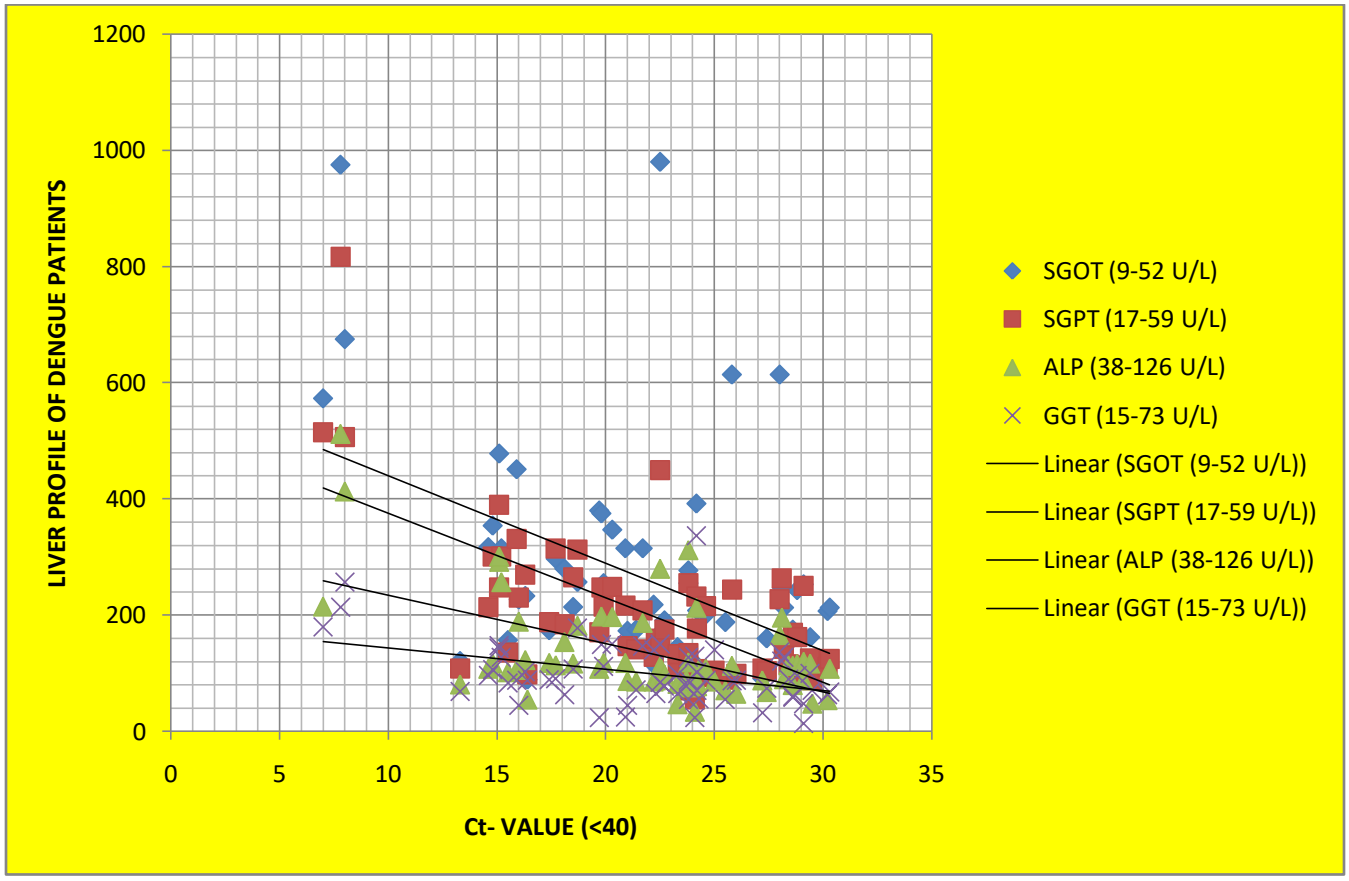
Table (15) correlation of Liver profile with dengue fever.

Correlation of liver enzymes with Dengue			
PARAMETER	MEAN±SD	'r' - value	Strength
SGOT	249.26±158.56	-0.449	S
SGPT	184.97±109.91	-0.649	VS
ALP	125.91±74.52	-0.539	S
GGT	103.09±5.37	-0.377	M

S=strong, VS= very strong, M= moderate

A negative correlation is represented between the liver profile and the thermocycler values of the RT-PCR machine which states that lower the Ct- value the more is the viral load and more is the infectivity. Hence more is the hepato-toxicity.

Graph (12) A negative correlation is represented between the liver profile and dengue (thermocycler value).



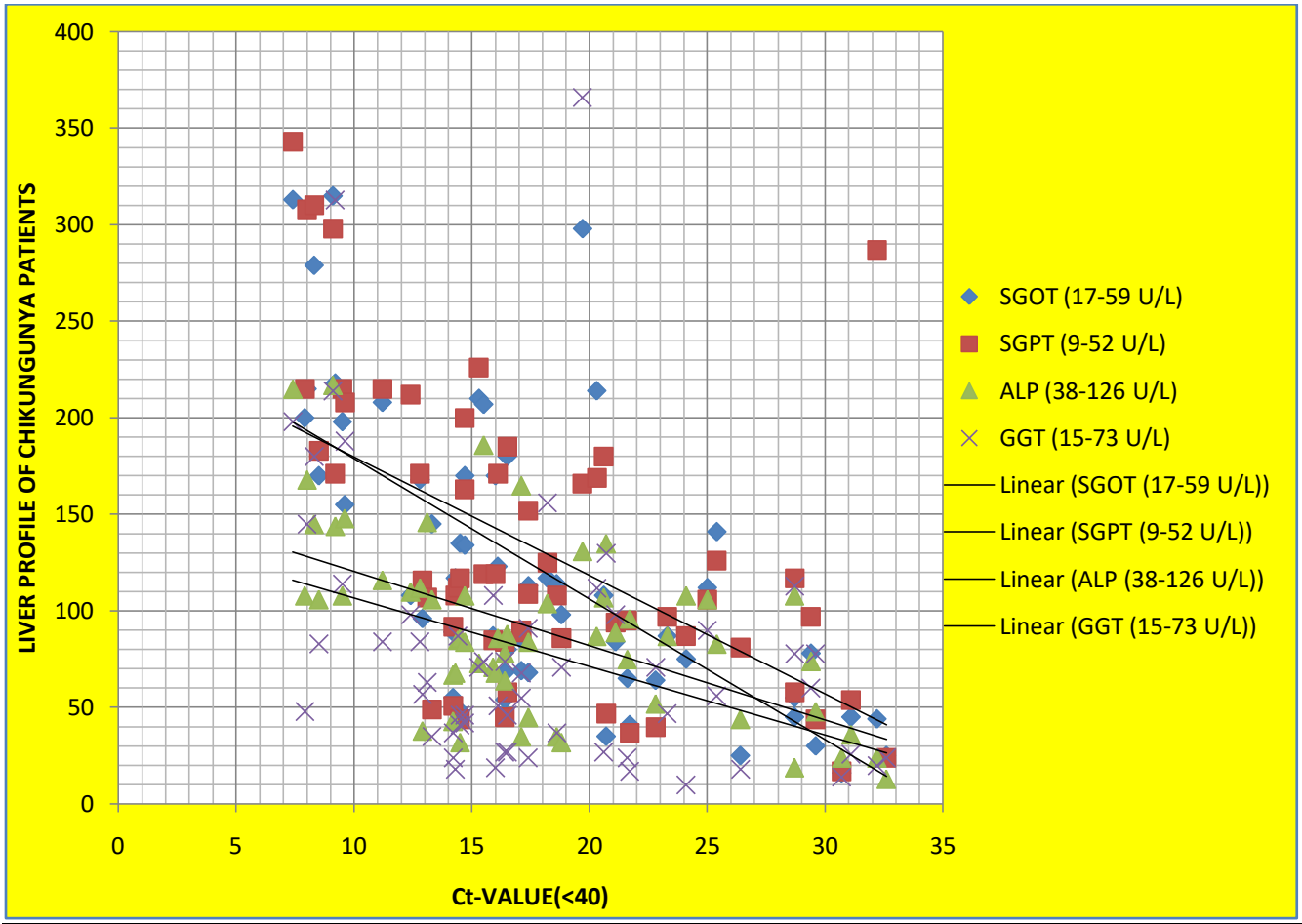
Result shows that the Ct- value (thermocycler value) for chikungunya virus is negatively correlated with the liver enzymes. As it shows if the Ct value goes down, the liver enzymes are found to be raised. The result is Tabulated in Table (16)graphically represented in Graph (13).

Table (16) Correlation of liver enzymes with the Ct- value for Chikungunya

Correlation of liver enzymes with the Ct- value for Chikungunya			
PARAMETER	MEAN±SD	'r' - value	Strength
SGOT	121.47±74.10	-0.661	VS
SGPT	131.37±77.09	-0.536	S
ALP	90.12±45.95	-0.565	S
GGT	78.72±68.54	-0.348	M

VS= very strong, S=strong, M=moderate

Graph (13) Negative correlation between chikungunya Ct- value and liver profile.



The *'r'* values for correlation between Ct-value and renal enzymes are tabulated below. The correlation could be found as: '+' = indicates positive correlation, '-'= indicates negative correlation.

Table (17) Strength of correlaion.

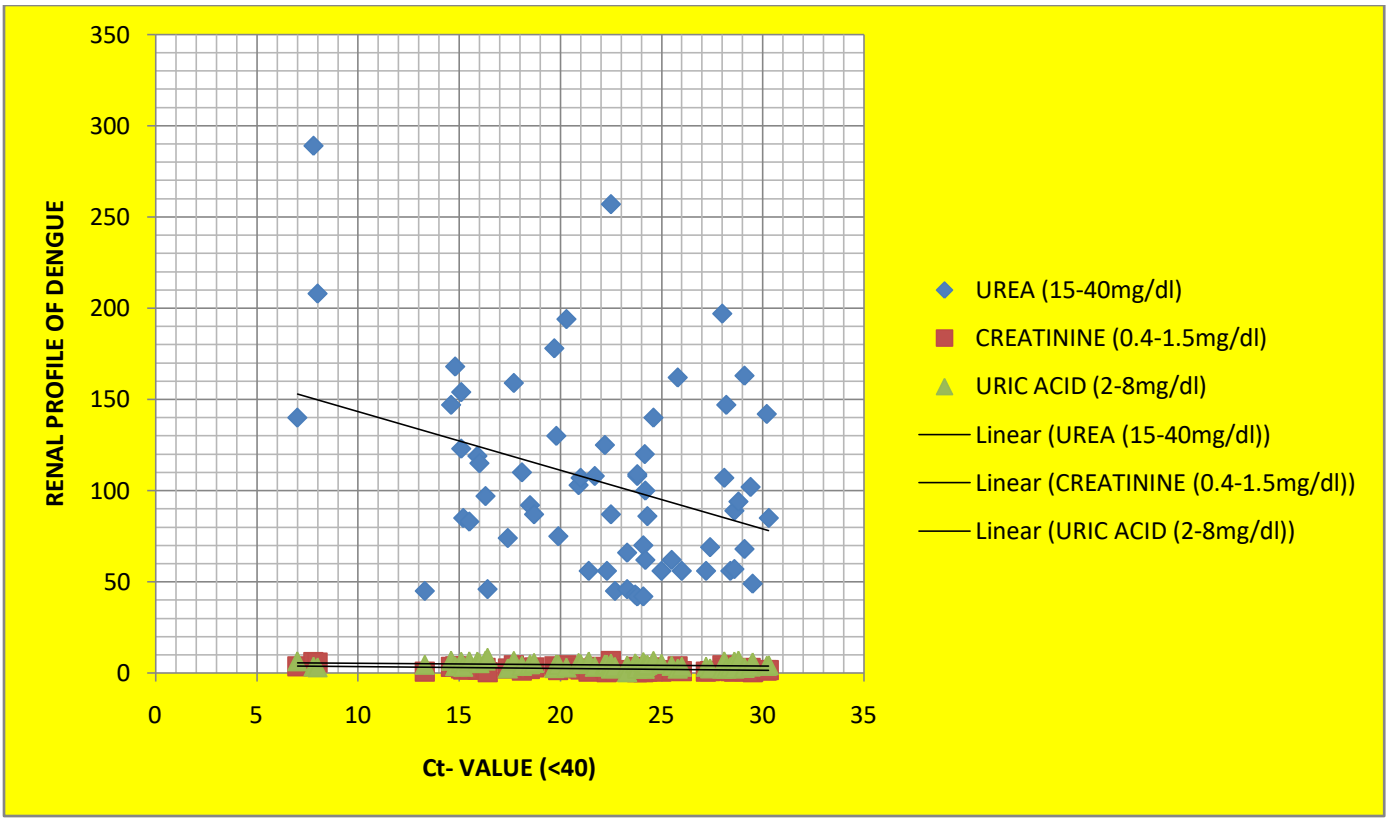
correlation *'r'* value	Strength of correlation
0	No correlation
< -2	Weak correlation
-2 to -4	Moderate correlation
-4 to -6	Strong correlation
>-6	Very strong correlation

A negative correlation is found between the Ct-value and renal profile. the results are tabulated below and represented graphically with the scatter plot.

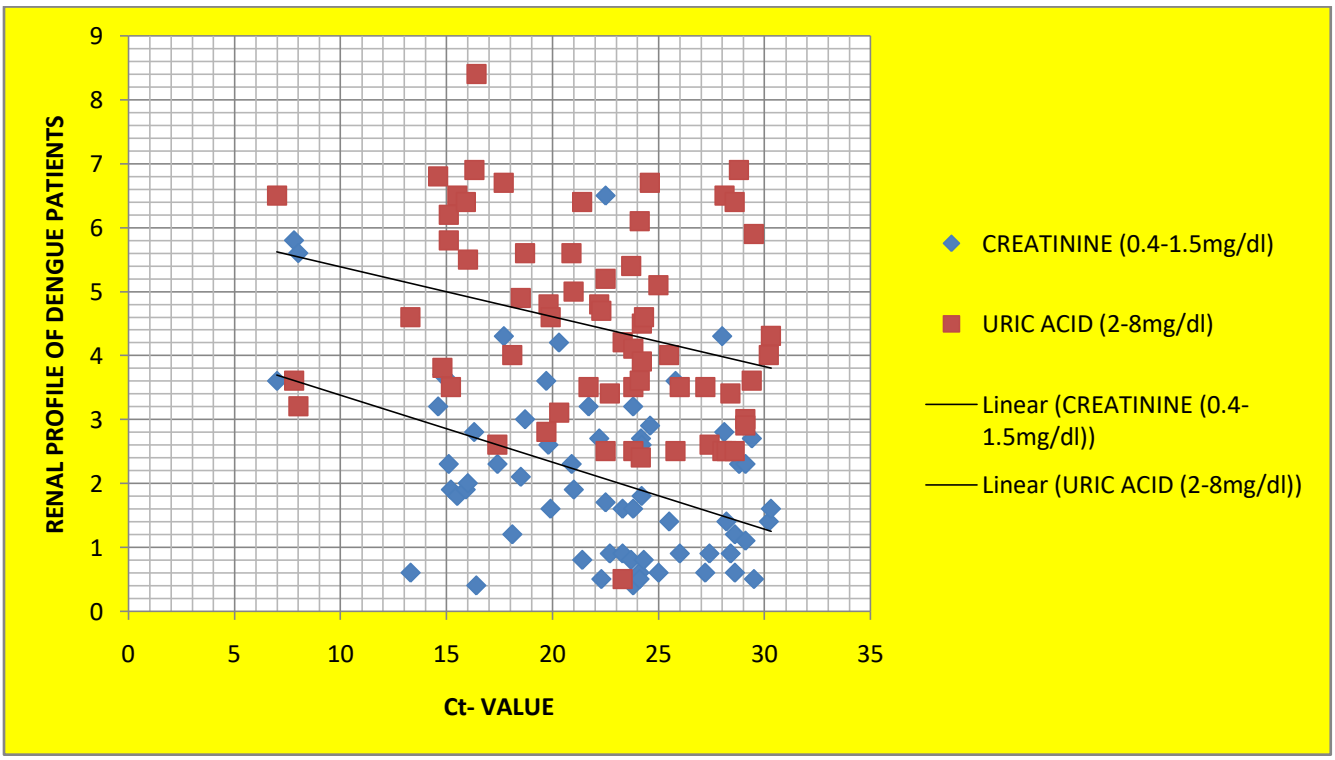
Table (18) Correlation of Renal Profile with dengue fever.

Correlation of Renal enzymes with the Ct- value for Dengue fever			
Parameter	mean±SD	'r' value	Significance
Urea(mg/dl)	116.41±54.83	-0.345	M
Creatinine(mg/dl)	2.39±1.33	-0.428	S
Uric acid(mg/dl)	4.50±1.51	-0.202	W

M=moderate, S=strong, W=weak.



graph (14) Negative correlation between chikungunya Ct-value and renal profile.



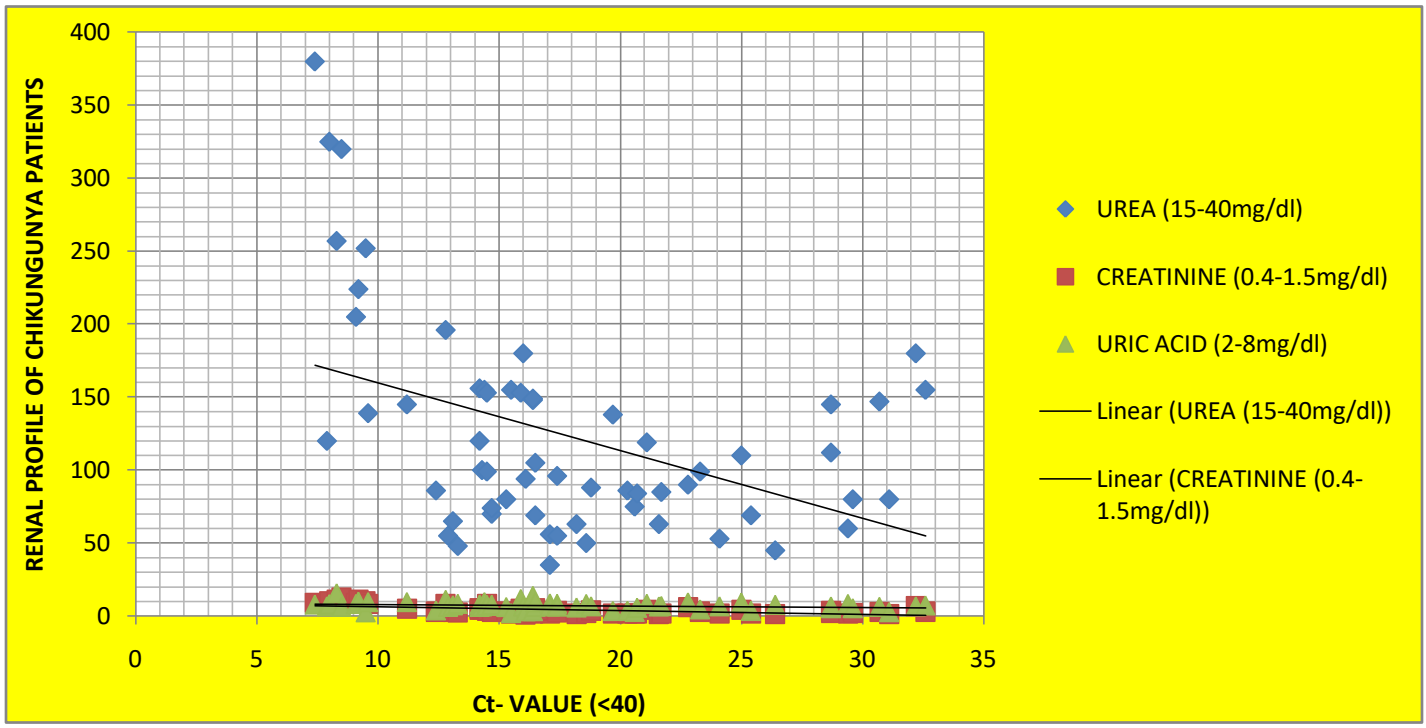
Graph (15) Negative correlation between chikungunya Ct-value and renal profile.

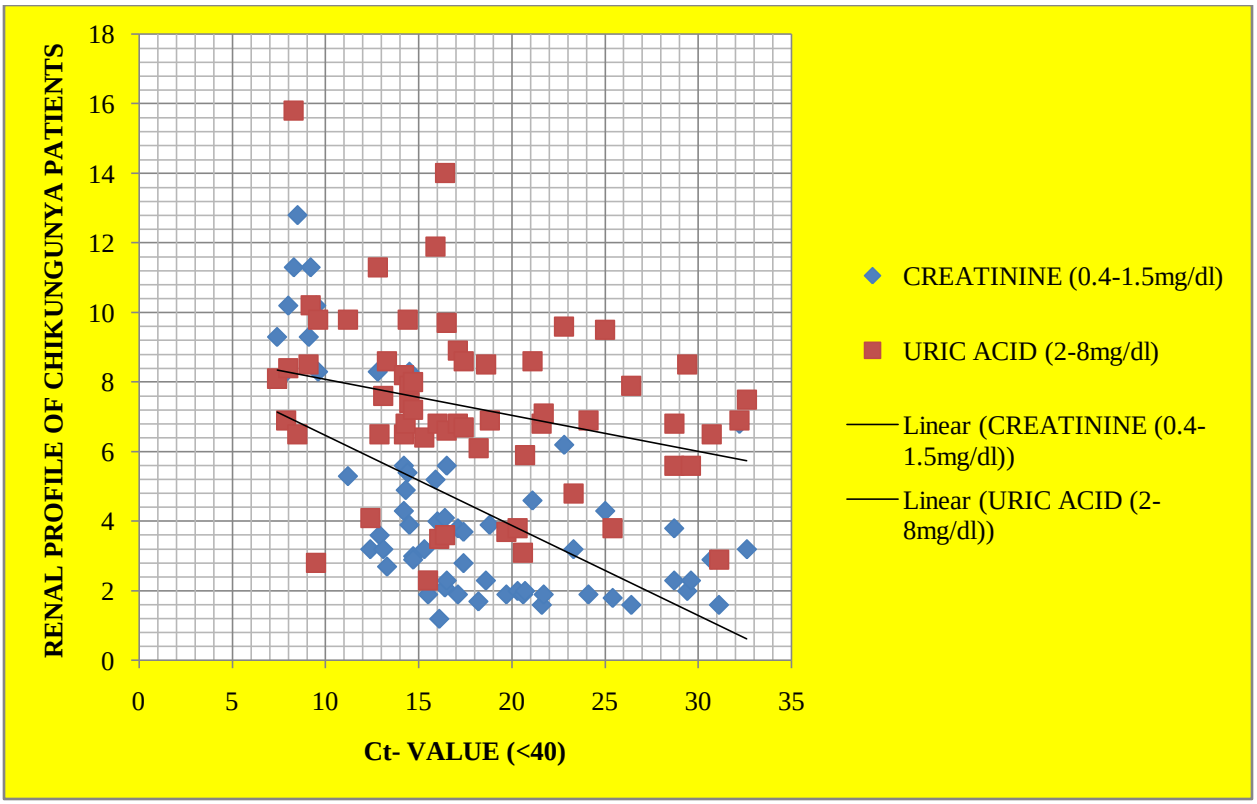
Table (19) Correlation of Renal enzymes with the Ct- value for Chikungunya fever

Correlation of Renal enzymes with the Ct- value for Chikungunya fever			
Parameter	mean±SD	'r' value	Strength
Urea(mg/dl)	123.25±72.14	-0.434	S
Creatinine(mg/dl)	4.42±2.92	-0.595	S
Uric acid(mg/dl)	7.26±2.5	-0.270	M

S=strong, M=moderate.

Graph (16) Correlation of Renal enzymes with the Ct- value for Chikungunya fever





Graph (17) Correlation of Renal enzymes with the Ct- value for Chikungunya fever

DISCUSSION

In this study, we found that DENV-2 serotype is mostly occurred in Dengue Fever cases reported from SMIH Hospital, although DENV-1 and DENV-3 were also detected in lesser dengue patients.

This may be because of that reasons:-

1) Environmental conditions of northern India, especially in Uttarakhand are very favoring for the growth of the DENV.

2) In the rainy seasons the water lodging is very easily occurred in the pot holes, near the river catchment areas, and also in the construction sites of the city which also helps in promoting the growth of the Aedes mosquitoes.

3) SMI hospital also covers the patients, who are always on greater risk of dengue fever from border states of Uttarakhand like Himanchal Pradesh, Haryana, Uttar Pradesh(west), Delhi NCR areas from U.P. where because of above mentioned reasons like Industrialization, River catchment areas, poor Socio-economic status, lack of awareness about the disease and disease controlling programs, Illiteracy, large number of Laborer class who are exposed to repetitive bites of infected mosquito are residing.

In addition, we established that the CHIKV which is also spread by the Aedes mosquito also have the proper climatic conditions over here in Uttarakhand. CHIKV requires temperature of about 22ºC to 28ºC for his growth. In Uttarakhand the temperature rises above 28ºC only between the months of May to August and the temperature goes below 22ºC near to month of December to March, so CHIKV spreads infection in biphasic manner at the change of season. A high vector density as seen in the post monsoon season accentuates the transmission. Chikungunya fever epidemics display cyclical and seasonal trends. There is an inter-epidemic period of 4-8 years (sometimes as long as 20 years) also seen sometimes. But its occurrence found less in the early half of the year because of the low rainfall season so the vector mosquito Aedes is not found at that time.

An important aspect of this study is the use simple Laboratory Parameters like Liver function tests and Renal function tests to distinguish between Dengue and Chikungunya fever. Different hospitals, with a distinct and more rural catchment areas serving for the Dengue and Chikungunya patients but early diagnosis of Dengue and Chikungunya is important for the early treatment of the disease.

Our models show high involvement of liver damage in Dengue Fever as compared to Chikungunya Fever on the other hand renal involvement was more found in Chikungunya Fever although both the diseases are clinically similar to an extent.

Our models did not show an improved % agreement over the WHO definition of Dengue fever and Dengue Hemorrhagic Fever. However, this is not surprising when considering that the physician used these laboratory parameters as an indicator for differentiate Dengue fever with Chikungunya Fever.

The main limitations to our study were that the study design limits the number of patients who developed severe dengue illness. Our study was also limited to SMI hospitals patients only. However, this reflects what is seen in Uttarakhand, where the majority of dengue cases are because of DENV-2 serotype. This is further area of research that why the serotype 2 is is most prevalent over here in Uttarakhand. Use of any prior or concomitant hepatotoxic drugs was not ruled out. Also patients with hepatitis A and E could not be excluded as the tests were expensive and are not routinely perform in the hospital. Patients suffering from secondary infection were not excluded which can cause bias in the results. Other markers of liver damage like prothrombin time, activated partial thromboplastin time, albumin and total proteins should also be looked into and the effect of dengue infections on these and their relation with elevation of transaminases should be studied. The involvements of healthy controls were not there due to ethical committee conditions.

Murgue *et al.* found that when dengue cases were classified according to severity score (developed after close examination of clinical and laboratory data), the 50

most-severe cases were characterized by hemorrhage, decreased platelet count, and associated hepatic disorders, of which 17 were DF cases as classified by WHO criteria (103). Specifically, the most severe DF cases were characterized by severe hemorrhage, miscellaneous (cardiac, renal, pulmonary) manifestations, and elevated serum Transaminase levels (103). Expanded dengue syndrome was coined by WHO in the year 2012 to describe cases which do not fall into either dengue shock syndrome or dengue hemorrhagic fever. **Mohanty B. *et al***. described the atypical manifestations noted in expanded dengue are multisystemic and multifaceted with organ involvement, such as liver, brain, heart, kidney, and CNS. Patients with involvement of hepatic system may present with features of asymptomatic elevation of liver enzymes, fulminant hepatic failure, acute pancreatitis, acalculous cholecystitis, peritonitis, sub acute intestinal obstruction (SAIO), and rupture of spleen. **Lee *et al*.** observed transaminases elevated in 30% of patients, whereas they observed it in 57.5% of cases (104). **Somia iqtadar *et al***. reported that Hepatomegaly was commoner in patients of DHF (60%) as compared to DF (40%) but the difference was not found to be significant statistically. 57% of total patients developed liver dysfunction. Liver functions profile analysis showed that liver dysfunction developed more in DF than in DHF (38.15 vs. 18.6%). AST was found more commonly elevated as compared to ALT levels (85% vs. 51%). ALT had mean value of 69.22, while in DHF it was 103.71. Male patients in age

group of 31-40 years and those having DF had more frequent elevations of ALT (32.7% versus 18.2%). Male patients in age group of 31-40 years and those having DF had more frequent elevations of ALT (32.7% versus 18.2%) (105). ***Mishra et al.*** also demonstrated these three serotypes (DENV-1 ,DENV-2, DENV-3) circulating during the period of 2009 to 2013 in Uttar Pradesh (west) which is also seen in our results so this could be due to the population migration between two states (106). Since the clinical features of the DENV and CHIKV diseases are usually common so chance of misdiagnosis and co-infection is also present in dengue endemic regions as the vector for both is same. In the Indian setting, while screening, considering both the dengue and the chikungunya infections is necessary, because the clinical presentation of symptoms are similar and the outcome may vary (107). In a study carried out in Bangkok, ***Kuo et al.*** 104 cases of Dengue classified as classic dengue, DHF and DSS, LFT explained the most severely ill patients had greater aminotransferases. In children, 70% had Hepatomegaly, and aminotransferases and ALP levels were high compared to normal. So, AST and ALT are important factors for severity of infection (108). With respect to the pharmacological treatment of dengue symptomatology, the association of liver toxicity of this virus should be taken into consideration, as well as avoiding the hepatotoxic drugs. ***Lee et al.*** demonstrated in a retrospective study carried out at Singapore on 690 dengue patients that elevated AST and ALT

occurred in 86% and 46% respectively. Mean for AST and ALT values were significantly higher with increasing dengue severity by both WHO 1997 and 2009 criteria (109).

Chronological analysis of published CHIKV epidemic reports depicts the emergence and re-emergence of CHIKV across continents over time. A single mutation in the viral genome of CHIKV resulted in advantageous changes to the virus by increasing its fitness and ability to adapt to Ae. Albopictus, a new vector that is more tolerant to cold temperature, subsequently changing the global distribution and risk profile of CHIKV [110]. The majority of people infected with chikungunya virus become symptomatic. The incubation period is typically 3–7 days (range, 1–12 days). The disease is most often characterized by acute onset of fever (typically >39°C [102°F]) and polyarthralgia. Joint symptoms are usually bilateral and symmetric, and can be severe and debilitating. Other symptoms may include headache, myalgia, arthritis, conjunctivitis, nausea/vomiting, or maculopapular rash. Clinical laboratory findings can include lymphopenia, thrombocytopenia, elevated creatinine, and elevated hepatic transaminases (2 to 3 fold) (111). No vaccine or specific antiviral treatment for Chikungunya fever is available. All the cases are treated symptomatically -rest, oral (ORS) or intravenous fluids, and non-steroidal anti-inflammatory drugs (ibuprofen, naproxen, acetaminophen, or paracetamol) which relieve the symptoms of fever

and aching. ***Anna et al.*** demonstrated that the liver involvement is there in acute chikungunya infection as the severity increases the renal damage is worsening leads to acute nephritis (112). ***Sison et al.*** demonstrated that clinician diagnosis of chikungunya and dengue fever based on result of liver enzymes AST, ALT and ALP and evident that liver damage is significant higher among dengue fever patients as compared to chikungunya fever patients. Kidney function tests, though may have a potential but less reliable because thrombocytopenia is common and blood urea levels are always raised for both conditions (113).

CONCLUSION AND SUMMARY

Dengue and Chikungunya are two mosquito born arboviral and alpha viral diseases endemic to most tropical and sub-tropical countries. Both illnesses represents a severe flu like symptomatology which included the severe headache, nausea, joint

pain, vomiting, skin rash, Joint pain is specific in Chikungunya, Bleeding diathesis are common among Dengue patients. Both the diseases are enough capable of masking person disabled for work up to 1 to 2 weeks. Liver and Renal derangement is seen in both Dengue and Chikungunya but with different severities. The clinical picture of both diseases are almost same. So An institution based prospective observational study was conducted in the Biochemistry department in collaboration with the department of Medicine of Shri Mahant Indiresh Hospital, a tertiary care Hospital attached to Shri Guru Ram Rai Institute Of Medical and Health Sciences Dehradun, Uttarakhand. The study includes 100 confirmed cases of dengue fever and 60 confirmed cases of Chikungunya, Both are RNA viruses and Molecularly Characterized via Real Time Polymerase Chain Reaction (RTPCR). Then RNA detected confirmed patient is correlated with Liver Profile and Renal profile. The present study concluded that:

- Out of 100 confirmed Dengue cases 64 RNA were extracted 36 RNA was degraded.
- Out of 64 dengue cases 26 were females(40%) and 38(60%) were males.
- Out of 60 confirmed cases of Chikungunya 30(50%) were males and 30(50%) were females.
- The DENV-2 was the most frequent serotype detected 32 (50%).

- The DENV-3 was found with the highest viral load having the thermocycler value of 20.3±5.4±1.3.
- DENV-2 affects males (20.24±5.21) more as compared to females (25.67±5.6) with highly significant p value =0.008.
- A strong negative correlation was observed between cT- value of dengue cases and SGOT with '*r*' value -0.449.
- A very strong negative correlation was observed between cT- value of dengue cases and SGPT with '*r*' value -0.649.
- A strong negative correlation was observed between cT- value of dengue cases and ALP with '*r*' value -0.539.
- A moderate negative correlation was observed between cT- value of dengue cases and GGT with '*r*' value -0.377.
- A very strong negative correlation was observed between cT- value of Chikungunya cases and SGOT with '*r*' value -0.661.
- A strong negative correlation was observed between cT- value of Chikungunya cases and SGPT with '*r*' value -0.536.
- A strong negative correlation was observed between cT- value of Chikungunya cases and ALP with '*r*' value -0.565.
- A Moderate negative correlation was observed between cT- value of Chikungunya cases and GGT with '*r*' value -0.348.

- A moderate negative correlation was observed between cT- value of dengue cases and Urea with '*r*' value -0.345.
- A strong negative correlation was observed between cT- value of dengue cases and Creatinine '*r*' value -0.428.
- A weak negative correlation was observed between cT- value of dengue cases and Uric acid with '*r*' value -0.202.
- A strong negative correlation was observed between cT- value of Chikungunya cases and Urea '*r*' value -0.434.
- A strong negative correlation was observed between cT- value of Chikungunya cases and Creatinine '*r*' value -0.595.
- A moderate negative correlation was observed between cT- value of Chikungunya cases and Ureic acid '*r*' value -0.270.

The present study also concluded that dengue virus showed hepatotoxic effects which is directly proportional to the infectivity of dengue virus. More the viral load of dengue virus more is the hepatotoxicity. Chikungunya virus also showed the hepatotoxicity but hepatotoxicity of chikungunya virus is less severe with respect to dengue virus. On the other hand, the dengue virus also deranged the renal profile but the creatinine level is more affected in case of Chikungunya with respect to the Dengue illness. So the renal toxicity is more with the Chikungunya fever. The

levels of AST was found to be on higher side than that of ALT level in the dengue patients . Blood urea is elevated in both dengue and chikungunya.

It should be mentioned that this study had some limitations. This research had a relatively small sample size. Prospective studies which are conducted on random samples from multiple centers, which have larger sample size can be necessary to validate our data. Additionally, blood samples from the controls were not included in our study.

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