Serotyping and Genetic Assessment of Dengue Virus Circulating in Lahore

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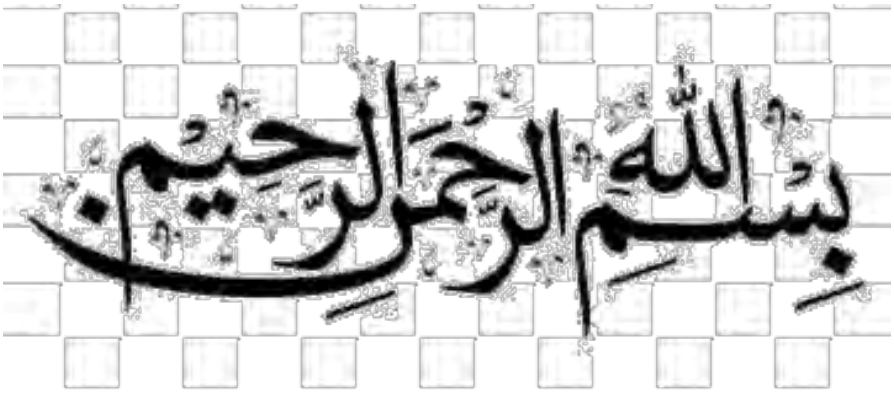

In the name of Allah, The Most Gracious, The Most Merciful

DECLARATION OF ORIGINALITY

I hereby declare that the work **"Serotyping and Genetic Assessment of Dengue Virus Circulating in Lahore"** accomplished in this thesis is the result of my own research carried out in ***Infectious Diseases and Molecular Pathology laboratory***, Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan. This research study has not been published previously nor does it contain any material from the published resources that can be considered as a violation of international copyright law. Furthermore, I also declare that I am aware of the term "copy right" and plagiarism. If, any copyright violation is found in this research work, I will be responsible for the consequence of any such violation.

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DEDICATION

I dedicate this dissertation, wholeheartedly, to my beloved parents, siblings, and my respected supervisor. Without their prayers, support and guidance, accomplishment of this work would not have been possible for me.

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I am overwhelmed in all the humbleness and beholden to Allah Almighty who is The Omniscient and guided me through this research to put the ideas into something concrete. Countless mercy on the Holy Prophet Mohammad (S.A.W).

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How can I forget the most beautiful people in my life Saad and my brother who have always got my back in my darkest moments and lifted my spirit to not give up in face of any challenging situation.

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LIST OF ABBREVIATIONS

5'CS	5′ Cyclization Sequences
ADE	Antibody-Dependent Enhancement
cHP	C-Coding Hairpins
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
dNTPs	Deoxyribonucleotide Triphosphates
DSS	Dengue Shock Syndrome
NLS	Nuclear Localization Sequences
NS1	Non-structural Protein 1
NS2A	Non-structural Protein 2A
NS2B	Non-structural Protein 2B
NS3	Non-structural Protein 3
NS4A	Non-structural Protein 4A
NS4B	Non-structural Protein 4B
NS5	Non-structural Protein 5
nsSNP	Non-synonymous Single Nucleotide Polymorphisms
NTPase	Nucleoside Triphosphatase
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RdRp	RNA-dependant RNA Polymerase
RTP	RTP
SL	Stem-loop
UTRs	Untranslated Regions

Abstract

Dengue virus (DENV) belongs to the family *Flaviviridae*, genus *Flavivirus.* It is one of the major health threats in tropical and sub-tropical regions of the world, including Pakistan, with increasing incidence and spread over the past decade. First case of dengue fever was reported in 1994 in Pakistan and since 2010, Pakistan is facing annual outbreaks. Dengue virus works by attaching to the cell surface and injecting its genome into the host cell which is then replicated by host cell machinery and infect other cells. The attachment and injecting of the genome is aided by structural proteins of the virus which are capsid protein (C), pre-membrane protein (preM) and envelope protein (E). The primary objective of this investigation was to assess CprM gene from DENV prevalent in Lahore. For this purpose, blood samples from 112 patients admitted in hospitals in Lahore, Pakistan were collected. Viral RNA was isolated and sequencing of CprM gene was done. We report serotype-1 from the outbreak-2022 in Lahore. Then, phylogenetic analysis, mutational analysis and protein structure prediction analysis were performed. The results of the study revealed 3 potent amino acid substitutions resulting in amino acid substitutions at L215I, S227F, and K236T in the CprM region, potentially effecting the structure of CprM protein. The effect of identified amino acid substitutions which were validated via *in-silico* tools could be further verified in a population level study and more detailed studies can be done to assess the relationship of amino acid substitution with the severity of disease.

CHAPTER 1

INTRODUCTION

1. Introduction

Dengue fever is brought on by the dengue virus or DENV, which is a member of the *Flaviviridae* family and contains a single strand of positive-sense RNA. This virus is transferred from one individual to another by Aedes mosquito's bite that is already infected with the virus (WHO, 2019). Dengue fever represents a significant risk to public health all around the globe. About three billion people are afflicted by this condition, which accounts for forty percent of the world's population and is present in more than one hundred countries across the globe. The tropical and subtropical regions are the most severely impacted, and between one hundred and four hundred million people are infected annually. During the course of the previous two decades, the number of cases of dengue fever has grown by more than a factor of eight (Nanaware *et al*., 2021). A few of the causes for this growth include lax rules for the management of mosquito populations, reforestation efforts, climate change, and global warming. A severe infection with the Dengue virus may produce symptoms such as discomfort and pain in the abdomen, vomiting, hematemesis, epistaxis, melena, weariness, and fatigue, which can ultimately result in hemorrhagic fever and the failure of the organs (Roy & Bhattacharjee, 2021).

There are four distinct serotypes of the Dengue virus that may be found in various parts of the globe. They are capable of causing anything from a simple illness that clears up on its own to the severe Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS), that is accountable for around 20 thousand fatalities each year. The development of conventional vaccines is making headway, but the co-circulation of many serotypes and Antibody-Dependent Enhancement (ADE) proceedings are limiting the progress that can be made (Xu *et al*., 2016). As a result of these factors, the Dengue virus (DENV) maintains its status as one of the most fascinating viruses in the study of infectious biology. There are four serotypes of the Dengue virus that are well-known to the general public. They are DENV-1, DENV-2, DENV-3, and DENV-4. Nevertheless, a fifth serotype was lately discovered in the year 2013 (Mustafa *et al*., 2015). Despite the fact that the DENV serotypes share around 65% of their characteristics, an infection with one of these serotypes may cause a wide variety of clinical symptoms.

The Dengue virus is an encased positive-sense and single-stranded RNA virus possessing a nucleocapsid with icosahedral structure that is enclosed by the lipid bilayer. It has an irregular spherical form. The DENV genome, which is 11 kilobases long and can serve as mRNA, has Untranslated Regions (UTRs) at 5' and 3' ends bordering the Open Reading Frame (ORF), which is comparable to what is seen in eukaryotic genomes. The translation onset takes place at the type I 5' cap, which is located at the 3' end. The 3' end does not have a poly-A tail but rather a stem-loop in its place. There are C-coding hairpins (cHP), 5′ cyclization sequences (5′CS), downstream and upstream regions regarding AUG that are represented as the 5′UAR for AUG region in upstream direction and 5'DAR for AUG region in downstream direction, as well as Stem-Loop (SL) structures within the 5'UTR. These structures are referred to as SLA and SLB, respectively. Inside the C protein encoding area may be found the DAR, cHP, and 5′ coding sequence. In each of the four DENV serotypes, the 5'UTR consists of sequences ranging from 95 to 101 nucleotides, but the length of the 3'UTRs varies depending on the serotype (Harapan *et al*., 2020).

A polyprotein is encoded by the ORF that is flanked by UTRs. This polyprotein is the precursor of 10 mature proteins. There are three structural proteins and seven non-structural proteins that are produced as a result of processing this polyprotein both during and after translation. Capsid (C), pre-Membrane (prM) and Envelope (E) proteins are the components that make up the structural of a virus particle that are engaged in the encapsulation of RNA of the virus, also known as the formation of nucleocapsids, membrane formation, development, and envelopment of the virion. As their names imply, these proteins are the part of the structure of a virus particle. The proteins that do not form the structure (non-structural) NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 all exhibit a diversity of different enzymatic activities, all of which are now being studied for their many functions in the life cycle of an infectious agent (Harapan *et al*., 2020; Murugesan & Manoharan, 2020).

To illustrate a few of the functions that NS proteins perform: NS5 is an RNA-dependent RNA polymerase (RdRp) that is produced by the virus. It also has nuclear localization sequences (NLS) and is active as an S-adenosyl methionine methyltransferase (MTase).

NS3 has activities known as RNA-triphosphatase (RTP), nucleoside triphosphatase (NTPase), and helices. These activities provide the capacity for both NS3 and NS5 to make significant contributions to the process of RNA replication. In viral replication, assembly, and release, NS2B, along with other NS proteins like as NS1, NS2A, NS4, and NS4B, serves a variety of tasks (Nanaware *et al*., 2021).

Dengue fever has become a major public health concern in Pakistan in recent years. According to the World Health Organization (WHO), the first recorded incidence of dengue fever in Pakistan was in 1994. However, it was not until November 2005 that the yearly epidemic pattern of dengue began in Karachi. Prior to 2006, dengue was only found in a limited number of areas in Pakistan. Since 2010, Pakistan has been facing annual dengue outbreaks, with the number of cases peaking during the post-monsoon season (Yousaf *et al*., 2021). The rapid spread of dengue to new areas of Pakistan is attributed to Aedes mosquitoes transmitting the virus from dengue-infected areas to unaffected areas. Additionally, vertical transmission may also be contributing to the spread of the disease (Rasheed, Butlin, *et al*., 2013).

Pakistan is known to be an endemic region for dengue fever. The most notable outbreak occurred between September and December 2019, resulting in 53,498 cases and 95 deaths. Unfortunately, the incidence of dengue fever has continued in Pakistan, with a total of 48,906 cases and 183 deaths being reported between 1 January to 25 November 2021. These cases were reported from several provinces, including Khyber Pakhtunkhwa, Balochistan, Sindh, Punjab, the federally administered Islamabad Capital Territory (ICT), and Azad Jammu and Kashmir autonomous territories (AJK) (Khatri *et al*., 2022).

The province with the highest number of reported dengue cases as of 25 November was Punjab, with more than 24 thousand cases and 127 deaths. This province alone accounted for 49.4% and 69.4% of all reported cases and deaths, respectively. Most of the deaths in Punjab were reported in the Lahore district. Khyber Pakhtunkhwa had the second-largest number of reported cases, with more than 10 thousand cases and 10 deaths. In Sindh province, around 5,500 cases and 24 deaths were reported. ICT reported more than 5

thousand cases and 21 deaths. Balochistan province reported nearly 2 thousand cases, and AJK reported almost 2 thousand cases, with one death (WHO, 2021).

The incidence of dengue fever in Pakistan is a cause for concern, and efforts to control its spread are ongoing. These efforts include mosquito control measures such as fumigation and elimination of breeding sites, public awareness campaigns, and improvements to the healthcare infrastructure. It is essential to continue these efforts to combat the spread of dengue fever in Pakistan and protect the health and well-being of the population (J. Khan *et al*., 2022).

The situation in Pakistan is alarming, and efforts are being made to combat the spread of dengue fever. The government has implemented various measures, including fumigation, mosquito control programs, and public awareness campaigns, to control the disease. Despite these efforts, however, the number of cases continues to rise each year. It is essential that Pakistan takes effective steps to prevent and control the spread of dengue fever. This includes improving healthcare infrastructure, providing accurate and timely information to the public, and increasing awareness about the importance of preventive measures such as using mosquito nets, repellents, and avoiding areas with standing water. With coordinated efforts, Pakistan can successfully combat the spread of dengue fever and protect its citizens from this deadly disease (S. Khan, 2022).

Aims and Objectives

1) To amplify and sequence the CprM gene of DENV strains circulating in Lahore.
2) To assess the serotype of the virus using CprM gene nucleotide sequence.
3) To assess genetic diversity of DENV strains circulating in Pakistan with those reported in other regions of the world.

CHAPTER 2

LITERATURE REVIEW

2. Literature Review

2.1. Dengue Fever and Dengue Virus

DENV is a virus, with a single-stranded RNA, that belongs to the family Flaviviridae and is spread to *Homo sapiens* via the bites of infected *Aedes* mosquitoes. The virus is comprised of four distinct serotypes, DEN-1, DEN-2, DEN-3, and DEN-4, each of which can cause a range of clinical symptoms ranging from mild febrile illness to severe hemorrhagic fever (Murugesan & Manoharan, 2020).

Dengue-like disease epidemics have been recognized for over two centuries and have been reported in various regions around the globe, including Asia, the Americas, and Africa. The first recorded outbreak of dengue fever occurred in Asia in the 1770s, and since then, numerous epidemics have been reported in different parts of the world. For instance, in the early 1900s, outbreaks of dengue fever were reported along the Atlantic and Gulf coastlines of the United States and in the Caribbean (Simmons *et al*., 2012).

Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are severe variants of dengue fever that were first identified in the Philippines in 1954 and have subsequently spread throughout Southeast Asian countries (Guzman *et al*., 2010). DHF/DSS is a severe cause of infection and fatality in infants in the tropical region of Asia, and in the absence of effective treatment, it is becoming more common in the Western Hemisphere. DHF/DSS is characterized by bleeding, plasma leakage, and low platelet count, which can lead to shock and death if not treated promptly (Halstead & Cohen, 2015).

Dengue fever is a widespread tropical illness that is endemic to many regions of the world, including Southeast Asia, Africa, and the Americas. In extreme cases, this acute febrile sickness can be fatal, resulting in dengue hemorrhagic shock, which has a mortality rate of up to 50%. Dengue is an arbovirus that causes approximately 390 million worst dengue infections annually, with an estimated 96 million of them manifesting clinically in almost 129 countries worldwide (WHO, 2019). The burden of dengue fever is highest in Southeast Asia and the Western Pacific, where an estimated 70% of the disease burden occurs, but

the disease is also prevalent in many parts of Africa, the Americas, and the Eastern Mediterranean (Messina *et al*., 2014; Zerfu *et al*., 2023a).

2.2. Evolutionary History and Origin of Dengue Virus

The evolutionary history of dengue virus is a complex and ongoing subject of study. It is believed that the virus originated in the sylvatic (wild) cycle, where it infected non-human primates and was transmitted by Aedes mosquitoes in forested areas of Africa and Asia. Over time, the virus adapted to infect and spread among humans and urban environments, leading to the emergence of the epidemic cycle (Johari *et al*., 2019).

Studies have suggested that all four serotypes of DENV are thought to have their sylvatic ancestral lineage sheltered in Malaysia, with sylvatic DENVs from Africa and Asia using nonhuman hosts as primates and Aedes mosquitoes as their vectors in forests. These sylvatic DENVs are assumed to be the origins of urban transmission, and all serotypes of DENV are believed to have emerged about 1,000 years ago (Isa *et al*., 2021).

Recent research has shed light on the evolution and spread of each serotype of DENV. For example, DENV-1 is believed to have originated in Asian countries before spreading to African and American countries. DENV-2 evolved from its sylvatic ancestor, which emerged about 400-600 years ago. The first report of DENV-2 was made in the Americas in 1953, in Nigeria in 1964, and in Asian countries in 1944. DENV-3 was first discovered in 1953 in Asia, reported in America in 1963, and found in Africa during 1984-1985. DENV-4 was initially discovered in the Americas in 1981 and Asia in 1953 (Harapan *et al*., 2020; N. Sirisena *et al*., 2021).

The history of dengue virus infections in humans can be traced back to the late 18th century when major epidemics occurred in Asia, Africa, and North America. However, it is believed that the virus may have existed in human populations for much longer, with evidence of association between sylvatic DENV and humans found in retrospective serological studies of human communities residing in forests (Silva *et al*., 2020; Wilder-Smith *et al*., 2013).

The emergence of urban transmission and the spread of dengue virus in human populations have been linked to the destruction of forests and the expansion of human settlements. Aedes albopictus and other peri-domestic species of mosquitoes have been identified as primary vectors of viral transmission across tropical Asia's cities, towns, and villages as a result of human and economic migration. Entomological studies have also suggested that *Aedes furcifer*, which spreads into rural and peri-domestic areas, may be the common linking vector between different types of sylvatic DENV and human populations found near forest areas (Mayer *et al*., 2017; Rezza, 2014).

Overall, the evolutionary history and origin of dengue virus are complex and ongoing subjects of study. While noteworthy development has been made in comprehending the emergence and spread of the virus, much remains to be learned about the factors that drive its transmission and how it can be effectively controlled.

2.3. Dengue Classification

Dengue fever can be classified into three categories according to World Health Organization issued guidelines published in the years 1975 and 1977 which are: Undifferentiated Fever, Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) (Srikiatkhachorn *et al*., 2011). The derails of each type is given below:

2.3.1. Undifferentiated Fever

In South Asian nations in specifically, undifferentiated fever is a frequent reason for people to seek medical attention from health care practitioners. Fever deprived of a clear focus of infection based on the physical examination and medical history is the definition of this condition. It poses significant difficulties in both diagnosis and treatment, making it a huge public health burden (N. Ali *et al*., 2020). The major infectious trigger for acute undifferentiated fever is dengue, which causes a significant economic burden and results in the administration of presumptive broad-spectrum antibiotics with no proof supporting their efficacy (Sundén-Cullberg *et al*., 2017).

2.3.2. Dengue Fever (DF)

The classic form of dengue fever, sometimes known as "break bone fever," manifests itself as an abrupt start of high fever anywhere between 3 and 14 days after being bitten by an aedes mosquito (Juckett, 2015). A forehead pain, retro-orbital discomfort, muscle aches, arthralgias, hemorrhagic signs, rash, and a decreased white blood cell count are some of the symptoms that may be experienced. In addition, the patient might report of nausea and loss of appetite. When acute symptoms are present, they typically last for approximately one week; however, symptoms such as fatigue, malaise, and anorexia might continue for many weeks. A significant majority of dengue infections either do not cause any symptoms at all or create very mild symptoms. This is particularly true in youngsters and in people who have no previous record of a dengue infection in the past (Schaefer *et al*., 2022).

2.3.3. Dengue Hemorrhagic Fever (DHF)

DHF is a severe form of dengue fever caused by the same viruses that cause typical dengue fever. DHF is characterized by a high fever that lasts from 2 to 7 days and at least two of the following symptoms: severe headache, retro-orbital pain (pain behind the eyes), joint and muscle pain, nausea, vomiting, rash, and mild bleeding. DHF can also cause severe bleeding, a sudden drop in blood pressure (shock), and death. The onset of DHF typically occurs around the time when the fever is resolving or has resolved (Mallhi *et al*., 2015).

The development of DHF usually follows a previous dengue infection. The hazard of acquiring DHF is greater through a secondary infection with a diverse serotype of dengue virus. The body's immune system produces antibodies against the virus during the first infection, but these antibodies do not provide immunity against the other three serotypes of dengue viruses (P. Bhatt *et al*., 2021). When a person is infected with a different serotype, the immune system can overreact, producing antibodies that form immune complexes that can activate the complement system and lead to vascular damage, increased vascular permeability, and plasma leakage. Plasma leakage is responsible for the severe fluid imbalance and hemorrhagic manifestations that occur in DHF. DHF can occur in all age groups, but children under the age of 15 are at a higher risk of severe disease (de Almeida *et al*., 2017).

Early diagnosis and appropriate clinical managing of DHF can minimize the danger of complications and death. Treatment for DHF typically includes fluid and electrolyte replacement, close monitoring of vital signs, and blood transfusions if necessary. Early recognition of the warning signs of DHF is essential, as early intervention can prevent the disease's progression to the severe form. Dengue hemorrhagic fever can be a life-threatening illness, but with prompt medical attention, most people with DHF recover fully (Leowattana & Leowattana, 2021).

2.3.4. Dengue Shock Syndrome (DSS)

DSS is a severe and life-threatening complication of dengue hemorrhagic fever, which can lead to shock and death if not managed promptly. It typically occurs in patients with a secondary infection with a different serotype of dengue virus (Alejandria, 2015; Rajapakse, 2011). The pathogenesis of DSS involves a complex interplay between viral, host, and environmental factors. During a secondary dengue infection, the immune system produces antibodies to the new serotype of the virus, which can bind to the virus but not neutralize it. Instead, the virus-antibody complexes are taken up by macrophages and other immune cells, leading to the release of cytokines and other pro-inflammatory mediators. This cascade of events can cause an rise in vascular penetrability, preceding to leakage of plasma, hypovolemia, and shock (Samarasekara & Munasinghe, 2018).

2.4. Epidemiology of Dengue

More than one hundred nations have been found to be afflicted by dengue virus, putting around 3.6 billion people throughout the globe at danger of being infected with the disease (Diamond & Pierson, 2015). Yearly epidemics of dengue fever has been observed in the Australia, Africa, America, and Asia (CDC, 2014), and tourists from endemic countries are at risk of contracting the disease. The prevalence of dengue fever has grown by a factor of thirty over the last half century. Not only do these epidemics have enormous consequences on public health, but they also generate substantial economic losses in the nations that are impacted, including Pakistan (Shabbir *et al*., 2020).

2.4.1. Epidemiology in Western Region

North America had its first verified case of dengue fever in the year 1780 (Dang *et al*., 2020). In 2010, there were approximately 1.6 million cases of dengue recorded in North and South America, with 49,000 cases classified as severe. In 2016, the United States had the greatest epidemic of dengue fever, with over 2.38 million cases; yet, Brazil was the nation with the highest number of dengue fever infections overall. Around three million cases of dengue fever were recorded in the United States in 2019 (PAHO, 2019).

2.4.2. Epidemiology in Asian Region

The industrialization of Southeast Asian countries, notably following World War II, has been a key contributor to the proliferation of dengue fever epidemics in those nations. The Philippines was the location of the first two confirmed cases of DHF, which were documented in 1953 and 1956, correspondingly. Since then, Southeast Asian nations including the Philippines, Thailand, Bangkok, Bhutan, Brunei, Cambodian islands, East Timor, Malaysia, Laos, Indonesia, Burma, Singapore, and Vietnam have had recurrent outbreaks of dengue fever on an annual basis (Roy & Bhattacharjee, 2021). In the years 2009 and 2010, the bulk of cases of dengue in Indonesia were caused by serotype 4, but in 2013, acute dengue was recorded as a result of infection with serotype 3. This was the period in which Indonesia seemed to have the second-highest proportion of dengue cases behind Brazil (Lardo *et al*., 2016; Taslim *et al*., 2018). The most common strain of dengue virus, serotype 1, was found in Indonesia between 2007 and 2010 (Sasmono *et al*., 2015).

2.4.3. Epidemiology in Pakistan

It is believed that dengue fever is spreading across Pakistan. In 1960, when the populace of Pakistan was believed to be 45.9 million, Hub, which is located in the Baluchistan Province, was the location where the initial dengue incidence was documented. The overall number of dengue incidence that were recorded throughout the time period of 1960–1980 was just 12 (Rasheed, Boots, *et al*., 2013; Rasheed, Butlin, *et al*., 2013). In 1994, Karachi was the location where the first dengue epidemic that was verified both serologically and virologically was reported [10]. Since 1960, Pakistan's population has expanded to 188.2 million (2014), while the total number of dengue cases that have been documented has

climbed up to 74,495, with 690 deaths reported. It is difficult to understand the variables that are leading to the widespread spread of dengue virus as well as the upsurge in the quantity of cases for dengue (J. Khan *et al*., 2018).

The World Health Organization (WHO) estimates that 48,906 instances of the disease, including 183 fatalities, have been registered in Pakistan for the year 2021. The situation is very concerning as a result of the growing number of cases. In every region of the nation, DAWN news reports that there are daily reports of an increase in the number of persons who test positive for dengue fever. Since January, Punjab has recorded a total of 24,146 cases as of November 25, 2021, with the city of Lahore being the most severely impacted. In a same fashion, Khyber Pakhtunkhwa reported 10,223 instances, Sindh reported 5,548 cases, the federally managed ICT recorded 5,261 cases, Baluchistan recorded 20,54 cases, and AJK reported 1,674 cases (DAWN, 2021). If appropriate preventative steps are not done, the situation will become much more dire, particularly in the province of Punjab (U. Khan & Azeem, 2022).

2.5. Morphology and Genome of Dengue Virus

Dengue Virus, much like the various *flaviviruses*, is an RNA virus with a single strand and a positive sense of direction. This virus is encased in an icosahedral nucleocapsid and has a lipid envelope around it. The mature virion has a diameter of around 50 nm (Roy & Bhattacharjee, 2021). The genome has a length of 10.7 kilobases and has a single lengthy ORF that is translated into a polyprotein with about 3388 amino acids (Shrivastava *et al*., 2018; Zeng *et al*., 2018). Each four serotypes of DV have had their whole genomes sequenced, and this information is available. The nucleocapsid or core protein (C), a membrane associated protein (M), and an envelope protein (E) are the structural proteins that are encoded by the genome. The genome also contains seven nonstructural (NS) protein genes in addition to the three structural protein genes (Murugesan & Manoharan, 2020). The nonstructural proteins assist in the generation of new viruses after the virus has entered the cell, whereas the structural proteins are responsible for forming the coat of the virus and transporting RNA to the cells it infects (Rosales Ramirez & Ludert, 2019). The

sequence of the genome is as follows: 5'-C-PrM (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (Yergolkar *et al*., 2017).

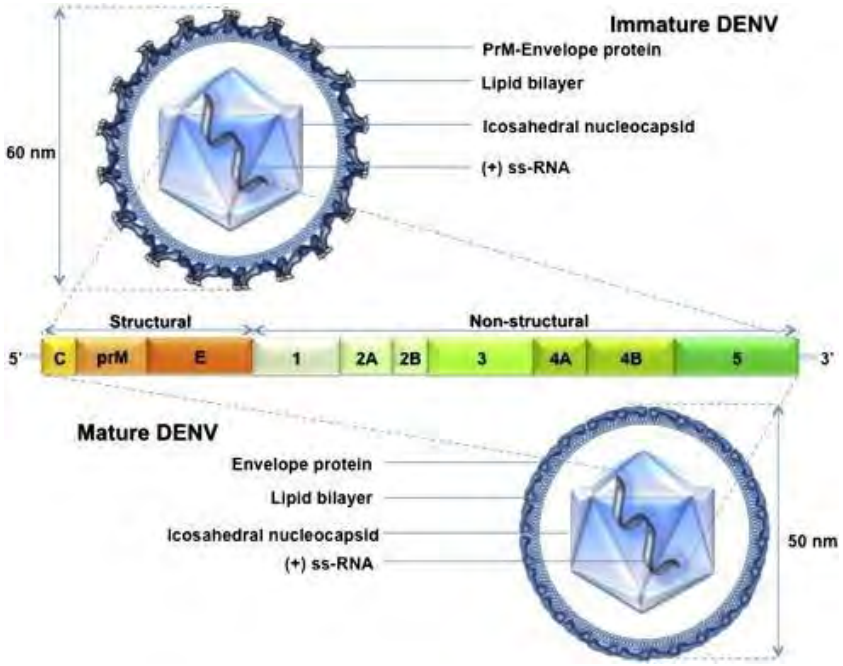


Figure 0.1 *Morphology of dengue virus Source:* (Herrero *et al*., 2013; Pant, 2017)

A matured dengue virion has a nucleocapsid that is isometric and has diameter of around 20 to 30 nm. Its nucleo-capsid is surrounded by an envelope of lipid that measures 10 nm in depth. The envelope is made up of a lipid bilayer, in which an E component and a M part are both encapsulated. The envelope protein, whose size ranges from 51,000 to 59,000 Daltons and facilitates adhesion, union, and entry, and the internal matrix protein, whose size ranges from roughly 8,500 to 9,000 Daltons and is not glycosylated, are both non-glycosylated. In the majority of *Flavi* viruses, the envelope protein is glycosylated and may be seen exposed on the surface of the virion. The lipid makeup of the envelope is determined by the lipid makeup of the host cell membrane, which is where the virus buds off from (Pant, 2017).

2.6. Proteomics of Dengue Virus

The proteomics of Dengue Virus is divided into two categories depending on their functions. The first one is structural proteins and the second one is non-structural proteins.

2.6.1. Structural Proteins

2.6.1.1. Core or Capsid Protein (C)

The dengue virus has a crucially important structural component known as capsid protein. It contributes to the formation of the virus's outer shell and plays a role in the virus's ability to maintain its genetic material (RNA). Capsid protein is essential to the generation of infectious virion as well as the assembly and packaging of the viral genome (Dwivedi *et al*., 2017). This role is played by capsid protein in both processes. It is also necessary for the early stages of viral infection because it has a role in the control of viral replication and is essential for those stages. It has been shown that the capsid protein interacts with other viral proteins as well as components that are present in the host cell, and it is believed that this interaction contributes to the virus' ability to evade the immune system. It has been proposed as a viable technique for the development of antiviral medications for the dengue virus that targeting the capsid protein might be an effective method (Byk & Gamarnik, 2016).

2.6.1.2. Membrane or Pre-Membrane Protein (M or preM)

The membrane (M) and pre-membrane (prM) proteins are structural proteins of the dengue virus that play an important role in viral assembly and maturation. These proteins are denoted by the letters M and prM, respectively. Whereas the M protein is the one that is in charge of attaching the viral envelope to the viral nucleocapsid, the prM protein is the one that helps ensure that the viral envelope proteins fold correctly and are assembled correctly (Li & Kang, 2022).

In order to generate the mature M protein, the prM protein must first be cleaved by a host protease over the course of the viral replication cycle. This cleavage is necessary for the structural rearrangements that take place throughout the maturation process of the virus, which eventually leads to the generation of infectious virions (Cruz-Oliveira *et al*., 2015).

In addition to this, the immunological evasion that the dengue virus engages in is facilitated by the prM protein. It protects the fusion protein by acting as a shield, preventing the host immune system from recognizing the protein as foreign. This is due to the fact that the fusion protein is highly immunogenic and has the ability to generate a robust immune response, both of which may result in the virus being neutralized. The prM protein enables the virus to circumvent the immune system and successfully establish an infection by acting as a cover for the fusion protein (Lee *et al*., 2022).

2.6.1.3. Envelope Protein (E)

The envelope (E) protein is an important structural protein of dengue virus. It plays a crucial role in viral entry into host cells by mediating fusion of the viral and host cell membranes. The E protein consists of three domains: domain I, domain II, and domain III. Domain III is responsible for receptor binding and determines the virus's host range and tropism. Once the E protein binds to its receptor on the host cell surface, it triggers a conformational change that exposes a fusion peptide in domain II. This peptide then inserts into the host cell membrane, and the virus fuses with the host cell, releasing the viral genome into the cell (Pitcher *et al*., 2015).

In addition to its role in viral entry, the E protein also plays a critical role in inducing a host immune response. The protein contains multiple epitopes recognized by the host's immune system, making it an important target for vaccine development. However, due to the existence of four dengue virus serotypes, each with distinct epitopes, creating a vaccine that targets all four of the serotypes has proven challenging. Nonetheless, attempts to form a safe and successful vaccine for dengue targeting the E protein are ongoing (Wang *et al*., 2021).

2.6.2. Non-structural Proteins

2.6.2.1. NS1

The flavivirus NS1 protein is a versatile, conserved, N-linked non - structural protein with six conserved intramolecular disulfide linkages. It has a molecular weight of around 48 kilodaltons (Meng *et al*., 2015). These linkages are synthesized on the surface of the cell

and then released into the nearby environment, where they engage in immune evasive functions. Additionally, it was found that the NS1 protein performs a vital part in the reproduction of DENV (Fan *et al*., 2014). NS1 is first synthesized as a monomer, then undergoes dimerization following post-translational modification inside the core of the endoplasmic reticulum (ER), is processed in the trans-Golgi apparatus, and is finally released into the extracellular environment as a hexameric lipoprotein particle (Muller & Young, 2013). Hexamers of NS1 have a rich concentration center of lipid and are kept organized by mild hydrophobic contacts. These relations cause the hexamers to break into dimers when they come into contact with nonionic cleansers. NS1 may be released into the extracellular environment when aberrant conditions are present, and it has been found at concentrations of up to 50 g/ml in the sera of certain DENV-contaminated individuals. It has been shown that the NS1 protein may be expressed on the surface of the plasma membrane by using a variety of molecular processes. The released NS1 attaches to the plasma membrane of cells when it recognizes sulfated glycosaminoglycans as a component of the membrane (Dwivedi *et al*., 2017).

2.6.2.2. NS2A

The majority of the species that belong to the flavivirus family have a hydrophobic nonstructural protein 2A (NS2A) protein that has a mass of 22 kilodaltons (Xie *et al*., 2015). DENV's NS2A protein is a crucial component of the viral reproduction unit; it is essential to the process of virion production and inhibits the immunological reaction of the host (Dwivedi *et al*., 2017). It has been discovered that there are two distinct groups of NS2A protein (Xie *et al*., 2015). One set, which was shown to be responsible for DENV RNA synthesis and was found to be localized in the viral reproduction unit, while the other set, which was discovered to be localized in the virion assembly location and was identified as being engaged in the virion assembly (Xie *et al*., 2015). The DENV hydrophobic protein of NS2A is made up of five transmembrane parts that span the lipid bilayer of the membrane of the ER (Meng *et al*., 2015).

2.6.2.3. NS2B and NS3

DENV's NS3 is an enzyme with multiple functionalities that carries functions important in viral RNA replication and packaging (Shannon *et al*., 2016). These functions include helicase, nucleoside 5'-triphosphatase (NTPase), and RNA 5'-triphosphatase (RTPase). At a resolution of 3.15 angstroms, the crystalline framework of the whole molecule of NS3 together with 18 repeats of the NS2B cofactor has been discovered (Dwivedi *et al*., 2017). Its structure is made up of two different domains: the ATPase/helicase domain and the serine protease N-terminal domain. In order for this protein's protease domain to be activated, it must engage with an NS2B cofactor, which results in the formation of a complex known as NS2B-NS3 protease. It has been shown that the NS2B-NS3 protease complex is responsible for cleaving the DENV polyprotein (Dwivedi *et al*., 2017). The structure–activity connections of DENV NS2B-NS3 proteases are very consistent across all of the various serotypes. As a result, NS2B-NS3 protease is the most promising strong therapeutic target for the creation of efficient medicines versus all DENV serotypes (Cabarcas-Montalvo *et al*., 2016; H. Wu *et al*., 2015). The portion of NS3 that is located at its carboxyl terminus is responsible for three distinct enzymatic activities: RTPase activity, NTPase activity, and helicase activity (McCullagh & Davidson, 2016). Current research suggests that the C-terminal 50 amino-acid residues of the NS3 protein are crucial for interaction of NS3 and NS5 as well as viral proliferation (Tay *et al*., 2015).

2.6.2.4. NS4A and NS4B

NS 4A and 4B are the essential proteins of the membrane of Dengue virus, which perform numerous roles in Dengue virus reproduction and virus–host associations. Cellular vimentin is responsible for regulating the development of DENV replication complex (Teo & Chu, 2014). This is achieved by molecular interaction with Dengue virus NS4A. Both the NS4A and NS4B proteins are essential components of the complex that is linked with the ER membrane for replication. NS4A protein is comprised of 127 amino acids and has two transmembrane domains (TMDs) (Zou *et al*., 2015). It has been discovered that the initial transmembrane domain of 48 amino acids is important in the creation of an amphipathic helix that facilitates oligomerization. There are three TMDs present in the

NS4B protein, which has a total of 248 amino acids (Stern *et al*., 2013). NS4B binds with the helicase domain of NS3 in DENV and is responsible for dissociating NS3 from single-stranded RNAs. NS4A and NS4B proteins are linked together by a homologous peptide that is 23 amino acids long and has a molecular weight of 2000 (2K). The activity of NS4A in modifying the ER membrane may be controlled by the 2K peptide in various flaviviruses through a variety of different regulatory methods. The cleavage between NS4A and 2K by the protease produced by the virus is a prerequisite for the signalase produced by the host, which is necessary for the cleavage of the connection among 2K and NS4B (Zou *et al*., 2015). There is an interaction between a region of NS4A consisting of 40 to 76 amino acids and a region of NS4B consisting of 84 to 146 amino acids. Certain amino acid residues have been shown to have a link between virus assembly and NS4A-NS4B contact, which supports the biological relevance of the NS4A-NS4B interaction, according to a functional analysis research that was conducted. These mutations in these amino acid sequence areas prevent NS4A and NS4B from interacting with one another, which may result in a suppression of the replication (Naik & Wu, 2015). Given the physiological significance of the NS4A-NS4B interaction, it is reasonable to hypothesize that the development of anti-dengue drugs might include the pursuit of inhibitors of this connection (Dwivedi *et al*., 2017).

2.6.2.5. NS5

NS5 is the most extensively conserved and the biggest of the DENV proteins. It performs two crucial roles, the first of which is the functioning of RNA-dependent RNA polymerase (RdRp), which is essential for the replication process of the virus (Iglesias *et al*., 2011). The second factor is RNA methyltransferase (MTase) operation, which plays a key role in RNA capping throughout polyprotein translation (Klema *et al*., 2016). Over the process of replication of viruses, NS5 also contributes to the formation of an RNA replicase complex together with NS3 in the membrane of the endoplasmic reticulum. After replication is complete, NS5 separates from NS3 and then moves to the nucleus. Nuclear translocation has only been seen in DENV-2 and -3 serotypes (Hannemann *et al*., 2013), according to previous research. Experiments using yeast two-hybrid (Y2H) reveal that nuclear

translocation may take place due to the fact that the nuclear import receptor importin-β-competes with DENV-NS3 for interaction with NS5. Even though the accumulation of NS5 in the nucleus does not appear to be necessary for viral replication (A. Kumar *et al*., 2013), it does happen to be connected to an improve in the generation of the cytokine IL-8, which has been shown to be formerly associated with serious cases of dengue (Bhatnagar *et al*., 2021).

Recently has been a lot of curiosity in targeting NS5 for the development of vaccines and anti-viral therapies since it plays a crucial role in viral replication and is an important focus for cytotoxic T cell responses (Alves *et al*., 2016). Mutational analysis of the NS5-MTase domain uncovered a number of residues that are expected to play an important role in viral replication. In the early stages of the life cycle of the virus, the process of 2'-O-methylation of viral RNA is absolutely necessary for the inhibition of immune reactions from the host. Removal of the 2′-O-MTase by altering just one amino acid (E216A) culminates to an earlier stimulation of anti-viral reactions such as RIG-I, IL-8, and IFIT2 resulting in an attenuation of viral activity (Chang *et al*., 2016). Large-scale in vitro screening has led to the discovery of a number of compounds that block the function of MTase and RdRp (Lim *et al*., 2015). Furthermore, NS5 collaborates with host proteins like STAT2, which are essential for type 1 interferon (IFN-I) signaling and innate reactions, and it blocks host anti-viral responses (reviewed recently by (El Sahili & Lescar, 2017). Additionally, current high-throughput investigations in an array of research frameworks, in addition to bioinformatic evaluations, indicate that NS5 communicates with a wide range of host proteins (Amemiya *et al*., 2019; El Sahili & Lescar, 2017).

2.7. Replication of Dengue Virus

The first step in the replication cycle is the attachment of the virus to the host cell surface. The dengue virus envelope protein (E protein) binds to specific receptors on the surface of the host cell. Once the virus has attached to the cell surface, it enters the cell via receptor-mediated endocytosis. The virus is taken up into a vesicle called an endosome. The acidic environment of the endosome triggers a conformational change in the E protein, which allows the virus to fuse with the endosome membrane and release its RNA genome into

the cytoplasm of the host cell (O'Connor *et al*., 2021). Following that, the dengue virus genome is a single-stranded positive-sense RNA molecule. The viral RNA is translated into a polyprotein upon release into the cytoplasm, which is processed by viral and host proteases into individual structural and non-structural proteins. Then the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) then assemble into a replication complex, which replicates the viral RNA genome. After that, the structural proteins (C, prM, and E) are synthesized and transported to the endoplasmic reticulum (ER) membrane. The prM protein is cleaved by a host protease, which results in the formation of mature virions. The mature virions are then transported through the secretory pathway and released from the host cell via exocytosis (Nanaware *et al*., 2021).

The replication cycle of dengue virus can occur in a diversity of types of cells, comprising of skin cells, immune cells, and liver cells. The virus can cause a range of clinical manifestations, from mild dengue fever to severe dengue hemorrhagic fever and dengue shock syndrome (Zerfu *et al*., 2023). Understanding the replication cycle of dengue virus is crucial for developing effective antiviral therapies and vaccines to control the spread of this disease.

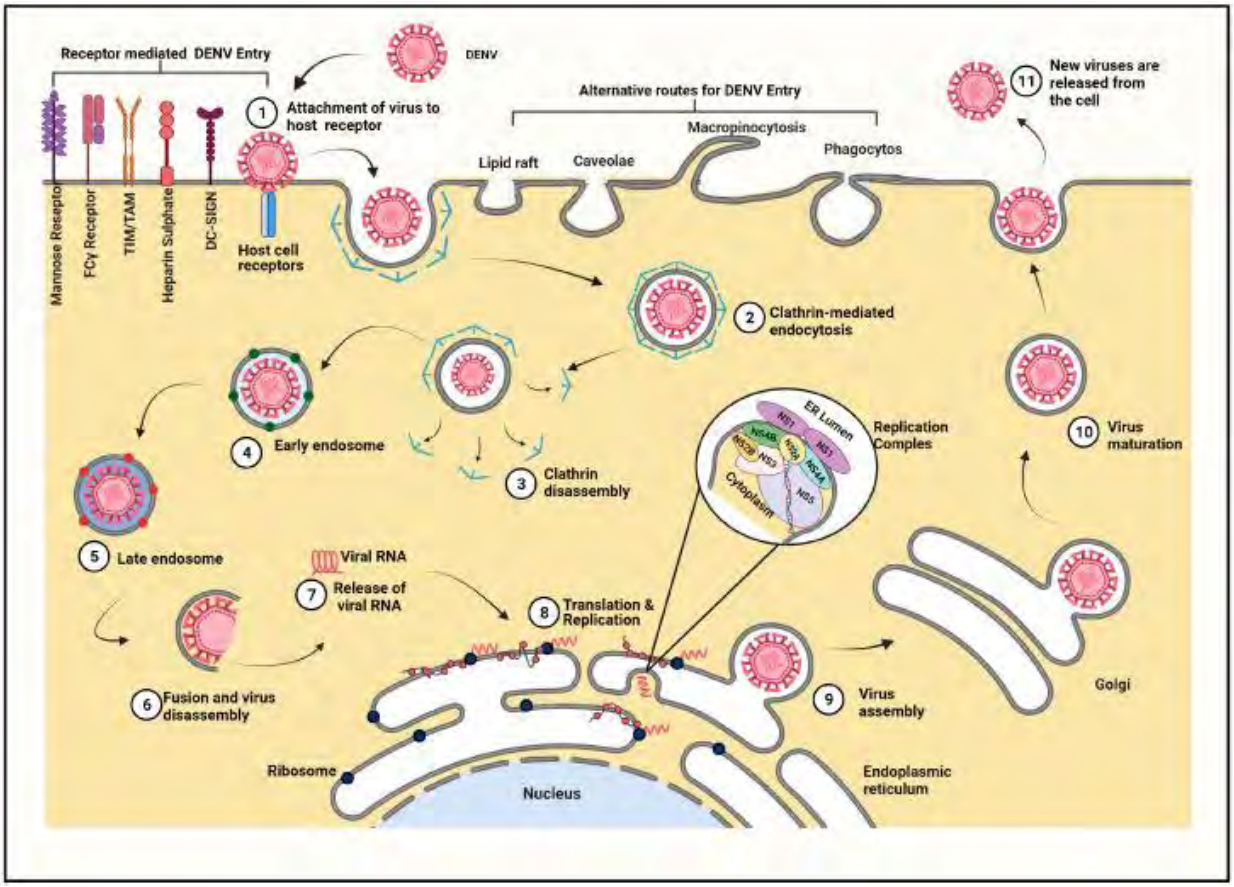


Figure 0.2 *Replication Cycle of DENV. Source:* (Nanaware *et al*., 2021)

2.8. Vectors

Aedes aegypti and *Aedes albopictus* are the two main vectors of dengue virus. Both *Aedes aegypti* and *Aedes albopictus*, which are two of the most important DENV vector species, have successfully adapted to living in urban areas. In residential neighborhoods that have inadequate cleanliness, it is common to discover larvae of both species living in abandoned containers (Dowling *et al*., 2013; Paul *et al*., 2018; Walker *et al*., 2018). A elevated danger of infection is generally connected with socio-economic issues and fast suburbanization lacking sufficient infrastructure (S. Bhatt *et al*., 2013). This is one of the explanations why high infection danger is frequently linked with these characteristics. Because of this, urban development in tropical climates leads to a rise in the number of vectors that are present. The previous species reproduces nearly solely in man-made environments in urban settings

(Rajarethinam *et al*., 2020), but the later species may also reproduce in tree holes and bamboo that has been chopped or broken (Medeiros-Sousa *et al*., 2015).

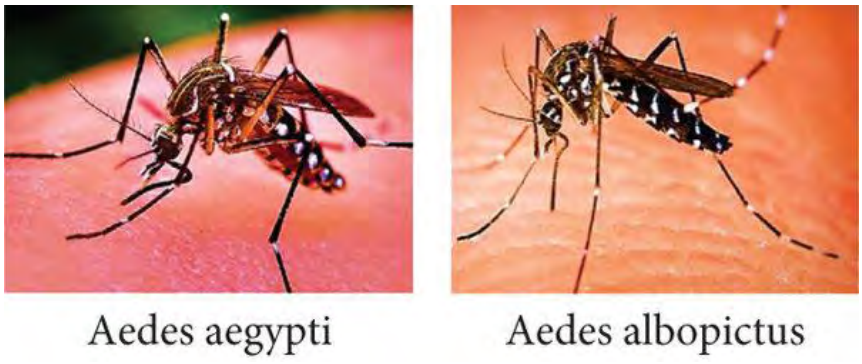


Figure 0.3 *Vectors of DENV are Aedes aegypti and Aedes albopictus.*

The majority of the feeding that each species of vector does takes place throughout the day. Even while they typically show a bimodal peak activity pattern in the early morning and early evening (Chen *et al*., 2014), the peak hours might shift depending on the characteristics of the surrounding environment (Unlu *et al*., 2021). The danger of infection is not confined to residential settings since it is possible for individuals to spend the majority of the day away from their homes. Residents of Ho Chi Minh City, Vietnam, who spent more time at home were found to have a lower likelihood of having IgM positive with DENV, according to research that was conducted there (Anders *et al*., 2015). Dengue fever broke out in downtown Tokyo in 2014, and virtually all of the people who had the disease had been to the same parks at some point during the epidemic (Kutsuna *et al*., 2015). Moreover, the populations of *Aedes albopictus* in the parks had extraordinarily high biting densities and infection rates (Kobayashi *et al*., 2018; Tsuda *et al*., 2016). It is very uncommon for urban parks to include a large number of abandoned containers, and the presence of plants in these areas makes them an ideal habitat for *A. albopictus*. Even though the scale of the outbreak in Tokyo was much smaller in comparison to outbreaks in tropical regions, the incident suggests that urban parks also may enable strenuous transmission in

endemic regions. This is because a lot of individuals of all age brackets attend parks during the daylight hours when the vectors are operative (Huynh & Minakawa, 2022).

2.9. Transmission

The transmission of dengue virus occurs when a female mosquito bites an infected person, and the virus enters the mosquito's bloodstream. The virus then replicates and infects the mosquito's salivary glands. Once the virus has reached the salivary glands, the mosquito can transmit the virus to a new host when it bites again. Transmission can also occur through blood transfusion, organ transplantation, or from mother to child during childbirth or breastfeeding. However, these modes of transmission are relatively rare compared to transmission by mosquito bite (Mukherjee *et al*., 2019).

It is important to note that humans cannot transmit dengue virus to each other directly. A person with dengue fever can only transmit the virus to mosquitoes, which can then transmit the virus to another person (Carrington & Simmons, 2014).

2.10. Pathogenesis

The risk of DENV infection is often higher in tropical and subtropical climes everywhere in the globe, particularly in urban and semiurban areas. DENV infection may strike individuals of any age if they are bitten by a mosquito that has been infected with the virus. Infection with DENV may lead to dandy a high body temperature, breakbone fever, and dengue hemorrhagic fever. In extreme circumstances, dengue shock syndrome can develop as a result of DENV infection. The environment in tropical regions in Asia and South America is particularly conducive to DENV virus epidemics during the wet season, which coincides with the rainy season. DENV is typically transmitted from human to human by female Aedes mosquitoes that have been infected with the virus. Despite the fact that humans are incapable of passing on DENV to one another, the virus can be passed on through blood transfusions when an infected individual gives blood to another individual who is not infected and is healthy (Eick *et al*., 2019; Kulkarni *et al*., 2019).

2.11. Clinical Symptoms

Initial clinical signs of DVI vary from patient to patient, and first symptoms are not disease-specific in and of themselves. The majority of cases of dengue fever are asymptomatic or extremely mild, and are characterised by an indistinct fever, which may or may not be accompanied by a rash, particularly in babies and young kids. It is possible for adults and older kids to acquire a mild febrile syndrome or a classic case of DF. These conditions include a high temperature, severe headaches, fatigue, joint pain, discomfort behind the orbits, and a maculopapular rash. These first symptoms of DF are quite similar to those of chikungunya fever. Dengue fever is the mildest type of clinical dengue infection; nevertheless, since there is such a wide range of signs and symptoms associated with dengue, the World Health Organisation (WHO) has proposed that there should not be a precise clinical definition for DF. In some epidemics, DF may be followed with symptoms related to bleeding, including epistaxis, gingival bleeding, gastrointestinal bleeding, hematuria, and menorrhagia. Other bleeding complications include hematuria and hematuria (Khosavanna *et al*., 2021).

2.12. Treatment

Currently, there is no specific antiviral medication for the treatment of dengue. Therefore, the management of dengue is primarily focused on supportive care to alleviate symptoms and prevent complications. Treatment options may vary depending on the severity of the disease and the patient's overall health status (Obi *et al*., 2021).

2.12.1. Mild Dengue Fever

Patients with mild dengue fever are advised to rest, drink plenty of fluids, and take medications such as acetaminophen or paracetamol to relieve fever and pain. It is important to avoid taking aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen or naproxen, as these can increase the risk of bleeding. Patients should also be monitored closely for signs of dehydration (Kellstein & Fernandes, 2019).

2.12.2. Severe Dengue Fever

Severe dengue fever, also known as dengue hemorrhagic fever or dengue shock syndrome, requires hospitalization and close monitoring. Treatment focuses on maintaining blood volume and preventing shock. Intravenous (IV) fluids and electrolytes may be given to replace fluids lost due to fever and vomiting. Blood transfusions may be necessary to manage severe bleeding (Alejandria, 2015).

In some cases, platelet transfusions may be given to prevent or manage bleeding (Kaur & Kaur, 2014). However, platelet transfusions are controversial as they can increase the risk of developing severe dengue (Kularatne *et al*., 2023).

2.12.3. Prevention

Prevention of dengue is mainly focused on controlling the mosquito population and preventing mosquito bites. Measures include using insect repellents, wearing protective clothing, and using mosquito nets. Mosquito breeding sites should be eliminated, such as stagnant water sources and uncovered water storage containers. In areas where dengue is endemic, a vaccine is available that can protect against all four serotypes of dengue. The vaccine is recommended for individuals aged 9-45 years who have had previous dengue infection (Rather *et al*., 2017).

2.13. Vaccine

Vaccines are an important tool in preventing the spread of infectious diseases, and several vaccines have been developed for dengue. The first dengue vaccine, Dengvaxia, was approved in 2015 by the World Health Organization (WHO) and has been licensed for use in several countries, including the Philippines, Brazil, and Mexico. Dengvaxia is a live attenuated tetravalent vaccine, meaning it contains weakened forms of all four dengue virus serotypes. The vaccine is given in three doses, six months apart, to individuals aged 9-45 years who live in dengue-endemic areas. The vaccine has been shown to be effective in preventing dengue infection in clinical trials, with an overall efficacy of around 60%. However, its efficacy varies depending on the age and serostatus of the individual receiving

the vaccine, as well as the dengue serotypes circulating in the population (Tully & Griffiths, 2021).

In addition to Dengvaxia, several other dengue vaccine candidates are currently in development. These include:

TAK-003: a live attenuated tetravalent vaccine developed by Takeda Pharmaceutical Company. The vaccine is currently undergoing phase 3 clinical trials and has shown promising results in preventing dengue infection in previous trials (Patel *et al*., 2023; Tricou *et al*., 2023).

TV003/TV005: live attenuated tetravalent vaccines developed by the National Institutes of Health (NIH) in the United States. These vaccines are currently undergoing phase 2 clinical trials and have shown good safety and immunogenicity profiles (Hou *et al*., 2022).

DENVax: a live attenuated tetravalent vaccine developed by Butantan Institute in Brazil. The vaccine is currently undergoing phase 3 clinical trials and has shown promising results in previous trials (Torres-Flores *et al*., 2022).

LAV Delta-30: a live attenuated dengue vaccine developed by the University of North Carolina at Chapel Hill. The vaccine has shown promising results in preclinical studies and is currently undergoing phase 1 clinical trials (B. Wu *et al*., 2023).

It is important to note that the development of dengue vaccines has been challenging due to the complex nature of dengue virus infection and the need for a vaccine that is effective against all four dengue serotypes. However, with the recent approval of Dengvaxia and the ongoing development of other vaccine candidates, there is hope for effective prevention and control of dengue in the future.

CHAPTER 3

MATERIAL AND METHODS

3. Material and Methods

3.1. Sample Collection

In the present study, serum specimens obtained from individuals who tested positive for dengue were procured from Services Hospital, Mayo Hospital, and Children Hospital located in Lahore. A cohort of 112 individuals who tested positive for Dengue were included in this study, with 78 being male and 34 being female. The age range of the participants spanned from 12 to 81 years. All the individuals involved in this investigation provided their agreement on written consent forms. In the course of our research, we refrained from revealing the identities and any other personally identifiable information of the individuals involved.

3.2. Inclusion Criteria

The inclusion of Dengue positive sample, which was confirmed by the clinical manifestations, presence of IgG, IgM and NS1 antibodies against dengue virus, was observed. The patients included in the current study were living in Lahore.

3.3. Exclusion Criteria

Patients who tested negative for dengue were excluded from the study.

3.4. Statistical Analysis

Statistical analysis was performed on data sets obtained from laboratory tests. Descriptive tests were performed (sample size (N), minimum value, maximum value, mean, and standard deviation) on different variables Age, HCT, WBC, Plts, IgM, _IgG, and Num_NS1. The Pearson Chi-Square, Likelihood Ratio Chi-Square, and Linear-by-Linear Association tests were applied between two categorical variable, IgM antibodies, IgG antibodies with Gender (male and female). P value below 0.05 was considered significant.

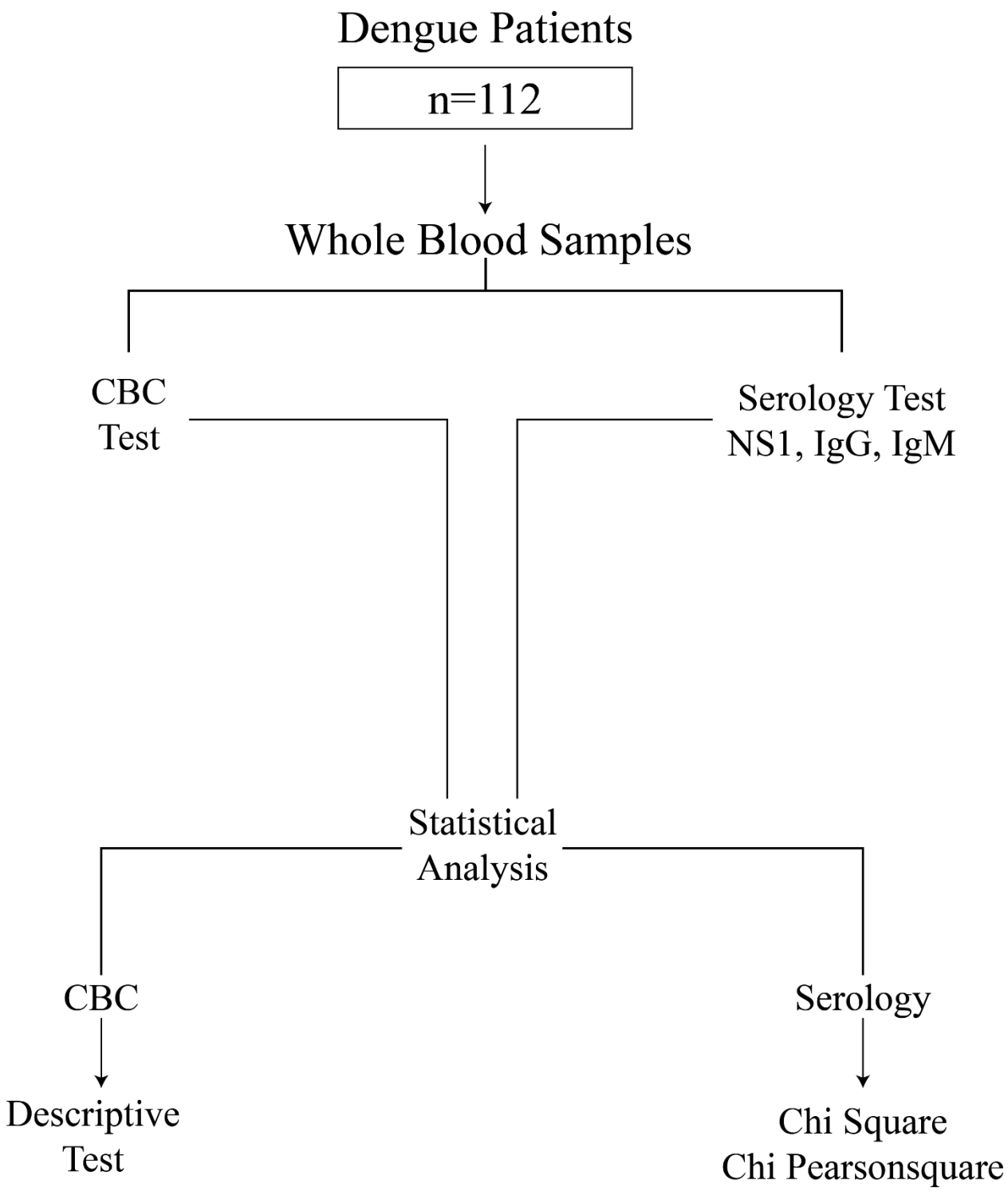


Figure 0.1 *Flow chart of Statistical Analysis*

3.5. Primer designing for the CprM gene of dengue

The primers utilized in the current investigation were consistent with those previously documented in the study conducted by Fatima *et al*. (2011) for the purpose of amplifying the nucleotide sequence. The primer sets underwent additional confirmation and validation using Primer3, a widely utilized primer design software (http://bioinfo.ut.ee/primer3/), as well as Primer Blast, an online tool provided by the National Center for Biotechnology Information (NCBI) specifically designed for *in silico* validation of primers (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers underwent modifications in accordance with the nucleotide sequences that were recently documented. The CprM region of the Dengue-1 virus was employed as the designated reference sequence. The nomenclature, oligonucleotide sequence, and resultant product size of the primers exhibiting optimal *in-silico* specificity are listed herewith.

Serial Number	Primers	Oligonucleotide sequence: 5`-3`	Product Size	Reference
1	CprM F-Primer	TCAATATGCTGAAACGCGWGAGAAACCG	511 bp	(Fatima *et al*., 2011)
2	CprM R-Primer	TTGCACCARCARTCWATGTCTTCWGGYTC		

Table 0.1 *Name, oligonucleotide sequence, and product size of the primers having maximum in-silico specificity.*

3.6. RNA Extraction

The extraction of Dengue virus RNA was performed using the Viral DNA/RNA extraction mini kit (WizPrepTM, South Korea) in accordance with the established manufacturer's protocol. This protocol was employed to effectively isolate the serum from dengue-positive

blood samples. The blood samples were carefully aliquoted into a 1.5ml Eppendorf tube, ensuring proper containment. Subsequently, the tube was subjected to centrifugation at a speed of 8000 revolutions per minute (rpm) for a duration of 1 minute. Following centrifugation, two distinct layers were observed, with the uppermost layer being the serum. This serum was subsequently transferred into a freshly autoclaved microfuge tube. Initially, aseptic microcentrifuge tubes were appropriately designated utilizing precise laboratory codes. The 200µl serum samples contained in the appropriately labeled microfuge tubes were introduced into the experimental setup. Subsequently, the process of sample lysis was initiated by adding 50µl of proteinase k (an enzyme known for its ability to degrade proteins) and viral lysis buffer. The resulting mixture was vorted, and homogenized. In the context of the Viral DNA/RNA extraction procedure, it is necessary to introduce 200 µl of Viral Lysis Buffer into the designated tube, followed by the addition of 15 µl of carrier RNA. These components were subsequently combined with the sample within the tube and thoroughly mixed for a duration of 10-15 seconds through vertexing. The sample mixture underwent incubation for a duration of 10 minutes at a temperature of 66°C within a pre-heated block. Following this incubation step, an amount of 280µl of ethanol (absolute) was inserted into each tube and promptly mixed to avoid the occurrence of ethanol precipitation resulting from its concentration. Subsequently, the sample mixture was carefully transferred into the designated column provided in the kit, followed by centrifugation at a force of 5000g for a duration of 1 minute. Following the completion of centrifugation, the resulting flowthrough was discarded. The column was then subjected to a thorough washing process using 500µl of wash buffer 1, followed by another round of centrifugation at 5000g for 1 minute. Once again, the resulting flowthrough was discarded. The column was subjected to a second round of washing using 500µl of wash buffer-2, which was included in the experimental kit. Subsequently, the column was subjected to centrifugation at a force of 5000g for a duration of 1 minute, following which the flowthrough was discarded. Once more, the column was subjected to a thorough washing process using 500µl of wash buffer-2. Subsequently, it was subjected to centrifugation at a force of 14000g for a duration of 3 minutes. The resulting liquid, known as the flow through, was deemed unnecessary and subsequently discarded. The column was carefully

transferred into a fresh microfuge tube, followed by the addition of 30-50 µl of elusion buffer. The final mixture was exposed to centrifugation at a force of 5000g for a minute duration. The resultant viral RNA was then appropriately stored at a temperature of -20°C.

3.7. Dengue Complementary DNA (cDNA) Synthesis

cDNA was synthesized through the utilization of the extracted RNA as a starting point. The experimental procedure involved the utilization of the First strand cDNA Synthesis Kit, or Revert Aid kit, manufactured by ThermoScientific. The constituents utilized for complementary DNA (cDNA) synthesis encompassed the subsequent components: A total of 8 microliters (µl) of extracted RNA was combined with 2 µl of either random hexamer or gene-specific reverse primers. Additionally, 2 µl of double distilled water, 2 µl of deoxyribonucleotide triphosphates (dNTPs) at a concentration of 10 millimolar (mM), 4 µl of a 5x reaction buffer, 1 µl of RevertAid reverse transcriptase (RT) enzyme with a concentration of 200 units per microliter (U/µl), and 1 µl of RiboLock RNase Inhibitor with a concentration of 20 units per microliter (U/µl) were gently mixed together. The total reaction volume was then adjusted to 20 µl. The reaction mixture components were thoroughly combined and subjected to incubation at 25°C for 5 minutes, subsequently followed by incubation at 42°C for 60 minutes, then at 45°C for 30 minutes. Finally, the reaction was terminated by incubating at 70°C for 5 minutes.

Table 0.2 *Components of cDNA Reversed transcription-PCR mixture.*

Serial no	Reagents	Quantity
1	5x reaction buffer	4µl
2	Random hexamer primers/ R. Primer	2µl
3	10 mM dNTP Mix	2µl
4	Template RNA	8µl
5	ddH2O	2µl
6	RiboLock (20U/µL)	1µl
7	RevertAid RT enzyme (200 U/µL)	1µl
	Total volume	**20µl**

3.8. PCR amplification of dengue CprM gene

Nested PCR was performed to amplify the CprM gene of the dengue virus. The PCR Master Mix used was 2X Phusion High-Fidelity from ThermoScientific. The initial PCR reaction mixture consisted of 10µl of 2X Phusion High Fidelity Master Mix, along with 1.5µl of forward primer, 1.5µl of Reversed primer, 1µl of double-distilled water (ddH2O), and 6µl of complementary DNA (cDNA) template. These components were combined to achieve a total reaction volume of 20µl, as specified in table 3.3. The cyclic conditions required for polymerase chain reaction (PCR) are as follows: The thermal cycling conditions involve an initial denaturation step at a 95°C temperature of for a 5 minutes duration. This is accompanied by a series of 35 cycles, each consisting of denaturation at 95°C for 45 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 1 minute. Finally, a final extension step is performed at 72°C for a period of 10 minutes. The subsequent amplification step of polymerase chain reaction (PCR) was executed employing primers that are complementary to internal regions of the target DNA sequence. The DNA template utilized for this round of PCR was obtained from the product generated during the initial amplification step. The second round of polymerase chain reaction (PCR) was conducted using identical reaction reagents as the first round, with slight adjustments. Specifically, 2µl of the first round PCR product was utilized as the template, along with 5µl of double-distilled water (ddH2O). The same set of forward and reverse primers (table 3.1), along with their corresponding concentrations employed in the first round PCR, were added as specified in Table 3.3. The cumulative reaction volume for both iterations was 20 microliters. Table 6 provides the concentration details of the various components employed in the first and second rounds of polymerase chain reaction (PCR).

Table 0.3 *Reaction mix for carrying out 1st round PCR.*

Serial number	Reagents	Volume
1	Phusion Master Mix	10µl
2	Forward Primer	1.5 µl

3	Reverse Primer	1.5 µl
4	Double distilled water	1 µl
5	Template	6 µl
	Final volume	**20 µl**

Table 0.4 *Reaction mix for carrying out 2nd round PCR.*

Serial number	Reagents	Volume
1	Phusion Master Mix	10µl
2	Forward Primer	1.5 µl
3	Reverse Primer	1.5 µl
4	Double distilled water	5 µl
5	Template	2 µl
	Final volume	**20 µl**

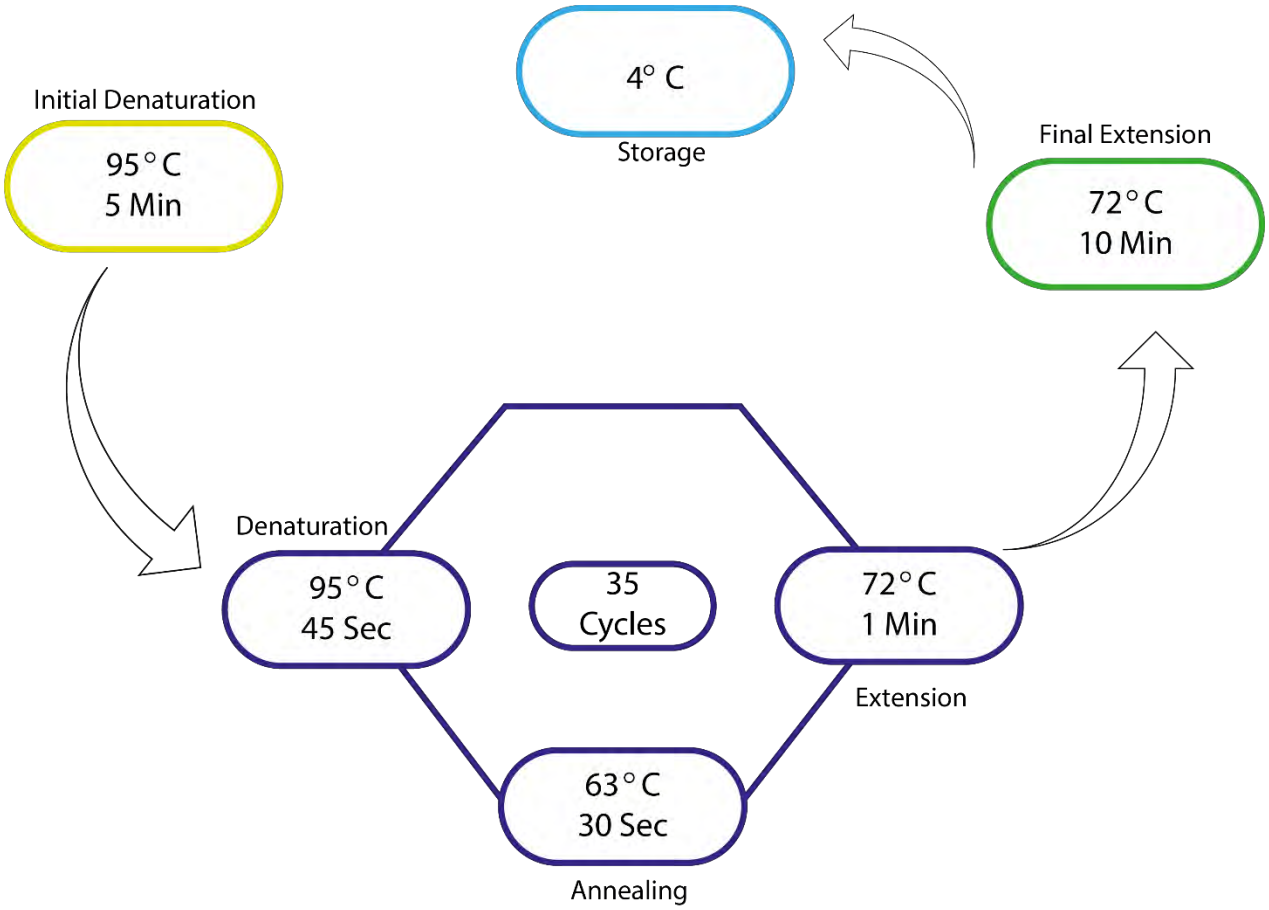


Figure 0.2 *PCR settings for the first and second rounds of the cycle. Utilizing gradient PCR allowed for the parameters to be tuned.*

3.9. Preparation of TAE buffer

In order to perform the synthesis of the 10X TAE buffer, a total of 54 grams of tris-base was solubilized in 350 milliliters of deionized water. Subsequently, a precise quantity of 27.5 grams of boric acid and 4.65 grams of ethylenediaminetetraacetic acid (EDTA) were meticulously introduced into the solution, followed by thorough homogenization. Subsequently, an aliquot of 150 milliliters of deionized water was introduced into the amalgamation, thereby achieving a final volume of 500 milliliters. Subsequently, a 1X solution was generated by diluting 30ml of the 10X TAE buffer with 270ml of deionized water.

3.10. Gel Electrophoresis

A 2% agarose gel was prepared by combining 0.8 grams of agarose with 40 millilitres of 1X TAE buffer. The conical flask was subjected to microwave irradiation for a duration of 30 seconds in order to thermally activate the crude powder, followed by subsequent cooling to ambient temperature. Next, a volume of 2µl of Ethidium bromide was introduced to the agarose solution in order to facilitate visualization using a UV transilluminator. The agarose solution was carefully transferred into a gel casting tray, ensuring the comb was properly positioned. The solution was then left undisturbed for approximately 10 to 15 minutes to undergo solidification. Following the process of solidification, the gel was subsequently placed within a gel tank for further handling. The gel tank was supplemented with 1x TAE buffer, which served as the running buffer during the experiment. The PCR products that underwent amplification were combined with 2 µl of loading dye and subsequently introduced into the wells of the gel. A 1000 bp gene ruler was meticulously loaded into a distinct well to facilitate the comparison of amplified PCR products in terms of their size. Following this procedure, electrophoresis was conducted using a gel matrix, applying a voltage of 90 volts for a duration of 30 minutes. After the completion of the gel electrophoresis process, the gel was subjected to examination under a UV transilluminator in order to visualize the amplified products. The DNA band was excised from the gel and the amplified product was purified/eluted using GeneJet Gel Extraction Kit (ThermoScientific).

Table 0.5 *.Components for agarose Gel .*

Serial #	Components of 2% Agarose gel	Amount
1	1X TAE buffer	40 ml
2	Agarose	0.8g
3	Ethidium bromide solution	2 µl

3.11. Sequencing of Dengue CprM Gene

The eluted product obtained from the polymerase chain reaction (PCR) was subjected to sequencing analysis using gene-specific primers targeting the CprM junction. A total volume of 4µl of the forward primer was used, along with 8µl of the purified DNA product, for the sanger sequencing procedure. The molecular sequence data was subjected to alignment using sequence alignment software, specifically BioEdit. The molecular sequencing results were subjected to analysis using sequence alignment software, specifically BioEdit. A nucleotide sequence in FASTA format was acquired for subsequent analysis.

3.12. BLAST Analysis

The present investigation involved the comparison of the QAU-N1 study sequences with the established dengue nucleotide sequence available in the National Center for Biotechnology Information (NCBI) GenBank database. This comparison was conducted utilizing the Basic Local Alignment Search Tool (BLAST), which can be accessed at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

3.13. Phylogenetic Analysis

To assess the homology between the recent CprM gene sequences and other dengue sequences from Pakistan, as well as reference sequences from around the globe, a search was conducted in the NCBI GenBank using nucleotide BLAST. The alignment of all the accessible sequences was performed using the MEGA 11 software, as described by Tamura *et al*. (2021). The construction of the phylogenetic tree was completed. The estimation of the history of evolution was conducted via the consumption of the "Maximum Likelihood" method.

3.14. Mutational Analysis

The CprM gene was chosen for molecular analysis in order to elucidate any genetic variations that could potentially contribute to the virulence of dengue virus. In this experiment, we opted to focus on the protein sequence of CprM's highly variable region. To accomplish this, we performed amplification of the sequence and subsequently

employed the BLASTx tool for analysis. The query sequence underwent alignment with multiple closely related target proteins, and the variations in the sequence were identified using BLASTx. The confirmation of the significance of mutations identified by BLASTx was further validated through the utilization of additional software tools, thereby reinforcing the association between these mutations and their potential impact on disease progression.

3.14.1. PolyPhen-2

PolyPhen-2, a web-based resource accessible at http://genetics.bwh.harvard.edu/pph2/, serves as a valuable tool employed in the assessment of the potential impacts of amino acid substitution on the intricate architecture and dynamic functionality of proteins (Choudhury *et al*., 2021). By utilizing the protein FASTA sequence, along with the wild type and substituted amino acids as input, this computational model is able to make predictions regarding the impact of amino acid substitutions on the protein's functionality. The score values span a continuum from 0 to 1, serving as an indicator of the mutational impact, with values falling within the range of 0 to 0.2 denoting a benign effect, those within 0.2 to 0.85 suggesting a potential damaging effect, and scores within 0.85 to 1 indicating a higher likelihood of causing damage. If the score approaches unity, amino acid substitutions are postulated to exhibit a higher degree of deleteriousness.

3.14.2. PhD SNP

It is a web-based server that is accessible at https://snps.biofold.org/phd-snp/phd-snp.html which serves as a valuable instrument in the field of molecular biology for predicting the detrimental impact of an amino acid substitution on protein structure and function. The input consists of a protein FASTA sequence, accompanied by the position of a substitution event and the resulting new residue. The model has ability to make predictions regarding the pathogenicity of single amino acid substitution. The model distinguishes between wildtype and current study sequences that are associated with disease and those that have no discernible impact, thereby aiding in the identification of potentially harmful genetic variations (Capriotti & Fariselli, 2017).

3.14.3. PROVEAN

PROVEAN, a web-based application accessible at http://provean.jcvi.org/index.php, is a computational tool employed in the field of molecular biology to forecast the potential impact of amino acid substitutions on protein functionality (Choudhury *et al*., 2022). The nomenclature of the mutation or variant is supplied as the input for the protein FASTA sequence. In the realm of molecular biology, outcomes can be broadly classified into two distinct categories, namely deleterious and neutral. A mutation exhibiting a score surpassing the designated cut off value is classified as deleterious, whereas a mutation displaying a score exceeding the cut off value is categorized as neutral.

3.14.4. I-MUTANT 3.0

I-Mutant 3.0, a web server based on Support Vector Machines (SVM), was employed to forecast the impact of amino acid substitutions on protein stability. This was accomplished through the examination of both the amino acid sequence and protein structure. These modifications can exert a substantial influence on the energy landscapes associated with protein stability. Consequently, the protein's stability can be either enhanced or reduced. I-MUTANT 3.0, a computational tool, leverages ProTherm data to scrutinize alterations in energy and prognosticate the impact on a protein's stability, discerning whether it has undergone augmentation or diminishment. By employing this particular methodology, one can potentially anticipate the impact of a mutation on both the conformation and activity of a protein. I-MUTANT is a computational tool utilized in molecular biology that accepts protein sequences as its input. Additionally, it requires the specification of the substitution site and the residue that is to be replaced. Subsequently, it generates informative results which are communicated to the user via email. The subject line of the email may either indicate a increase in stability or a decrease in stability (Soremekun *et al*., 2021).

3.14.5. MUpro

MUpro, a web tool based on Support Vector Machines (SVM), is employed for the prediction of alterations in protein stability resulting from a single site mutation. The input data for the software includes protein sequences, along with mutation sites, the native amino acid, and the substituted amino acid. Upon processing, the software generates online

outputs that depict alterations in both the structural conformation and functional stability of the protein (Masso & Vaisman, 2010).

3.16. Evolutionary Effect of Amino Acid Substitutions

The missense mutation has a significant impact on the evolutionary trajectory of organisms. This type of mutation occurs when a single nucleotide substitution in the DNA sequence leads to the incorporation of a different amino acid in the resulting protein. As a result, the protein's structure and function Single nucleotide polymorphisms (SNPs) are discerned through the utilization of ConSurf, an online tool employed in the assessment of the evolutionary conservation of amino acid residues within proteins. This tool facilitates a comparison between the aforementioned conservation and the 25 established structures available in the Protein Data Bank (PDB). The 4Site algorithm, as described by Ashkenazy *et al*. (2016), serves the purpose of assessing the degree of evolutionary conservation exhibited by individual amino acids.

3.17. Surface Accessibility

The determination of the amino acid residues that are most likely to be exposed (hydrophilic) and those that are most likely to be buried (hydrophobic) was conducted using NetSurfP v.1.1, a computational tool available at http://www.cbs.dtu.dk/services/NetSurfP-1.1/. The server employed in this study was utilized for the purpose of forecasting the surface accessibility of protein structure, as described by Vidya *et al*. in their publication from 2014.

3.18. Prediction of Protein Secondary Structure

In order to anticipate the secondary structure of the CprM region across all isolates and the reference sequence (accession number: QBA57538.1), we employed a self-optimized prediction method (SOPMA) through an online server. SOPMA, also known as Self-Optimized Prediction Method from Alignment, has been elucidated to enhance the efficacy in prognosticating the secondary conformation of polypeptides.

3.19. 3-D Modeling

The protein structure models of CprM were generated utilizing a methodology based on predicting inter-residue distances. These models were subsequently improved through the application of molecular dynamics (MD) simulations. For the estimation of inter-residue distances and orientations, we employed Robetta, a computational tool widely utilized in the field of molecular biology, to generate tertiary structure models. For each modified protein, a total of five three-dimensional models were meticulously constructed. Among these models, the one with the lowest energy was selected to proceed to the subsequent refining phase (Heo & Feig, 2020).

3.20. Refinement and Verification

The GalaxyRefine tool (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE), a web-based application, employs iterative molecular dynamics simulations to enhance the quality of the three-dimensional protein structure acquired through Robetta (Srivastava *et al*., 2020). Ultimately, the confirmation of the 3-dimensional conformation is achieved through the utilization of Errat, a tool accessible at https://saves.mbi.ucla.edu/. This tool effectively assesses and establishes the congruence between the 3D model and the specific arrangement of amino acids within the sequence with the help of crystallography (Hamid *et al*., 2021).

3.21. Superimposition and Structural Analysis

To facilitate the observation of conformational alterations in the three-dimensional representation of the mutated protein, we employed PyMol Visualizer, a software tool commonly utilized in molecular biology research. This enabled us to visually compare and align the wild-type protein models with their corresponding mutated counterparts. PyMOL, a software widely used in the field of molecular biology, facilitates the production of visually appealing and accurate three-dimensional representations of protein sequences (Rigsby & Parker, 2016). Additionally, HOPE (https://www3.cmbi.umcn.nl/hope/), which retrieves data from PDB and UniProt database, and compares the results with BLAST and develop a homology with Yasara (Tosh *et al*. 2015).

Chapter 4

RESULTS

4. Results

In the present study, a total of 112 samples exhibiting clinical indications of dengue infection were procured. The study group consisted of individuals spanning a range of ages, encompassing 78 male individuals and 34 female individuals. The comprehensive patient information by age is provided in Figure 4.1. The distribution of age groups is represented in percentage in Figure 4.2.

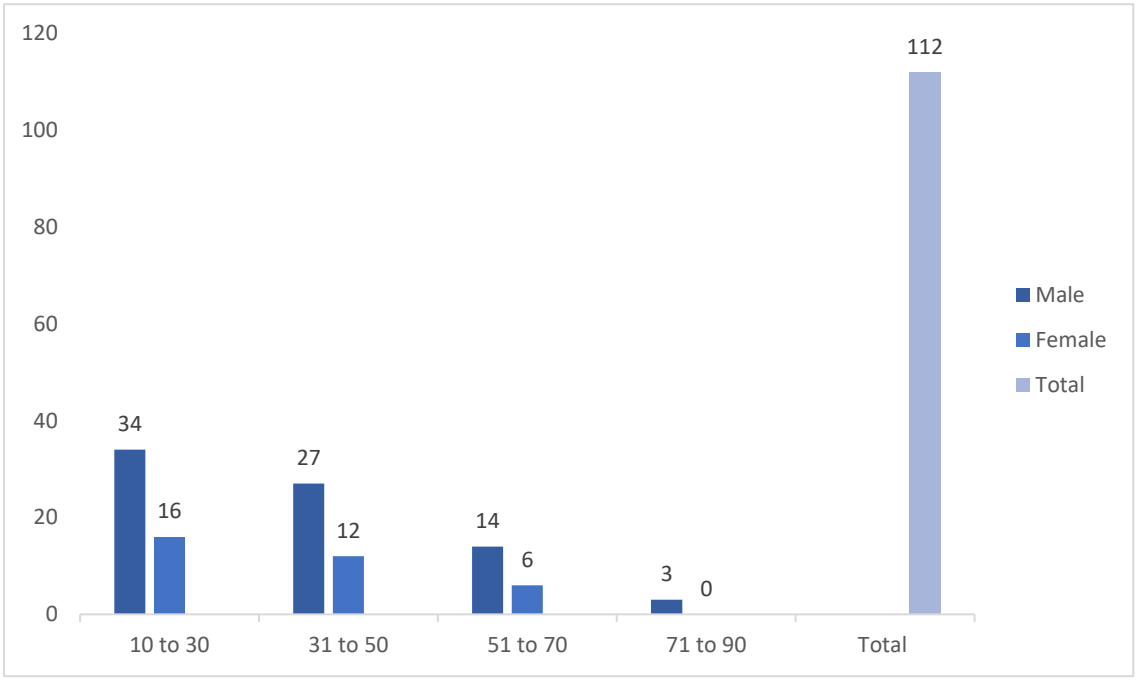


Figure 0.1 *Age and gender wise representation of studied individuals*

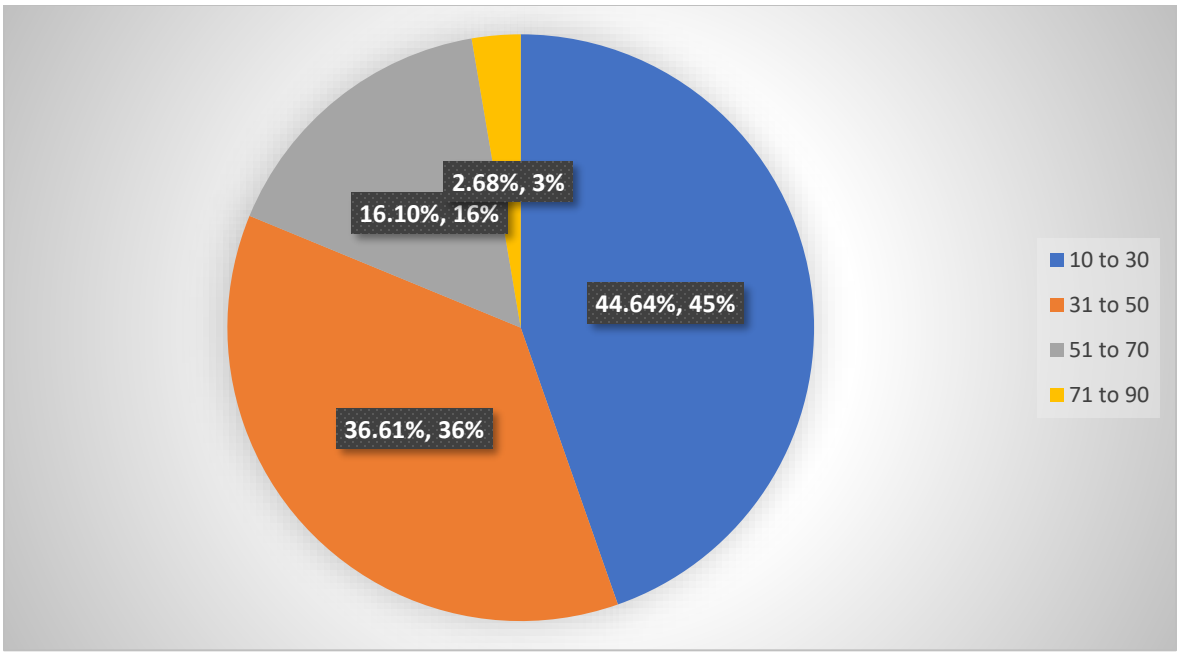


Figure 0.2 *Age-wise percentage of the infected individuals. Most of the patients (45%) were from the age group 10-30 years.*

4.1. Statistical Analysis

The variables (Age, HCT, WBC, Plts, IgM, _IgG, and Num_NS1) are listed in the Table 4.9, along with the descriptive statistics: sample size (N), minimum value, maximum value, mean, and standard deviation. This summary offers an overview of each variable's central tendency and distribution in the dataset.

4.1.1. Complete blood count parameters

The study group (n= 112) consisted of individuals with a range of ages, encompassing 78 males, and 34 females. The study consisted complete blood parameters of dengue patients, out of which the average HCT count of dengue patients was 42.48 ±21.03, WBC (5.63 ± 4.052) and average Platelet Count (53.54 ± 60.315). Furthermore, the average measurements of IgM were 1.55 ±0.918, IgG (1.30 ± 0.524) and NS1(1.33 ± 0.589). This summary offers an overview of each variable's central tendency and distribution in the dataset.

Table 0.1 *Comparison of complete blood parameters of dengue patients.*

Variable	N	Minimum	Maximum	Mean ± S.D
Age	112	12	81	35.63 ±16.037
HCT	112	19	203	42.48 ± 21.083
WBC	112	1	33	5.63 ±4.052
Plts	112	1	314	53.54 ± 60.314

4.1.2. Serology Analysis

The Pearson Chi-Square, Likelihood Ratio Chi-Square, and Linear-by-Linear Association tests were applied between two categorical variable, IgM antibodies, IgG antibodies with Gender (male and female). The findings indicated that there is no statistically significant relationship between these two variables under consideration. Association tests all have p-values larger than the conventional significance level of 0.05, suggesting that we do not reject the null hypothesis. As a result, based on this Chi-Square test, we do not have enough information to infer that there is a significant link between the two variables. However, because the predicted numbers in several cells were fewer than 5, more inquiry and care are urged when interpreting these data.

Table 0.2 *Serological assessment for anti-dengue IgM antibodies (n = 27).*

			IgM		Total
			Negative	Positive	
Gender	F	Count	8	10	18
		% of Total	44%	55%	66%

	M	Count	4	15	19
		% of Total	21%	79%	70%
Total		Count	23	4	27
		% of Total	85%	14.8%	100%

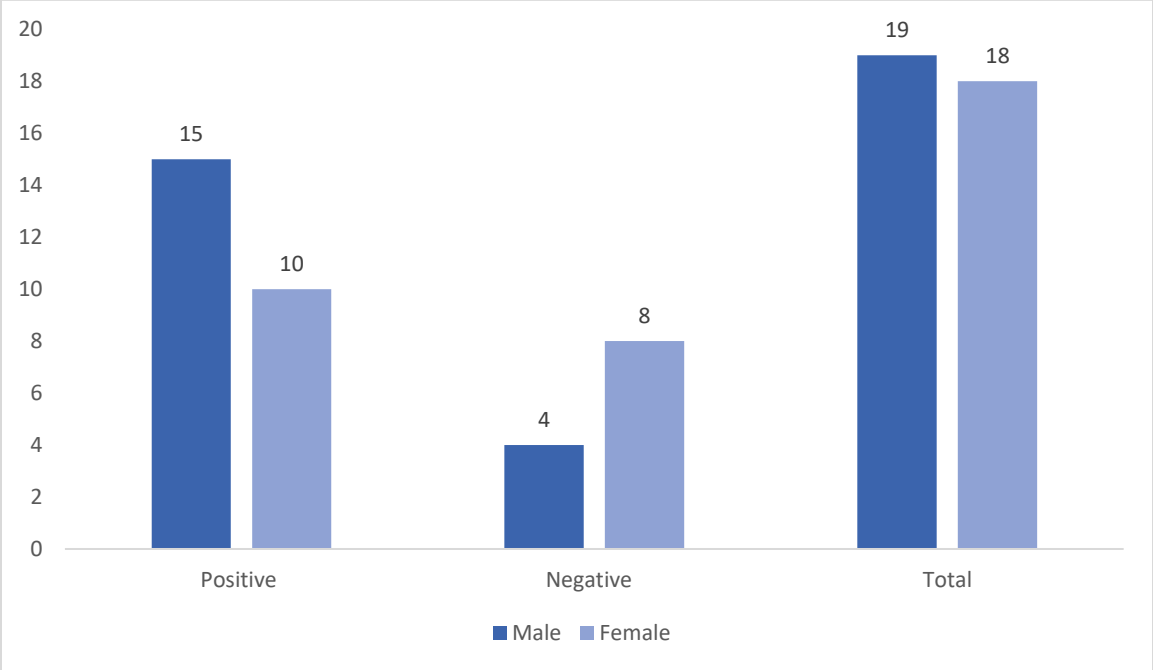


Figure 0.3 *Gender-wise Anti-dengue IgM identification in the study patients (n=27).*

Table 0.3 *Serological assessment for anti-dengue IgG antibodies (n = 27).*

			IgG		Total
			Negative	Positive	
Gender	F	Count	7	1	8
		% of Total	87.5%	12.5%	29.6%
	M	Count	17	2	19
		% of Total	89.4%	42.1%	70.3%
Total		Count	21	6	27
		% of Total	77.7%	22%	100.0%

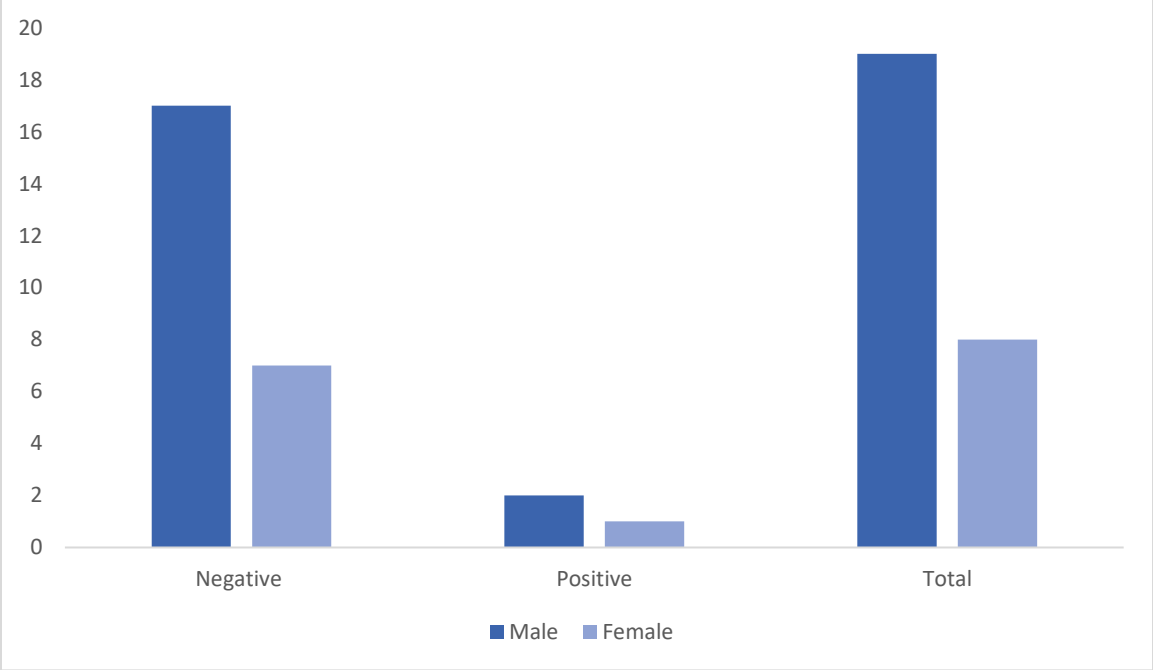


Figure 0.4 *Gender wise Anti-dengue IgG identification in the study patients (n=27).*

4.2. Results of PCR Amplified Products

Following the polymerase chain reaction (PCR) procedure, the resulting amplified products were subjected to electrophoresis on a 2% agarose gel medium in order to validate their presence and integrity. Positive amplification of the targeted CprM gene was observed in all of the samples. The amplified product exhibited a consistent band size of approximately 511 base pairs (bp) across all samples, as observed in Figure 4.5, in comparison to the 1 kilobase (kb) DNA ladder.

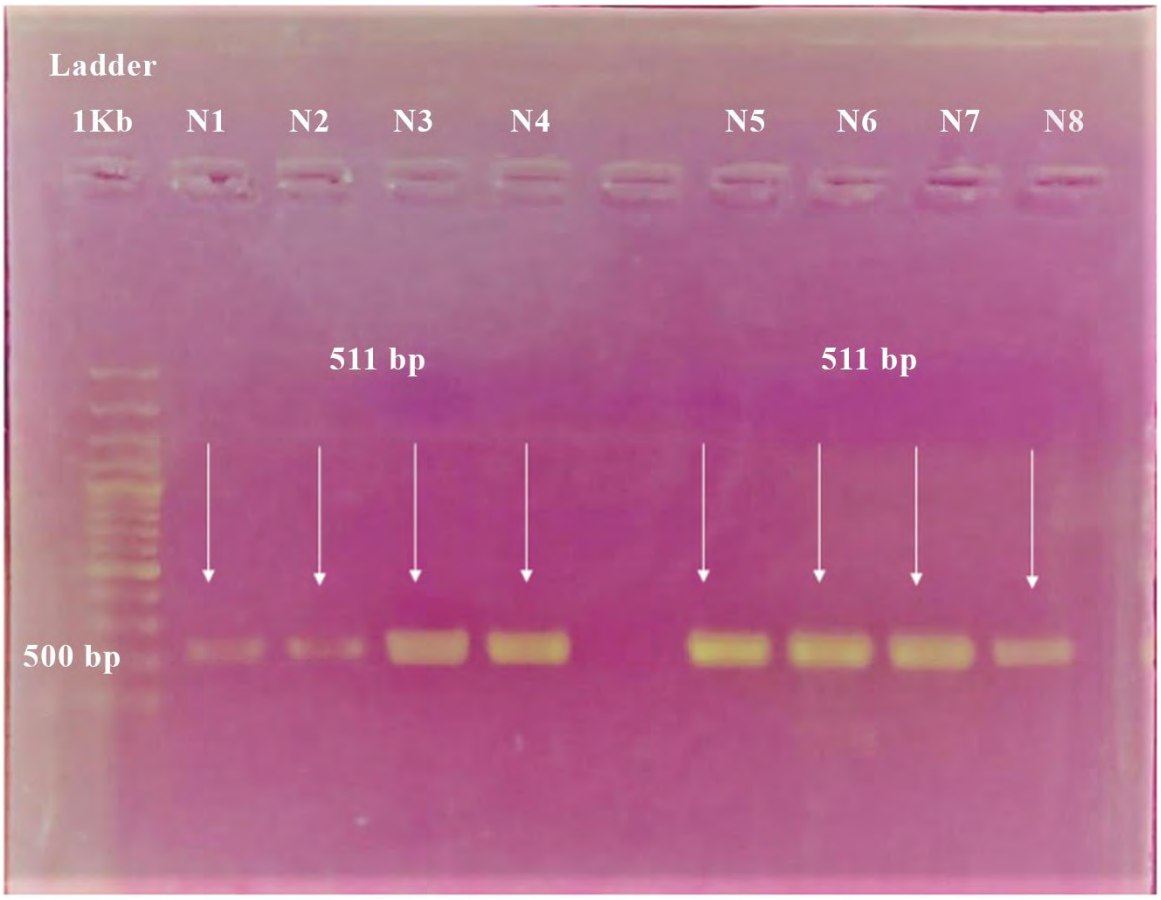


Figure 0.5 *Gel electrophoresis and visualization of amplified products. Lane 1: 1000bp ladder, Lane N1 to lane N8 represents amplified PCR products. Samples were labeled. Negative control sample is shown between N4 and N5.*

4.3. Sequencing of PCR products:

The amplified DNA fragments obtained through polymerase chain reaction (PCR) were meticulously excised from the agarose gel matrix, ensuring the removal of any impurities or contaminants. Subsequently, a purification process was employed to isolate the PCR products, employing state-of-the-art techniques. Finally, the purified DNA samples were dispatched to a specialized sequencing facility, which was entrusted with the task of performing the Sangar sequencing. The DNA sequences derived from the one samples were subsequently employed for molecular typing and subsequent post-sequencing analysis. Figure 4.6 depicts the chromatogram representing the nucleotide sequence of the

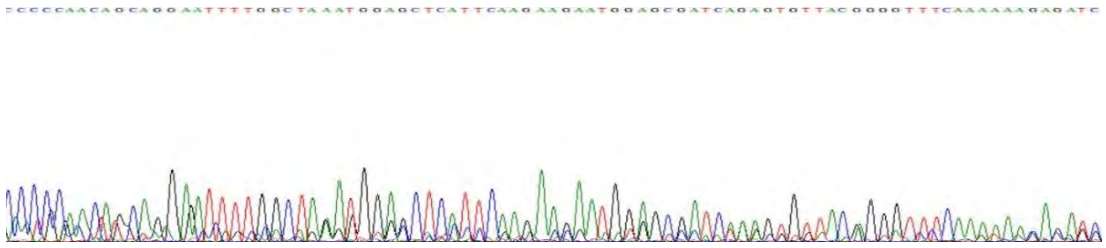


Figure 0.6 *Nucleotide sequencing results of CprM gene of Dengue virus*

isolate QAUN1.

4.4. Phylogenetic Analysis:

To better understand the genetic diversity, we conducted sequencing analysis targeting the gene junction of DENV1 isolates derived from the Lahore locality. A comprehensive collection comprising 39 nucleotide reference sequences of the DENV1 has been successfully procured from the esteemed National Center for Biotechnology Information (NCBI) database. The application of the Maximum Likelihood (ML) method was employed to infer the phylogenetic relationships and reconstruct the evolutionary history of the biological entities under investigation. The phylogenetic analysis was conducted utilizing the Maximum Likelihood method within the MEGA 11 software program. A total of 51 nucleotide reference sequences were encompassed within the scope of this investigation. The present investigation into the sequence of isolate QAUN1 has revealed that its phylogenetic relationship is observed to be closely associated with the dengue virus isolate identified by Accession numbers MG840572 and MG840545. The findings of this

investigation have revealed a significant genetic affinity between our DENV1 CprM gene isolates and previously documented viral isolates originating from China. Based on its phylogenetic placement within the tree, the isolate currently under investigation, QAUN1, exhibits characteristics that suggest it is a distinct isolate within an emerging clade, as depicted in Figure 4.7.

Figure 0.7 *Phylogenetic tree of the CprM nucleotide sequences of studied isolates (n=1) with the other reference sequences (n=39) from different countries of the world. The current study viral isolates and reference sequences are represented as red.*

4.5. Mutational Analysis:

There were 3 *Amino acid substitutions*, in sequenced exons, which were verified and analysed computationally to forecast their potential consequence on protein function and structure.

4.5.1. Functional Score Prediction of Identified amino acid substitutions

Sequence-based predicted *amino acid substitutions* were validated through online servers; SIFT, Polyphen-2, PhD-SNP and PROVEAN. All these tools classify the *amino acid substitutions* into deleterious or neutral, based on amino acid sequence and their physical properties. According to SIFT and PROVEAN, 2 out of 3 *amino acid substitutions* were affecting the protein function, while PhD-SNP predicted all *amino acid substitutions* to be disease causing. Further analysis with Polyphen-2 predicted that 1 out of 3 *amino acid substitutions* are estimated to be deleterious. Moreover, protein stability was predicted by I-Mutant and MUpro. According to their estimation, 2 *amino acid substitutions* are resulting in evident decrease in protein stability, while 1 amino acid substitution is enhancing protein stability (Table 4.4). Based on the degree of potency and deleteriousness, total 3 *amino acid substitutions*; L215I, S227F and K236T were selected for further *in-silico* investigation.

Table 0.4 *Functional Score Prediction of Identified amino acid substitutions*

Mutation Name	PROVEAN	Poly-phen-2	PhD-SNP	SIFT	I-MUTANT	MUpro
L215I	Neutral -0.32	Benign 0.269	Disease	Tolerated 0.35	Increase Stability	Decrease stability -0.878
S227F	Deleterious -7.08	Possibly Damaging 0.856	Disease	Affect Protein structure 0.00	Decrease stability	Increase Stability 0.0817
K236T	Deleterious -3.54	Benign 0.048	Disease	Affect protein structure	Decrease stability	Decrease Stability -0.6554

0.01

4.6. Evolutionary effect of amino acid substitutions

Amino acid substitutions present in conserved regions are considered to be more dangerous and disease causing than amino acid substitutions present in non-conserved area. The evolutionary conservation of amino acid substitutions was investigated, using Consurf. Results indicated that all residues are present in conserved area (Conservation score of 6–9). Table 4.5 shows the ConSurf prediction score along with conserved portions.

Table 0.5 *Evolutionary effect of along with their conservancy score and surface accessibility properties*

Serial No.	Mutation Name	Score	Color	Conservancy
1	L215I	-0.262	6	Conserved
2	S227F	-0.467	6	Conserved
3	K236T	-0.469	7	Conserved

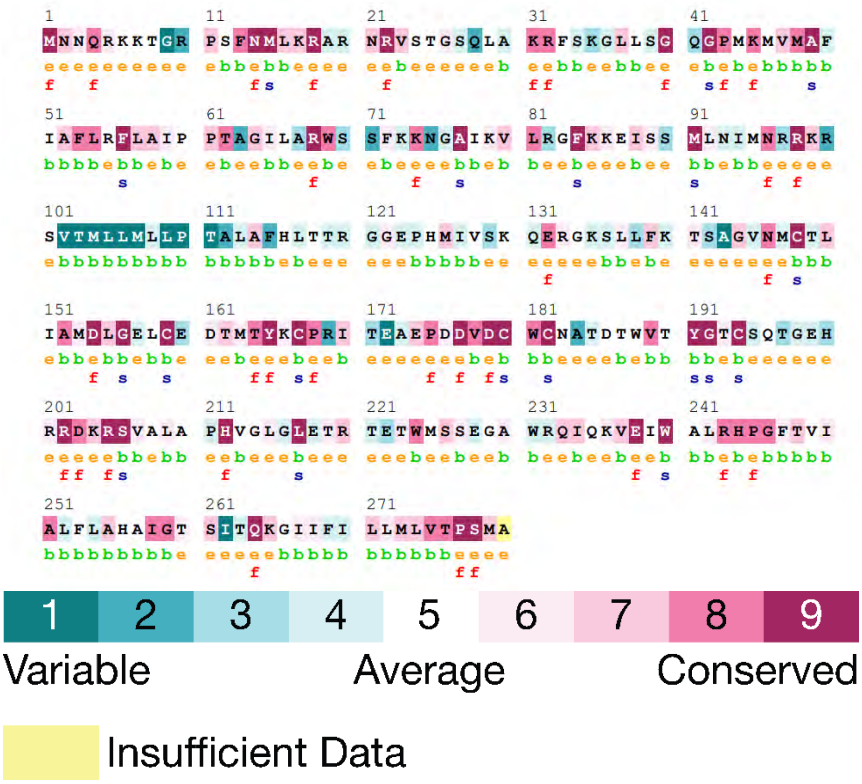


Figure 0.8 *Estimations of the evolutionary rate for each position of nucleic acid sequences in identified amino acid sequences.*

4.7. Surface Accessibility of missense SNPs/amino acid substitutions:

The preservation of the three-dimensional structure of proteins and their interactions depends on amino acid exposure, whether Hydrophobic (buried) or Hydrophilic (exposed) to the surface. Each surface accessibility prediction in NetSurfP v.1.1, which is based on artificial neural networks, is given a reliability score. The reliability score in the form of Z-score shows the most effective measurements and differentiate all residues from buried to exposed amino acid residues.

Table 0.6 *Surface Accessibility of amino acid substitution along with their Relative and Absolute surface accessibility*

Serial No.	Amino Acid Substitutions	Type of Residue (wild type)	Type or residue (mutated)	Relative Surface Accessibility (RSA)	Absolute Surface Accessibility	Z-Fit Scores for RSA Prediction
1	L215I	Exposed	Exposed	0.389	93.554	-1.168
2	S227F	Buried	Buried	0.144	20.218	-0.694
3	K236T	Buried	Exposed	0.096	19.347	0.120

4.8. Protein Structure and Function Prediction:

An online server SOPMA was employed for the prediction of secondary structure of the reference protein CprM region of DENV1 under the accession number (QBA57538.1). The server used bioinformatics approaches that attempt to determine the local secondary structures of proteins based only on their amino acid sequence. Reference structure protein has an alpha helix of 41.07%, Beta turn of 5.36%, extended strand of 15.00%, random coils of 38.57% as shown in the Figure 4.9. This shows that conserved regions are present which maintain the secondary structure of CprM protein. The secondary structure of amino acid substitutions, L215I has an alpha helix of 41.79%, Beta turn of 5.00%, extended strand of 15.36%, random coils of 37.86% as shown in the Figure 4.10. S227F has an alpha helix of 45.36%, Beta turn of 5.00%, extended strand of 14.64%, random coils of 35.00% as shown in the Figure 4.11. While the secondary structure of K236T has an alpha helix of 45.36%, Beta turn of 5.00%, extended strand of 14.64%, random coils of 35.00% as shown in Figure 4.12.

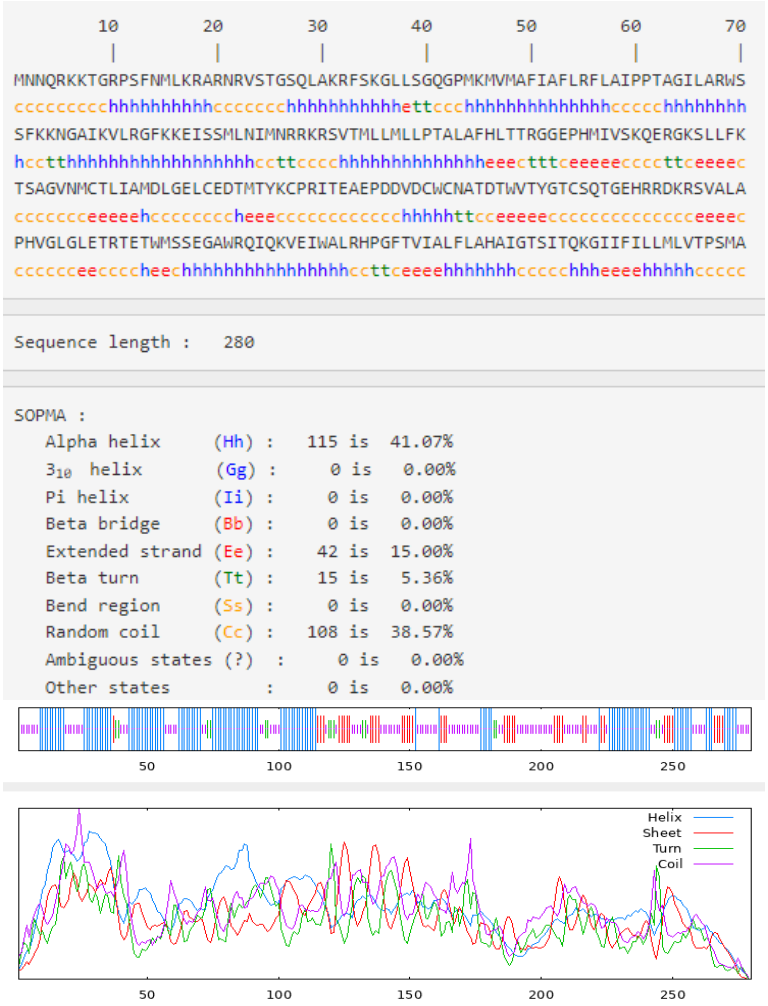


Figure 0.9 *Secondary structure of the reference sequence accession number: (MH891771.1). Different motifs and parts of CprM region i.e. Alpha helix, Beta turn & Beta Bridge, random coils are represented along with their percentage.*

Figure 0.10 *Secondary structure of the reference sequence (accession number : MH891771.1) having L215I amino acid substitution, Different motifs and parts of CprM region i.e. Alpha helix, Beta turn & Beta Bridge, random coils are presented along with their percentage.*

Figure 0.11 *Secondary structure of the reference sequence (accession number: MH891771.1) having S227F amino acid substitution, Different motifs and parts of CprM region i.e. Alpha helix, Beta turn & Beta Bridge, random coils are presented along with their percentage.*

Figure 0.12. *Secondary structure of the reference sequence (accession number: MH891771.1) having K236T amino acid substitution, Different motifs and parts of CprM region i.e. Alpha helix, Beta turn & Beta Bridge, random coils are presented along with their percentage.*

4.9. Protein Modeling and Validation

3-D protein structure of amino acid sequences of CprM and reference sequence (accession number: MH891771.1) were generated using Robetta online modeling tool (https://robetta.bakerlab.org/queue.php). The PYMOL software was used to visualize the 3-D structures and made possible to compare the structures of reference isolate and current study isolates. The 3-D structure of the reference and all the isolates are shown in the Figure 4.13, Figure 4.14, Figure 4.15 and Figure 4.16, respectively. The structural prediction of proteins allows us to assess the substitution of amino acid easier and clear. The amino acid substitution of variant isolates was highlighted according to their specific site and compared with reference protein structure.

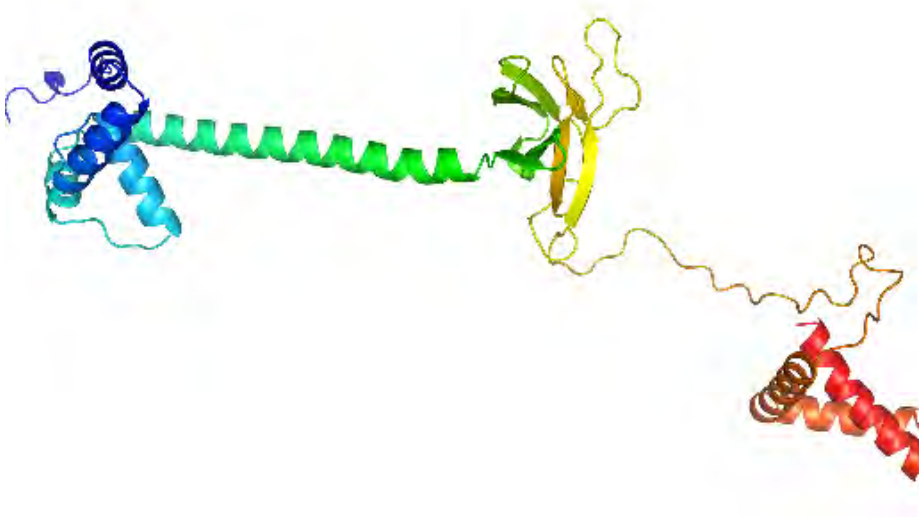


Figure 0.13 *3D structure visualization of CprM by using PyMOL. Alpha helix is represented as red, beta sheets are represented as yellow and coils and loops are represented as green.*

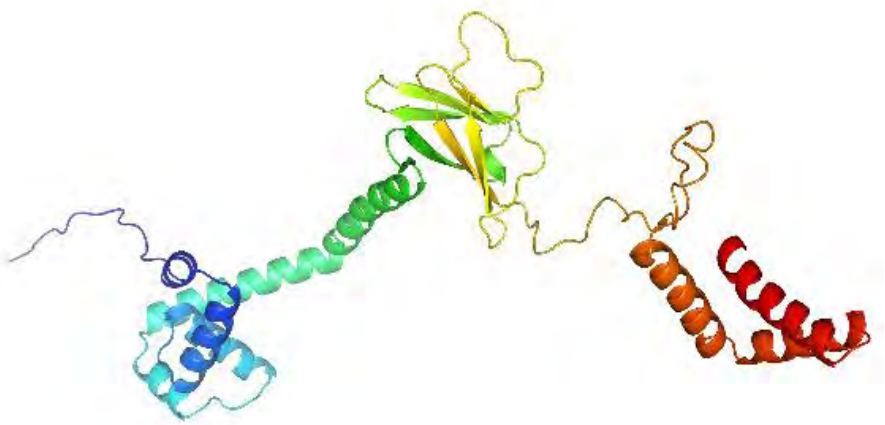


Figure 0.14 *Visualization of 3D structure of mutated CprM of amino acid substitution L215I by PyMOL.*

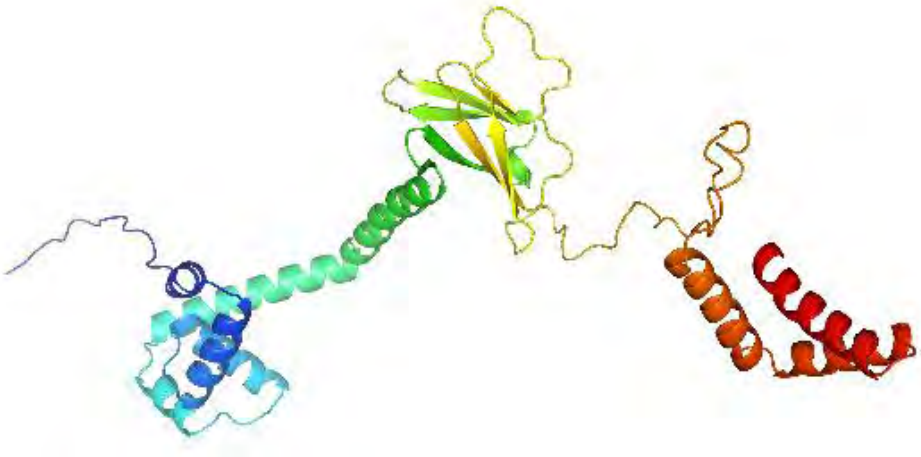


Figure 0.15 *Visualization of 3D structure of mutated CprM of amino acid substitution S227F by PyMOL..*

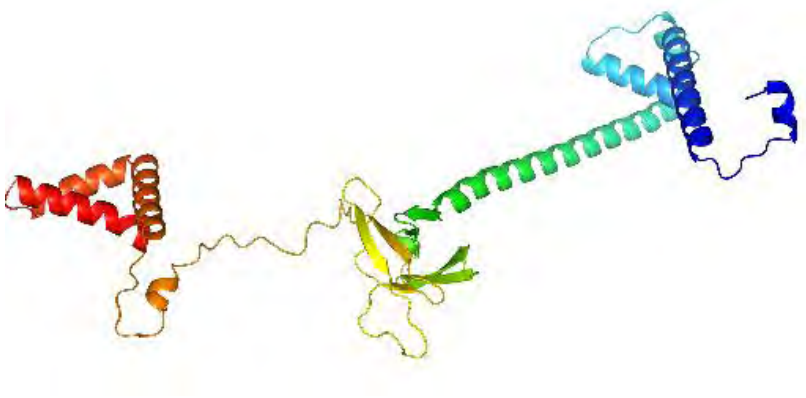


Figure 0.16 *Visualization of 3D structure of mutated CprM of amino acid substitution K236T by PyMOL.*

4.10. Refinement and verification of 3D structure

Following the selection of protein models, refinement of the 3D structure and further verification were carried out using several software to get them closer to accuracy for future computational investigations. Refinement of the predicted 3D structure from Robetta was carried out by the application of Galaxy Refine. Quality of the model was improved in terms of both contact of interface and orientation of the inter-protein. 3D models predicted by Robetta and Galaxy Refine is described in Table 4.7, Table 4.8, Table 4.9 and Table 4.10.

Table 0.7 *Refinement of protein structure of reference (accession number: MH891771.1).*

Model	GDT-HA	RMSD	MolProbity	Clash score	Poor Rotamers	Rama favored
Initial	1	0	0.918	1.7	0	98.6
MODEL 1	0.975	0.346	1.429	7.9	0.4	98.9
MODEL 2	0.983	0.326	1.373	6.8	0.8	98.6
MODEL 3	0.9795	0.32	1.309	5.7	0	98.6
MODEL 4	0.9812	0.338	1.349	6.3	0.8	98.6
MODEL 5	0.9759	0.356	1.429	7.9	0.4	98.6

Table 0.8 *Refinement of mutated protein structure having L215I substitution.*

Model	GDT-HA	RMSD	MolProbity	Clash score	Poor Rotamers	Rama favored
Initial	1	0	1.078	2	0	97.5
MODEL 1	0.9696	0.375	1.385	6.3	0	97.8
MODEL 2	0.9759	0.368	1.486	8.3	0	97.8
MODEL 3	0.9661	0.376	1.433	7.2	0	97.8
MODEL 4	0.9696	0.382	1.53	6.8	0	97.1
MODEL 5	0.9723	0.385	1.592	9.2	0.4	97.5

Table 0.9 *Refinement of mutated protein structure having S227F substitution.*

Model	GDT-HA	RMSD	MolProbity	Clash score	Poor rotamers	Rama favored
Initial	1	0	1.078	2	0	97.5
MODEL 1	0.975	0.381	1.449	8.3	0	98.2
MODEL 2	0.9768	0.378	1.408	7.4	0.4	98.2
MODEL 3	0.9786	0.351	1.465	6.5	0.4	97.5
MODEL 4	0.9679	0.401	1.496	8.5	0.4	97.8
MODEL 5	0.9768	0.353	1.489	7	0	97.5

Table 0.10 *Refinement of mutated protein structure having K236T substitution.*

Model	GDT-HA	RMSD	MolProbity	Clash score	Poor rotamers	Rama favored
Initial	1	0	1.088	1.5	0	96.8
MODEL 1	0.9741	0.343	1.27	4.6	0.4	97.8
MODEL 2	0.9705	0.363	1.386	6.3	0.4	97.8
MODEL 3	0.9768	0.344	1.453	6.3	0.4	97.5
MODEL 4	0.9705	0.364	1.413	5.7	0.4	97.5
MODEL 5	0.9723	0.353	1.36	5.9	0.4	97.8

Furthermore, ERRAT assess the compatibility of sequence of amino acids with the previously predicted 3D structures. The ERRAT score for the 3D models were 97.52, 95.29, 96.3 and 98.1, which fit well in a high range model as shown in Figure 4.17, Figure 4.18, Figure 4.19 and Figure 4.20.

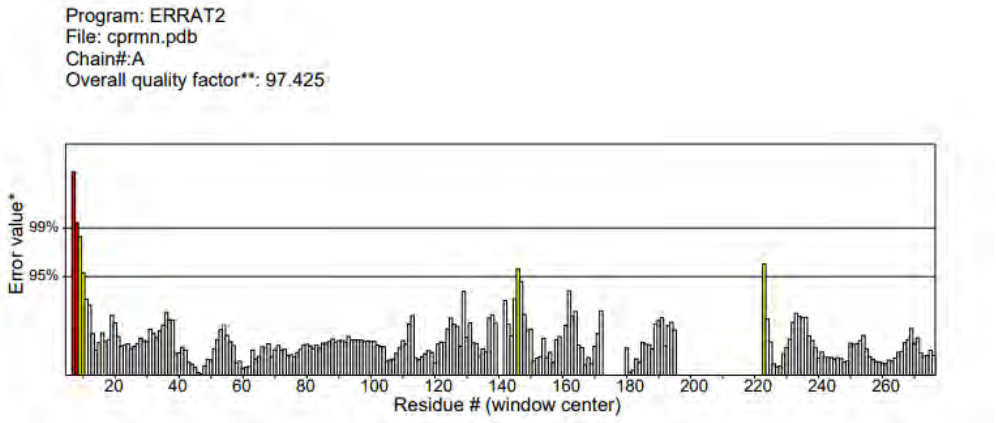


Figure 0.17 *Structural verification and evaluation of CprM reference protein(accession number: MH891771.1) through ERRAT.*

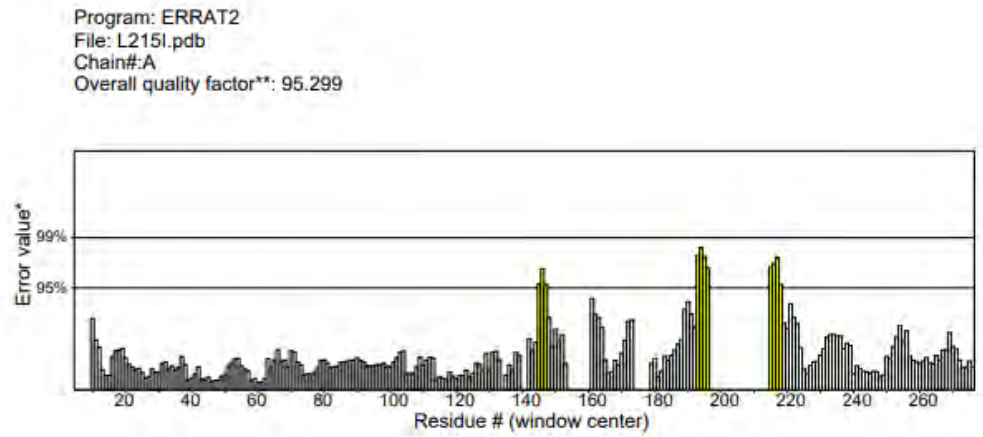


Figure 0.18 *Verification of mutated protein structure with L215I substitution through Verify ERRAT.*

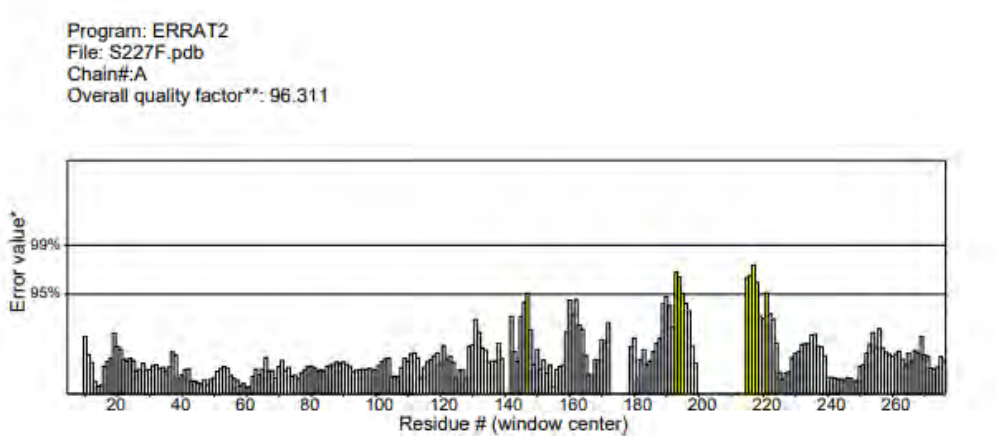


Figure 0.19 *Verification of mutated protein structure with S227F substitution through Verify ERRAT.*

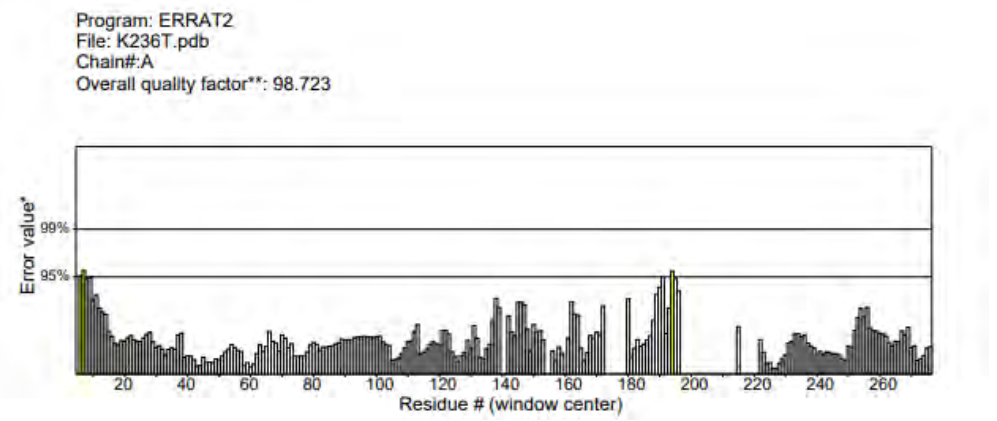


Figure 0.20 *Verification of mutated protein structure with L215I substitution through Verify ERRAT.*

4.11. Superimposition and Visualization:

All mutated models were compared to HOPE results and placed on a reference 3D model (accession number: MH891771.1) to highlight the positions of amino acid residues and side chain differences between the reference model (accession number: MH891771.1) and mutated models. PyMOL was used to superimpose the mutant and normal 3D models and shown in Figure 4.21 Figure 4.22, and Figure 4.23.

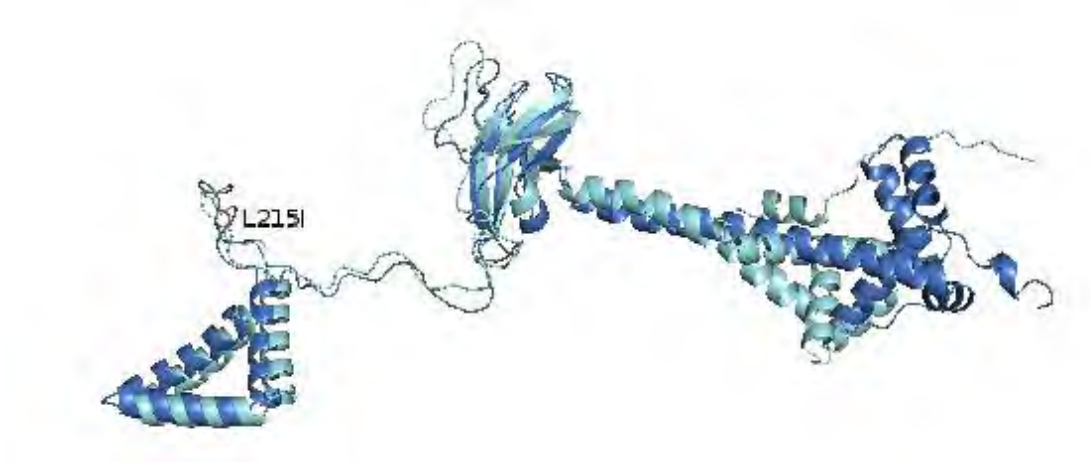


Figure 0.21 *Visualization and super impossibility of reference protein model(accession number: MH891771.1) and mutated model with L215I substitution.*

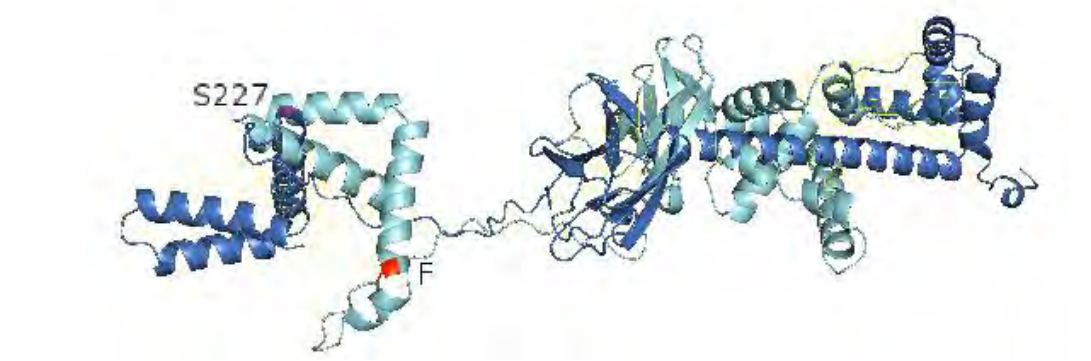


Figure 0.22 *Visualization and super impossibility of reference protein model (accession number: MH891771.1) and mutated model with S227F substitution.*

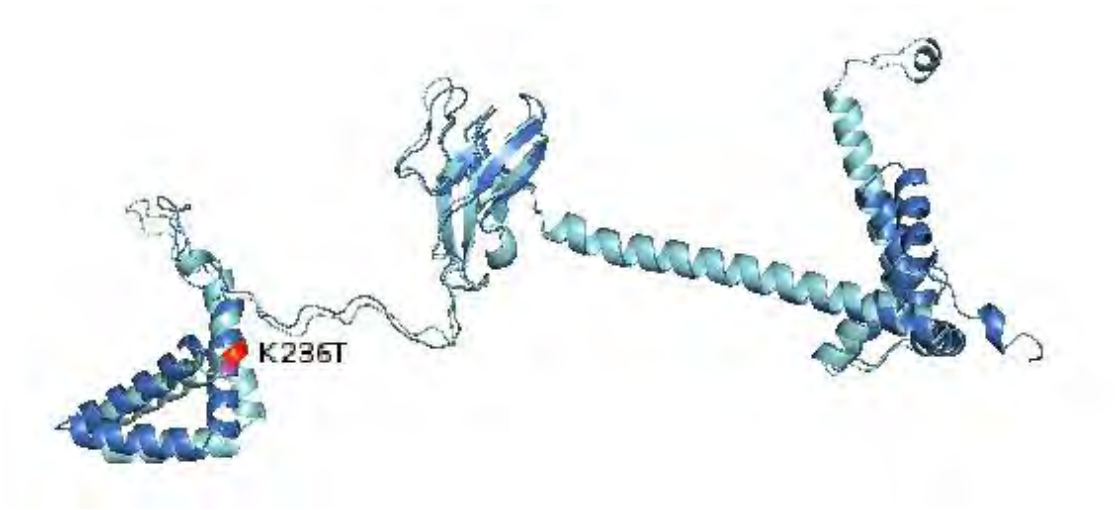
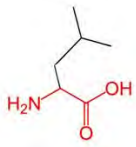
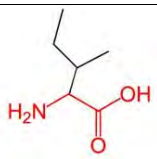
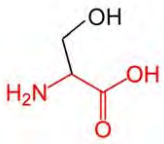
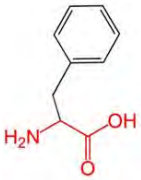
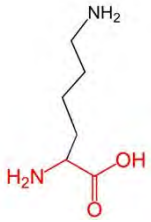
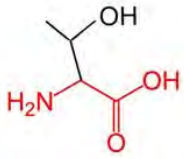


Figure 0.23 *Visualization and super impossibility of reference protein model (accession number: MH891771.1) and mutated model with K236T substitution*

4.12. Analysis of Structural Effects of amino acid substitutions on Protein

HOPE (https://www3.cmbi.umcn.nl/hope/), develops a comparison between reference model and mutated protein models on the basis of conservancy and amino acid properties (Table 4.11). An evaluation report on the basis of specific size, charge densities, molecular interactions, hydrophobicity or hydrophilicity values and visualized structural and functional effect has been evaluated in discussion.

Table 0.11 *Structural effects of amino acid substitution on CprM protein. The term "Not Damaging" mean that the substitution is not significantly altering the structure of the protein or vice versa.*

Sr. No	Mutation	Wild Type Structure	Mutated Structure	Effect of Mutation	Effect on the basis of conservancy
1	L215I			Loss of interactions and destabilize the conformation. Create bumps.	Not Damaging
2	S227F			The mutant residue is bigger than the wild-type residue. Loss of hydrogen bonds and/or disturb correct folding.	Damaging
3	K236T			The mutant residue is smaller than the wild-type residue The wild-type residue charge is positive, the mutant residue charge is neutral. Loss of interactions with other molecules or residues. Loss of hydrogen bonds and/or disturb correct folding.	Probably Damaging

CHAPTER 5

DISCUSSION

5. Discussion

Dengue fever, an arboviral illness caused by a positive-sense single-stranded RNA virus, is horizontally transmitted to the human host via the hematophagous activities of *Aedes* mosquitoes that have been previously infected with the pathogen. According to the World Health Organization (WHO), Pakistan has reported a total of 48,906 cases of the aforementioned disease in the year 2021, with 183 unfortunate fatalities. The prevailing circumstances are highly disconcerting due to the escalating incidence of cases. Since the onset of the year, the province of Punjab has meticulously documented a cumulative tally of 24,146 instances as of the 25th of November in the year 2021. Notably, the urban center of Lahore has emerged as the epicenter of the most profound illness. In each geographical locality across the country, it has been consistently documented a surge in the frequency of individuals exhibiting positive results for dengue fever on a daily basis (DAWN, 2021; U. Khan & Azeem, 2022).

During the period from July 2022 to August 2023, a total of 112 individuals with ages ranging from 12 to 81 years were included. The average age was 35.63 years with a standard deviation of 16.037, suggesting a wide age range. Frequent clinical features such as DHF, Gastrointestinal bleeding, vomiting, and abdominal pain were common in these patients (Ahmed *et al*., 2008). According to other studies, increased HCT levels indicate plasma leakage, a major indicator of Dengue Fever (Wisanuvej *et al*., 2021). Our results predicted that the average HCT was 42.48, with a standard deviation of 21.083, indicating a substantial variation in hematocrit levels (Narayanan *et al*., 2002). Furthermore, the platelet count was very low in patients with dengue, which is evident from our results that the average platelet count was 53.54, with a relatively high standard deviation of 60.314. This indicates a wide dispersion in platelet counts. Similarly, the mean WBC count was 5.63, indicating decreased production of white blood cells in patients with dengue (Chaloemwong *et al*., 2018).

During laboratory examinations, IgM antibodies were detected in 25 patients, followed by IgG antibodies in 3, NS1 in 6, and NS1 along with IgG and IgM antibodies in 2 patients. Out of 27 patients, for IgM, among males, there are 15 positive cases and 10 positive cases

among females, indicating higher proportion of IgM positive antibodies in males (M. Kumar *et al*., 2020). However, owing to the small amount of data and the expected count in some cells, less than 5 (67%), it is important to consider these results cautiously (P=0.372).

Furthermore, for IgG, among females, there was one patient with IgG-positive antibodies and two male patients with positive IgG antibodies. These results suggest no significant association between gender and IgG status (p=0.882), which is consistent with other studies conducted in KPK (A. Ali *et al*., 2013).

For IgG, among females (F), there were 26 negative cases, 7 positive cases, and 1 intermediate case. Among males (M), there were 59 negative cases, 17 positive cases, and 2 intermediate cases. This indicates that more males had positive IgG levels than females did. Moreover, five males and one female were found to be seropositive by NS1, as indicated by another study, that males are more affected by dengue than females (Neralwar *et al*., 2017). However, due to limited data and expected counts below 5 in 50.0 % of cells, a firm conclusion could not be drawn (p=0.430). In the current analysis, we evaluated potential amino acid substitutions in the CprM gene of the Dengue Virus. Variations in CprM may be associated with virulence or drug resistance. SIFT prediction was used to deduce amino acid substitutions generated by the three amino acid substitution. SIFT predicted two amino acid substitutions, S227F and K236T, to be deleterious based on the degree of conservation of amino acid residues in sequence alignments produced from closely related sequences and collected by PSI-BLAST [28]. PROVEAN predicted two substitutions, S227F and K236T, to be damaging (Schulpis *et al*., 2017), and the structural impact of these substitutions was estimated by Polyphen-2, which predicted one substitution, S227F was possibly damaging, and two substitutions, L215I and K236T, did not have any impact on protein structure (Kumari & Rameshwari, 2022). Additionally, the PhD-SNP identified all three amino acid substitutions that are accepted to be disease-associated (Kamaraj *et al*., 2015).

Manual concurrence of all the amino acid substitutions studied was performed using different software. All three substitutions, L215I, S227F, and K236T, deleterious in almost every software, were selected for further verification. The effect of these amino acid substitutions on protein stability was determined through I-Mutant 3.0 and MUpro, which predicted the difference in Gibbs free energy between wild-type and mutant proteins, thus revealing changes in protein stability (Beg *et al*., 2018; Zhao *et al*., 2022). Of the three amino acid substitutions, I-Mutant showed, S227F and K236T and MUpro showed L215I and K236T to decrease the stability of CprM protein.

With these predictions, we focused on three amino acid substitutions that were predicted to be potential candidates for stability. Using further computational algorithms, Consurf found that all three amino acid substitutions (L215I, S227F, and K236T) were present in the conserved regions of the protein (conservation score 6-9) (Portelli *et al*., 2021). NetSurf predicted that 1/3 substitutions (L215I) were exposed and 2/4 substitutions (S227F and K236T) were buried in the protein structure (Karthikeyan *et al*., 2020).

3D models, were generated by Robetta (Zhao *et al*., 2023) and the RMSD (Root Mean Square Deviation) scores, developed by Galaxy Refine has given the quantitative measurements of the similarity between two superimposed atomic coordinates of normal and mutated residues (Das *et al*., 2023). The RMSD values of substitutions, L215I, and S227F were highly deviated, affecting the functionality and stability of the protein. Additionally, PyMOL predicted considerable structural deviation of mutated CprM 3D protein models for all three amino acid substitutions compared to the wild-type CprM 3D protein model (Zhao *et al*., 2022).

Moreover, the HOPE server provides a more precise understanding of the variations in structural and functional contexts (Agrahari *et al*., 2019). The mutant amino acid residue (S227F) was found to be larger than that of the wild-type, which may result in the development of bumps and incorrect protein-protein interactions. In contrast, one mutant amino acid residue, K236T, was found to be smaller in comparison to the wild-type, which might result in an empty space in the protein core, leading to the loss of external interaction.

The difference in nature and conservation of mutant and wild-type amino acid residues affects molecular interactions. The HOPE results revealed that 1 amino acid substitution K236T showed a charge shift from neutral to positive, which ultimately led to repulsion with ligands or loss of hydrophobic interactions on the protein surface. Furthermore, based on conservation, HOPE predicted that S227F and K236T residues are located near a highly conserved region, which may damage protein structure and function.

The current investigation explores the potential implications of amino acid substitutions within the CprM gene of the Dengue Virus. The study posits that the observed genetic variations may conceivably exert a substantial influence on the virulence of the virus as well as its resistance to antiviral therapeutics. These profound insights hold immense significance as they possess the potential to enhance our comprehension of disease severity and facilitate the formulation of highly specific therapeutic approaches. The investigation utilizes a range of computational methodologies to anticipate the consequences of the identified amino acid substitutions on the conformation and activity of the protein. Based on the collective predictions, it is evident that the amino acid substitutions S227F, K236T, and L215I are anticipated to have deleterious consequences on the protein's functional characteristics. This hypothesis is in concordance with the notion that these genetic variations may potentially induce functional alterations that could influence the virus's capacity to engage with host cells and elude the immune system. Subsequent investigations pertaining to the conservations and structural ramifications of the amino acid substitutions unveil their occurrence within conserved domains of the protein. This observation implies that these genetic variations may possess functional implications. The utilization of various structural analyses, such as the assessment of Root Mean Square Deviation (RMSD) and the implementation of 3D modeling techniques, effectively highlight the plausible perturbation in both protein stability and functionality that arises as a consequence of these amino acid substitutions. The aforementioned discoveries serve to enhance our holistic comprehension of the plausible molecular mechanisms that underlie their profound influence.

However, it is important to acknowledge that this study does possess certain limitations. The utilization of computational predictions, although highly advantageous, introduces a certain level of uncertainty. The aforementioned predictions may not comprehensively encompass the complexities inherent in genuine biological interactions, thereby underscoring the imperative nature of experimental validation to verify the veritable impacts of these amino acid substitutions on the behavioral patterns exhibited by the virus. Moreover, the analysis derives insights from a limited patient sample indicative of a small sample size. The aforementioned limitation has the potential to undermine the statistical robustness of the derived conclusions. The extensive spectrum of patient age groups and the inherent diversity in clinical phenotypes serve to imply the presence of heterogeneity within the patient cohort, thereby potentially introducing confounding variables that could impact the observed associations between genetic variations and disease phenotypes.

Conclusion

In conclusion, the present investigation provides captivating revelations regarding the plausible ramifications of amino acid substitutions within the CprM gene of the Dengue Virus. The observed genetic variations possess the potential to exert profound ramifications on the virulence of the virus as well as its resistance towards therapeutic agents. Although computational tools offer valuable predictive insights, it is imperative to exercise caution when interpreting the results due to the reliance on these tools and the limited sample size of patients. The investigation highlights the significance of empirical verification in substantiating the true influence of these amino acid substitutions on protein dynamics. By virtue of its methodology, this study establishes a fundamental framework for subsequent investigations pertaining to the intricate molecular pathways dictating the behavior of the Dengue Virus and its intricate interplay with the immune system of the host organism. In essence, this investigation propels our comprehension of the intricate dynamics between the virus and its host, thereby potentially laying the groundwork for enhanced disease control strategies and therapeutic interventions.

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