Phylogenetic Analysis of Dengue Virus Based on CprM Sequencing

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

Certificate of Approval

This is to certify that the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan accepts the dissertation entitled **"Phylogenetic Analysis of Dengue Virus Based on CprM Sequencing"** submitted by **Ms. Iffat Irshad** in its present form as satisfying the dissertation requirement for the Degree of Master of Philosophy in **Biotechnology**.

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DECLARATION OF ORIGINALITY

I hereby declare that the work ***"Phylogenetic Analysis of Dengue Virus Based on CprM Sequencing"*** 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, with all my heart, to my beloved father, mother, brother (TayyubAbbasi), sisters (Rizhat Abbasi, Talat Abbasi), cousins (Arslan Abbasi and Sajid Iqbal Abbasi), and my respected supervisor Dr. Muhammad Ali. Without their support, a bunch of sincere prayers, and sacrifices it would not have been possible for me to accomplish my work. Thank you for your being my comfort when things go wrong, The ones who encourage me, believe in me, change my weak to strong and for filling my heart with such a pride I feel like the happiest person alive.

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List of Abbreviations

%	Percentage
˚C	Centigrade
µl/µg	Microliter/Microgram
Aa	Amino acid
Bp	Base pair
DENV	Dengue- virus
DF	Dengue –fever
D-H-F	Dengue-hemorrhage-fever
DSS	Dengue-shock syndrome
JEV	Japanese-encephalitis virus
WNV	West-Nile-virus
TBEV	Tick-borne encephalitis virus
Ae. Aegypti	*Aedes Aegypti*
GIT	Gastrointestinal tract
TNF	Tumor Necrosis Factor
Min	Minute
PCR	Polymerase- chain- reaction
RNA	Ribonucleic- Acid
RT	Reverse -Transcriptase
Sec	Seconds
UTR	Untranslated Regions
WHO	World Health Organization
Cdna	Complementary DNA
DNA	Deoxyribonucleic acid
EDTA	Ethylene –Diamine- Tetra Acetate
ELISA	Enzyme Linked -Immunosorbent Assay
ORF	open-reading- frame
C	Capsid
E	Envelope
PrM	Precursor membrane protein
Centers for Disease Control	CDC

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Abstract

Dengue virus,a member of *Flavirus* causes an arboviral disease known as dengue fever. Dengue fever is an ailment that is transmitted by mosquitoes which is known as *Aedes aegpti* and up to some extent it is also spread by the *Aedes albopictus*. It is classified into distinct serotypes, mainly DENV-1, and DENV-2, DENV3, DENV-4, and DENV-5. More than half of the world's population are at high risk of DENV infection. In addition,up to 30–fold increase in dengue infection has been reported in the previous 50 years.The existing study was aimed to explore the CprM region of dengue virus in dengue positive isolates for nucleotide sequencing and its phylogenetic analysis in Pakistani population. In our study, blood samples from dengue infected patients were collected from Islamabad Pakistan. The samples were processed for RNA extraction followed by complementary DNA synthesis. Using high fidelity DNA polymerase, the CprM gene was amplified through nested PCR and the amplified product was further processed for nucleotide sequencing. In addition to the viral isolates from the current study, 68 refrence nucleotide sequences included in the study. Phylogenetic analysis of the current study sequence isolates (QAU IF- 1) (QAU-IF- 2) suggests clustering with a previously reported sequence from India with accession number (accession No. KU948528,KU948530).Further studies are required to explore amino-acid substitutions in functionally and structurally important regions of CprM protiens.

CHAPTER 1

INTRODUCTION

Introduction

The dengue virus is associated to *Flaviviridae* family and the *Flavivirus* genus, is the causative agent of dengue fever, a prevalent arboviral disease. The transmission of the virus to humans is primarily facilitated by two species of mosquitoes, *Aedes aegypti* and, up to some extent, *Aedes albopictus,* both serve as vectors for the RNA of the virus (De et al., 2022; Hasan et al., 2016). The flavivirus genus is composed of five distinct serotypes: DENV-1, DENV-2, DENV-3, DENV-4, and DENV-5. The serotype DENV-5 was initially reported in 2007 and primarily follows a sylvatic transmission cycle. However, there is limited information available about DENV-5 and further characterization is required (Wu et al., 2022).

The DENV genome is primarily comprised of a positive sense RNA and single-stranded molecule that measures almost 10,700 nucleotides in length. This RNA contains the substantial open reading frame, encoding for three structural-proteins, as well as seven non-structural (NS) proteins (Chang et al., 2016). Nonstructural proteins of the dengue virus are only expressed in infected cell and these nonstructural proteins are not part of mature virus particles. The proteins of dengue virus having structural role are part of mature virus particle but these protein have no role in replication of viral genome (Tuiskunen Bäck and Lundkvist, 2013).

In Pakistan dengue fever is common, but the maximum reported cases were almost post-monsoon (Ali et al., 2019). Currently, dengue is considered as most common and quickly spreading viral disease that affects humans. Dengue has now changed from rare disease that is problematic to primary public health with worst socio-economic consequences due to its increased prevalence and geographical expansion (Guzman and Harris, 2015). W.H.O considered dengue infection as serious global public health problem in different tropic and subtropics areas (Hasan et al., 2016).Annually, almost 50-million individuals are commonly estimated having dengue fever, with roughly 55000 people be in need of hospitalization, many of them are children. An average 2.5% of these people passed away. Mosquitoes which belong to the *Aedes* genus are considered as vectors of dengue in both rural and urban regions. Most significant mosquito vector of arboviruses is *Aedes aegypti* because it is generally anthropophilic and select habitats in close vicinity to humans (Rasheed et al., 2013). During the period of 1960 to 2010, a significant rise in dengue infections was observed worldwide, with an increase of thirty-fold. This was largely due to various factors such as a rapidly expanding

population, the effects of climate change, rapid urbanization, a lack of effective mosquito control measures, an increase in air travel and insufficient healthcare infrastructure (Hasan et al., 2016).

The clinical symptoms of dengue virus infections are varried and can range from a-symptomatic dengue fever to severe infection such as (DHF) or (DSS) (WHO, 2009). The commonly observed signs of dengue infection include abrupt feeling of high fever, stiffness in joints and muscles, myalgia, skin rashes, bleeding, and circulatory shock. While oral symptoms are infrequently seen in dengue, they may occur in some instances. (Hasan et al., 2016). Dengue is also called 'break bone fever' because of its un-survivable symptoms which include extreme pain in muscle and joint (Tuiskunen Bäck and Lundkvist, 2013). A quick onset of high fever indicates the beginning of classical–DF after an incubation time of 3-15 days (generally 5 to 8). In young children, dehydration may produce neurological issues and febrile seizures during the febrile phase of infection (WHO, 2009).

Pakistan is sub-tropical area and major hotspot for vector-borne illnesses like dengue hemorrhagic fever. Pakistan has different dengue serotypes which are circulating whole year, but their outbreak reached at peak during period of post-monsoon (September-November). Pakistan faced worst outbreak of dengue in 2011 with 21650 confirmed cases and almost 350 deaths are reported (Ahmad et al., 2017). Annually, almost 100 million confirmed dengue cases with greater than 390 million individuals having chance of infection are reported worldwide (Bhatt et al., 2013).

The symptoms of dengue gradually progress from mild to severe forms. DF is a terrible, influenza-like sickness that can sometimes be fatal and affects children, adolescents and adults of all ages. People who have a high temperature (over 40 °C) and other symptoms including a strong headache, muscle and joint discomfort, vomiting, swelling in glands, nausea, and extreme-level pain in body-part behind the eyes are at risk for dengue sickness (WHO, 2009). Deforestation, air travel, continue increase in growth rate of population, unplanned urbanization, poor health care facilities, lack of awareness about the disease spread among people, poor disease monitoring, global warming and difficulty in vector control are major factors which are involved in endemic threat of dengue (Guzman et al., 2010). Currently, no specific medication available for the treatment of emerging arboviral-disease (Kumar et al., 2013).

- ## Aims and objectives

1. To optimize amplification conditions for CprM region (partial genome) of dengue virus.

2. To perform phylogenetic analysis of dengue virus based on CprM sequencing.

CHAPTER 2

LITERATURE REVIEW

Literature Review

2.1 Dengue virus

Dengue virus is among the most significant mosquito-borne viruses responsible for human dengue disease (Wu et al., 2022; Xi et al., 2019). Dengue-like disease epidemics have been known for over two centuries and was widespread in Asia, along the Atlantic and Gulf coastlines of the United States, and in the Caribbean until the first part of this century. Dengue hemorrhagic fever/ dengue shock syndrome, a clinically severe variant of infection, was initially identify in the Philippines in Year 1954 and has subsequently spread throughout Southeast Asian countries. DSS/DHF is now a severe cause of infection and fatality in infants in the tropical region of Asia, in the absence of effective treatment, and becoming more common in the Western Hemisphere (Waterman and Gubler, 1989). Dengue fever is a widespread tropical illness. In extreme cases, this acute febrile sickness can be fatal, resulting in dengue hemorrhagic shock (Wiwanitkit, 2010). Dengue is an arbovirus that causes ~390 million worst dengue infections annually, with ninety-six million of them manifesting clinically in almost 129 countries worldwide (Umakanth and Suganthan, 2020)**.**

2.2 The origin of Dengue virus

Strong evidence suggests that DENV (sylvatic DENV) spread in wild primates in Africa and Asia before separately spreading to humans. Sylvatic DENVs from African or Asian continent, which use nonhuman hosts as primates and *Aedes* mosquitoes as their vectors in forests, are assumed to be the origins of urban-transmission. Sylvatic-DENVs first emerged about 1000 years back, and transmission occur in populations of human just recently became reported (Harapan et al., 2020). All serotypes of DENV are thought to have their sylvatic ancestral lineage sheltered in Malaysia (Messina et al., 2014; Villabona and Zanotto, 2013).

Different recent studies found that DENV-1 started in Asian countries and then spread to African and American countries. DENV-2 evolved from its sylvatic ancestor, almost four hundred to six hundred years ago. The first report of this serotype was made in the American country in 1953, in Africa (Nigeria) in 1964, and in Asian country in 1944 (Harapan et al., 2020). The initial DENV-3 cases were discovered in 1953 in Asian continent, reported in 1963 in the America, and found in African continent during 1984–1985. DENV-4 was initially

discovered in the Americas (Brazil, US Virgin Islands) in 1981 and Asia (the Philippines and Thailand) in 1953 (Messina et al., 2014).

2.3 Historical picture of Dengue virus

Any person living in areas where the virus is endemic, along with those who have recently been to such areas, should be doubted of having a history of dengue fever (Kularatne, 2015). It was considered that 1st report of major epidemics of diseases in Asian continent, Africa and North America continent caused by dengue in 1779 and 1780 (Halstead, 2008). Dengue viruses come out from the jungle and entered populous areas at certain points in the past, perhaps with the forest destruction and the expansion of human settlements. *Aedes albopictus* and other strongly linked peri-domestic Stegomyia species of mosquito are probably main vectors of viral transmission across tropical Asia's cities, towns and villages as a result of human and economic migration (Gubler, 2006). First evidence of association among sylvatic DENV and humans take place retrospective serologic studies including human communities that were residing in forests (Rudnick and Lim, 1986; Rudnick et al., 1986). According to entomological studies, *Aedes furcifer*, that spreads into-rural and peri-domestic areas, may be the common linking-vector between different types of sylvatic DENV and human-populations found near to the forest area (Diallo et al., 2003; Diallo et al., 2005).

2.4 Classification of Dengue

Dengue fever is classified into three types, according to the 1997 classification: DF, DHF and undifferentiated fever (Ranjit and Kissoon, 2011).

2.4.1 Undifferentiated fever

This stage can develop after the initial secondary infection but is most frequently associated with the primary infection. It is very hectic to clinically distinguish it from a range of other viral infections, and it frequently goes undetected (Hasan et al., 2016).

2.4.2 Dengue fever

The most frequent victims of DF are adults and older-children, and it can occur after both secondary as well as primary infections. Initially, most common symptoms are characterized by a biphasic, elevated body temperature lasting 3–7 days (Narayanan et al., 2002). Additional

indicators include appetite loss, metallic taste, severe headaches, myalgia, lassitude, aching joints, diarrhea and vomiting. Due to the association of pain in joints and myalgia, it is also recognized as break bone fever (Chen and Wilson, 2010; Whitehorn and Farrar, 2010). 50-82% of DF patients report a unique cutaneous rash (Itoda et al., 2006). After three to seven days of the illness the 2nd rash appears as an uncomplicated maculopapular or morbilliform rash (Waterman and Gubler, 1989; Radakovic-Fijan et al., 2002). The epidermal rash are frequently asymptomatic with Pruritis only occurs in 16–27% of individuals (Chadwick et al., 2006; Thomas et al., 2007).

2.4.3 Dengue hemorrhagic fever

The severe form of dengue fever is known as DHF (Sanyaolu et al., 2017). It occurs frequently when a secondary dengue illness occurs. However, due to maternally acquired dengue antibodies, It may also happen in newborns after a primary illness (Halstead et al., 2002). The suggested diagnostic criteria for DHF have been discussed in detail by Smith and Fairweather (Smith and Fairweather, 1988). Below is some of the basic information:

2.4.3.1 Clinical parameters

Acute febrile stage characterized by high body temperature lasting 2 to 17 days. Petechiae, gingival -epistaxis, purpura, ecchymosis, mucosal hemorrhagic, hematemesis or melena positive tourniquet, and hepatomegaly are the possible complication (Hasan et al., 2016).

2.4.3.2 Laboratory parameters

Thrombocytopenia i.e., the platelets count >100,000/cu mm. In DHF the hemorrhagic episodes have been linked with multiple factors which includes vasculopathy, platelet insufficiency and dysfunction, and abnormalities in blood coagulation pathways. Thrombocytopenia in DHF may be caused by decreased platelet formation and increased platelet destruction. Platelet malfunction makes blood vessels fragile, which can result in bleeding (Hasan et al., 2016). Usually, the clinical course of dengue hemorrhage fever is categorized in three stages: febrile, convalescent and leakage. The initial stage of the febrile sickness is characterized by a severe fever with an acute start, constitutional symptoms, and face erythema (Narayanan et al., 2002).The early febrile illness is identified by hemorrhagic tendencies and morbilliform rash (Richards et al., 1997).When the patient moves on to the plasma-leaking phase, the fever

disappears or returns to normal level after lasting for two to seven days (Srikiatkhachorn et al., 2007). In a rare case, gastrointestinal bleeding and severe ecchymosis followed by epistaxis are also noted, this stage is characterized by pallor, bradycardia, erythema, and confluent petechial rashes (Hasan et al., 2016).

2.4.4 Dengue shock syndrome

DSS is the term used to describe DHF with an irregular pulse, a low pulse pressure (20 mmHg), cold, restlessness, circumoral cyanosis and clammy skin. A significant fatality rate related to DSS is attributed to progressively increasing shock, dysfunction of multiorgan, and diffuse intravascular coagulation. With supportive treatment, the shock is short-lived, and the patient recovered quickly (Shivpuri and Shivpuri, 2011; Gurugama et al., 2010).

2.5 Various Serotypes of dengue virus exist in Pakistan

During 1994 outbreak DEBV 2 and DENV 3 were detected in Karachi (Khan et al., 2020). A monoclonal antibody-based ELISA analysis of a few individuals in 1998 indicated the presence of DENV1 and DENV2, while DENV3 was identified among the few examined patients in 2005. An epidemic in 2006 was once again dominated by DENV2 and DENV3. It was discovered that youngsters living in Karachi's slums having higher levels of anti-dengue IgM-antibody. Because these serotypes were found in all significant outbreaks in India, Bangladesh, and Sri Lanka, similar pattern can be seen in Pakistan. The DENV3 that caused the 2006 epidemic were prominently related to the DENV3 (subtype I) that caused the 2004 epidemic in New Delhi (Raheel et al., 2011). DENV2 and DENV 3 were reported during 2001 outbreak in Punjab (Ali et al., 2016). Over the past ten years, thousands of people infected with this lethal disease in several regions in Pakistan. Serotypes 1, 2, and 3 were the prevailing serotypes during 2013 outbreak in KP, while 2017 outbreak serotypes 2 and 3 were reported from Peshawar KP (Khan et al., 2020). DENV2 was the dominant serotype while DENV1 was also reported in some cases during 2019 outbreak (Malik et al., 2021). There is need for more detailed study to understand viral serotypes prevalent in Pakistan.

2.6 Genetic diversity of dengue virus

Genetic diversity is mostly limited with in every serotype, but it is still enough for clusters formation of variants-genotypes (Rico-Hesse, 1990). There are a number of factors that support

this biodiversity. Dengue virus is prone to mutation, as RNA dependent RNA polymerases (RdRp) are expected to yield almost one error/round of genome replication (Drake, 1993). Due to their high mutation rate, DENV serotypes have a diverse genetic makeup (Waman et al., 2017). The fourth serotypes of DENV can be further divided into separate genotypes that vary up to 3% and differentiate from one another by twenty five to forty percent at level of the amino acid (Guzman and Harris, 2015). As the RNA-dependent RNA-polymerase encoded by NS5 lacks proofreading, viral genome replication is more susceptible to error, leading to an antigenic and genetic diversity within and between DENV serotypes (Jenkins et al., 2002). The DENV RdRp causes errors during replication and introduces variations into the viral genome, like other RNA viruses. Thus, populations of genetically different viral isolates circulate in patients, sometimes known as "quasispecies"(Domingo and Holland, 1997;Wang et al., 2002).

2.7 Disease burden and Epidemiology of dengue

Dengue is the second utmost dangerous vector borne infection world-wide, after malarial infection, according to prevalence and fatality rates (Wu et al., 2010). The financial load of dengue has touched 2 billion USD in five American nations, such as Panama, Guatemala, and Brazil, Venezuela, El Salvador and three Southeast Asian countries with high dengue prevalence, including Cambodia, Thailand and Malaysia excluding expense on vector control, exceeding the normal cost of other diseases caused by different viruses. The annual burden has reached USD 950 million in Indonesia, Bhutan, Singapore, Brunei, Philippines, Cambodia, Myanmar, Vietnam, East Timor East and Taiwan province of China. Dengue has distinctive epidemiological patterns which are highly related to four serotypes of the virus. In several countries, all 4 serotypes of dengue virus are hyper-endemic, and these can continually co-circulate within a region (Shepard et al., 2013).

Dengue has a negative effect on human health as well as on the economies of world and nation. Infected travelers often carry the dengue virus from one place to another; if receptive vectors are present in these new regions, there is a possibility that local dengue virus transmission will establish itself. Due to the dengue virus's rising prevalence worldwide, about half the world's people are currently at risk. However, almost 100 to 400 million’s infections occur annually, above 80% are usually mild and a-symptomatic (W.H.O). Most of the countries with dengue endemic regions have seen a rise in dengue incidence during the COVID-19 pandemic,

including Argentina, Brazil, Colombia, Malaysia, Mexico, Thailand, the Philippines, Singapore, Indonesia, Vietnam, India, Nepal, Bangladesh, and Pakistan. From 2000 to 2019, 500,000 cases increased to 4.2 million cases, during the last 20 years the dengue cases have increased by almost 8 times. According to the findings of a recent study, Pakistan had a more than 16-fold increase in dengue incidence from 3204 cases in 2018 to 52485 cases in 2019. From February 2020, Pakistan has been combating COVID-19 alongside 221 other countries, while also attempting to combat increasing dengue transmission, because more than 10392 dengue cases were recorded in the country through September and October 2022 (Rana et al., 2022).

Dengue infection is most commonly spreading illness in Pakistan, which faces continuous spreading along with seasonal peaks as well. As compared to the four previous years, number of dengue cases reported in 2022 were significantly higher. This is a result of the worst floods the country has ever experienced, which began in mid-June. Between 1 January and 27 September 2022, Pakistan reported 25932 confirmed dengue cases and 62 deaths, with 74% of those cases occurring in September alone. With the recent flood crisis highly destroying the capability of national health system, there is considerable danger of critical health problem cause by dengue infection (W.H.O).

2.8 Factors influencing the incidence of or epidemics of dengue

Numerous investigations have been made to identify the causes of the high occurrence of dengue in some areas. A range of demographic, environmental, socioeconomic, and ecological factors are linked to the prevalence or outbreak of dengue (Harapan et al., 2020).

Table 1: Factors influencing dengue incidence (Harapan et al., 2020)

Factors influencing the dengue incidence or dengue outbreaks.	
Improper usage of vector controlling agent	(Heukelbach et al., 2001)
Lack of information	(Soghaier et al., 2014)
Higher container	(Pham et al., 2011)
Higher humidity, higher-temperature	(Pham et al., 2011; Goto et al., 2013)

2.9 Morphological pattern of DENV

The structural proteins shown in figure 1 shape the DENV virions' morphology. In the mature DENV virion, which is covered in a lipid-bilayer membrane that also contains the M and E proteins, the C protein encases the RNA genome. The prM protein is degraded during viral maturation in order to release its pr domain, and the M protein interacts with the mature virus's E protein (Kostyuchenko et al., 2013). By establishing connections with receptors and joining the endosomal membrane, the E protein enables cell entry. It is the primary protein that carries epitopes necessary for antibodies to be neutralized (Bressanelli et al., 2004). The formation of the replication complex and translational machinery are two key functions of nonstructural proteins in the translation and replication of RNA (Wilder-Smith et al., 2019).

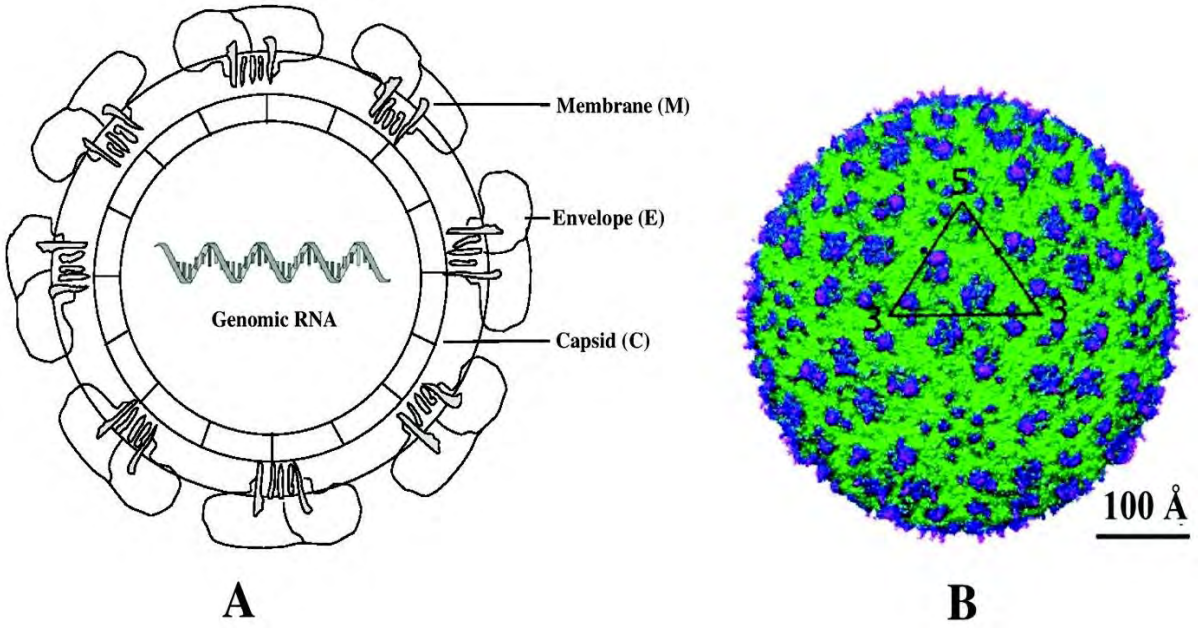

Figure 1: Morphology of DENV.

The dengue virus is shown in figure A as an enclosed, spherical particle with structural proteins, and in figure B as the virus's cryo-electron microscopic structure. The icosahedral asymmetric unit is displayed by the black triangle. The E protein is shown in blue. There is one asymmetric unit that contains all three E proteins. Scale bar equals 100 Å (Kostyuchenko et al., 2014; Roy and Bhattacharjee, 2021).

2.10 A brief overview about DENV Genome

This virus is a positive-stranded RNA with a genome that is approximately 11kbp in size. Its open reading frame encodes nearly 3000 amino acid polyprotein precursors, which is translated both cotranslationally and post-translationally by host and viral proteases. (Muñoz-Jordán et al.,

2003). Dengue contains three structural proteins and seven nonstructural proteins (Umareddy et al., 2007). Numerous biological processes, including viral replication, are carried out by these viral proteins (Shu and Huang, 2004). The interferon (IFN) signaling pathway is activated when a virus infects a person. But in dengue, interferon resistance emerges. The interferon cascade is blocked by nonstructural dengue proteins such NS2a, NS4a, and NS4b.NS4b inhibits the interferon cascade by preventing STAT-1 phosphorylation (Muñoz-Jordán et al., 2003).

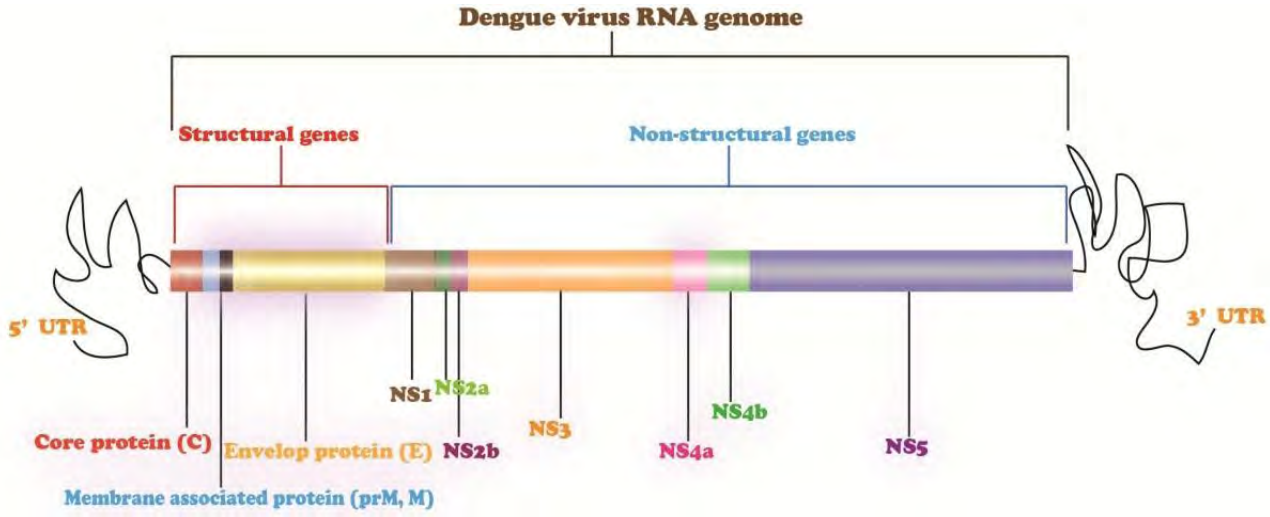

Figure 2: Representation of the genome of dengue virus.

It encodes ten viral proteins including three structural proteins that are responsible for the structure of the virus and its attachment to the host cell, and seven non-structural proteins that mediated replication of virus (Idrees and Ashfaq, 2012).

2.11 Dengue virus proteins and its major functions

2.11.1. Structural proteins of dengue virus

Dengue virus consists of three structural proteins known as Envelop proteins, Core proteins and membrane associated proteins. These proteins show significant role in forming the structure of dengue virus (Idrees and Ashfaq, 2012).

2.11.1.1. Core protein of dengue virus (C)

The core proteins C is located in nucleus (S.-H. Wang et al., 2002). The DENV core protein is a very basic protein that interacts with RNA to assemble the nucleocapsid (Ma et al., 2004).

2.11.1.2. Membrane associated protein (M)

These membrane glycoproteins are an important component of nucleo-capsids. These glycoproteins help the dengue virus's envelope proteins to generate mature virions. Typically, prM protein can be used to distinguish between distinct flaviviruse's antibody responses (Cardosa et al., 2002).

2.11.1.3. Envelop protein (E)

The envelope protein, which is often take place on the surface of the virus, is crucial for the virus ability to bind to the host cell via cell receptors including heparin sulphate Dc-SIGN.I. It is the most crucial protein for viral entrance into cells (Marks et al., 2001; Modis et al., 2004; Mondotte et al., 2007). Functions of structural proteins in DENV genome (Table 2).

2.11.2. Nonstructural proteins of DENV

The dengue virus genome codes for 7 non-structural proteins. Viral replication and other cellular function are mediated by these nonstructural proteins (Idrees and Ashfaq, 2012).

Table 2: Functions of structural proteins in DENV genome.

Gene	Function	References
Pre-membrane(prM)	Viral entry through membrane protein/protein interactions Infection enhancement, Proper E protein folding and its stability	(Konishi and Mason, 1993; Lindenbach and Rice, 2003; Nem.sio and Villalaín, 2014; Reddy and Sansom, 2016; Rodpothong et al., 2016; Smith et al., 2016; Wang et al., 1999)
Capsid (C)	packaging and release of the viral DNA, viral growth, Replication, RNA encapsidation	(Amberg and Rice, 1999; Clyde et al., 2008; Samsa et al., 2009; Clyde and Harris, 2006; Vasilakis et al., 2011; Samsa et al., 2012)
Envelope (E)	Cell attachment, Antigenicity, Immunological epitopes Infectivity	(Chen et al., 1997; Modis et al., 2004; Zhang et al., 2013)

2.11.2.1. Non-structural NS1 protein

NS1 is found on the surface of infected cells and can be a target for dengue virus infection (Jacobs et al., 2000). Despite being present at the location of RNA replication, the NS1 protein's precise function is yet unknown. Between the first- and ninth-days following infection, NS1 antigen was often detected. RT-PCR was still able to detect NS1 antigen despite the viral RNA test yielding negative findings. Identification of the NS1 protein may therefore facilitate the early detection of dengue infection (Alcon et al., 2002).

2.11.2.2. Non-structural NS2A protein

RNA replication and viral assembly are mostly mediated by the NS2A protein. Any NS2A protein mutation prevents viral replication. By preventing the transcription that is triggered by the IFN-promoter, NS2A performs the role of an interferon (IFN) antagonist (Muñoz-Jordán et al., 2003; Liu et al., 2004) If NS2A had a single amino acid mutation, this would reduce inhibitory effect of NS2 (Liu et al., 2004). NS2A may effectively break down the well-known NS1 protein to form a cis-acting protease or it might also give the cellular proteases that particularly cleave their junction recognition sequences (Falgout et al., 1989).

2.11.2.3. Non-structural NS3 protein

The 2nd largest non-structural protein, NS3 of DEN-2 contributes significantly to the regulation of polyprotein processing and RNA replication (Matusan et al., 2001; Teo and Wright, 1997). Together with its cofactor NS2B, the N-terminus of NS3 forms a chymotrypsin-like protease domain that, along with host-encoded proteases, catalyzes the cleavage of the viral polyprotein (Lindenbach et al., 2007). The type I interferon response is inhibited by the NS3 protease's cleavage of host proteins involved in the innate immune response pathway (Rodriguez-Madoz et al., 2010). NS3 is regarded as a key therapeutic target against dengue virus infection (Idrees and Ashfaq, 2012).

2.11.2.4. Non-structural NS4A protein

NS4A acting as significant role in the cycle of replication, membrane re-arrangements, and their control. In cell free system viral proteinase NS3 cleaves NS4A to form its N-terminus (Preugschat and Strauss, 1991).

2.11.2.5. Non-structural NS4B protein

When NS3 and NS4B interact, RNA replication is frequently regulated. NS4B inhibits IFN signaling by preventing the expression of (STAT 1), a signal-transducer and transcription activator, because NS4B interacts with IFN (Muñoz-Jordán et al., 2003).

2.11.2.6. Non-structural NS5 protein

NS5 is multidomain major dengue viral protein consist of approximately 900 amino acids (El Sahili and Lescar, 2017). At its C- and N-termini, respectively, NS5 has a polymerase domain and a methyltransferase (MTase) domain. NS5 assists in RNA replication because it has RNA dependent RNA- polymerase activity. Additionally, it takes role in the production of new RNA (Ackermann and Padmanabhan, 2001).

2.12 Life cycle of dengue virus

Mosquitoes, hematophagous vectors can carry 100s of viruses, are the etiological agents of diseases in animals and human. In nature, the life cycle of viruses is maintained between animals, particularly vertebrates and mosquitoes. Naive mosquito pick dengue viruses from an infected host. Then dengue virus rapidly proliferates in the mosquito's tissues. Then mosquito is considered as virus reservoir and can now act as active carrier of viruses when feeding on another naive host. In order to fully exploit the host, vector features and successfully cycle between two separate environments of the host, Mosquito borne viruses have formed sensitive and clever tactics (Wu et al., 2019). The life cycles of flavivirus are composed of numerous steps including 1) binding of virions to attachment molecules and receptors on the cell surface, followed by endocytosis, which internalizes the virions. 2) Viral glycoproteins facilitate the fusing of viral and cellular membranes because of the endosome's low pH level, which causes the virion to disassemble and release viral RNA (vRNA) into the cytoplasm. 3) Viral and cellular proteases break down the polyprotein that results from the translation of vRNA, and 4). The genomic RNA is replicated by the viral NS proteins. 5) The (ER) membrane of endoplasmic reticulum is the site of virus assembly, where vRNA and C protein are encapsulated by the glycoproteins and ER membrane to make immature dengue virus particles. 6) The virus become mature when secretory pathway transports immature virus particles and, prM is cleaved by furin

in the acidic trans-Golgi network. (TGN which causes the virus to mature. 7) The Matured virus is then come out from the cell (Tuiskunen Bäck and Lundkvist, 2013).

Many researchers, including ones on viral protein processing and genome replication, have been carried out to better understand the dengue virus life cycle. Dengue virus envelop protein facilitates the attachment of the virus to its receptor (E). Dengue serotypes 1-4 often bind to the mannose receptor, DC-SIGN/L-SIGN, heparan sulphate, and nLc4Cer in mammalian cells (Hidari and Suzuki, 2011). Viral particles are merged into acidic lysosomes by receptor-mediated endocytosis when the virus initially binds to certain receptors on the surface of the host cell. RNA causes the viral proteins to form when the viral particles split from the host cell (figure. 4). Viral RNA begins to replicate to form an antisense strand that is subsequently used to create new sense stranded molecules when all necessary proteins have been produced. After just a few hours of infection, tens of thousands of copies are created, leading to cell damage and even death. Cells include a number of elements that speed up the dengue virus infection cycle, such as virally encoded RNA-dependent RNA polymerase (RdRps) (Filomatori et al., 2006).

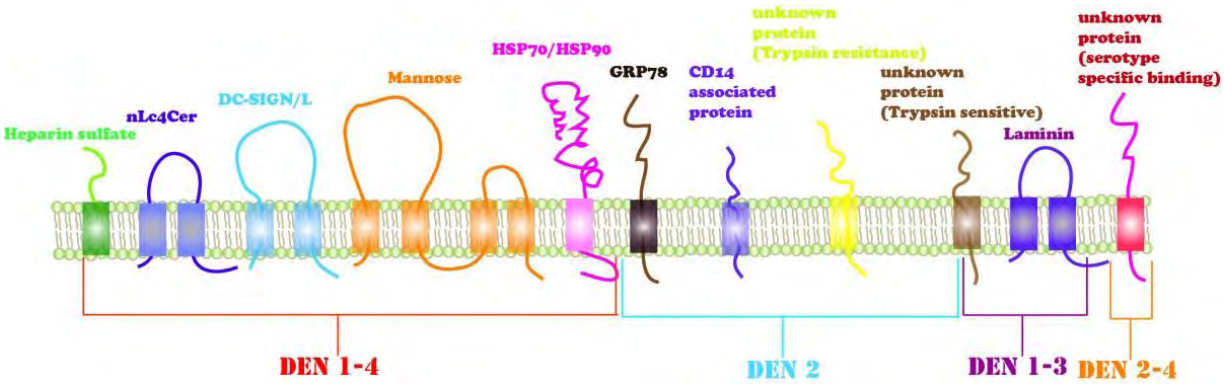

Figure 3: DENV cell entry receptors (Idrees and Ashfaq, 2012).

Dengue serotypes 1-4 often bind to the mannose receptor, DC-SIGN/L-SIGN, heparan sulphate, and nLc4Cer in mammalian cells

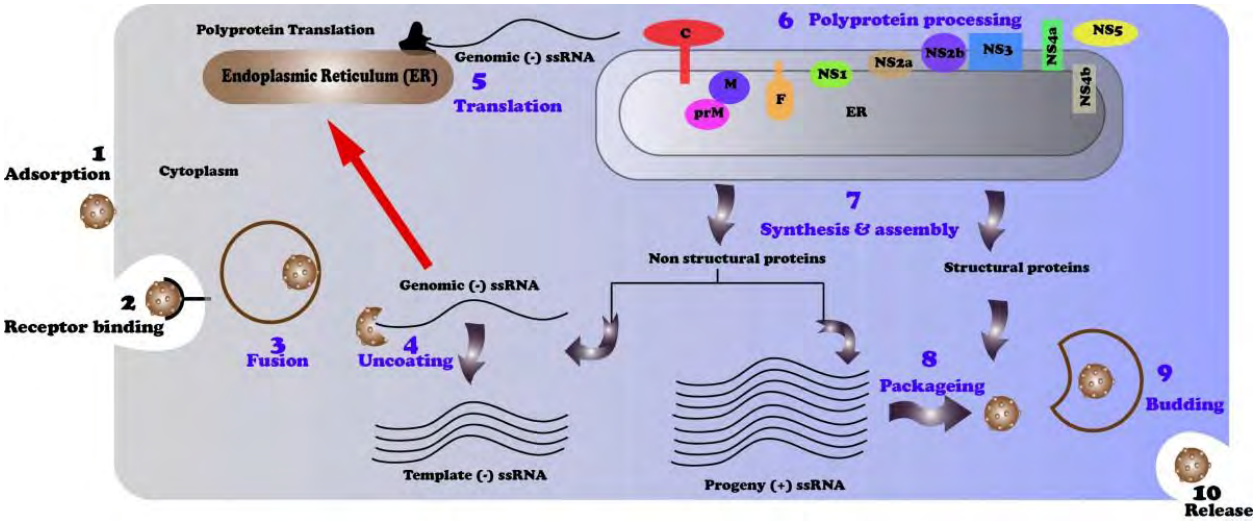

Figure 4: Life cycle of dengue virus (Idrees and Ashfaq, 2012).

The life cycles of flavivirus are composed of numerous steps including Adsorption,Receptor binding,fusion,uncoating,translation,poly protein processing,synthesis and assembly.

2.13 Pathogenesis of Dengue

Four dengue virus serotypes shows almost 65 to 70 percent sequences similarity, indicating a strong evolutionary relationship between them (Weaver and Vasilakis, 2009). The initial stage of an infection caused by a certain serotype is known as primary infection. The majority of early stage infections are asymptomatic .The term "secondary dengue infection" refers to a subsequent dengue infection with a different serotype, which can result in clinical symptoms that can be fatal (Bhatt et al., 2021; Guzman et al., 2013; Mathew and Rothman, 2008). In the pathogenesis of dengue viral infection, the intricate interaction between the host gene, the virus, and the host immune system is important. Host variables such anti-DENV NS1 antibodies, memory cross-reactive T cells, autoimmunity, antibody-dependent enhancement (ADE), and genetic diversity have a substantial impact on a disease's vulnerability. Severe dengue's pathophysiology involved the NS1 protein and anti-DENV NS1- antibodies. The generation of cytokines by immune cells as a result of antibody-dependent enhancement (ADE) via Fc receptors raises vascular permeability and impairs the function of vascular endothelial cells. The genomic variety of the dengue virus and sub genomic flavivirus RNA (sfRNA), which occupies the host immune response, are viral indicators of disease severity. Antibodies against the DENV NS1 antigen, E proteins, and prM region are produced as a result of dengue infection and can cross-react with platelet cells, integrin, and plasminogen. In addition, a number of host gene polymorphisms, genetic factors, and viral factors are involved in the pathogenesis of DENV

(Bhatt et al., 2021). Figure 5 illustrates various elements that are crucial in the pathophysiology of dengue virus infection. According to epidemiological and clinical research investigations, infants born to mothers having immunity to the disease have primary and secondary dengue infections as well as dengue vascular permeability syndrome (Bhatt et al., 2021).

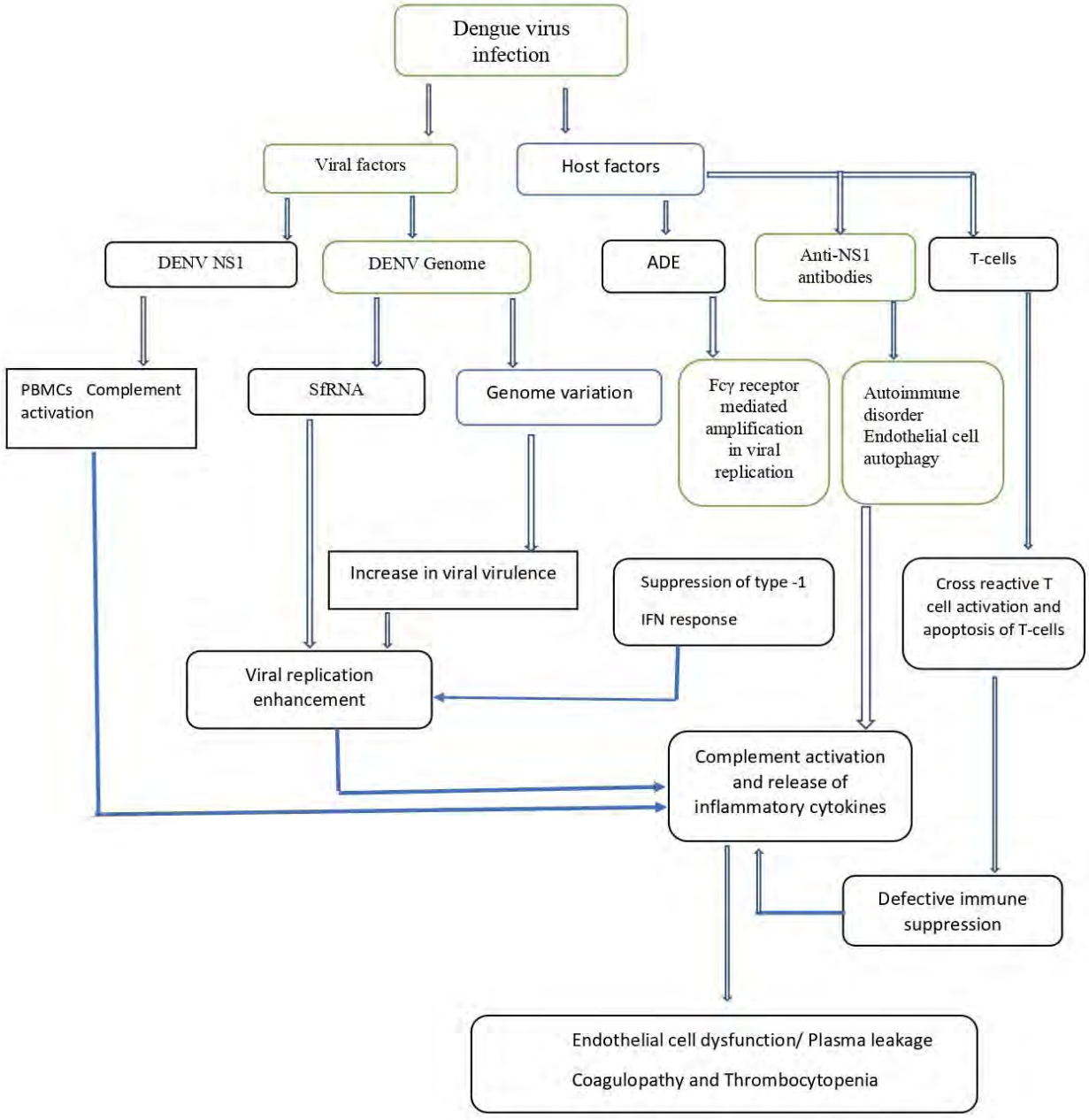

Figure 5: Pathogenesis of DENV.

Complex interaction of host and viral factors during pathogenesis of dengue virus infection (Bhatt et al., 2021).

2.14 Transmission of dengue virus infection

Aedes albopictus and *A. aegypti* are the primary *Aedes* species of mosquito responsible for transmitting dengue among humans. In some situations, organ transplantation or blood transfusions from infected person can potentially contribute to dengue transmission; there is evidence that a pregnant woman who has the virus can pass it on to her fetus. The transmission from person-to-person have been described, but they are incredibly uncommon (Islam et al., 2017). Mosquitoes in the wild pick DENV by feeding on a host who is viremic as shown in the figure 5. The midgut of the mosquito serves as the first site of DENV infection after the blood meal. When the bite occurs, saliva is released, causing the virus to move to the salivary glands where it continues to replicate before being spread to the next victim (Merkling et al., 2020). A. Aegypti mosquito, which typically picks the virus by ingesting the blood of a person who has the disease and transmits it to another person who is not afflicted. In houses, schools, and other places in towns or cities, the female mosquito typically lays her eggs in water containers. Within just ten days, these eggs develop into adults. The potential for spawning in either an open or a closed water storage system is a trait of dengue mosquitoes. Dengue can also grow in containers like buckets, tanks, flower vases, drums, and water collection systems that collect rainwater (Jahan, 2011).

Figure 6*: Aedes albopictus* mosquito feeding on blood (CDC).

Aedes albopictus and *A. aegypti* are the primary *Aedes* species of mosquito responsible for transmitting dengue among humans.

The spread of dengue around the world as a result of migration, international travel, and two horrific world wars, shipping and speedy development increase dengue transmission in nearly

all South East Asian nations, resulting in the spread of the severe form of the disease and a more than 30 times increase in dengue rates worldwide from 1960-2010. Deforestation, growing population, un-planned urbanization, air travel, unsatisfactory public health care facilities, a nonexistence of public awareness of the disease, poor disease surveillance, challenges in controlling vectors, and global warming are all significant contributing factors to the endemic threat for dengue transmission (Ahmad et al., 2017). DENV is spread by the sylvatic transmission cycle in forested areas and by the human transmission cycle in urban areas. These transmission cycles are distinct from one another from an ecological and evolutionary standpoint (Chen and Vasilakis, 2011).

While dengue is primarily spread between humans in urban settings, it is more seldom spread between non-human primates and humans in forested areas (Simmons et al., 2012). Dengue virus transmit vertically from mosquitoes to their offspring during the dry season is vital for maintaining both the human and the sylvatic transmission cycle (Chen and Vasilakis, 2011). There have also been reports of dengue virus transmission thru non vector methods, such as blood transfusions, bone marrow transplants, and perinatal and intrapartum transmission (Chen and Wilson, 2016). Due to transmission complexity of dengue virus, eradication of dengue virus become challenging (Chen and Wilson, 2016).

2.15 Symptoms of Dengue infection

A person who contracts the dengue virus may experience a range of different- symptoms, from a mild, self-limiting fever to a serious, perhaps fatal condition. According to the WHO's 1997 dengue guideline, the clinical signs and symptoms of DENV infection have range from minor level illness to classical (DF), (DHF), and dengue shock syndrome (WHO, 2009). Table (3) shows commonly reported symptoms of dengue (Lee et al., 2020; Wang et al., 2019) (Kuo et al., 2018)**.**

Table 3: Commonly reported clinical symptoms of dengue virus infection (Wang et al., 2020).

Category of (DEV) infection	**Symptoms of DEV infection**	**Common Duration**
Dengue fever	Nausea Severe pain in joint and muscle Severe headache Rash Fever Retro-orbital pain "Flu-like" syndrome	Duration range from 2-7 days
Dengue Hemorrhagic Fever	Leakage of Plasma Vomiting Severe pain in abdomen Restlessness Raise in hematocrit levels Pleural effusion Bleeding Thrombocytopenia Sudden decrease in temperature	After 3 to 5 days duration of fever
Dengue Shock Syndrome (DSS)	Temperature usually reaches 37.5 -38 °C. Cardiorespiratory failure and cardiac arrest Hypotension Organ impairment Reduction in platelet count lead to hypovolemic shock	After 3 to 5 days of fever

2.16 Diagnosis of dengue virus

During febrile illness, non-specific symptoms that can appear at wider range, the diagnosis of dengue on the basis of clinical signs is inadequate. There is sensitive and targeted diagnostic technology that is ideal for usage at specific phases of illness. The initial phases of dengue disease can be identified by virus isolation, detection of RNA through nucleic acid amplification assays (NAAT) or the discovery of an antigen like NS1 (up to five days). After 5 days of infection, dengue virus RNA and antigens may not be detected since just the viremia has

decreased and antibody responses have begun to rise. At this point, serological techniques for IgM or IgG detection of specific antibodies are appropriate (W.H.O, 2009).

2.16.1. Virus Isolation

Dengue virus diagnosis can be verified using viral isolation. A range of cell types, such as the mosquito cell line or the mammalian cell lines can be used to inoculate clinical samples onto (Ae. albopictus) Whole blood, serum, and plasma may be utilized as clinical specimens for the virus isolation process. Most usually when anything bad happens, clinical specimens can be made from homogenized tissue (Peeling et al., 2010). After the inoculation-phase and incubation -phase, a confirmation test, such as RT-PCR required. Several guidelines must be adhered to when isolating viruses: (1) it is necessary to have well-established lab facilities and skilled staff. (2) Incubation and conformational testing require a minimum of seven days. (3) The best time for collection of samples is only available while the sickness is acute. (4) Viral culture is not appropriate for low viral loads (Parkash and Shueb, 2015).

2.16.2. Tests for Amplification of Nucleic Acid

Nucleic acid amplification techniques, which can easily detect DENV RNA in a clinical sample twenty-four to 48 hours after the infection, can be used to diagnosis the acute stage of dengue sickness. Real-time, isothermal-amplification techniques, and Real-time PCR are the most widely used procedures (Lanciotti et al., 1992).

One-step multiplex RT-PCR techniques, which combine four serotype-specific oligonucleotide primers in a single reaction tube, can be used to perform RT-PCR. The one-step pan-flaviviruses quantitative RT-PCR assay and the nested RT-PCR method (Patel et al., 2013; Vina-Rodriguez et al., 2017; Waggoner et al., 2013). The sensitivity of RT-PCR methods changes from 80% to 100% depending on the genomic areas that primers are targeting, the serotyping method used, and the method for amplifying or detecting PCR results (WHO,2009). The multiplex -real-time RT-PCR assay can be used to swiftly determine the viral-load of a clinical sample (Vaughn et al., 2000). Expensive tools and materials are needed, as well as trained technicians to perform this test (WHO,2009).

2.16.3. Detection of Antigens

Up to 9 days after the initial infection, new NS1 focused ELISA and fast immunochromatographic assays have made it possible to distinguish between main and secondary dengue virus infection. In comparison to Panbio's ELISA kit, which had 99% specificity and 66% sensitivity, Platelia's NS1 ELISA kit has sensitivity and specificity of 74% and 99% respectively (Guzman, Jaenisch, et al., 2010). One more meta-analysis found that the NS1 antigen detection-based IC assay has a marginally higher sensitivity than ELISA (71% vs. 67%). For both confirmation and screening of dengue virus infection, NS1-based assays often have better diagnostic capabilities (Zhang et al., 2014). However, there are several issues with this test. NS1-based assays are less sensitive in case of secondary infections (Shan et al., 2015; Zhang et al., 2014).

2.16.4. Serological Tests

In developing nations, serological tests like the (HI) assay and ELISA to identify IgM and IgG are more frequently used to diagnose dengue because they are simple to perform, affordable, and stable with the required specimens at room temperature. The E protein's capacity to precipitate red blood cells is what the HI assay depends on (RBCs). This precipitate is inhabited by anti-DENV antibodies detected in sera, and the HI assay quantifies the degree of this habitation (WHO, 2009). The HI test has some flaws that render it useless, like the following: (1) It is unable to distinguish between immunoglobulin isotypes (IgM vs. IgG) or infections caused by DENV and other related *Flaviviruses*; (2) Chemical and heating pretreatment may be necessary to remove nonspecific inhibitors from the clinical specimen; and (3) Each serotype requires a different optimal pH of RBCs, necessitating the use of multiple pH buffers (Parkash and Hanim Shueb, 2015). The ELISA IgM detection method's sensitivity and specificity are 90% and 98%, respectively, when serum is obtained five days following the commencement of a fever (Peeling et al., 2010).

A recent study found that the sensitivity and specificity of IgM-based assays are significantly impacted by the quality of the antigen used in commercially available kits (Hunsperger et al., 2009). This assay does have certain disadvantages, though, like labor-intensiveness, workload, and low throughput. To address these problems, a new generation of PRNT-based assays has

been created, including the enzyme-linked immune-sorbent spot micro neutralization test and a specific type of ELISA-based micro neutralization test (Rodrigo et al., 2009; Vorndam and Beltran, 2002).

2.17 Treatment

Currently, dengue does not have any known specific treatment or cures. The goals of current therapy options are to reduced complication rates and symptom severity. One such essential therapy in the treatment of dengue is fluid therapy. For dengue fever, oral fluid replacement is sufficient; however, intravenous fluid replacement for shock prevention is necessary in case of severe dengue (Wiwanitkit, 2010).FDA approval for a particular medicine to treat dengue fever has not yet been granted in the US. Clinical trials have also been conducted on additional potential anti-dengue medications that focus on the virus or the host. These medications include oral prednisolone as an anti-inflammatory, carbazochrome sodium sulfonate to stop capillary leakage, lovastatin (statin) as an anti-DENV and an anti-inflammatory at the endothelium, and others (Martínez-Gutierrez et al., 2011; Tam et al., 2012; Tassniyom et al., 1997). Trials have also been conducted with additional anti-DENV medications, including chloroquine (Tricou et al., 2010), balapiravir (a nucleoside analogue and polymerase inhibitor) (Nguyen et al., 2013), and celgosivir (a glucosidase I inhibitor) (Low et al., 2014).

There are many challenges and difficulties in development of suitable anti-dengue therapy (Chan and Ooi, 2015). Finding an antibiotic that is effective against each of the four DENV serotypes has proven to be highly tough and demanding because there is a significant (30–35%) variance in the amino acid sequence between them. Intravenous injection is chosen since it is difficult to develop antibodies that are similarly protective against all serotypes (Gu and Shi, 2014). Additionally, the lack of a reliable animal model that perfectly reflects the etiology of the human dengue virus hinders the development of safe and efficient therapeutics (Bos et al., 2018).

2.18 Vector control and prevention

Controlling the vector is the main method of preventing dengue infection. Environmental changes, chemical control employing insecticides and larvicides, and biological control can all be used to achieve this. Natural and artificial breeding grounds for vectors, such as containers and improperly run waste facilities, are reduced or removed as part of environmental

interventions (Buhler et al., 2019). Although vector resistance has been observed in various areas, insecticides have been used for decades to chemically control dengue vectors However, during dengue outbreaks, this method continues to be the core of vector control (Chang et al., 2011). A thorough investigation revealed that while interior residual spraying and indoor space spraying had a very good impact on lowering the adult mosquito population, they had less of an impact on the number of larvae (Samuel et al., 2017).

Other chemical control techniques, such as larvicide- or insecticide-treated bed nets, window and door screens, and water container coverings, showed varying effects on vector abundance as determined by the Breteau index and container index (Andersson et al., 2017). Modern, integrated vector management strategies have also been developed recently. These strategies use cutting-edge biological control methods like paratransgenesis, sterile insect techniques, and the creation of genetically modified vectors. Use of insect repellent, wearing clothes that covers the entire body, using mosquito bed nets, and using window screens are a few behavioural protective methods for reducing human exposure to mosquitoes. However, because the majority of these solutions rely on compliance and community support, poor community support and low compliance make them challenging to implement (Harapan et al., 2020).

2.19 Vaccines

Government and policy makers begin to investigate the potential advantages of dengue control programmes employing vaccination approaches in dengue hyperendemic zones (DeRoeck et al., 2003). The CYD-TDV dengue vaccine from Sanofi Pasteur recently got approval in a number of nations (Harapan et al., 2020). Tetravalent Dengue Vaccine, formerly known as DENVax, is a chimeric tetravalent vaccine created by Takeda Vaccines Inc. based on dengue-2 PDK-53 (Osorio et al., 2011). Two Phase I studies looked into the efficacy, immunogenicity, and safety of TDV in individuals without a history of flavivirus infection. These investigations found no adverse reactions to the vaccination, but it was shown that TDV induced high viral loads in those who had never had dengue previously. TDV also triggered the emergence of N-A-B to all dengue serotypes in good health persons who had never been exposed to the virus before (George et al., 2015; Osorio et al., 2014). A live attenuated dengue vaccine, such as TV003/TV005, is another potential dengue vaccine. This vaccine is a mixture of potential monovalent vaccines (Durbin et al., 2013). In Brazil and Thailand, Phase II trials are now being

conducted to assess the safety and immunogenicity of TVOO3 and TV005 (Schwartz et al., 2015).

The dengue vaccine D1ME100, which provide protection against all four serotypes, was created by the Naval Medical Research Center in the United States (Beckett et al., 2011). Phase I clinical studies are currently being conducted to assess two additional dengue vaccines, TDENV PIV (a tetravalent purified inactivated vaccine) and V180, a recombinant subunit vaccine based on the DENV wild type prM and truncated E protein (DEN-80E) (Schwartz et al., 2015). However, due to a number of challenges, no dengue vaccine that has undergone testing has met well-established criteria: (a) Genetic variation among dengue virus serotypes. (b) DENV evolution is very fast as well as so unpredictable, resulting in many strains for each serotype. This makes it extremely difficult to create a vaccine that can trigger effective immune response against all-serotypes of varied genotypes (Bos et al., 2018).

CHAPTER 3

MATERIAL AND METHODS

Material and Methods

3.1 Sample Collection

In this study, serum samples from dengue positive patients were acquired from Holy Family Hospital Rawalpindi and SURF Hospital Islamabad. A total of 60 Dengue positive patient's serum was collected, of which 30 were male and 30 were females under the age 1-20 years to 60 -80 years. All the participants in this study signed written consent. In our study, we did not disclose name and other personal information of the patients.

3.2 Inclusion criteria

Dengue positive samples confirmed by IgG, IgM and NS1 antibodies were included. Those patients were included who were willing to participate in our research study.

3.3 Exclusion Criteria

Dengue negative patients were excluded from the study.

3.4 Primer designing for the CprM gene of dengue

The previously reported primers were used in the present study (Fatima et al., 2011) for sequence amplification. The primers sets were further confirmed/validated through Primer3 (http://bioinfo.ut.ee/primer3/) and Primer Blast (NCBI primer designing online tool) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were then modified according to the recently reported nucleotide sequences. Dengue-1 virus CprM region was used as a reference sequence. Name, oligonucleotide sequence, and product size of the primers having maximum *in-silico* specificity are listed below (Table 4.).

Table 4: Name, oligonucleotide sequence, and product size of the primers having maximum *in-silico* specificity.

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		

3.5 RNA Extraction

Viral DNA/RNA extraction mini kit (WizPrepTM) was used for the extraction of Dengue RNA, following the standard manufacturer's protocol to separate the serum from dengue-positive blood. The blood was transferred to an Eppendorf tube of 1.5ml and centrifuged for 1 minute at 8000 rpm. After centrifugation, two layers were formed, then we transfer the upper layer i.e., serum into a new autoclaved microfuge tube. First, sterile microfuge tubes were labeled using specific lab codes. The labeled microfuge tubes of 200µl serum samples were added after that sample lysis started by the addition of 50µl proteinase k and then the sample were mixed thoroughly. From the Viral DNA/RNA extraction mini add 200 µl of VL buffer and then 15µl of carrier RNA were added into the tube containing sample and were mixed for 10-15 sec by vertexing. The sample mixture was incubated for 10 mins at 66°C in pre-heated block, after the incubation step, 280µl of the absolute ethanol was added into each tube and were mixed immediately to prevent precipitation of ethanol due to its concentration. After that the sample mixture was transfer into the column provided in the kit and was centrifuged at 5000g for 1 min, after the centrifugation the flowthrough was discarded and washed the column with 500µl of wash buffer 1, and was centrifuge again for 1min at 5000g, and the flowthrough was discarded. The column was washed again with 500µl of wash buffer-2 provided in the kit and was centrifuged at 5000g for 1 min and discarded the flowthrough. Again, the column was washed

by 500µl wash buffer-2 and centrifuged at 140000g for 3 minutes, the flow through was discarded. The column was transferred into a new microfuge tube, 30-50 µl of elusion buffer was added and centrifuge for 1 min at 5000g, the viral RNA collected was stored at -20°C.

3.6 Dengue complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) were synthesized by using the extracted RNA as a template. First strand cDNA Synthesis Kit (Revert Aid, Thermo Scientific) was used. The components for cDNA synthesis were as follows: 8µl of extracted RNA along with 2µl Random hexamer or gene specific reverse primers, 2µl double distilled water, 2µl dNTP (10 mM), 4µl 5x reaction buffer, 1µl RevertAid RT enzyme (200 U/µL) and 1µl RiboLock (20U/µL) RNase Inhibitor were mixed gently and total reaction volume adjusted to 20 µl as shown in Table 5. All the components of reaction mixture were mixed and incubated for 5min on 25°C, followed by 42°C for 60 min, 45°C for 30 minutes and finally reaction was ended at 70ºC for 5 mins as shown in Figure 7.

Table 5: 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**

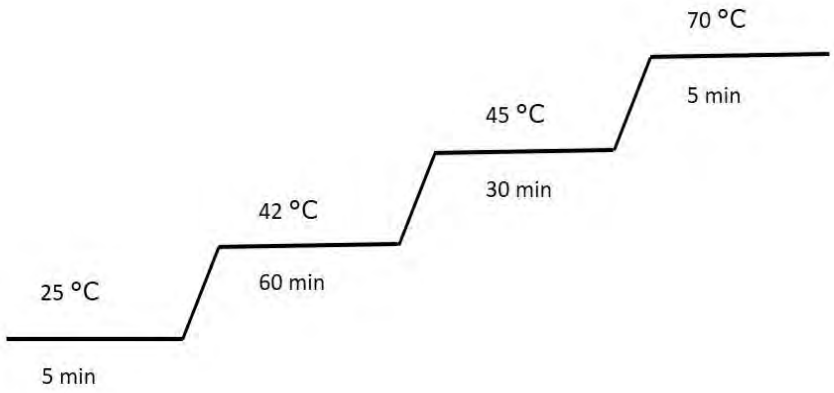

Figure 7: Representing the conditions for cDNA synthesis

The optimized condition for cDNA synthesis was 25 °C for 5 mins, 42 °C for 60 mins. 45 °C for 30 mins and 75 °C for 5 mins.

3.7 PCR amplification of dengue CprM gene

For dengue CprM gene amplification nested PCR was carried out and PCR Master Mix (2X Phusion High-Fidelity, Thermo Scientific) was used. The reaction mixture of PCR first round included 2X Phusion High Fidelity Master Mix 10µl,1.5µl of D1aF, 1.5µl of D1aR, 1µl of ddH2O and 6µl cDna template, which makes a total volume of 20µl reaction as given in Table 6. The cyclic condition for PCR were as follows: 95°C for 5mins following 35 cycles of 95°C for 45 seconds, 63°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 10 min as shown in Figure 8. The reaction was hold at 4°C for infinity. The second round of PCR was performed by using nested primers and the template used was the first-round product. Second round PCR contained the same reaction reagents as in first round with minor modification, such as 2µl first round PCR product were used as template ,5µl ddH2O, same set of forward and Reverse primers and their same conc as used in 1st round PCR were added as given in Table 7. The total reaction volume for both rounds were 20µl. The concentration of the different components used in PCR 1st and 2nd round was presented in Table 6 respectively. While the condition for PCR cycles is given in the Figure 8.

Table 6: 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 7: 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**

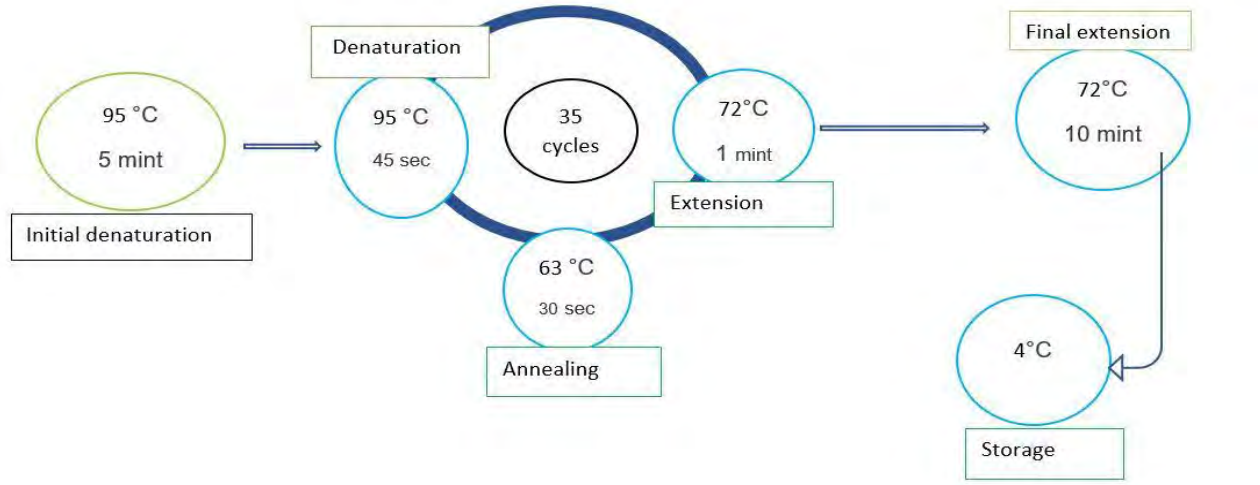

Figure 8: Representation of optimized conditions of Polymerase Chain Reaction.

These are Cyclic conditions for 1st and 2nd round of PCR. The conditions were optimized by using gradient PCR.

3.8 Preparation of TAE buffer

To synthesize the 10x TAE buffer, 54g of tris-base was dissolved in 350 ml of deionized water. Then 27.5 gram of boric acid and 4.65g of EDTA were added to it and mixed thoroughly. After that 150 ml of deionized water was added to the mixture for making final volume of 500ml. Then from 10x TBE buffer, 1x was prepared by the addition of 270 ml of deionized water into 30ml of the 10x TBE buffer.

3.9 Gel electrophoresis

A 2% agarose gel was made by mixing of 0.8 g of agarose with 40 ml of 1X TAE buffer for confirmation of amplified PCR products.The conical flask was kept in microwave oven for 30 seconds duration to heat the crude powder and then cooldown at room temprature.Then 2µl of Ethidium bromide was added to it for visualizing under UV transilluminator. The agarose solution was pouredoff into gelcasting tray with a well-placed comb in it and allowed for soldification for almost 10 to 15 minutes duration. After solidification it was kept in gel tank.In the gel tank,1x TAE buffer was also used as running buffer. The amplified PCR products was mixed with 2 µl loading dye and loaded into the wells of gel. A 1000 base pair gene ruler was

loaded carefully in a separate well in order to compare the size of amplified PCR products. After this step ,gel was carried out at 90v for 30 minutes duration.Upon completion of Gelelectrophoresis process, gel was examined in UV transilluminator for visualization of amplified products.

Table 8: Components for gel electrophoresis procedure

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

3.10 DNA Extraction/Elution from Agarose gel

To elute the ampilified fragments from the agarose gel, the Gene JET gene extraction Kit (Thermo scientific) was used. The target DNA gel fragment was cutout from the gel by using disposable smooth razor blade and required fragment was kept in autoclaved microcentrifuge tuble which was pre-weighted. The weight of excised gel was recorded by using digital balance. After that the binding buffer was added to the tube as 1:1 (weight:volume) i.e., 50µl binding buffer was added to 50 mg of gel slice and heat up the gel slice and binding buffer mixture at 60ºC for 10 min using heat block. After the mixture had been heated hundred% iso-propanol was added to completely solubilize it. Then transferred upto 800µl solubilized gel solution to the column and cenrifuge for 1min on 12000 rpm. After discarding the flow-through, Then 100µl more binding buffer was supplemented for sequencing purposes to the column. The mixture was centrifuged again on 12000 rpm for 60 seconds and discarded the flow-through. 700µl Wash Buffer was added and centrifuged for 1min on 12000 rpm and discard flow-through. The empty columns were then centrifuged for 1min on a 12000 rpm to eliminate remaining wash buffer. The column was subsequently transferred to a sterile microfuge tube and 35µl elution buffer was added. This was centrifuged on 12000 rpm for 1min, column removed, and

purified DNA fragment stored for further processing at -20 °C. For confirmation, 5µl of obtained DNA was again run-on agarose gel (1.5%) and visualized via UV transilluminator (Figure 9).

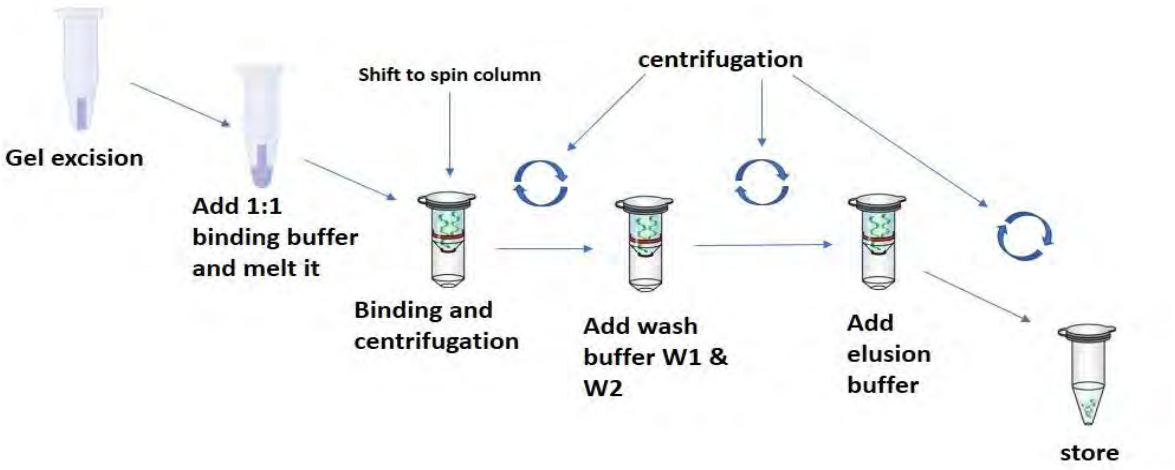

Figure 9: Gel purification of amplified PCR products.

To elute the ampilified fragments from the agarose gel, the Gene JET gene extraction Kit (Thermo scientific) was used.

3.11 Sequencing of Dengue CprM Gene

The PCR eluted product was sequenced, with CprM junction gene specific primers (Forward) of 4µl, and 8µl of purified DNA product through sanger sequencing.The molecular sequence findings were aligned by using sequence alignment software (BioEdit). Through sequence alignment software (BioEdit) the molecular sequencing results were analyzed. FASTA format of nucleotide sequence was obtained for further analysis.

3.12 BLAST Analysis

The current study sequence (QAU-IF1, QAU-IF2) was compared with the known dengue nucleotide sequence present in NCBI GenBank using the Basic Local Alignment Search TooL (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.13 Phylogenetic Analysis

For the homology of the recent sequences of the CprM gene with other sequences of the dengue of pakistan as well as refrence sequences from the rest of the world were searched in the NCBI genbank through nucleotide BLAST. All the available sequences were aligned through MEGA 11 software (Tamura et al., 2021). The phylogenetic tree was constructed. The evolutionary history was estimated by using the Maximum likelihood method.

Chapter 4

RESULTS

Results

In this study, 60 clinically suspected dengue positive samples were collected. The patients were of different ages including 28 males and 32 females.The details of the patients are given in Figure 10. Age wise percentage is given in Figure 11. All the samples were positive for both IgG and IgM antibodies.

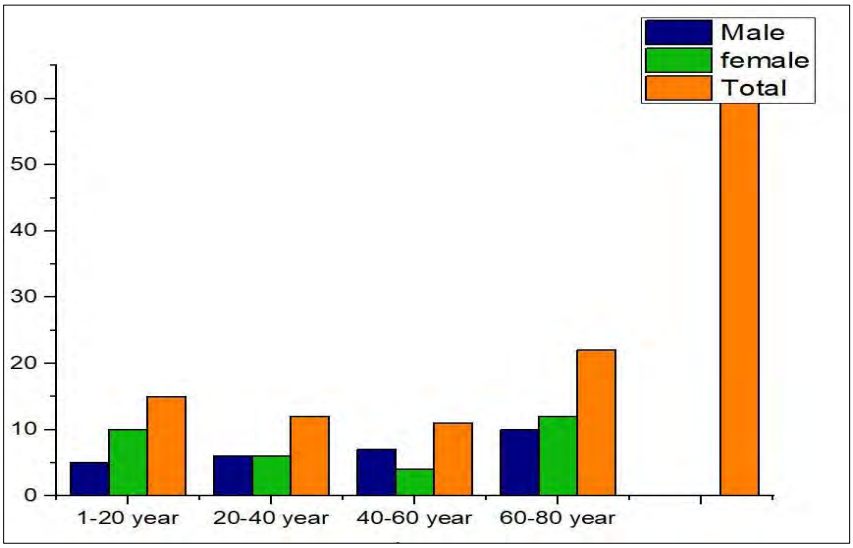

Figure 10: Age and gender wise representation of studied individuals.

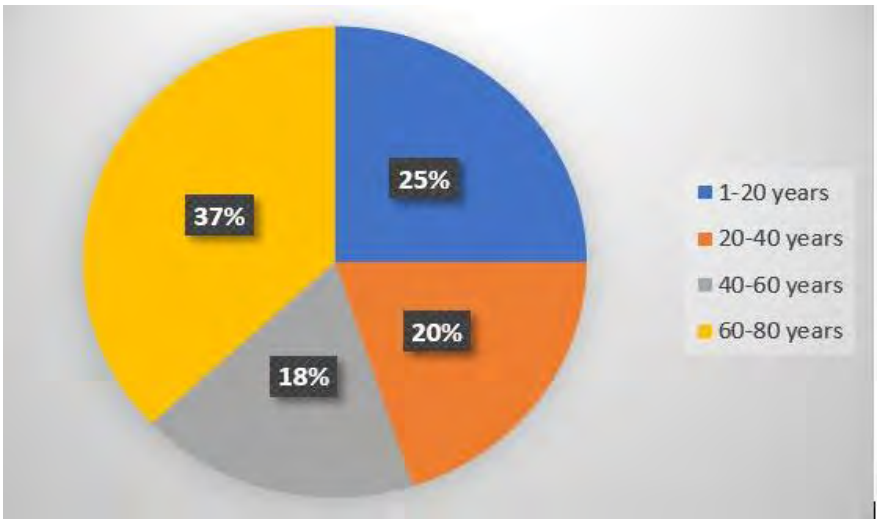

Figure 11: Age –wise percentage of infected individuals.

Age-wise percentage of the infected individuals. Most of the patients (37%) were from the age group 20-40 years.

4.1 Results of PCR amplified products

After the PCR reaction the amplified products run on 1.5 % of agarose gel for confirmation. All the samples were found positive for amplification of targeted CprM gene. The band size of amplified product was approximately 511 bp for all the samples when compared with the 1kb DNA ladder as shown in **Figure 12.**

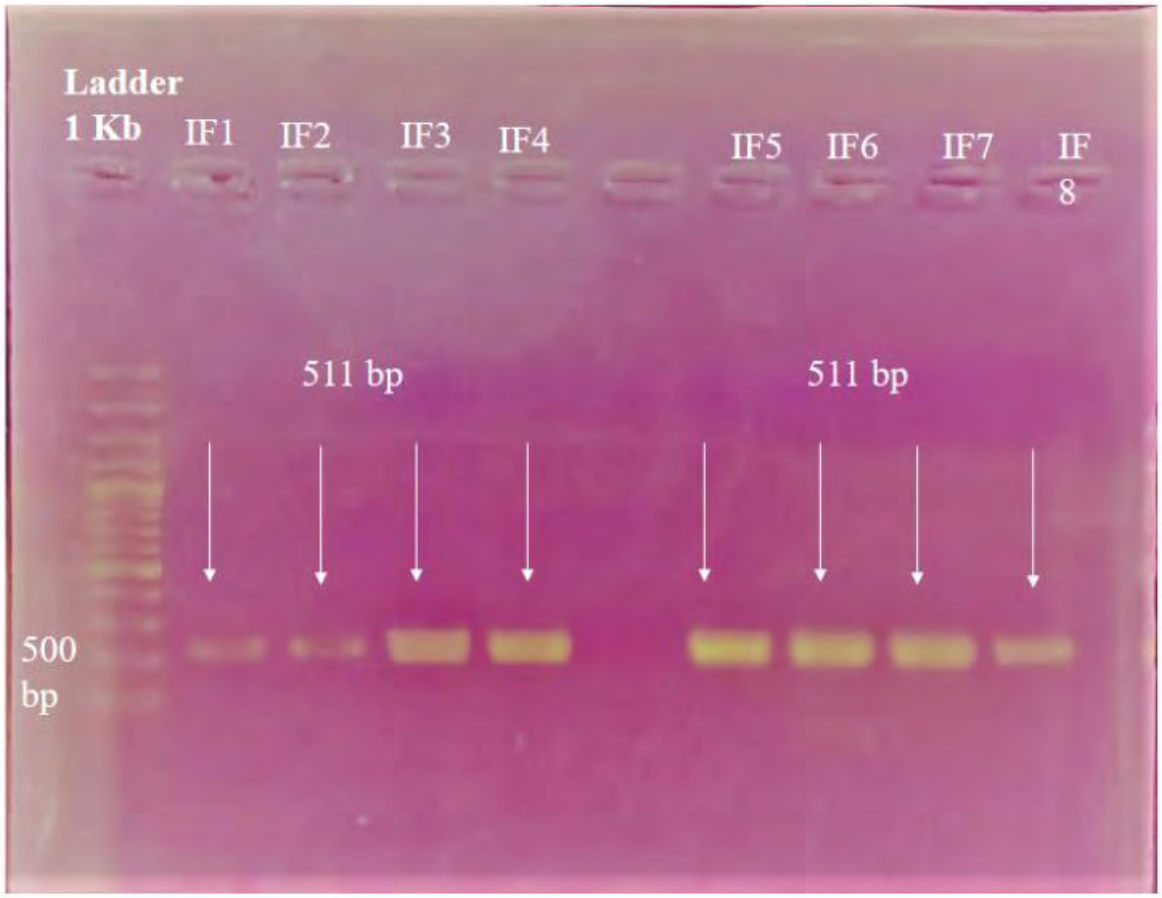

Figure 12: Gel electrophoresis and visualization of amplified products.

Lane 1: 1000bp ladder, Lane 1 to lane 8 represents amplified PCR products. Samples were labeled as IF1, IF2 and IF3, IF4, IF5, IF6, IF7 and IF8 respectively.

4.2 Sequencing of PCR products

The PCR products were excised, purified from the gel and sent to sequencing facility (outsourced). The DNA sequences of the 2 samples were further used for molecular typing and post sequencing analysis.The **Figure 13** representative of the chromatogram of nucleotide sequence of the isolates QAUIF1 and QAUIF2.

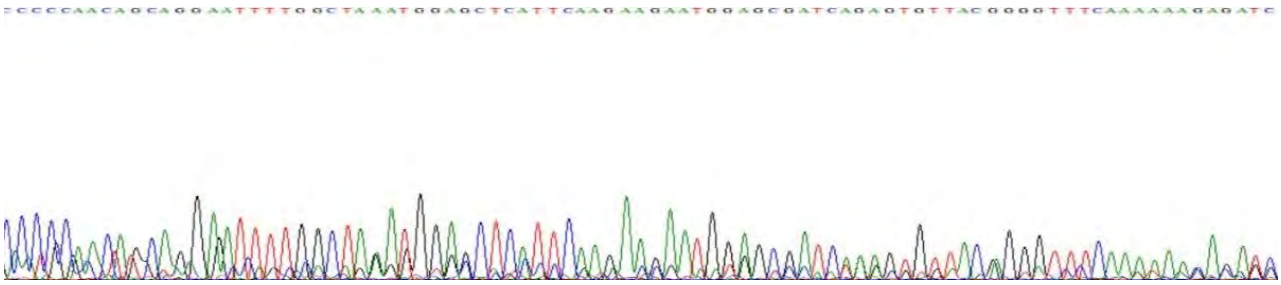

Figure 13: Nucleotide sequencing results of Cprm gene of Dengue virus

4.3 Phylogenetic Analysis

For understanding the genetic variability, we performed sequencing of the selected gene junction of DENV1 samples obtained from Islamabad region. A total of 68 nucleotide reference sequences of the DENV1 have been acquired from the NCBI database. The Maximum likelihood method was used to predict evolutionary history. Phylogenetic analysis was carried out by using Maximum likelihood method in MEGA 11 program. There were 68 nucleotide reference sequences included in the study. Current study sequences for isolates QAU-IF1, QAU-IF2 indicated that their phylogeny were found clustered with dengue virus isolate Accession no: KU948528.1,KU948530.1. This study demonstrated that our DENV1 CprM gene isolates are mostly related to viral isolates reported from India (Arunachal Pradesh). Based on their location in the phylogenetic tree, current studied isolates (QAU-IF1, QAU-IF2) could be considered as phylogenetically distinct isolates and emerging clade as shown in Figure 14.

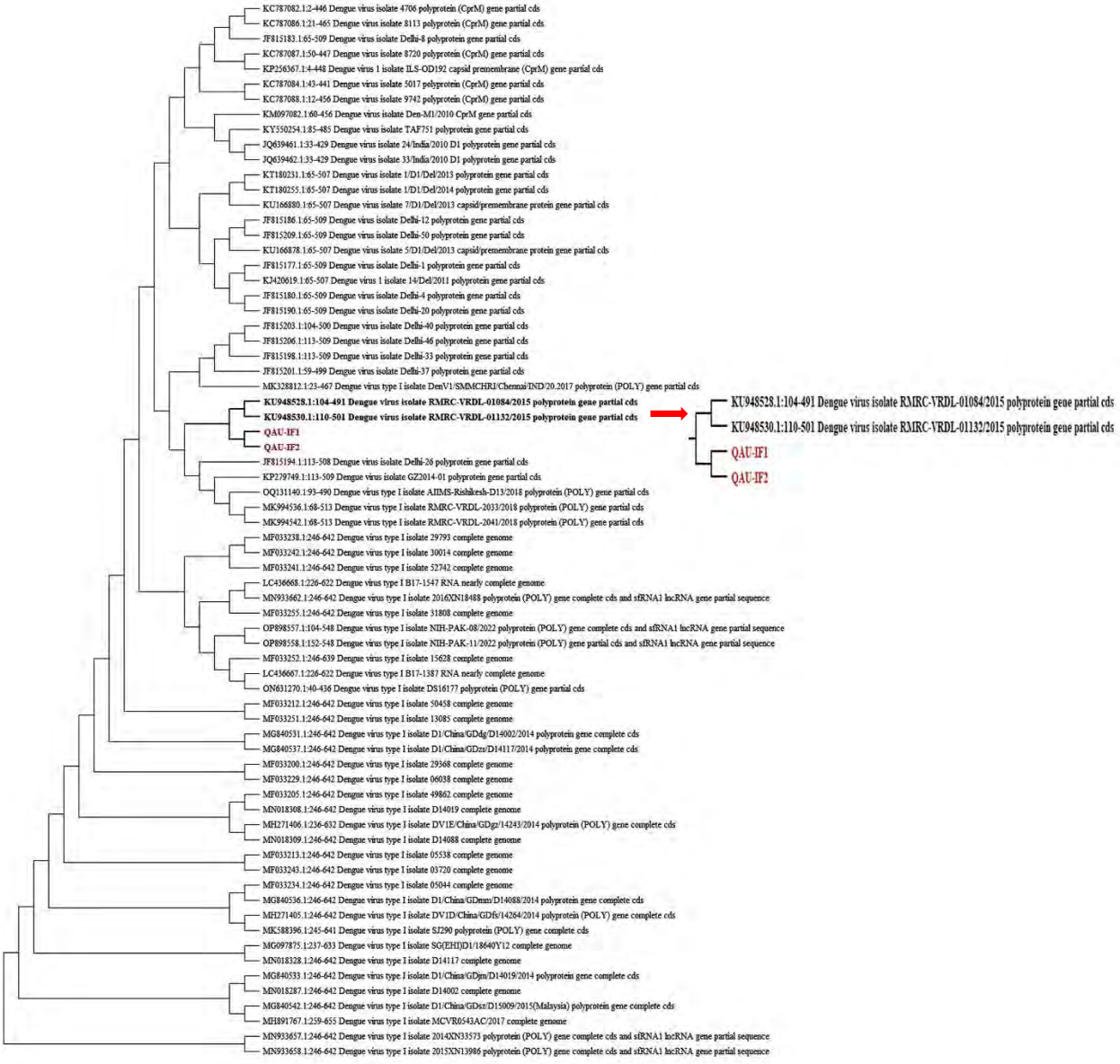

Figure 14: Phylogenetic Analysis of CprM gene of DENV

Phylogenetic tree of the CprM nucleotide sequences of studied isolates (n=2) with the other reference sequences (n=68) from different countries of the world. The current study viral isolates are labelled as red, while reference sequences are represented in black colour.

CHAPTER 5

DISCUSSION

Discussion

Dengue is an emerging infectious disease and is causing huge outbreaks around the globe. It belongs to *Flaviviridae* family, and has four different but closely related serotypes including DENV-1, DENV-2, DENV-3, and DENV-4 (Khan et al., 2020) DENV-5 is another serotype initially identified in 2007 and found to follow only the sylvatic cycle (Shrivastava et al., 2018). WHO has identified dengue fever as an emerging public health issue with a fast-rising global incidence in past decade. Dengue virus causes acute febrile sickness that is known as dengue fever. Dengue fever is one of the most notorious of all other arboviral infections (Parveen et al., 2019).

Once people get infected, they become the primary dengue multipliers and vectors, and they can transmit the infection to uninfected mosquitoes. Currently, the control of mosquito population is considered as effective strategy to hinder the transmission of DENV-2. Over the past decade, thousands of people got infected with this lethal disease in several regions of Pakistan, and they even lost their lives. According to WHO report, over 50,000 cases were reported from Punjab in 2011, about 8546 people were infected in 2013 in KP with 33 mortalities reported in district Swat. The most prevalent serotypes included Serotypes 1, 2, and 3 during this period. There is ambiguity about the prevalence of dengue in Pakistan due to suboptimal diagnostic facilities. Most of the cases are asymptomatic or minor and self-managed, that's why the actual number of dengue cases is misjudged. Much of the cases are also mistreated as other febrile diseases (Gorbalenya and Lauber, 2017).

Since 1994 multiple outbreaks of the dengue virus occurred throughout Pakistan. All of these outbreaks result in severe morbidity and mortality. Most reports from Pakistan that study 2007, 2008 and 2009 outbreaks showed that DENV-2 and DENV-3 were the most predominant serotypes in these outbreaks (Fatima et al., 2011). While the current study that was conducted in 2022 reported the serotype DENV-1 to be most prevalent in the Islamabad region of Pakistan. Outbreaks of 2007 to 2009 witnessed the serotype DENV-2 is more prevalent in these outbreaks than DENV-3.

Table 9: Previously reported isolates of dengue virus.

Time span for isolation of sample	Total numbers of sample collection	Samples tested positive	Type of serotype reported			Reference
			Serotype 3 reported in sample	Serotype 2 reported in sample	Serotype 1 reported in sample	
2007	114	5	1	4	0	Fatima et al.,2011
2008	66	8	5	8	0	
2009	7	7	1	6	0	
2017	703	268	42	152	2	Shahid et al., 2017

A study by Jamil and colleagues conducted in 2005 in Karachi first reported the serotype 3 (Jamil et al., 2007). Hamayoun et al also reported DENV-3 from Lahore during the 2008 outbreak (Humayoun, et al., 2010). Behara and his colleagues reported serological studies of dengue virus during 2018-2019 outbreak of dengue. Among four serological types, there was 16% probability of dengue virus type 1 in 64 serological test samples (Behera et al., 2023). In comparison to these studies the present study reported DENV-1 in 2022 outbreak that shows the divergence of the DENV-1 from DENV-3.

In present study we also characterize samples on molecular level. We characterize (13.3%) that 8 out of 60 samples on molecular level. This may be due to the abundance of only suspected patients or patients with initial stage of infection. Fatima et al characterized (17.5%) of their total samples which is 20 out of 114. The reason behind this was told to be due to the sampling of the pre viremic patients rather than post viremic patients. Maximum characterization is advocated when samples collected from serological confirmed patients by several studies. Molecular studies from multiple researchers approves the serum to be used for the RNA isolation of the dengue virus which is further confirmed through PCR (Fatima et al., 2011). The present study also uses the serum for the RNA isolation followed CprM amplification through conventional PCR.

Dengue fever is equally prevalent among all the age groups, according to previous studies, some may be more susceptible to the infection than others. Patients between the ages of 21 and 40 were found to develop dengue fever more frequently. The age range between 21 and 40 had the highest incidence of DENV infection, according to past research conducted in Delhi, which had

found similar results. Several studies from South India found that dengue fever was more common in younger age groups (0 to 18 years) than in adults (Parveen et al., 2019). Muhammad Shahid and his colleagues conducted study in dengue virus prevalence and molecular analysis in 2013 epidemic in KP and Punjab, Pakistan. In total, 703 serologically positive suspected patients from various major Pakistani hospitals were registered in this study, with 214 female and 489 male participants. Overall, 38% of samples tested positive for PCR (268). Serotype 1, 2 and 3 were all present in 0.74 %, 56.71% and 15.67% of the 268 PCR-positive patients. Just 72 suspected individuals had serotype 2 and 3 concurrent infection (Shahid et al., 2017).

In the current study 60, (ICT) positive samples were collected (30 males, 30 females). These samples were confirmed and processed for CprM gene amplification by PCR. Dengue virus was most prevalent among the age group 60-80 including 22 patients (37%), followed by age group 01-20 years with 15 patients (25%), and age group 20-40 with 12 patients (20%), while the age group 40-60 with 11 patients (18%). All the samples were found serotype 1 of dengue (Table 10).

Table 10: Age-wise percent prevalence of dengue virus in the studied patients (n = 60).

Serial #	**Age-group**	**Percentage of patients tested positive for dengue**	**Type of dengue-serotype reported**
1	1-20	25%	Serotype-1
2	20-40	20%	
3	40-60	18%	
4	60-80	37%	

Phylogenetic analysis of viruses is a significant molecular technique for ancestor investigations, molecular tracking, carrying out various genetic analyses. It also provides information about the evolutionary history and hierarchy of viruses. (Gorbalenya and Lauber, 2017). Dengue

serotypes have been categorized into multiple genotypes and clades due to the high mutation rate of *Flaviviruses* (Shrivastava et al., 2018).

Dash PK et al., reported a phylogenetic analysis on the dengue serotypes DENV-2 during the 2007-2009 traced to be genotype *IV*. The present study also performed phylogenetic analysis of DENV-1 which was found to link with those reported from India. That studies also confirm the prevalence of the DENV-2 in China, Indonesia, and northern India. Worldwide studies on the prevalence of the dengue serotypes revealed no specific or definite pattern that is followed by the serotypes (Dash et al., 2004). The phylogenetic analysis from Pakistan unveil that the serotype circulating during the 2005 pandemic grouped together with monophyletic clade belonging to Indian subcontinent lineage IVb of cosmopolitan genotype IV. Another Pakistani study based on envelope protein sequencing found to be related to monophyletic clade that belong to Indian isolate of the year 1991 and 1996 (Khan et al., 2013).

In current study, core-pre-membrane gene (CprM) gene isolates were analyzed for phylogenetic and evolutionary relationships. Phylogenetic trees of current sequences were constructed by MEGA 11. All query sequences (viral isolate QAU-IF1, and QAU-IF2) assembled with the different clusters according to phylogenetic analysis of nucleotide sequences of the CprM gene, indicating that these sequences QAU-IF1, QAU-IF2, have been evolved and related to dengue virus isolates accession no: KU948528 and KU948530 While making clustered with these polyprotein sequences. The study demonstrates that DENV1 CprM gene isolates are also closely related to viral isolates reported from India. This study suggests that isolated sequences are more closely inter- related and distinct from other sequences. Moreover, isolated sequence can potentially be emerging. Further studies are required to understand possible amino-acid substitutions in functionally and structurally important regions of CprM. As no specific medication is still available for dengue infection in Pakistan, there are lot of research needed for designing vaccination strategies. In addition, molecular assessment of dengue virus can be helpful in finding drug targets and possible molecules to be used as therapies.

Conclusion

Dengue is still widely prevalent and uncontrolled in many countries, including Pakistan. Dengue prevalence in Pakistan currently seems unstoppable. In the current study, dengue CprM region was optimized, amplified and sequenced. Its phylogenetic analysis reveals that the current sequences (isolates QAU-IF1, QAU-IF 2) formed separate clusters but showed similarities with indian sequences, dengue virus isolate with accession number:KU94852 and KU948530. Further studies are required to analyze functionally and structurally important regions of CprM protiens.

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