Design of Molecular Diagnostic Assays for Detection of Dengue in Pakistan

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CERTIFICATE

This is to certify that the work carried out by **Ms. Maryam Gulraiz** (Registration No. 00000127928), **Ms. Alishbah Maqsood** (Registration No.00000151021), **Ms. Karishma Ali** (Registration No. 00000200443), **Ms. Rehma Khanam** (Registration No. 00000201160) has been completed under my supervision in the Molecular Immunology Laboratory of Atta-ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), H-12, Islamabad, Pakistan. It has further been found complete in all respects as per NUST Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award of PhD degree. It is further certified that necessary amendments as pointed out, during final presentation of the scholar, have also been incorporated in the said thesis.

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Dedicated to our all our friends, family, faculty and anyone who aims to seek knowledge.

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List of Abbreviations

IgM, IgG	Immunoglobulins M and G
WHO	World Health organization
EIP	Extrinsic Incubation Period
PCR	Polymerase Chain Reaction
EDTA	Ethylene-diamante-tetra-acetic acid
CDC	Centre for disease control and prevention
DENV	Dengue virus
DHF	Dengue hemorrhagic fever
prM	Membrane protein
NIH	National Institute of Health
ViPR	Virus Pathogen Database and Analysis Resource
uL	Microliter
NS3	Non-structural protein-3
APC	Antigen presenting cells
Env	Envelop Proteins

Abstract

Dengue disease is endemic in more than a hundred countries worldwide. The prevalent situation in Pakistan is a similar challenge, with two serotypes predominantly occurring in Karachi, Khyber Pakhtunkhwa, Punjab and Sindh. Assays for the amplification and detection of dengue genome are based on the subtypes found globally, and in Pakistan, kits imported from abroad are costly and non-specific to the particular strains prevalent in the population. This study involved the retrieval of *pol* and *NS3* sequences from DENV2 serotype, and *env* sequences from DENV3 serotype using consensus sequences occurring in Pakistan. Degenerate primers were designed and optimized to allow the detection and diagnosis of these components in infected sera samples. Viral RNA was extracted from the patient's blood and converted to cDNA prior to amplification. The primers were optimized to determine the most effective working temperatures and to establish the assay's validity. Degeneracy allowed for a wider scope of detection of uncommon dengue sequences by providing multiple potential sites of complementarity. The results of this study provide a means for the effective, inexpensive and specific detection of dengue in Pakistan to help provide accurate clinical diagnosis and to distinguish between dengue and other Flaviviridae with similar clinical presentations circulating in the area.

Chapter 1: Introduction

1.1 Dengue Virus Infection

Dengue is a mosquito-borne viral disease, transmitted by female mosquitos mainly of the species Aedes aegypti. They are responsible for a wide spectrum of disease ranging from asymptomatic to severe flu-like symptoms. It is found in tropical and sub-tropical climates worldwide, mostly in urban and semi-urban areas. According to a report of WHO (World Health Organization) estimates that 3.9 billion people are at risk of infection with dengue viruses. Also, there are an estimated 100-400 million infections each year. In Pakistan, 120 confirmed cases of dengue fever, including 75 deaths, were reported from the four provinces (KP, Punjab, Baluchistan, and Sindh), Islamabad, and AJK, in 2019. (Pakistan: Dengue Outbreak, 2019)

1.2 Dengue Strains Worldwide

The first case of dengue was reported in 1943 in japan and Hawaii. DENV1 occurrences by time period. DENV1 was first reported in 1943 in French Polynesia and Japan, followed by reports in Hawaii in 1944 and 1945. Malaysia and Thailand have reported many consecutive years of DENV2 occurrence since the early 1960. There are four strains of dengue worldwide named DEN-1, DEN-2, DEN-3, and DEN-4. These four viruses are called serotypes because each has different interactions with the antibodies in human blood serum. The four dengue viruses are similar — they share approximately 65% of their genomes — but even within a single serotype, there is some genetic variation. However these four serotypes are widely spread across the word, mostly present in the tropical and subtropical regions of the world. It is predominant in more

than 100 countries and forty percent of population live in those we high risk areas. (CDC). the threat of a possible outbreak of dengue now exists in Europe; local transmission was reported for the first time in France and Croatia in 2010. (Bhatt, 2013)

In USA, 122 cases of dengue were reported in 2019. There has been a 30-fold increase in global incidence over the past 50 years. In 2015, 1181 deaths were reported in America alone. (Medscape). Since the beginning of 2020, the majority of the cases is reported by Brazil, Paraguay and Colombia.

In 2019, more than 45,000 people were infected with dengue in Pakistan. And most cases were reported in Rawalpindi and Islamabad. Sindh has seen the most fatalities from dengue, with over 30 recorded deaths. (WHO, 19 Nov 2019).

The ancestor of these viruses has been postulated to have emerged about 1 000 years ago in an infectious cycle involving non-human primates and mosquitoes, with transmission to humans having occurred independently for all four virus types only a few hundred years ago. (Villabona-Arenas, 2013)

Dengue has distinct epidemiological patterns, associated with the four serotypes of the virus. These can co-circulate within a region, and indeed many countries are hyper-endemic for all four serotypes. Dengue has an alarming impact on both human health and the global and national economies. DENV is frequently transported from one place to another by infected travellers; when susceptible vectors are present in these new areas, there is the potential for local transmission to be established.

The four serotypes are present globally, and have a huge impact on the economic burden worldwide.

1.3 Transmission of Dengue infection

In Mosquito-to-human transmission, the transmission of the virus occurs once the infected female mosquito bites, mainly it is the Aedes aegypti mosquito. After feeding on a DENV-infected person, the virus replicates in the mosquito midget, before it disseminates to the salivary glands. The time it takes from ingesting the virus to actual transmission to a new host is termed the extrinsic incubation period (EIP), this is the time taken after the mosquito ingests the virus to actual to a new host. The EIP is about 8-12 days long, depending on the temperature which should be between 25-28°C. There are a number of factors that influence the EIP such as the temperature, virus genotype, and initial viral concentration. Once virulent the mosquito is able to transmit this deadly virus for the rest of its life.

In Human-to-mosquito transmission, mosquitoes can become infected from people who are already infected with dengue. These people can be symptomatic with severe dengue infection or completely asymptomatic. This occurs 2 days before someone shows symptoms of the illness up to 2 days after the fever has resolved.

The vermeil can last for 12 days but most people are vermin for about 4 to 5 days. The risk increases when a person is tested positive and have high fever, or other symptoms of dengue which includes rash, pain behind the eyes, muscle and joint pains, nausea, vomiting, swollen glands. The risk decreases when there are high levels of DENV-specific antibodies.

The infection is also spread through mosquito vectors. According to a report published by WHO, there has also been cases where there is a possibility of maternal transmission. But the rates of vertical transmission is very low. And babies born to mothers with

dengue infection may suffer from pre-birth, low birth weight and other fatal distress. (Jane P. Messina, 2014)

1.4 Dengue Virus Genome and Structure

The virus is roughly spherical in shape with 50nm in diameter. Dengue virus is enveloped single positive-sense RNA virus because it can be directly translated into proteins. The viral genome encodes 10 genes. The long polypeptide is cut into 10 proteins. . The nucleocapsid is surrounded by a membrane called the viral envelope and a lipid bilayer. The nucleocapsid consist of the viral genome and C proteins. And the nucleocapsid is surrounded by a viral envelop, a lipid bilayer that is taken from the host. The 10 proteins are the capsid (C), envelope (E), and membrane (M) proteins. Seven are non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These non-structural proteins play roles in viral replication and assembly. The E and M proteins span the lipid bilayer. These proteins controls the viral entry into the host. There are 180 copies of the E and M proteins that are present in the lipid bilayer. These form a protective outer layer proteins. (Beasley, 2008)

1.5 Therapies against Dengue Virus

Current standard of care treatment for dengue is mainly supportive but there are also other various therapeutics available that are under research. There are very few treatments available for dengue infection. Currently, there are only therapy and fluid administration.

Symptomatic therapy is limited to acetaminophen as an analgesic and antipyretic in which uses of aspirin and non-steroidal anti-inflammatory drugs is prohibited to avoid bleeding risk due to thrombocytopenia.

In Fluid administration depends on the severity of the infection. If the patient is able, oral administration is sufficient. In more severe cases, such as severe haemorrhagic fever or other severe symptoms, hospitalizations and administration of fluids by intravenous routes is necessary. In case of severe bleeding or vascular leakage, more aggressive fluid administration and blood transfusion is required. Doctors need to monitor fluid resuscitation, should be well-titrated. In case of excessive fluid administration during recovery phase, patients tend to develop fluid overload manifested by pulmonary oedema or ascites.

1.5.1 Development of therapeutics targeting the virus

The goal for therapeutics for DENV infection is to create an antiviral that could inhibit viral replication, reduce transmissibility, or reduce disease severity and should have a safe profile that can be taken rally as most patients are with mild symptoms. A number of drugs have been tested on humans with very little success so far, though there are still some drugs under investigation. Antivirals with both DENV and human host targets as mechanisms of action are discussed below.

1.5.1.2 Antiviral drugs

The genome of DENV measures around 11 kilogausses with a single open reading frame with 3 structural and 7 non-structural proteins. These proteins help in virus replication cycle and are prime targets of antiviral drug discovery. There are many therapeutics that have targeted the viral proteins. Due to the similarity between the RNA polymerases of both hepatitis C and DENV scientists decided to use the nucleoside analogue balapiravir which inhibits the RNA polymerase of hepatitis C and DENV. But it proved to be not so effective against DENV. Its antiviral ability was poor.

Similarly, NITD-008, a nucleoside inhibitor targeting RNA-dependent RNA polymerase, showed a strong inhibitory effect against all 4 DENV serotypes. But it had severe side effects such as acute renewal toxicity observed in preclinical trials. Other DENV specific drugs targets NS2b, NS3, NS4b, and capsid proteins or halting other stages of the replication cycle.

Celgosivir, an alpha-glycosidase antagonist demonstrated impaired folding and trapping of viral NS1 in the endoplasmic reticulum. Studies on the drug pharmacokinetics and dosage suggested that increasing the drug dosage may improve the efficacy of this drug.

Statins have both antiviral and anti-inflammatory properties, considering the importance of membrane lipids in the Flavivirus life cycle, statins were hypothesized to interfere with viral replication. But the clinical trials in humans did not demonstrate any benefit in vermeil or disease course. (Mohamad Fadhli Bin Masri, 2019)

Chloroquine is most well-known for its role as an anti-malarial. In the case of DENV, it has been demonstrated to inhibit virus replication by preventing the proteolysis processing of viral proteins. In addition, similar to statins, chloroquine has an added anti-inflammatory function that could theoretically also be of clinical benefit. There were successful animal trials but failed human trials. Overall, neither study showed any significant improvement in disease progression to severe dengue.

Ivermectin, an anti-parasitic drug, commonly used to treat nematode infections, shown to have anti-DENV antiviral activity. The antiviral activity of ivermectin relies on its ability to inhibit host nuclear import receptors, importing α and β, which is necessary in DENV replication. A clinical trial is currently being conducted to assess the efficacy of ivermectin against dengue.

1.5.1.3 Other potential therapies

Papaya leaf extracts (PLE) as a potential homeopathic therapy have been suggested to have a diverse range of beneficial activities during dengue disease. The PLE have no antiviral activity, but they have shown anti-inflammatory and platelet augmenting activities. Vitamin E supplementation along with standard therapy have shown to be beneficial in liver functions and recovery in platelet counts during dengue infection. (Rathore, 2019).

Chapter 2: Literature Review

2.1 Dengue virus infection

Infections caused by dengue virus are the most common mosquito borne illnesses and its symptoms vary from being very mild to dengue hemorrhagic fever (DHF). It is transmitted through the bite of infected mosquito and a person cannot get the infection directly from the infected person. Most of the cases of DHF are observed in children under age of 15 years. The common symptoms in DHF are

- High fever
- Abdominal pain
- Bleeding problems
- Myalgia
- Flushing
- Retro-orbital headache
- Fatigue, Nausea and Vomiting

The characteristic symptoms begin after 4-6 days of infection and they retain up to 10 days. Initially the symptoms are very mild and are very similar to other viral diseases so they are often mistaken as normal flu or any other viral infection .Younger children and the people with first exposure tend to show mild illness but it also depends on the immune system of the person , people with weakened immune system due to other illnesses or the ones with multiple exposure to dengue virus infection are more prone to dengue hemorrhagic fever. Dengue hemorrhagic fever is a very complicated condition characterized by very high fever and serious damage to lymph node which can cause bleeding from nose and gums and subsequently failure of circulatory system.

If the condition persists this can lead to shock due to excessive bleeding and can cause Dengue Shock Syndrome (DSS).

DSS is mainly due to endothelial dysfunction which leads to myocardial dysfunction. Increased membrane permeability in this state causes dehydration, ultimately leading to Shock. DSS is associated with very high mortality rate as there is no specific therapy available to increase survival. (Retini, 2019)

2.2 Global prevalence

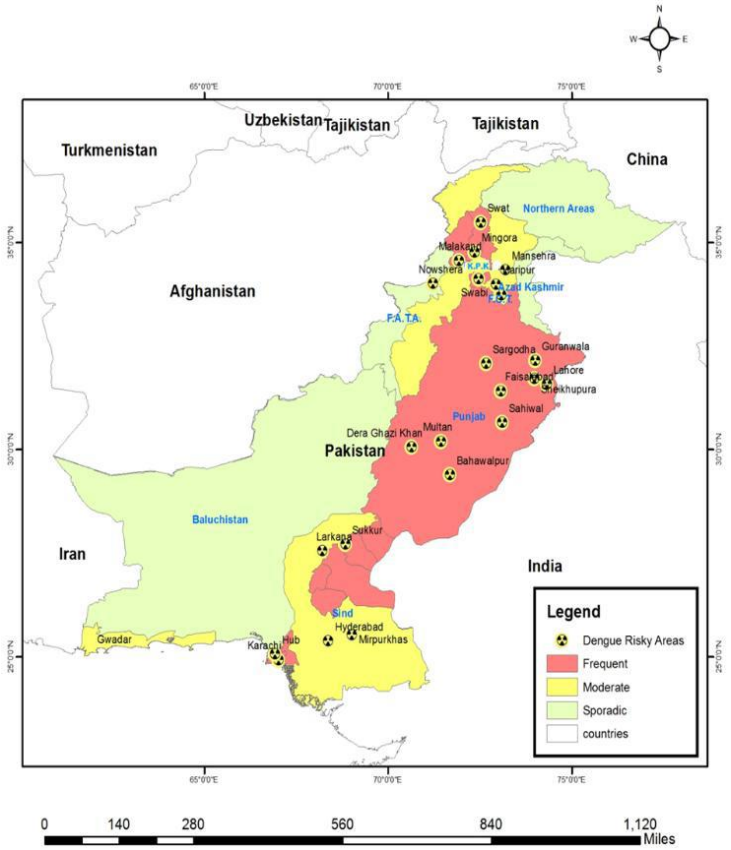
In the 19th century , it was considered as sporadic which causes epidemics for longer intervals but a very distinct change in pattern has been observed and now dengue is considered the most important viral mosquito borne disease with 30-fold increase in the outbreaks.

According to an estimate , each year 400 million people get infected with dengue virus and 96 million people show illness due to infection and the greatest risk lies in the tropical areas due to the vector mosquito. (Retini, 2019)The tropical areas with highest risk include

- Indian subcontinent
- Southeast Asia
- China
- Taiwan
- Caribbean
- South Africa
- Central America

2.2.1 Prevalence in Pakistan

In 1994, first epidemic of dengue virus was observed and after 1994 there was a sudden increase in the frequency of epidemics until 2004 but now every year Pakistan faces the problem of dengue virus infection. (Hamid, 2017) **Error! Not a valid bookmark self-reference.** shows the frequency of dengue epidemic in Pakistan



Pakistan is the most important region in South East Asia and climate of Pakistan is very diverse as there are very distinct four seasons; summer, winter, spring and the monsoon season which is usually from June to September every year. But this duration varies from one locality to another locality. Monsoon climate is the point of discussion because in this duration dengue epidemics are very frequent due to the circulating dengue virus serotypes. In Pakistan, first case of dengue virus was reported in 1980.

And it has become a serious public health concern in Pakistan since 2005. First case of dengue hemorrhagic fever was from Karachi and was reported in 1940. In 2019 epidemic total 47,120 cases and 75 deaths were reported. (WHO, 2019)

2.2.2 Dengue specie distribution

There is very little data available about the diversity of vector in Pakistan but total 104 species have been reported in Pakistan. Both *aedes aegypti* and *aedes albopictus* have been identified in several areas of Punjab especially in central Punjab area. But aedes aegypti is the most prevalent in all the urban and rural areas. The major issue is in the control of these mosquitoes.

According to a study dengue is most frequent in three provinces: Punjab, Sindh and KPK. And statistical analysis shows that DEN-2, DEN-3 are the most frequent and mixed infection of DEN-2, DEN-3 also exists.

2.3 Dengue virus serotypes

Dengue infection is caused by four different types of dengue virus and they are very closely related to each other and are classified as DEN-1, DEN-2, DEN-3 and DEN-4. They are called as different serotypes because they differ in their interaction with the immunoglobulins in the serum of the infected person. They share approximately 65% similarity in their genetic makeup. But variation also exists among the same serotype. Despite all these variations, all these serotypes, upon infection give rise to a similar set of symptoms giving rise to characteristic dengue virus infection.

DEN-1 and DEN-2 were first identified in the central America region and all the four serotypes were present in the southeast Asia region. Now all these serotypes are circulating in the tropical and subtropical regions of world. There is also a hypothesis

about the origin of virus in non-human primates 500 – 1000 years ago and then it jumped into humans in Africa and southeast Asia regions.

When a person is infected with one serotype, it provides him/her immunity against the other serotypes, but it is not permanent, it is only for 2-3 months. After that the person can get infected with the rest of serotypes. According to a research, people who have early history of infection are at a higher risk of subsequent infection.

2.4 Dengue Genome

The genome of dengue virus consists of a positive sense single stranded RNA molecule. The positive sense strand has the ability to get directly translated into proteins. The genome encodes a single polyprotein which is then cleaved into multiple proteins. (Kuhn, 2002) There are total 10 proteins and they are classified as structural (C ,M,E) and nonstructural proteins as shown in Figure 2 .Three proteins make up the structural component and seven are the nonstructural ones and are responsible mainly in the assembly after replication phase.

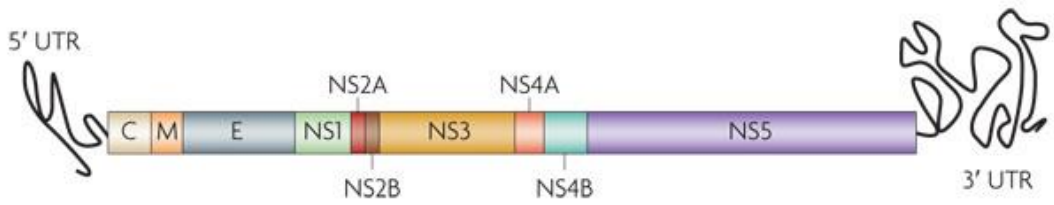


Figure 2 Dengue genome

2.4.1 Capsid gene (C)

This gene encodes capsid protein which is highly basic and is of 12kDa size. This capsid protein forms homodimers and has affinity for both lipids and nucleic acids. There are also four alpha helices in the capsid structure and a distinct hydrophobic cleft. Capsid

protein is translated first in the infection cycle and is very important in assembly for the formation of nucleocapsid and among the four serotypes there are very less conserved regions. (A.Byk, 2017)

2.4.2 Membrane gene (M)

The M protein is very essential for the maturation of viral particles. This M protein basically consists of seven beta-strands and the stability of this protein is due to disulfide bonds. And it also functions as a protective coat for inner hydrophobic core. (Perera R, 2008)

2.4.3 Envelope gene (E)

This protein forms the outer covering of the virus and is mainly responsible for the immunogenicity. It is the main site of interaction of antibodies. It is basically multifunctional as it is also responsible for the specific attachment of virus particle to host cell and to mediate its fusion with cell membrane for entry.

This proteins is composed of three domains and they show the most conservancy among all flaviviruses. (Amarilla, 2009) The components which are responsible to determine host range , specificity are mostly present in the third domain.

2.4.4 Nonstructural proteins

There are 7 nonstructural proteins which include

- NS1
- NS2A
- NS2B
- NS3A
- NS3B
- NS4

- NS5

2.4.5 NS1

NS1 is a monomer and is present on the membrane which is usually secreted in the patient's blood upon infection and has high molecular weight due to high level of glycosyl residues. It is very crucial in the replication cycle of virus. Among all the nonstructural proteins , the main target for the early detection of infection is the amount of NS1 in the patient's blood through ELISA. (Norazharuddin, 2018)

2.4.6 NS2

The NS2 region gives rise to two subunits: NS2A and NS2B. The main functions of this protein are in viral RNA synthesis in the infection cycle and to suppress the immune system in the body by interaction with interferons (Norazharuddin, 2018). It is also present in the final step of assembly of virion particles. During release of the virions from the infected cells, NS2B alters the membrane permeability to facilitate the release.

2.4.7 NS3

The main function of NS3 is in the replication of virus so it is of great importance for the antiviral strategies in control of the infection. It acts as a serine protease and has helicase activity. It mediates the replication by uncoiling the double stranded molecule formed during replication and is fueled by the hydrolysis of the nucleotide phosphates. It is the second largest nonstructural protein according to size. Due to all these critical functions in replication, researchers are very interested in it to block replication. (Norazharuddin, 2018)

2.4.8 NS4

There is very little information about the subunits of NS4 but along with acting as enzymes they act as scaffold for the replication complex in the replication cycle of the

virus. NS4 also provides stability to the replication complex and protects the cells from dying during the infection cell. Overall, it plays an indirect role in the replication of the virus. (Norazharuddin, 2018)

2.4.9 NS5

This is the largest nonstructural protein and is the most conserved among flaviviruses. It is very important in the replication and performs the function of capping as it is a methyltransferase. It is localized inside the nucleus of the infected cell so it is supposed to inhibit the host's immune response during the infection hence promoting the survival of the virus inside the cell.. (Norazharuddin, 2018)

2.5 Dengue virus life cycle

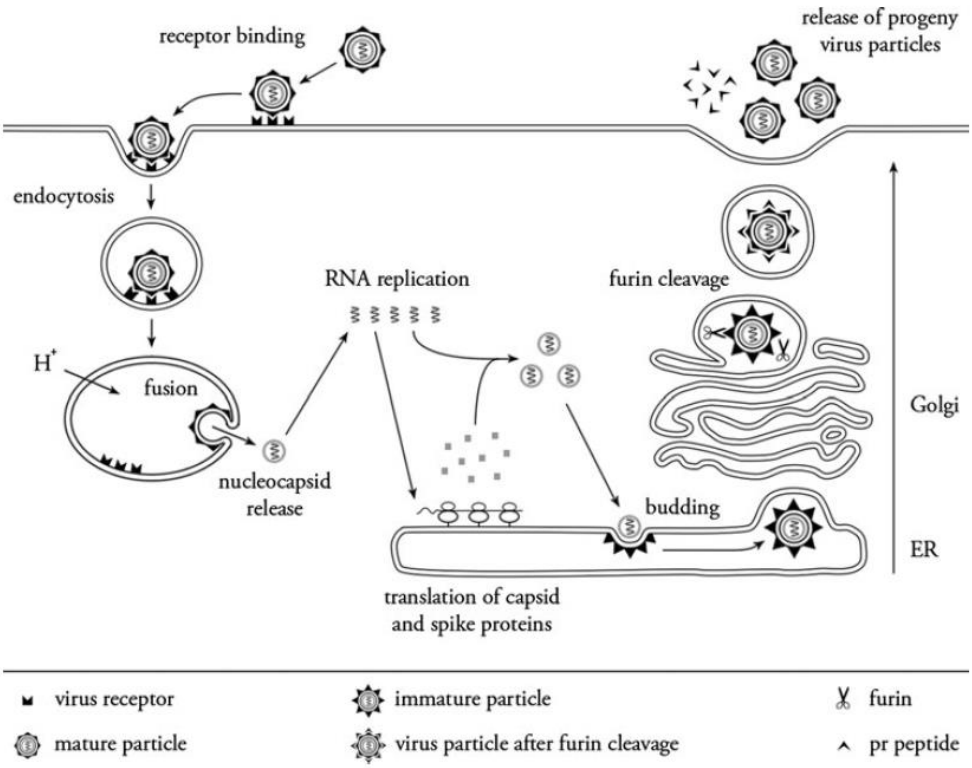
The life cycle of dengue virus can be described in multiple steps as show in Figure 3 Dengue virus life cycle.

2.5.1 Attachment and entry

This is the first step of the infection cycle where viral surface proteins interact with the receptors on the target cells. This specific recognition and attachment mediates the initial entry of virus inside the cell. In DENV infection the primary targets of the virus are the phagocytes specifically the antigen presenting cells (APC's) which include macrophages and dendritic cells. They can also attach to the dendritic cells specific to skin which are called as Langerhans cells. (Rodenhuis-Zybert, 2010)

In insects and mosquitoes, initially the cells of midgut are infected and then it progresses to other body parts. DENV is also known to infect many human cell lines including HUVEC , EC304 etc. In humans several studies suggest that DENV uses more than one receptor on the target cell for its movement across the membrane and

Figure 3 Dengue virus life cycle



many receptors have been identified which have shown the ability to interact with DENV. These receptors include Heparan sulfate , laminin receptors , c-type Lectin

receptors , Mannose receptors , intracellular adhesion molecules and Heat shock protein. They all facilitate the virus for attachment to the target. After this receptor – virus interaction, the entry of the DENV is through clattering-mediated endocytosis. All flaviviruses follow this strategy for entry. But studies suggest that different strains of DENV can employ different strategies for entry other than clattering coated vesicles. After entry there is formation of an early endosome which is then matured to initiate the fusion of viral particle with the endosomal membrane as shown in Figure 3 Dengue virus life cycle

.There are many ongoing research about the structural and molecular mechanisms involved in the fusion process but until now it has been said that the pH environment is the triggering factor for fusion. The domains of the E protein change their conformation and form a hairpin loop structure which forces the viral particle and eventually fusion takes place.

2.5.2 Replication and assembly of virion particles

After the fusion of the viral particle with the endosomal membrane , the viral nucleocapsid is released into the cytosol. In the cytosol , the nucleocapsid is uncoated to release the genomic RNA. As the RNA is positive sense so it is directly translated into a polyprotein molecule. Viral proteases then act on this polyprotein molecule to give rise to three structural i.e. Capsid protein (C) , Envelope protein (E) and Membrane protein (prim) and seven nonstructural proteins (NS1 , NS2A , NS2B , NS3 , NS4A , NS4B and NS5). These proteins then undergo post translational modification to become fully functional . E protein is glycosylated at specific residues which mediates its proper folding and the specific confirmation of its domains. After the structural proteins , the nonstructural proteins enable the multiplication of the genome. When multiple copies of the genome are formed , the Capsid proteins interacts

with the RNA strand to form nucleocapsid particles. The E and prim proteins are conjugated into the ER membrane and they form heterodimers. These heterodimers are then converted into trimers and this trimer formation creates a curved surface which facilitates the budding of the viral particles .

When newly formed viral particles were studied , it revealed that they contain 180 copies of E/prM heterodimers which were present on surface as 60 trimer spikes.

After the assembly of the viral particles , the specific acidic pH facilities the specific Furin molecule to act on Arginine residues of prM protein as shown in figure. This protein is then cleaved into Membrane associated M protein and a pr peptide. These two peptides perform their function as chaperones and stabilize the E protein in the whole budding / secretory pathway. (Rodenhuis-Zybert, 2010)

2.6 Diagnostic Strategies

Dengue virus can cause dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which is characterized by severe manifestations and is often associated with secondary infections so it is very critical to diagnose. Diagnosis is also important for case confirmation (DF or DHF/DSS), to differentiate dengue from other diseases such as leptospirosis, rubella, and other flavivirus infections, and for the clinical management and evaluation of patients with severe disease. The use of good dengue diagnostic tools is critical for laboratory confirmation of DHF/DSS, including the number of case fatalities, determining which strains are involved, and to derive estimates of total cases in epidemics.

2.6.1 Serological diagnosis

2.6.1.1 Antibody detection

In case of dengue infection , the first immune response of the host/patient is the production of IgM antibody. After IgM , IgG is the second one to appear and its amount is very low in the first week of infection and antibody titer begins to increase after the first week of infection. But in secondary cases of dengue , there is rapid increase in amount of antibody in comparison to primary infection. According to an American health organization , 80% of patients have detectable amount of antibody by day 6-10. And in approximately 95% of cases this antibody persists for about 90 days.

This anti-Dengue IgM is detected through ELISA technique. But there is a limitation because 10% false negative reports and 1.7% false positives has been observed in different cases so it is not very specific and also the time span in which these antibodies appear depends on the immune system of the affected individual. Different types of techniques such as dot- ELISA , capture ELISA have been developed and many commercial kits are also available but the accuracy really depends on the time frame in which antibodies are detectable in the serum. The presence of IgM in the body is an indicator for recent infection and is not helpful to determine the specific serotype of the dengue. The major issue with antibody detection is that they are not very specific to dengue virus as they have affinity for other flaviviruses like yellow fever and encephalitis. (G N Malavige, 2004)

2.6.1.2 Antigen detection

The most common technique ELISA is used as a rapid diagnostic test due to its high efficiency. There are others methods for antigen detection like immunofluorescence and radio immunoassay but they are not used in routine diagnostics due to their less

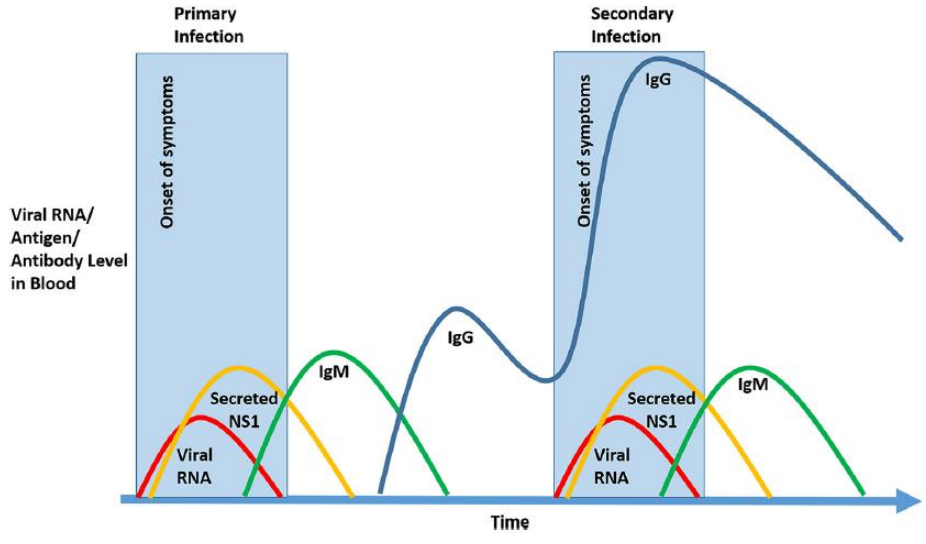
sensitivity. ELISA, in comparison to these immunoassays show 90% sensitivity and 98% specificity that why it is more accepted as a diagnostic tool.

In case of Dengue infection, NS1 is used as an antigenic marker because it is present in very large amounts in the patient's serum especially in the acute phase of the infection. Several personalized kits have been developed by scientists which have different sensitivity and specificity. Studies show that NS1 can also be used as a marker for early diagnosis and for diagnosis of Dengue hemorrhagic fever...

2.6.1.3 Genome detection

For detection of viral RNA , Polymerase chain reaction (PCR) is used. In case of dengue , first the RNA is converted into cDNA which id then further amplified using region specific primers. Due to specific primers, it is very helpful in serotype recognition and also for detection and amplification of specific regions in the genome.

For detection of different regions or different serotypes, a diverse range of primers are used and the protocol of PCR also varies along with that. PCR is a very reliable diagnostic tool because it can detect the viral RNA in stored samples too. As it is able to differentiate between multiple strains so it can diagnose multiple infections of dengue



in the host. Over time scientists have developed different protocols for PCR to increase the specificity and sensitivity. Normally RT-PCR is used and some methodologies for real time PCR have also been developed to reduce the time consumption and for rapid diagnosis in the early stages of the infection . (G N Malavige, 2004)

Figure 4 Levels of different biomarkers in Dengue infection describes the level of IgG, IgM, viral RNA and NS1 at different times of the

infection. As the graph depicts , the first marker which can be detected is the viral RNA , then the antigen and the last to appear is the antibody which is the least specific for dengue virus. Also the level of NS1 varies from patient to patient, some secrete it in large amounts so it is detectable but in some patients its level is not detectable. (Junxiong Pang, 2017)

2.6.2 Recent challenges in Dengue diagnosis

Instead of development in the diagnostic tools and strategies , still there are a lot of limitation in early and on time diagnosis of dengue infection. The major challenges linked with the dengue diagnosis are as follows:

- Dengue detection through antibody detection is very non-specific and also it requires testing to be done in the specific time frame in which antibodies are detectable and this is very challenging. Another issue is the affinity of these antibodies for other flaviviruses so it does not confirms dengue infection.
- For viral RNA detection which is the most specific, requires PCR. The challenging thing in nucleic acid detection is the expensive equipment and careful monitoring and proper optimization of the protocol so it is very critical. Also it is time consuming and is a huge barrier in the early diagnosis of the infection.

2.7 Future Prospects

Dengue fever is a very common mosquito borne illness in tropical and subtropical areas. It has been observed that most of the developing countries where dengue is a serious problem are lacking in their diagnostic facilities for dengue virus and as a result there are more severe cases of dengue because they are not being detected in the early phases and it progresses to worst cases. Whereas the developed countries where dengue is not a serious problem , they have developed diagnostic kits for dengue. In case of epidemics when these developing countries have to import these kits, it becomes very expensive and due to low budget diagnostic facilities are not available in every locality. Same is the case in Pakistan , where lot of people die due to non-specific diagnosis of dengue. So there is a need in such developing countries to develop their own specific and low costing kits so that they can control the situation in case of epidemics. The development of kits really depends on the research sector of the country and it also requires funding in the specific research area. (Pooi-Fong Wonga, 2019)

Diagnosis of dengue is important for facilitation of patient management in epidemics and for proper measures in case of severe dengue hemorrhagic fever and dengue shock syndrome. Overall situation suggests that the specific techniques for detection of dengue are not difficult to handle and are also not very lengthy but the major issue is in the cost so there is a need for development of such strategies which ensure the availability and the affordability in terms of low cost.

Chapter 3: Methodology

3.1 Assay Design

3.1.1 Sequence retrieval

We retrieve polyprotein (*pol*) and non-structural protein-3 (*NS3*) sequences of DENV2 serotype, and envelope (*env*) sequences of DENV3 serotype from the NCBI database using Pakistan isolates. Each category of nucleotide sequences was compiled in FASTA format.

3.1.2 Sequence alignment

The sequences were run through Mega X software and aligned using ClusalW to determine their conserved region. A full genome sequence of DENV2 or DENV3 was inserted and the sequences re-aligned to determine their position. We ran these sequences through the Bio Edit software to determine the numerical positions of their conserved regions for primer design. Conserved regions of at least 300 bps were selected to design primers.

3.1.3 Degenerate Primer design

A set of five primers – two forward, two reverse and one cDNA primer – were designed from each of the three regions: *pol, NS3* and *env*. A minimal of 200 bps product between forward and reverse primers, F1 and R1, was standardized using Bio Edit. F2 and R2 primers overlapped for the *pol* and *NS3* sequences. The reverse complement tool was used to design R1, R2 and cDNA primers, while the F1 and F2 primers were kept unchanged. Degeneracy among the sequences was determined and substituted by

IUPAC codes. The primers were ordered on Thermo Fisher to be further optimized in the lab.

The primers were run through BLAST on the Virus Pathogen Database and Analysis Resource (ViPR) to determine the primers' GC content, hairpin loop, degree of self-complementarity and primer-dimer formation, as well their specificity to the gene region. The OligoCalc online portal was employed to determine their melting temperatures, and these were compared with manually calculated values computed through the standard formula.

3.2 DNA library

8 non-infected blood samples were obtained from healthy individuals at the National University of Sciences and Technology and used to create the DNA library. It served the purpose of acting as control samples for validation of in-house assay. The demographical information of each non-infected blood donor was recorded, including name, age, sex, blood group and date of collection. DNAs were extracted using the phenol-chloroform method. Three protocols were employed to determine which one yielded the darkest band of DNA, and the third protocol was found to best deliver on that criteria. It was used on all samples Another fourth protocol of DNA extraction from the Qiagen Kit was used to extract DNA from two samples for the library The purity and concentration of each DNA extraction was determined using Nano Drop., and they were amplified for GAPDH, TGF-B, IL-1 and IL-18 through polymerase chain reaction and run on gel to obtain bands.

3.2.1 DNA Extraction

3.2.1.1 Protocol 1

In the one-day protocol, 100uL of blood was taken in an Eppendorf tube and, using a micropipette, 400ul of lysis buffer was added into it and the mixture was centrifuged at 5000g for 5 minutes. 200uL of solution A was added and the mixture centrifuged again at 10,000 rpm for 2 minutes until it separated into to two distinct layers. The supernatant was discarded and solution A was added again, and the process repeated, until the pellet became transparent. Mechanical action on the pellet using a micropipette tip allowed us to obtain a clear pellet. 200uL of solution B was added and the Eppendorf was left in the incubator at 65^{o}C for 20 minutes. 20uL of proteinase K and 20ul of 20% SDS were now added, and the mixture incubated again at 55^{o}C for 20 minutes to allow for digestion action at optimal temperature. 300ul of phenol : chloroform: isoamyl alcohol were added in the ratio 25:24:1 and this mixture centrifuged at 10,000 rpm for 8 minutes. Two layers became visible in the tube, and the upper layer was separated into a new Eppendorf tube with 40uL of sodium acetate and 300uL of chilled Isopropanol added inside with a small-tip micropipette. The mixture stored for 20 minutes at -20^{o}C. Centrifugation for 8 minutes at 13,000 rpm was followed by washing the pellet with 200uL of chilled ethanol after discarding the supernatant. A final centrifugation at 8000 rpm for 2 minutes followed this step, after which the supernatant was removed again, and followed by leaving the pellet to dry on a paper towel for an hour. Finally, 30uL of nuclease-free water added and the Eppendorf tube with the precipitated DNA stored at -20^{o}C.

3.2.1.2 Protocol 2

The two-day protocol started with 750uL blood in an Eppendorf to which 750uL of fresh solution A was carefully added, and allowed to rest at room temperature for 5-10 minutes to permit activity. The tube was centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet submerged again in 400uL of solution A, followed by another centrifugation at 13,000 rpm for 1 minute. After discarding excess supernatant, the nuclear pellet was resuspended in 400uL of solution B, 5uL of proteinase K and 12uL of 20% SDS, and left to sit in an incubator at 37^{o}C overnight. On day two, 250uL of phenol and 250uL of chloroform: isoamyl alcohol in the ratio 24:1 was added to the Eppendorf using a micropipette. The solution was centrifuged for 10 minutes at 13,000 rpm. The upper layer was collected in a new tube and 500uL of chloroform: isoamyl alcohol were added again in the ratio 24:1. The blend was left to centrifuge at 13,000 rpm for 10 minutes. The upper layer was then carefully drawn with a micropipette and transferred to a new tube. Its DNA was precipitated out with 55uL of sodium acetate (3M, pH 6) and 500uL of isopropanol, and the tube inverted several times. Centrifugation at 13,000 rpm for 10 minutes was followed by addition of 200uL of 70% ethanol to wash the DNA a second time, and the entire mixture was centrifuged at 13,000 rpm for 7 minutes. The ethanol was discarded and the Eppendorf left to dry for 30 minutes, before adding 100uL of TE buffer to solubilize DNA. The transparent pellet of DNA was stored in the freezer at -20^{o}C.

3.2.1.3 Protocol 3

Protocol 3 was used to extract DNA from all patient samples. This technique yielded the thickest, most visible bands. Here, a 750uL sample of blood was taken in an

Eppendorf tube and combined with 750u L of solution A, after which the tube was inverted 4-6 times to mix the contents thoroughly together. It was rested for 10 minutes at room temperature, then taken to centrifuge at 13,000 rpm 10 minutes. The supernatant was discarded after the mixture split into two distinct layers, and the pellet resuspended in 400u L of solution B. The pellet was dislodged/ dissolved by applying mechanical force and tapping the tube repeatedly on the counter to shake it well, and the tube was centrifuged again at 13,000 rpm for 10 minutes. The pale red supernatant was discarded and the pellet resuspended in 400uL of solution B, 12uL of 20% SDS and 5uL of proteinase K, added with a small-tip micropipette, for overnight incubation at 37^{o}C to allow enzymatic activity. The next day, 250uL of phenol solution and 250uL of chloroform and isoamyl alcohol in the ratio 24:1 were mixed together separately and added to the Eppendorf tube. These allow the coagulation of proteins and separate the organic and aqueous phases of the solution. The mixture was centrifuged at 13,000 rpm for 10 minutes. The aqueous phase was transferred to a separate Eppendorf, and 55uL of sodium acetate along with 500uL of isopropanol were added to it. The tubes were inverted to precipitate DNA into a visible pellet. Centrifugation at 13,000 rpm 2 minutes was followed by discarding of the supernatant, and resuspension of the solution in 200uL of 70% ethanol, which allows for pure bands of DNA to be extracted. The mixture was centrifuged at 13,000 rpm for 8 minutes, and left inverted to dry for 1 hour on a paper towel. The obtained DNA precipitate was submerged in 100uL nuclease-free water to store at -20^{o}C in the freezer.

3.2.1.3 Protocol 4 (Qiagen Kit)

In an Eppendorf tube, we started with 400µl of lysis solution to which 20µl of proteinase K was added in addition to 2ml whole blood. The mixture was briefly spun on a vortex and incubated afterwards at a temperature of 56^{o}C for 10 minutes. Vortex was applied

to the tube occasionally, or alternative methods of shaking water bath or thermomixer were employed. This aided the digestive activity of proteinase K. Later, 2uL of 96-100% ethanol was pipetted into the tube, and vortex was applied. The tube was transferred to a Gene-Jet column, and centrifuged for 1 minute at 6000xg. The tube with Flow-thought solution was discarded. This tube was now put into a 2ml collection tube, together with 500uL of wash buffer 1 (with ethanol added), and centrifuged at 8000xg for 1 minute. The flow-through tube was discarded, and the small tube placed back in collection tube. Run-off was discarded. 500ul of wash buffer 2 (without ethanol added) was pipetted, and the mixture centrifuged at 12000xg for a maximum of 3 minutes. If residue was seen after revolutions, the liquid in the collection tube was discarded and tube re-spun for 1 minute at maximum speed. The collection tube was discarded, and the column was transferred to a 1.5ml microcentrifuge tube. 200ul of elution buffer was added to the center of membrane to effectively elute genomic DNA. The column was incubated for 2 minutes at room temperate and centrifuged again at 8000xg for 1 minute.

For maximum DNA precipitation, the previous step was repeated with another 200uL of elution buffer. To obtain maximum concentration of DNA, 50-100 uL elution buffer would be used instead of 200uL, but the volume of the resulting mixture would be less...

The purification column was discarded, and the DNA was obtained in the elute underneath. It was transferred to a new column. The DNA was used immediately or stored at -20^{o}C in the freezer.

3.2.2 Nano Drop

To determine the purity and concentration of DNA, Nano Drop was blanked with nuclease-free water or TE buffer (subject to the medium used to dissolve DNA). The

procedure were standardized to path length ‘auto’. 1uL of the DNA sample was dropped onto the screen. The A260/A280 value was noted to determine purity of the mixture (should ideally lie near 1.8). Its concentration and absorbance spectrum were also recorded.

3.2.3 Polymerase Chain Reaction

The samples were amplified for GAPDH, TGF-B, IL-10 and IL-18 genes, whose primers were previously optimized in the healthcare laboratory. TGF-B, IL-10 and IL-18 each have two single-nucleotide polymorphisms, namely CT, AG, and AC respectively. The standard PCR recipe employed for TGF-B, IL-10 and IL-18 involved using a micropipette to carefully add 8.8uL of nuclease-free water in a small PCR tube, followed by 2uL of 10x buffer and 2uL of 25mM MgCl2. 1.5uL of 10mM dNTPs were then added to the tube, followed by 1uL of forward primer, 1.2uL of reverse primer and 1uL of the respective SNP allele after they were thawed at room temperature. 2uL of the template DNA was added and 0.5uL of Taq polymerase is the final component to be added before inserting the tubes in a PCR machine to begin the polymerization reaction. A total reaction volume of 20uL is obtained using this protocol. The annealing temperatures for TGF-B, IL-10 and IL-18 are 60^{o}C, 55^{o}C and 60^{o}C respectively.

For GAPDH, a slightly distinct recipe was used involving 15uL of nuclease-free water, 2.5uL of 10x buffer, followed by 2ul of MgCl2, and 1uL DNTPs, forward and reverse primer each. Following this addition, 2uL of template DNA and 0.5uL of Taq polymerase are added to complete the reaction volume of 25uL. The mixture is either refrigerated immediately or inserted into the PCR machine after adding Taq polymerase, since the amplification of DNA has already begun. Two annealing temperatures, 59^{o}C and 60^{o}C, were used for the GAPDH reactions.

The PCR tubes were briefly spun in a mini-spin centrifuge to settle their contents. The conditions to operate the PCR machine are listed in *Table 1* Conditions to operate PCR. The reaction volume of the mixture was set to 20uL or 25uL respectively according to the protocol and the PCR was set to stop at 4°C for ∞ time after it had run its course for 1 hour and 50 minutes.

Number of stage	**Temperature**	**Time duration**	**Cycles**
Stage 1	95°C	5 minutes	1 cycle
Stage 2	95°C	45 seconds	1 cycle
Stage 3 (Veriflex compartments)	50°C 55°C 57°C 60°C 60°C 66°C	45 seconds	35 cycles
Stage 4	72°C	45 seconds	1 cycle
Stage 5	72°C	7 minutes	1 cycle

Table 1 Conditions to operate PCR

3.2.4 Gel Electrophoresis

The amplified DNA was run on 2% gel that was prepared by dissolving 2g agarose powder into a 100ml TAE buffer. The mixture was melted for 1 minute in the microwave, and stirred with a glass rod for another minute until it cooled down slightly, but not enough to gel. 0.5uL of ethidium bromide was added using a micropipette and thoroughly stirred. The mixture was poured into the casting tray attached with a comb before it can begin to set. It was solidified at room temperature for 45 minutes.

1x TAE was poured into the electrophoresis compartment and the gel was submerged in it. 2.5uL of the loading dye was mixed into 5.5uL of the PCR product (DNA) and loaded carefully into the wells, ensuring that the tip of the pipette did not damage the agarose. The first well was loaded the last with 2.5uL 50-bps ladder, before the electrodes were attached to the apparatus and the gel was run at 80V for 45 minutes. The agarose was carefully removed from the casting tray and bands visualized under UV transilluminator.

3.3 Assay optimization

3.3.1 Blood sample collection

Human clinical samples of DENV2 and DENV3 positive patients were obtained from National Institute of Health (NIH) and stored in cryovials at -80^{o}C. The relevant clinical factors were recorded for each sample, with details including serotype, viral load and date of collection. The samples were handled with standard precautionary measures of Biosafety level 2.

3.3.2 Viral RNA Extraction

RNA extraction of viral dengue components from human plasma was extracted using the Qaigen RNeasy mini kit, which involves a few underlying steps such as lysis, washing, binding and elution.

The whole blood is split into its respective components, and the plasma retained for further use in RNA extraction. In the 1.5ml microcentrifuge tube provided in the kit, 560µl of each of the reagents, carrier RNA mixture and buffer AVL were added, followed by 140µl of retained plasma. The contents of the tube were thoroughly mixed in a pulse vortex and left to incubate at room temperature for 10 minutes to allow enzymatic breakdown of plasma proteins. Centrifugation followed this step, after which

560µl of 100% ethanol was added to the tube and the contents whirled again in pulse vortex for 15 minutes. A second configuration was followed by transferring of the mixture into the Qlamp mini column comprising of a 630µl tube and a 20ml collection tube at the bottom. The apparatus was centrifuged at 600xg for 1 minute to allow liquid to seep to the bottom as filtrate, which was discarded. The contents of the column were placed in a clean tube and subjected to another centrifugation to repeat the previous step.

The washing stage involved adding 500µl of Buffer AW1 into the tube and centrifuging it at 6000xg for 1minute. The clear filtrate was removed and replaced in a clean tube, to which 500µl of Buffer AW2 was added and another centrifugation of 20,000xg for 3 minutes was performed. The filtrate tube was placed in a new collection tube and 40µl of Buffer AVE was micropipetted inside. The mixture was incubated for 1 minute at room temperature, and centrifuged a final time to obtain an RNA concentrate that would be further directed towards cDNA synthesis.

3.3.3 cDNA synthesis

In order to perform reverse transcriptase of PCR to verify our primers against the extracted viral RNA, we converted RNA to complementary DNA that would be added with the primers into the PCR machine to bind to and amplify against them. This process would allow us to verify the workability of the primers and optimize their melting temperatures. The protocol followed to convert RNA to cDNA for each specific gene region using the RT-PCR First-Strand Synthesis system is briefed below. The specific primers used to bind to the region of interest are the cDNA primers we previously designed.

1µl of our specific cDNA primer was added into a PCR tube with 1µl of the10mM dNTPs. The extracted RNA was left at room temperature to thaw and then briefly centrifuged to homogenize its contents. 12µl of the RNA was added into the reaction mixture and heated at 65^{o}C for 5 minutes. The tube was incubated on an ice block for 1 minute. A master mix of cDNA synthesis was prepared meanwhile in a separate PCR tube that included 1µl of Superscript III RT, 1µl of 0.1M dithiothreitol, 4µl of 5X buffer and 1µl of RNase reagent from the kit. This mixture was added to the RNA tube and incubated for an hour at 50^{o}C.

An inactivation stage where the mixture was subjected to 70^{o}C for 15 minutes was followed to stop further action of reagents, and this cDNA mixture was immediately used for reverse transcriptase PCR. The remaining mixture was stored in the PCR tube at -20^{o}C.

3.3.4 Reverse Transcriptase PCR

The nested PCR technique was preferred over conventional PCR to cross-test the viral cDNA against the four sets of prepared primers for each region. Three regions from dengue, *pol*, *NS3* and *env*, were amplified with the respective designed primers. Two rounds of PCR reaction were run for the two forward and two reverse primers of each region. The protocol followed is as below.

5µl of buffer was pipetted into two tubes, the sample and the control. The addition of 1ul each of dNTPs and forward and reverse primers followed this step for both the tubes, along with 2ul of $MgCl_2$ and 34.6ml of nuclease-free water. 1µl of cDNA template was then added to the sample tube, followed by 0.4µl of *Taq* polymerase in

both tubes. These reaction tubes were set up in the nested PCR and run to obtain amplification.

3.3.5 Gel Electrophoresis

Amplified DNA were visualized on 2% gel agarose gel, using the above mentioned protocol and conditions.

Chapter 4: Results

4.1 Primer Design

4.1.1 Assay sequences

The respective positions and lengths of the primers formulated with the above procedures are as follows. Degenerate primers were designed to detect multiple DENV sequences deviating from the consensus sequence. The designed primers are listed in *Table 2 Primers for DENV 2 and 3 serotypes in Pakistan isolates.*

Serotype	**Region**	**Primer**	**Sequence (5'- 3')**	**Position**	**Length**
DENV 2	*Pol*	F1	AACCGSGTGTCAAKTGTGCA	158-177	19 bps
		F2	AAAYAGATTRTCACTTGGAAT	187-207	20 bps
		R1	CTCTTGTTTGCTGACGATCATG	476-497	21 bps
		R2	GGTTCTCCGTTGCGTGTGGTTA	449-470	21 bps
		cDNA	CCTTTCTCYTGTTTGCTGACG	482-502	20 bps
DENV 2	*NS3*	F1	TTTATGGCAATGGTGTCG	4967-4985	18 bps
		F2	ATCGTCGACAAAAAAGGA	4935-4953	18 bps
		R1	GCRTCGTTTTATGGCCTC	5153-5171	18 bps
		R2	GCCAGGATTAATGTTCTC	5172-5190	18 bps
		cDNA	GCCAGGATTRATYTTRTC	5190-5208	18 bps
DENV 3	*Env*	F1	TGCGGGGCACTTRAAGTGYAGAC	1776-1798	22 bps
		F2	GAAATCCAAAACTCAGGAGGCAC	1744-1766	22 bps
		R1	AACTTTTAGTTGACCATGTTCTT	2098-2120	22 bps
		R2	TAACCYTKCTACAAGCTCCGGT	2130-2152	22 bps
		cDNA	CCCACTACTTTTAACCTTATCCA	2297-2319	22 bps

Table 2 Primers for DENV 2 and 3 serotypes in Pakistan isolates

4.1.2 Design of *Pol* Primers

The following figure shows the conserved regions obtained for polyprotein sequences of Dengue-2 from Pakistan isolates. *Figure 5 Conserved regions of Pol isolates*.

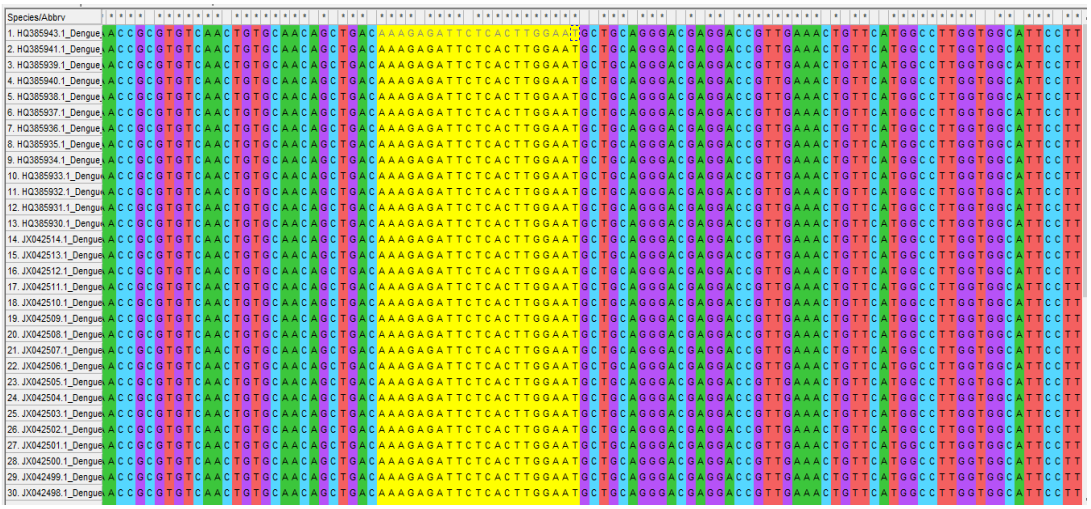


Figure 5 Conserved regions of Pol isolates

The Bio Edit tool helped determine the positions of the conserved regions used to develop primers, as shown in *Figure 6 Positions of nucleotides on Bio Edit*.

Figure 6 Positions of nucleotides on Bio Edit

After the primers were designed, they were run on OligoCalc tool to determine the following properties listed in *Table 3 Properties of Pol Gene Primers*.

Region	Primer	Tm	Hairpin structure	Self-complementarity	GC content
Pol	F1	51.8 – 53.8 °C	None	None	50 – 55%
	F2	42.6 – 46.5 °C	None	None	24 – 33%
	R1	53 °C	None	None	45%
	R2	56.7 °C	None	None	55%
	cDNA	52.4 – 54.4 °C	None	None	48 – 52%

Table 3 Properties of Pol Gene Primers

4.1.3 Primers of *NS3* Primers

The following figure shows the conserved regions obtained for non-structural protein 3 sequences of Dengue-2 from Pakistan isolates. *Figure7 Conserved regions of NS3 isolates*.

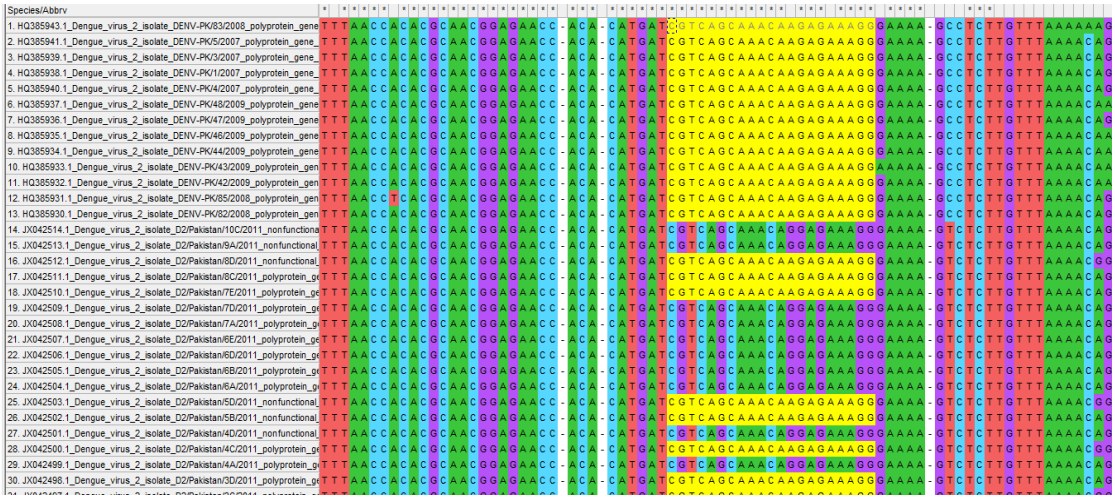


Figure7 Conserved regions of NS3 isolates

These primers were also run on OligoCalc tool to determine their properties shown in *Table 4 Properties of NS3 Gene Primers.*

Region	Primer	Tm	Hairpin structure	Self-complementarity	GC content
NS3	F1	45.8 °C	None	None	44%
	F2	43.5 °C	None	None	39%
	R1	48 – 50.3 °C	None	None	50 –56%
	R2	45.8 °C	None	None	44%
	cDNA	41.2 – 48 °C	None	None	33 – 50%

Table 4 Properties of NS3 Gene Primers

4.1.4 Primers of *Env* Primers

The following screenshots show the conserved regions obtained for envelope sequences of Dengue-3 from Pakistan isolates. *Figure 8 Conserved regions of Env isolates.*

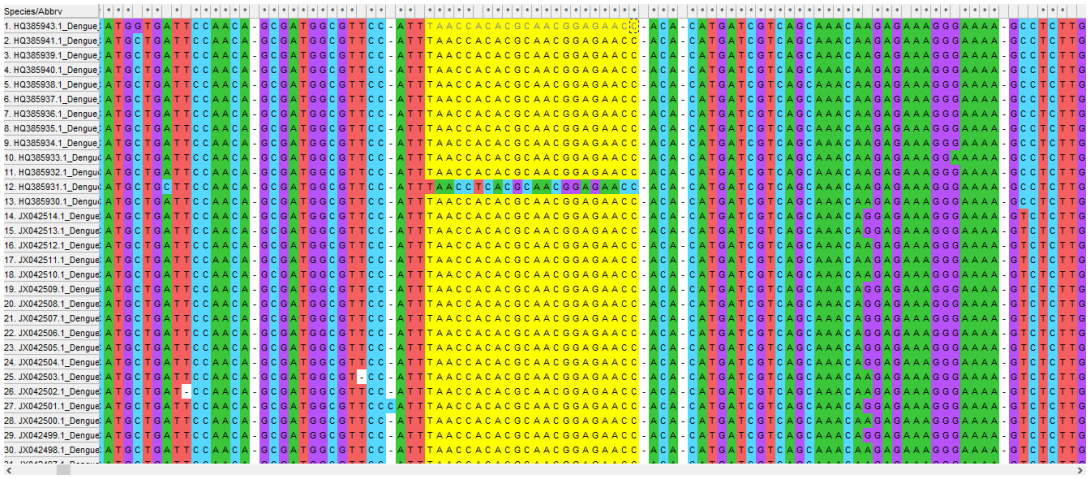


Figure 8 Conserved regions of Env isolates

These last set of primers were also run on OligoCalc tool to determine their properties shown in *Table 5 Properties of Env Gene Primers*.

Region	Primer	Tm	Hairpin structure	Self-complementarity	GC content
Env	F1	57.1 – 60.6 °C	None	None	52 – 61%
	F2	55.3 °C	None	None	48%
	R1	48.1 °C	None	None	30%
	R2	53 – 56.7 °C	None	None	45 –55%
	cDNA	51.7 °C	None	None	39%

Table 5 Properties of Env Gene Primers

4.2 DNA Extraction for Library

4.2.1 Extraction using Protocol 4 (Qiagen Kit)

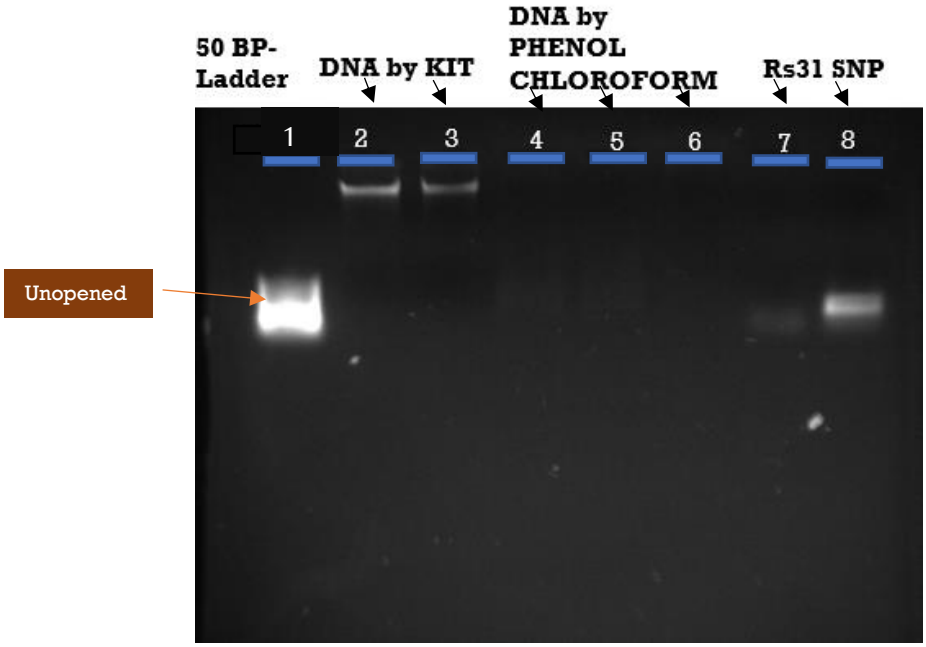


Figure 9 PCR results from Kit Extraction on 1x Gel

4. 2.2 Extraction using Protocol 1

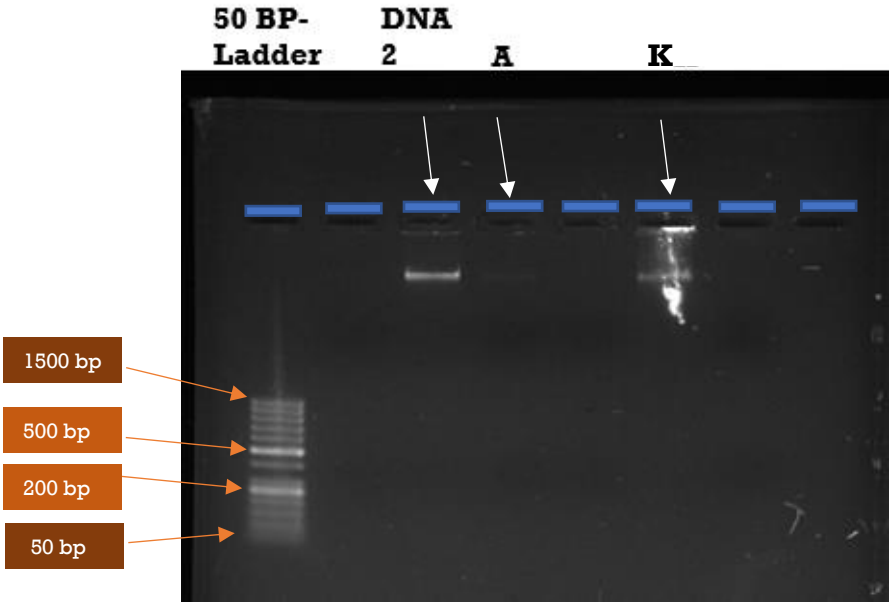


Figure 10 DNA samples from Protocol 1 Extraction on 1.5x Gel

4.2.3 Extraction using Protocol 2

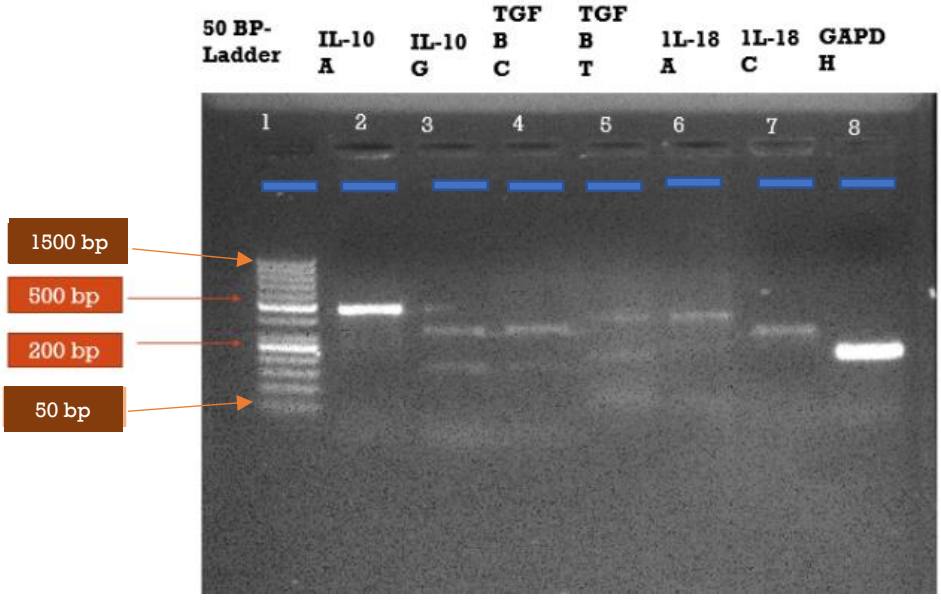


Figure 11 PCR results from Unknown Extraction on 2x Gel

4. 2.4 Extraction using Protocol 3

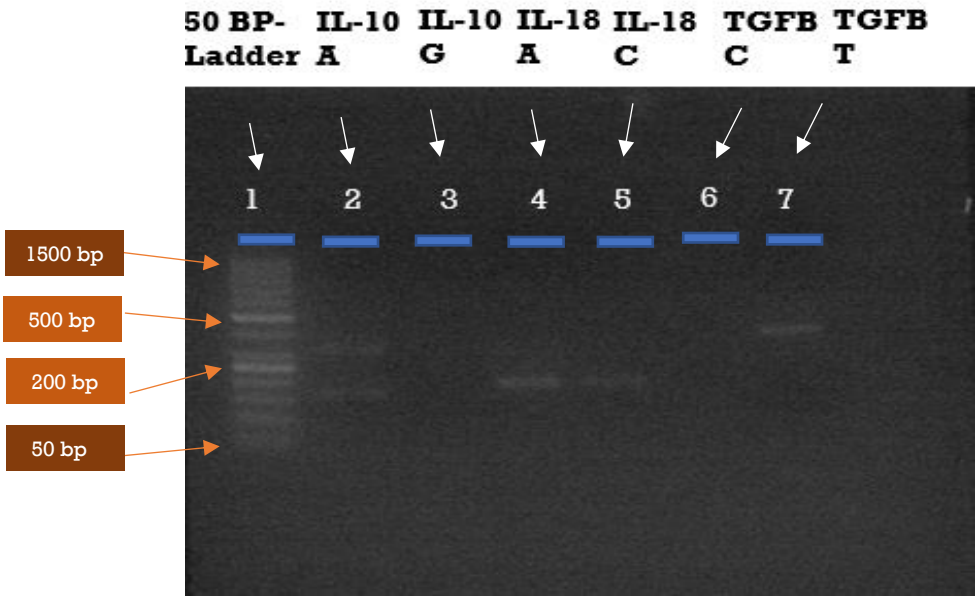


Figure 12 PCR results from Protocol 3 Extraction on 2x Gel

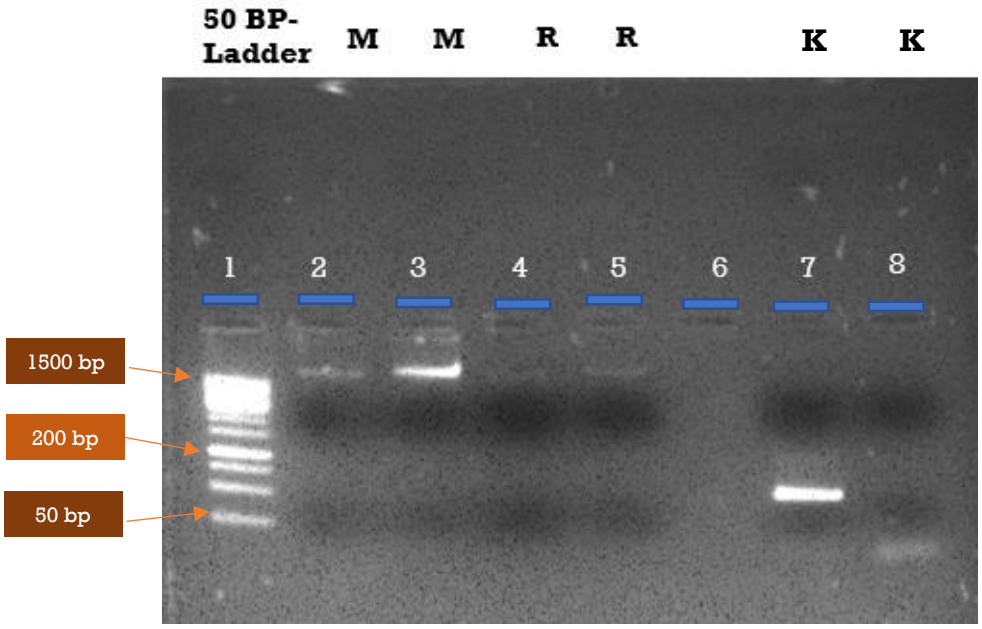


Figure 13 DNA samples from Protocol 3 on 2x Gel

Chapter 5: Discussion

Comparative analysis of diagnostic strategies used in countries with frequent dengue endemics.

5.1 Comparison of four serological and two molecular methods in Managua, Nicaragua

A study was conducted in which sensitivity and specificity of 4 serological methods (IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) and an inhibition ELISA method (IEM), NIAID-BEI MAC-ELISA and BOB ELISA (NS1 blockade-of-binding ELISA)) and 2 molecular methods (rRT-PCR assays) were evaluated for detection of ZIKA infection in 301 participants of Pediatric Dengue Cohort Study (PDCS). Among these 303 individuals 97 had cases of RT-PCR DENV confirmed infections (all serotypes). 127 showed ZIKV – positive cases (65 DENV-immune patients while 62 DENV-naïve) (Balmaseda et al., 2018)

Table 6 and *Table 7* are showing results of molecular and serological methods for Dengue virus detection respectively.

Methods	Brand	Specificity
RT-PCR	ZCD	100%
RT-PCR	Trioplex	100%

Table 6 Specificity of two different RT-PCR

Methods	Brand	Specificity
ELISA	CNDR MAC ELISA	79.5-90.5%
ELISA	NIAID BEI MAC ELISA	76.3-88.1%
ELISA	NS1 BOB ELISA	87.2-96.3%
ELISA	Inhibition ELISA	94.8-98.8%

Table 7 Specificity of serological methods

Based on the specificity and sensitivity both MAC-ELISA and RT-PCR are approved as in vitro diagnostic tests.

5.2 Comparison of Three rapid diagnostic tests in Myanmar

A study was conducted in Myanmar (Jang et al., 2019) and total 220 samples were assessed in this study. In these three commercially available rapid tests were compared which are ;

- The Humasis Dengue Combo NS1 & IgG/IgM
- SD Bioline Dengue Duo NS1 Ag & IgG/IgM
- CareUS Dengue Combo NS1 and IgM/IgG kits

Their sensitivity and specificity for NS1 (*Table 8*), IgM (*Table 9*) and IgG (*Table* 9) is given below.

For nonstructural protein	**SD Bioline Dengue Duo NS1 Ag & IgG/Ig**	**CareUS Dengue Combo NS1 and IgM/IgG kits** .	**The Humasis Dengue Combo NS1 & IgG/IgM**
Sensitivity	48.62%	79.82%	63.30%
Specificity	100%	100%	100%

Table 8 Sensitivity and specificity of rapid diagnostic tests for nonstructural proteins

For IgM	**SD Bioline Dengue Duo NS1 Ag & IgG/IgM**	**CareUS Dengue Combo NS1 and IgM/IgG kits**	**The Humasis Dengue Combo NS1 & IgG/IgM**
Sensitivity	60.55%	89.91%	51.38%
Specificity	100%	100%	98.21

Table 9 Sensitivity and specificity of rapid diagnostic tests for IgM

For IgG	**SD Bioline Dengue Duo NS1 Ag & IgG/IgM**	**CareUS Dengue Combo NS1 and IgM/IgG kits**	**The Humasis Dengue Combo NS1 & IgG/IgM**
Sensitivity	77.98%	82.57%	72.48%
Specificity	100%	100%	95.24%

Table 10 Sensitivity and specificity of rapid diagnostic tests for IgG

It was observed that IgM kits were more sensitive as compared to NS1 Ag or IgG kits. But if NS1 and IgM is combined missed cases can be reduced. Therefore, we can say that NS1 Ag plus IgM dengue kits increase the accuracy of results. In the above mentioned study**, the CareUS Dengue Combo NS1 and IgM/IgG kit** presented higher accuracy as compared to qRT-PCR and ELISA results.

5.3 Comparison of Two Commercial Real-Time PCR Assays used in Thailand

A study was conducted in Thailand in which two of two real-time PCR assays (i.e. ***ab*TES DEN 5 qPCR kit** and **innuDETECT Dengue**) were compared (Saengsawang, Nathalang, Kamonsil, & Watanaveeradej, 2014).

Among 117 positive cases that were confirmed through nested RT-PCR, **innuDETECT** which is two step assay detected dengue virus (DENV) infection with

44.4% sensitivity while ***ab*TES DEN 5 qPCR kit** detected dengue virus (DENV) infections with **97.4%** sensitivity.

It is assumed that ***ab*TES kit** has the ability to replace nested RT-PCR for the rapid diagnosis of dengue virus because of its higher sensitivity.

Summary of results is given in Table 11.

Kitk	Total samples	Detected Samples	Sensitivity	Confirmation in RT-PCR
ab TES DEN	117	114	97.4%	95%
innuDETECT	117	52	44.4%	76.3%

Table 11 Comparison of ab TES DEN and innuDETECT based on their sensitivity

5.4 Comparison of diagnostic tools used in Malaysia

The major methods used in the diagnosis are

Virus isolation

Nucleic acid detection

NS1 antigen detection

In a study it is shown that the most specific tests done here in Malaysia with most specificity and sensitivity are NS1 antigen detection and PCR method. (DS, 2015)

Following is the table (Table 12) highlighting the comparative specificity and sensitivity:

Method	Sensitivity	Specificity
RT-PCR 2 step	99%	66%
Real time Taq Man	80%	90%
kkReal time 2 step Taq Man	92.8%	92.4%
Multiplex RT-PCR	99%	100%
RT-PCR 1 step	95.2%	20-90%
PanBio NS1 ELISA	91.6%	100%
SD NS1 ELISA	87.5%	94.64%

Table 12 Sensitivity and specificity of diagnostic tools in Malaysia

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