# Construction of multi-epitope based anti-dengue virus vaccine and its impact on cell mediated immune response *In vitro*.

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A thesis submitted in the partial fulfillment of the requirement for the degree of

**MS Healthcare Biotechnology**


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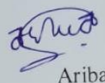

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# DEDICATION

*This thesis is dedicated to the battle of human v/s virus.*

# ACKNOWLEDGEMENT

"All praise is due to Allah, the Lord of the Worlds".

(Referencing ayah 1:2)

My passion towards research in applied biosciences has been flourished by the grace of ALLAH.

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# List of Acronyms

| | |
|---|---|
| DENV | Dengue Virus |
| ADE | Antibody dependent enhancement |
| mRNA | Messenger ribonucleic acid |
| IVT | *In vitro* transcribed |
| PBMC | Peripheral blood mononuclear cells |
| µg | Microgram |
| µl | Microliter |
| ng | Nanogram |
| BLAST | Basic local alignment search tool |
| Igκ | Immunoglobulin kappa |
| UTR | Untranslated regions |
| CTL | Cytotoxic T lymphocyte |
| ARCA | Anti-reverse capping analogue |
| rNTPs | Ribonucleotides |
| MgCl2 | Magnesium chloride |
| EPAP | E. coli poly adenosine polymerase |
| RT-PCR | Real-time polymerase chain reaction |
| TH1 | T helper 1 |
| IFN γ | Interferon gamma |
| IL-12 | Interlukin-12 |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |

# Abstract

The frequent epidemics and endemics caused by Dengue virus have imposed a threat on global health. The risk of antibody dependent enhancement (ADE) associated with four different serotypes of DENV is the footprint in serious illnesses like Dengue hemorrhagic fever and dengue shock syndrome. The death rate caused by DENV has also been escalated due to lack in treatment and inadequate prevention of the disease. There is an ultimate failure due to improper and trivial measures to control the vector (mosquito) spread. Such circumstances demand an effective vaccine to not only prevent the outbreaks, but also outrageous diseases caused by DENV.

Vaccination and immunization have been always a choice to prevent infectious disease. However, developing a vaccine against DENV is difficult as it has to effective against all of its serotypes. A tetravalent vaccine is not only a need to combat all of the circulating serotypes infections but to eliminate the risk of ADE.

To circumvent the laborious and expensive vaccine construction like live attenuated vaccines, mRNA has been selected to accomplish the objective. This research utilized multiple epitopes from DENV envelope, which is highly immunogenic, to construct mRNA vaccine against all serotypes of DENV. The mRNA was transcribed in vitro and transfected into peripheral blood mononuclear cells PBMCs to determine its immunogenicity. The in vitro transcribed mRNA proved to increase the mRNA levels of both T helper 1 (TH1) response inducer, interlukin-12 and TH1 cytokine interferon gamma which illustrates the generation of memory T lymphocytes. Thus, it is a promising candidate to be evaluated further as a vaccine against DENV.

# Introduction

Dengue virus is known to cause dengue fever, dengue hemorrhagic fever or dengue shock syndrome. It is a vector borne virus and transmitted by mosquito (Aedes aegypti) bite. It is a member of the genus, Flavivirus that also includes other insect borne viruses such as Tick-borne encephalitis virus, Zika virus, Japanese encephalitis virus, yellow fever virus and West Nile virus (Schaefer et al., 2022). Dengue fever is the most prevalent mosquito borne disease. Humans serve as its hosts, but its life cycle completes between mosquitoes and humans (Dengue Transmission | Learn Science at Scitable, n.d.). The number of people infected by it is 400 to 500 million per year. Whereas the cases of death record are 20,000 people annually (Dengue and Severe Dengue, n.d.)

There are four serotypes of Dengue due to distinct antigenic characteristics. Primary infection with one serotype causes Dengue fever which is also known as breakbone fever, and it confers immunity for the lifetime. Whereas a secondary infection with a different serotype leads to severe disease, dengue hemorrhagic fever or dengue shock syndrome, due to antibody dependent enhancement (Schaefer et al., 2022).

Electron microscopy of Dengue shows a round body with smooth surface. Surface bilayer of lipid is covered with another protein coat and the two surrounds the inner nucleocapsid (Roy & Bhattacharjee, 2021). Dengue is an RNA virus with positive sense single stranded genome. The genome length is 10 to 11 kb approximately (Murugesan & Manoharan, 2020). The genome codes for three structural proteins; Capsid C, Membrane M and Envelope E and seven non-structural proteins NS1, NS2A & B, NS3, NS4A & B & NS5. ER derive membrane embedding E and M proteins forms the outer layer into icosahedral symmetry (Perera & Kuhn, 2008). The non-structural proteins

are involved in replication process and hinderance of innate immunity (Avirutnan et al., 2006).

Dengue epidemics used to be reported among only few countries, earlier than 1970 (Dengue and Severe Dengue, n.d.). The situation now illustrates the endemic in 100 or more countries being affected with it risking 3.6 billion people approximately (Diamond & Pierson, 2015). America, Western Pacific regions, and South-East Asia account for the most severe outbreaks in all over the world (Dengue and Severe Dengue, n.d.).

Pakistan began to experience Dengue epidemics since 2010. In 2019 there was an increase in number of cases of infection and deaths. An endemic has again aroused this year in the regions of Sindh and Punjab (Pakistan, n.d.-a).

The primary infection occurs in monocytes and is caused by a single type of Dengue virus is characterized by a range of signs including, fever, and headache, body aches, rash and itching. The set of these acute infectious signs and symptoms are known as dengue fever (Mathew & Rothman, 2008). A secondary infection caused by different type of Dengue virus involves cross reactivity between pre-existing antibodies and the antigens. (Wollner & Richner, 2021). Dengue hemorrhagic fever begins with symptoms like that of dengue fever and leads to hemorrhagic indication due to rise in vascular permeability and thrombocytopenia. This condition turns to dengue shock syndrome with rapidly decreasing blood pressure and pulse rate (<20mm Hg). If the patient is left untreated with this condition, then soon death occurs due to undetectable blood pressure and pulse rate (Yeung et al., 2020).

The treatment against Dengue fever is the use of conventional antipyretics and pain killer. To date no antiviral drug is available to treat dengue specifically. Although

several studies have been found about some sulphated polysaccharides extracted from Seaweed, with high anti Dengue activity (Kothai et al., 2022). There is an unmet need of an effective vaccine for the eradication of fatal Dengue virus. There is only one licensed anti-dengue vaccine available, Dengvaxia. The drawbacks of vaccine include less efficacy due to different serotypes, and its approval to use in just 20 countries for only seropositive people against DENV. There are 5 other anti-dengue vaccines at different stages of clinical trials. All of these 6 vaccine formulations are tetravalent, composed of membrane and envelope proteins (Wollner & Richner, 2021). To avoid time-intensive approach of developing a potent vaccine against all serotypes, here the goal is to develop a multi-epitope-based vaccine that will be effective against all the serotypes of DENV.

An mRNA vaccine is composed of an antigenic code with a leading signal peptide sequence and are flanked by 5' and 3' UTR. The signal sequence codes for a cleavable peptide that translocate the nascent protein to endoplasmic reticulum for either secretion or membrane expression (Versteeg et al., 2019). It also contains a 5' cap and 3' poly adenosine tail to provide stability to the RNA. RNA vaccines do not require nuclear transfection as in the case of DNA vaccine. As soon as the RNA reaches inside the cell it undergoes translation with the help of cellular ribosomes. The antigens are then either expressed on the membrane or secreted, depending on their nature. RNA is itself immunogenic and the antigens expressed by it provide both robust innate and adaptive immunity.

To reduce the chances of innate immunity against mRNA it is modified by addition of Cap 1 structures which are not found in eukaryotes and replacement of common bases with modified bases such as pseudo uridine and pseudo cytidine. It not only diminishes the ubiquitous degradation mRNA but also improves translational efficiency (Hoernes

et al., 2016). To avoid rapid degradation of mRNA vaccines, it is not delivered naked. The current delivery mechanism involves the conjugation mRNA vaccine with cationic polymers, lipid nanoparticles and nano emulsions (Thess et al., 2015).

Determination of the vaccine immunogenicity requires animal model testing. Frequently, vaccine candidates proving to be successful in animal models, but fail in human trials. Such circumstances are a result of fundamental differences between humans and small animals' immune profiles (Delgado et al., 2009; Smith et al., 2017). The ideal system for evaluation of vaccine immunogenicity in should involve both human innate and adaptive immune responses. Recent studies have been reported the use of primary human monocyte derived dendritic cells and T cells for that purpose (Eneslätt et al., 2018; Tapia-Calle et al., 2019).

Following that model, the current study presents analysis of TH1 cytokines mRNA expression levels and proliferation of human peripheral blood mononuclear cells (PBMCs) after stimulation by mRNA vaccine against dengue. The mRNA vaccine is based on multiple epitopes from DENV envelope protein, targeting both T & B cells.

# Literature Review

## 2.1 Dengue Virus:

DENV belongs to family *Flaviviridae* with a +ve sense single stranded RNA genome (Roy & Bhattacharjee, 2021). It has 4 circulating serotypes worldwide, however, a 5$^{th}$ serotype has also been reported in Malaysia in 2007 (Mustafa et al., 2015). All 4 serotypes have been circulating across Pakistan with 2 & 3 being dominated (Khan et al., 2020). The serotypes shares only 65% similar genome and rest of the 35% contributes to antigenic shift (Sasmono et al., 2015).

### 2.1.1 Epidemiology:

Dengue virus causing Dengue fever, has been imposing serious public health threat for quite a long time (Bhatt et al., 2013). However, there has been negligence in eradicating and controlling its spread (Hotez et al., 2009). Being not reported, the true figure of total number of Dengue infection is difficult to be known. About 400 million infection per year and 100 million severe cases, approximately, has been estimated (*WHO | Dengue and Severe Dengue*, n.d.). The calculated deaths worldwide caused by DENV is around 22000 per year (Shepard et al., 2016). While infecting 3.9 billion people each year, DENV lays 128 countries at high risk (*WHO | Dengue and Severe Dengue*, n.d.).

In 1778, Indonesia & Egypt, and in 1780 North America experienced outbreaks of Dengue infections (W. Wu et al., 2011).

### 2.1.2 Transmission:

Mosquito species *Aedes aegypti* & *Aedes albopictus* are the primary and secondary vectors of DENV (Carrington & Simmons, 2014). There are two cycles of DENV transmission: sylvatic cycle and human cycle. The sylvatic cycle exists in non-human primates (Vasilakis et al., 2011), while the human cycle is transmission of all four serotypes exclusively humans (R. Chen & Vasilakis, 2011).

### 2.1.3 Prevalence in Pakistan:

Reported by national institute of health (NIH) in Pakistan, Dengue cases began to rise here in 2017 with 22,938 reported cases. In 2020, Pakistan faced again an outbreak of 24,547 DENV positive patients. Breaking the previous record, a sharp rise making the figure to 48,906 with 183 death was recorded in 2021. This year only Lahore was reported with 16580 cases alone with 257 deaths (*Pakistan*, n.d.). Addition to that around 5000 cases and 60 deaths more, from rest of the country has been recorded (Waheed, n.d.).

### 2.1.4 Structure:

Under the electron microscope it appears with smooth surface rounded body of about 50 nm in diameter. The outer surface is composed of lipid bilayer and the inside is the nucleocapsid core. The capsid C is covered with a membrane precursor prM and an out-envelope E. Its genome also code for 7 nonstructural protein (NS1, 2A, 2B, 3, 4A, 4B and 5) (Dwivedi et al., 2017).

Inside the lipid bilayer its approximately 11 kilobases long, ss +ve sense RNA genome is bound to capsid C protein. It is composed of a type 1 cap (m7GpppAmp) at the 5’ end.

Then the 5’ and 3’ ends are flanked by untranslated regions UTRs. Within the UTRs is packed the open reading frame. The open reading frame codes for all the structural and nonstructural protein into a single poly protein (Zeng et al., 2018). The polyprotein is then cleaved into distinct protein by both viral and cellular proteases (Constant et al., 2018).

### 2.1.5 Pathogenesis:

Dengue infection can be characterized into 3 types of clinical manifestation based on signs and symptoms. They are Dengue fever, Dengue hemorrhagic fever and Dengue shock syndrome.

The primary site of infection and replication of DENV is dendritic cells and then in monocytes, macrophages and lymphocytes. A primary DENV infection with a single serotype results in protective immunity against the same serotype. It is characterized by high fever, extreme body pain (due to which also called as breakbone fever), itching and may be rash and known as Dengue fever. The laboratory manifestation shows a drop in leucocytes along with lymphocytes.

A secondary infection with a different serotype results in antibody dependent enhancement ADE. It occurs due to the presence of cross-reactive antibodies in result of the primary infection with another serotype. Instead of neutralizing the virions, the cross-reactive antibodies bound to them are endocytosed by phagocytes and lead to increased infection. This scenario is clinically called Dengue hemorrhagic fever. During such infection hemostasis defects occur which result in high vascular permeability and bleeding. Blood clotting dysfunction occurs due to sharp drops in platelets count.

Dengue shock syndrome is a progression of Dengue hemorrhagic fever when the immediate treatment is not given. Soon death occurs due to undetectable blood pressure and pulse rate (<20mm Hg) (*Dengue Fever: Symptoms, Causes, and Treatments*, n.d.).

### 2.1.6 Prevention and Cure:

To date there isn't any FDA approved drug marketed to treat Dengue. To control the fever antipyretics and pain killers are given to the patients only. To relief dehydration electrolytes are also prescribed routinely (Schaefer et al., 2022).

Laboratorial research has been routinely performed to find the best candidate for cure of Dengue. Some sulfated polysaccharides derived from seaweed have been proved to combat DENV infection either by blocking its entry into the cells (Damonte et al., 2004). Certain phytochemicals and their derivates such as curcumin and glycyrrhizic acid have also been reported to possess anti-viral activity against DENV infection (Balasubramanian et al., 2019; Baltina et al., 2019). A combination of nucleosides (ribavirin, brequinar and INX-08189) was also found to be effective in interfering with its replication (Yeo et al., 2015).

### 2.1.7 Vaccines:

Along with the search of cure against DENV, an effective vaccine for protection against it has been focused by the research as well. The struggle to construct such began in 1920 but the ADE due to the presence of 4 serotypes has been a great challenge (McArthur et al., 2013). As a consequence of possessing RNA genome, DENV replication undergoes mutation of about $10^{-3}$ to $10^{-5}$. This phenomenon contributes to the emergence of new genotypes and may be serotypes, making it harder in its vaccine development (Dolan et al., 2021).

Until 2021 there was only one approved vaccine against DENV, Dengvaxia. It is commercially available to vaccinate 6 to 19 years old individuals. It is a live attenuated tetravalent chimeric vaccine. It is composed of yellow fever virus strain 17D of which the envelope E and membrane prM are replaced with that of the 4 serotypes of DENV (Yauch & Shresta, 2014). The vaccine contains considerable drawbacks including:

a. Only for use in specific age group.
b. Only for those who were previously infected with any of the serotype of DENV which require prior antibody screening.
c. Individuals with seronegative status are at a risk of more severe infection due to ADE (Ferguson et al., 2016).
d. Just 20 countries have licensed to use it (Thomas & Yoon, 2019).

Recently, QDENGA (TAK-003) manufactured by Indonesia has been approved to be used only in Indonesia. QDENGA (TAK-003) administration does not require prior anti-DENV antibody screening as it can be used among both seropositive and negative individuals. It is also a tetravalent live attenuated vaccine. The serotype 2 has been adjusted in providing antigens for all the serotypes. The overall vaccine efficacy to protect against DENV infection is 80.2% and to avoid hospitalization by 90.4%. It also does not prime the risk higher infection in seronegative patients (*New Dengue Vaccine Approved in Indonesia for Use Regardless of Prior Dengue Exposure*, n.d.). However, its limited use only in Indonesia still demands a vaccine to be available across all over the world.

Another promising DENV vaccine candidate is TV005/TV003, again a live attenuated tetravalent vaccine. Using directed mutagenesis the viral strain was attenuated and

comprise of all 4 serotypes antigens (Prompetchara et al., 2020). The preclinical testing recorded 90% neutralizing antibodies against DENV (Kirkpatrick et al., 2015).

Despite all of the advancement made in the development of a potent DENV vaccine, none is available in Pakistan to protect its people from a serious life threat. Being a demographically endemic region Pakistan relies only on mismanaged pest control which couldn't be the only prevention. So, a potent vaccine with easy to transport, less time consuming and cost-effective benefits is an unmet of Pakistan and other endemic regions worldwide.

## 2.2 mRNA Vaccines:

Being in research for about more than ten years RNA vaccines have opened their gateway to be commercialized in 2020 after the pandemic of COVID19. The 2 manufacturers Moderna® and Pfizer® are the only ones to be selling mRNA vaccines against SARS-CoV-2.

mRNA vaccine is a nucleic acid based vaccine that codes for antigens of pathogens to be protected against. mRNA vaccines are also made for cancer treatment. mRNA vaccine is a type of RNA vaccine that also includes self and trans amplifying/replicating vaccines.

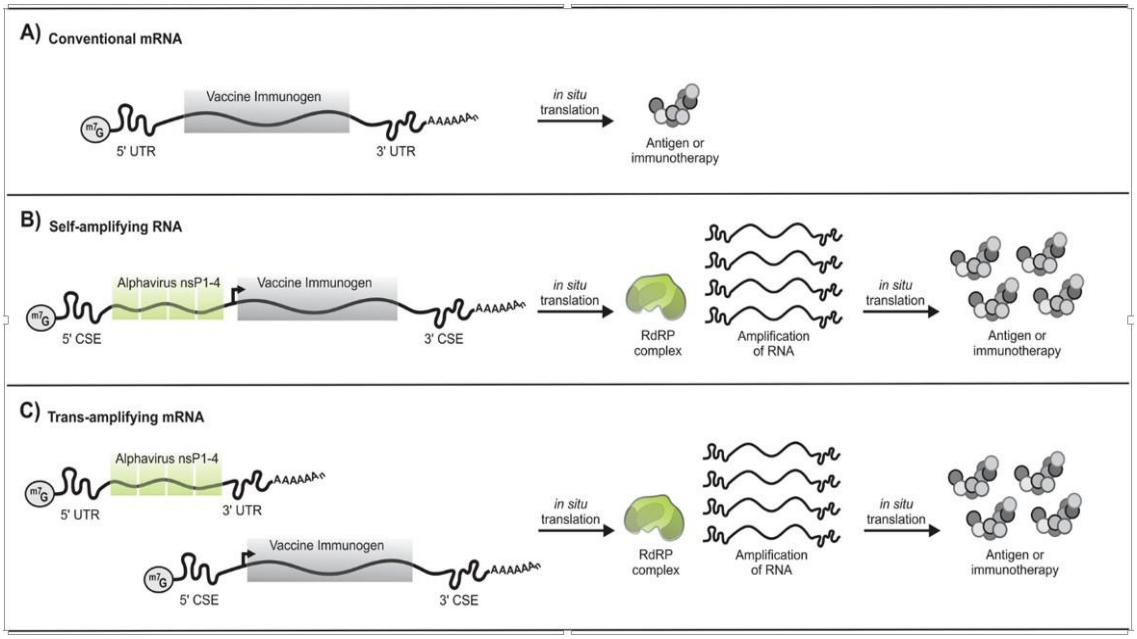


*Figure 2.1. A) A conventional mRNA vaccine without replication components. B) Self-amplifying RNA with self-replication components. C) Conventional mRNA with separate mRNA for providing replication components* (Bloom et al., 2021).

### 2.2.1 Advantages:

Considering mRNA vaccine instead of traditional vaccines is due to the following advantages:

**a. Safety:**

Unlike like attenuated & viral vector vaccines, RNA vaccine are without the risk of reverting back to the infectious state because of it is just a stretch of RNA. Moreover, DNA and recombinant vectors vaccines have a potential of RNA integration in human/animal genomes making the RNA exempted from this risk.

**b. Manufacture:**

RNA vaccine synthesis is quite simple as compared to the others as it is manufactured in a cell free environment thus making its production cheaper as well. The mass production of this synthetic nucleotide stretch is also quick and cheap.

**c. Efficacy:**

Unlike DNA based vaccines which require localization to nucleus and back the cytosol may contribute to less antigen production, RNAs are directly expressed in the cytosol making the chances of increased antigenic production.

To avoid rapid degradation mRNA it is delivered as conjugate with lipid based or Nano vehicles which makes its cellular transfer efficient (C. Zhang et al., 2019).

Usually protein-based vaccines, either synthetic peptides or subunits ones and killed/inactivated vaccines are poorly immunogenic without adjuvants. However, RNA vaccine doesn't require adjuvants for enhancing their immunogenicity because of the robust innate immune responses against RNA (Pardi et al., 2018).

All the above characteristics contribute to higher efficacy of RNA vaccines as compared to the other.

### 2.2.2 Mechanism of Action:

As stated above, mRNA vaccines are delivered in conjugation with non-viral vehicle such as lipid nanoparticles. LNP encapsulated mRNA enters the cell in the form of endosome. Inside the endosome low pH destabilize the ionisable lipid layer and thus making the mRNA to be released (Selby et al., 2017). As soon as the mRNA free falls in the cytoplasm the ribosome begins its translation into antigenic peptides. These antigens are endogenous

and thus MHC class I molecules presents them to the $CD8^+$ T cells. Some of the antigens are also secreted or expressed as trans-membrane molecules. The secreted antigens enable their uptake by the antigen presenting cells. These exogenous antigens are presented to $CD4^+$ T cells by the MHC class II molecules. As a consequence, cytokines are released and the antigens are presented to B cells to produce antigen specific antibodies (Cagigi & Loré, 2021).

The mRNA is also self-immunogenic and recognized by toll like receptors as part of innate immunity. TLR 7 and 8 recognize ssRNA while TLR 3 interacts with dsRNA molecules (Fang et al., 2022). It activates the pathways to the type I interferon and other pro-inflammatory cytokines production by activating TIR-domain containing adapter-inducing interferon β (TRIF) and mitochondrial antiviral signalling molecules (MAVS) (De Beuckelaer et al., 2017; Linares-Fernández et al., 2020). Briefly, mRNA vaccines are capable of inducing both innate and adaptive (cellular and humoral) immune responses.

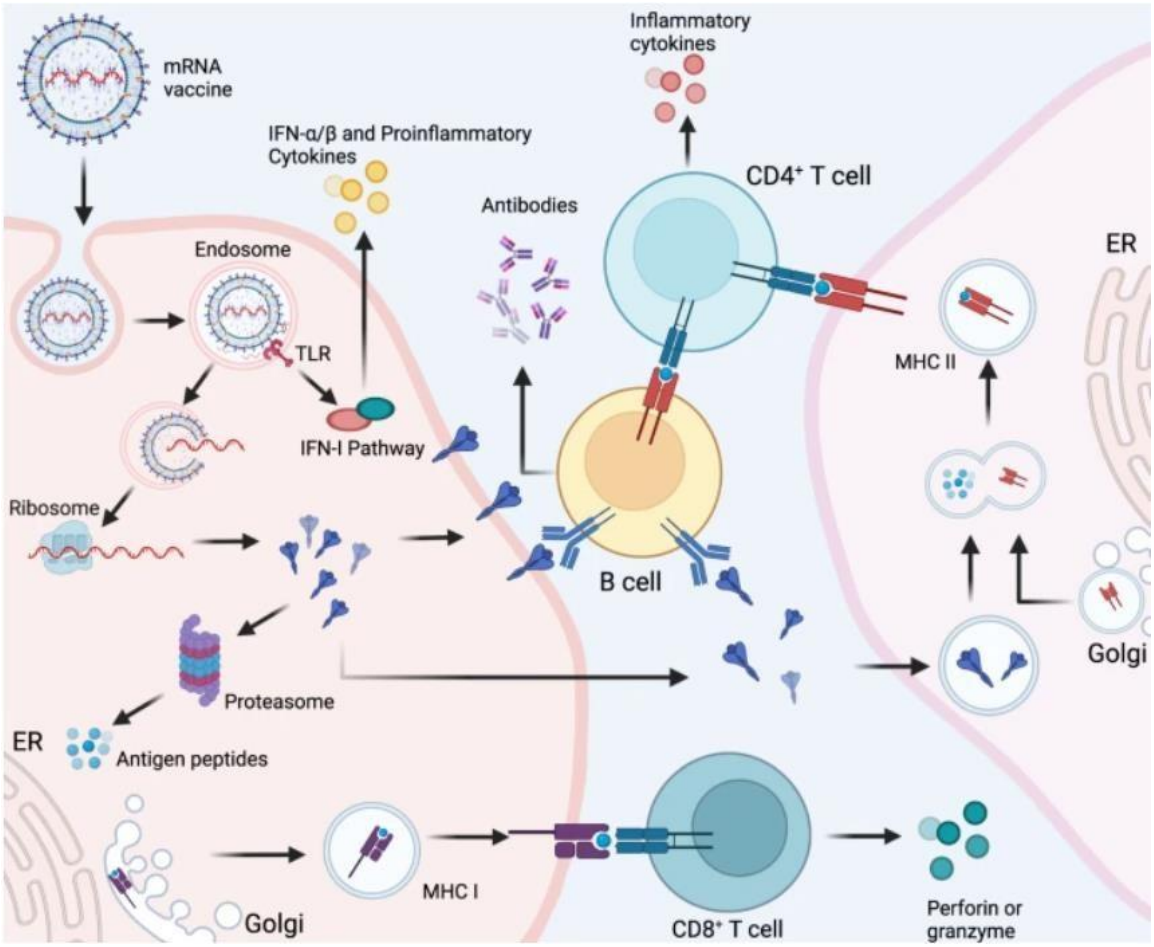


*Figure 2.2 Expression of mRNA vaccine and cellular and humoral immune responses against it.* (Fang et al., 2022). *mRNA vaccine enters a cell by endocytosis. After its releases from endosome it is translated by ribosome and forms endogenous antigens which are presented by MHC I to CD8+ T cell to generate cell mediated immune response. The secreted antigens are taken up by other cells and are presented by MHC II molecules as to exogenous antigens to the CD4+ T cells. The CD4+ T cells present them to the B cells to produce antigen specific antibodies.*

### 2.2.3 Composition:

mRNA vaccine or therapeutic mRNA are composed of:

a) Antigenic sequence (gene of interest)
b) A signal peptide sequence at 5' end of GOI
c) Kozek sequence (consensus eukaryotic start codon) 5' end of SP
d) Untranslated regions at both 5' and 3' ends
e) A poly adenosine (polyA) tail (can be added co and pre/post transcriptionally)
f) A CAP 1/2 structure or anti reverse CAP analogue (ARCA) (Pardi et al., 2018)

#### a) Antigenic sequence:

It is the codons for expressing antigens of pathogens into a protein sequence. That can be obtained by amplifying portion of interest from pathogen's genome through polymerase chain reaction PCR (Kadri, 2019) or by adding synthetic gene stretch (Hughes & Ellington, 2017), to the vaccine backbone.

#### b) Signal Peptide:

It is a short peptide sequence attached to the amino terminal of a protein. Their function is to direct the protein to its desired destination i.e trans- membrane or secreted outside the cell. SPs are of great importance in production of recombinant proteins. During translation of mRNA the signal peptide is cleaved off by signal peptidase (SPase) which acts on their cleavage site between SP and the protein. Then SP functions by localizing the nascent protein to the endoplasmic reticulum ER maturation of the protein takes place. After that the protein enters trans-membrane localization or secretory pathways (Owji et al., 2018).

### c) Untranslated regions:

Due to the unstable intrinsic property of mRNA, persistent expression of recombinant proteins is difficult to achieve. In order to secure the coding region and prolonged intracellular availability of the mRNA untranslated regions are required. They function by forming stem loops at both ends of open reading frame, which are highly stable. Inside the 5' UTR sequence is present that acts as internal ribosomal entry site IRES (Schuster & Hsieh, 2019).

As adapted from viruses 5' and 3' UTRs are provided with synthetic mRNAs to be efficiently expressed intracellular. The 5' UTRs help to bring the cellular ribosomes and other translational machinery for the IVT mRNA. Also, by constraining the regulatory and inhibitory mechanisms it protects the mRNA from gene silencing & thus increase the half-life of mRNA.

It has been reported, that the 3' UTRs in viruses contributes in enhanced gene encoding (Diamos et al., 2016). So, 3' UTRs has also been utilized and proved to improve the gene expression kinetics of the synthetic mRNAs (Orlandini von Niessen et al., 2019).

### d) CAP

A methylated structure called CAP is present at the 5' end of the mRNA to distinguish it from other RNAs. The role of CAP is to protect it from degradation by nucleases and bind to CAP binding proteins for translation initiation (Cowling, 2019).

For IVT mRNA, CAP 0 and CAP 1 structures are formed by utilizing capping enzymes (vaccinia virus capping system) (Brito et al., 2015). Addition of ARCA results in 70% of CAP 0 structure and 30% of 5' triphosphates (M. Z. Wu et al., 2020).

**e) Poly A Tail:**

Post transcription addition of poly adenine nucleotides to the mRNA is a natural process. In IVT mRNA, addition of poly A tail either pre or post transcription results in 50-200 nucleotides. Its role is to further increase the stability of mRNA and providing it similar features as that of cellular mRNA (Trepotec et al., 2019).

### 2.2.4 In vitro Transcription:

mRNA is synthesized by in vitro transcription of a dsDNA template that contain a phage (T7, T3 or Sp6) promoter at 5' end. In vitro transcription is cell free mRNA synthesis by addition of phage DNA- dependent RNA polymerase, necessary ribonucleotides (rNTPs) and $MgCl_2$ and ARCA can be added also at this time. If it wasn't capped and tailed before than vaccine capping enzyme and GTP for capping and E. coli poly adenosine polymerase (EPAP) with ATP for tailing would be added post transcription (Pardi et al., 2013).

### 2.2.5 Purification mRNA Vaccine:

There are several methods for purification of IVT mRNA for laboratorial use. They include lithium chloride precipitation, ammonium acetate precipitation, spin column chromatography, phenol-chloroform extraction and RNA gel excision.

Large scale production of IVT mRNA requires complex chromatographic techniques (HPLC/FPLC, Ion exchange chromatography). After chromatography, filter sterilization is performed and then it is stored in appropriate buffered condition (Blakney et al., 2021).

### 2.2.6 mRNA Vaccine Delivery Methods:

According to recent advancement in the mRNA vaccines, it has been successfully delivered in vivo by two methods:

i. Engineering of dendritic cells with mRNA and their injection to the donor (Benteyn et al., 2015).
ii. Direct injection mRNA to the recipient.

The former requires electroporation for transfection of DCs with mRNA. Electroporation results in open channel in form of pores to allow mRNA to cross the membrane. The ex vivo transfected DCs are then injected to the donor and yields in robust immune response against the vaccine. However, this method is expensive and time consuming (Kranz et al., 2016).

Direct injection of naked mRNA results in its ubiquitous degradation and requires a protective shield and delivery system to cross the cellular plasma membrane. To avoid it mRNA needs to be encapsulated in either virus like particles or non-viral delivery vehicles. The non-viral delivery vehicles are mentioned in figure 3, below:

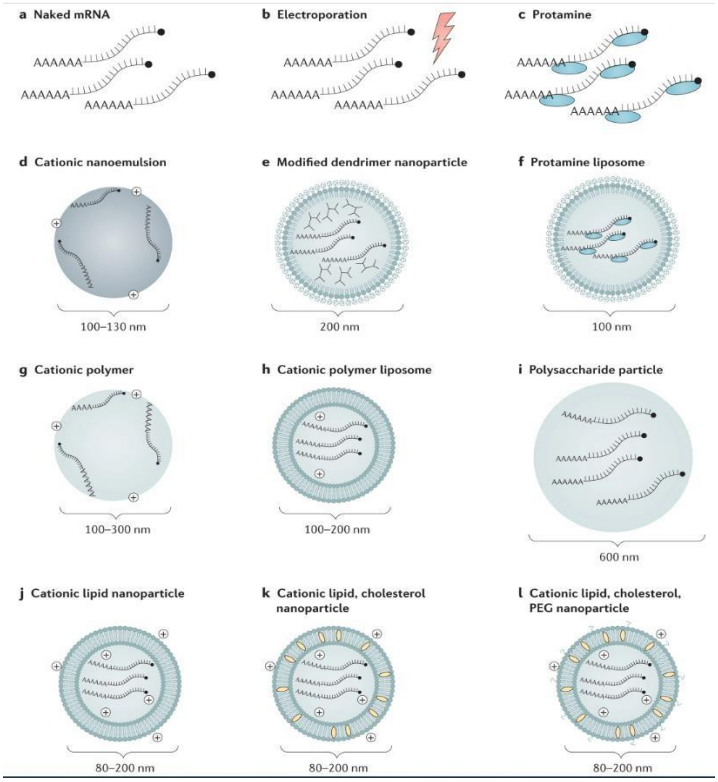


*Figure 2.3. Different non-viral mRNA delivery vehicle with their structures* (Pardi et al., 2018). *a) Naked mRNA, b) electroporation, c) Protamine, d) Cationic nanoemulsion, e) Modified dendrimer nanoparticle. f) Protamine liposome, g) Cationic polymer, h) Cationic polymer liposome, i) Polysaccharide particle, j) Cationic lipid nanoparticle, k) Cationic lipid cholesterol nanoparticle &l) Cationic lipid cholesterol, PEG nanoparticle.*

All of the above-mentioned delivery vehicles are cationic and encapsulate the anionic mRNA for efficient cellular uptake. Out of these the most widely used system is lipid nanoparticle with highest transfection efficiency (McKay et al., 2020).

## 2.3 PBMC Based Vaccine Analysis:

The pre-clinical testing of a vaccine includes immunogenicity monitoring which is mostly performed in vivo in animal models. Laboratorial research for vaccine immunogenicity

analysis relies on small animal such as rats. There are prominent distinguishable features between human and small animals' immune systems. Due to these fundamental differences the vaccines proven to be successful in animal trail result in failure in human trials, frequently. To circumvent those failures a testing system based on human immune features is an unmet need in the field of vaccinology (Tapia-Calle et al., 2019).

An ideal system for vaccine evaluation must consists of association between antigen presenting cells and T cells. Previous studies have shown successful use of peripheral blood mononuclear cells (PBMC) a model for that purpose (Etschel et al., 2012; Moncunill et al., 2020; Sasaki et al., 2018; Watkins et al., 2008). These studies included first the culture of PBMCs and then isolation of the required cell population e.g. monocyte derived dendritic cells or $CD4^+$ and $CD8^+$ T cells.

Due to time and budget constrains current study does not include the fractioning of PBMC and focuses only the TH1 cytokine expression evaluation at mRNA level following previous studies.

# Methodology

## 3.1 Antigen Sequence Retrieval:

Multi-epitope based antigenic sequences have been extracted from a computational study by (Islam et al., 2020), for anti DENV vaccine. They are comprised of 6 cytotoxic T lymphocytes and 2 B-cell epitopes.

## 3.2 Verification of Conservancy of Antigens:

Homology of the antigenic sequence with all four serotypes of Dengue were determined by protein BLAST® tool for the confirmation (*BLAST: Basic Local Alignment Search Tool*, n.d.).

## 3.3 Computational Vaccine Construction:

Following bioinformatics study (Islam et al., 2020), the 6 T cell epitopes were joint with GGGS linker with each other and the B cell epitopes, and the 2 B cell epitopes were joint with KK linkers each other.

## 3.4 Inclusion of Signal Peptide:

Signal peptide of human immunoglobulin kappa (Igκ) was added upstream of the antigens for effective translocation of the protein as reported previously (Hajam et al., 2020; Meyer et al., 2018). The cleavage of the signal peptide through signal peptidase activity was checked by SignalP - 5.0.

## 3.5 Reverse Translation of the Vaccine Construct:

The vaccine construct was reverse translated into double stranded DNA on SnapGene® (*SnapGene | Software for Everyday Molecular Biology*, n.d.). The codons used were optimized for eukaryotic system.

### 3.5.1 Addition of UTRs, T7 Promoter and Start Codon:

Untranslated regions (UTRs) (5′ UTR: 5′-AAATAAGAGAGAAAAGAAGA GTAAGAAGAAATATAAGAGCCACC-3′; 3′ UTR: 5′-TGATAATAGGCT GGAGCCTCGGTGGCCATGCTTCTTGCCCCTTGGGCCTCCCCCCAGCCCCTC CTCCCCTTCCTGCACCCGTACCCCCGTGGTCTTTGAATAAAGTCTGA-3′) were taken from a previous study (M. Zhang et al., 2020) and added to 5’ and 3’ end of the DNA template of vaccine construct. Then start codon ATG was added before signal peptide DNA sequence. Later 18 basepairs long DNA sequence of T7 promoter and prior to 5’ UTR sequence.

### Template DNA Amplification:

Template DNA suspension in Tris-HCl (pH 8.0), bought from Eurofins USA- Blue heron biotech, was amplified by polymerase chain reaction. The primers of the template were designed on SnapGene and validated on oligocalc: oligo nucleotide properties calculator.

Figure. Template DNA suspension in Tris-HCl.

Figure. Forward & reverse primers of Template.

The sequence of primers are as follows:

| Template DNA Primers | |
|---|---|
| **Forward Primers** | TAATACGACTCACTATAGAAATAAG |
| **Reverse Primers** | TCAGACTTTATTCAAAGACC |

The PCR was performed on a conventional thermo cycler by Applied Biosystems (model no. 2720). The amplification condition contained initial denaturation of 95°C for 5 minutes and 35 cycles of denaturation for 45 seconds, annealing for 53°C for 45 seconds, and extension at 72°C for 45 seconds with final extension at 72°C for 10 minutes. The reaction was composed of following components:

| | |
|---|---|
| Water | 8.5 µl |
| 2x Fermantas PCR master mix | 12.5 µl |
| Forward Primers | 1 µl |
| Reverse Primers | 1 µl |
| Template DNA | (55 ng) 2 µl |
| **Total** | **23 µl** |

Subsequently, to analyse the PCR product, 2% TAE (Tris, glacial acetic acid & EDTA buffer) agarose gel was prepared with 0.5 µg/ml ethidium bromide. 5 µl PCR mix was mixed with 3 µl of DNA loading dye and loaded into the well of the agarose gel. It was run with 100 bp DNA marker by Invitrogen for 45 min with 95 volts. The bands of PCR products along with marker under a UV transilluminator.

## 3.6 Invitro Transcription:

DNA template of the vaccine construct obtained from eurofins US, Blue Heron Biotech's Genemaker®, was invitro transcribed using mMESSAGE mMACHINE™ T7 Transcription Kit from Invitrogen™. According to product's user guide, briefly, the

transcription, capping and tailing of 350 ng of template DNA, was performed using two transcription reactions from the kit.

## 3.7 Purification of IVT mRNA:

The mRNA produced was purified by adding TURBO™ DNAse also provided in the kit and then wtih spin column and wash buffers of Nucleospin® RNA virus by Machery and Nagel. The mRNA suspension was mixed with equal volume of 75% ethanol and transferred to a spin column. The spin column was centrifuged at 12000 x g at 4°C for 30 seconds. This step lets the RNA to bind to the membrane of the spin column. Some of the RNA does not bind and remains in the flow through. Due to that the flow through was transferred into another tube and mixed with 200 ul of isopropanol and placed on ice. Then 500 ul of wash buffer RAW spin column bound to the mRNA and centrifuged at 12000 x g at 4°C for 30 seconds. The filtrate was discarded, and the remaining mRNA/ isopropanol suspension was applied on the spin column and centrifuged at 12000 x g at 4°C for 30 seconds. The filtrate was discarded. 300 ul of wash buffer RAW II (ethanol added) was applied on the spin column and centrifuged at 12000 x g at 4°C for 30 seconds. The filtrate was discarded and again after addition of 300 ul of wash buffer RAW II it was again added and centrifuged at 12000 x g at 4°C for 1 minute. Finally, 50 ul nuclease free water was applied on the spin column to elute mRNA, incubated on ice for 1 minute and then centrifuged at 12000 x g at 4°C for 1 minute. The purified mRNA was stored at -80°C.

## 3.8 mRNA Quantification using Nanodrop Spectrophotometer:

mRNA was quantified using Colibri microvolume spectrophotometer® with the guide of user's manual.

## 3.9 Denaturing Gel Electrophoresis for mRNA Quality Assessment:

Denaturing gel was prepared as describe previously (Aranda et al., 2012), with minor modification. 1.2% agarose gel was prepared in 1X TBE buffer and mixed with 0.5% commercially available bleach Clorox®. Gel was stained with 5 ul of 10 mg/ml of ethidium bromide.

3 ug of mRNA suspensions, before tailing and after tailing were mixed with 6X formaldehyde RNA loading dye (provided in the T7 transcription kit by Invitrogen™), in 1:2.5 ratio, respectively. The suspension was heated at 75°C for 10 min to denature secondary structures. Then placed at -20°C for 1 minute. The denaturing gel was placed in electrophoretic tank containing 1X TBE buffer. The mRNA samples of both the control i.e., pTRI-Xef and Dengue vaccine were loaded into different wells accompanied with 1 kb generuler® by Invitrogen™. The gel was run at 150V for 20 minutes. Then gel was assessed under UV transilluminator.

## 3.10 Isolation & Culture of Peripheral Blood Mononuclear Cells:

To evaluate the immunogenicity of the mRNA vaccine, PBMCs (peripheral blood mononuclear cells were isolated from 8 ml of whole blood using LSM™ Lymphocyte separation medium by MP Biomedicals™ according to the user's manual. The PBMCs were mixed with 1X RBC lysis buffer & incubated for 10 minutes, to eliminate the residual RBCs contamination. The suspension was centrifuged at 300 x g at room temperature for 10 and the washed with PBS. The PBMCs were then cultured for 24 hours at 37°C with 5% $CO_2$ supply, in RPMI 1640 medium with L-Glutamine and 25 mM HEPES by Gibco™, supplied with 10% heat inactivated human AB serum, 1% Penicillin-Streptomycin (5,000 U/ml) by Gibco™.

The human AB serum was prepared by clotting AB type blood in serum separator gel blood collection tubes for 30 minutes. The tube was centrifuged at 1500 x g for 10 minutes. Then serum was removed and heat inactivated at 56°C in a water bath on shaking for 30 min and later stored at -20°C until its use, following a previous study (Fante et al., 2021).

## 3.11 Electroporation of PBMCs with mRNA Vaccine:

The electroporation of PBMCs was performed using the methodology proposed by (Van Camp et al., 2010). 6 x $10^6$ cells were taken from the PBMCs culture and washed twice with serum free DMEM medium by Gibco™ and mixed with 6 µg of the mRNA. Later resuspended to 200µl with Opti-MEM by Gibco™. The suspension was transferred to 2mm Gene pulser/ Micropulser electroporation cuvettes by BIO-RAD. It was electroporated in a Gene pulser Xcell electroporation system by BIO-RAD with a single square-wave pulse at 500V for 5 milliseconds. Cells were also mock electroporated without mRNA with same conditions to be used as control.

### 3.12 Culture of Electroporated PBMCs:

Both the electroporated PBMCs with and without mRNA were resuspended in 1 ml complete medium (described above), & counted in a haemocytometer, separately. 10 µl of cell suspension were stained with 10 µl of 0.4% Trypan Blue dye solution by Gibco™ and observed under light microscope. The viable cells were counted in the four corner 1 $mm^2$ squares and in one in the centre. The number of cells per 1ml of cell suspension were calculated using the following formula:

Average number of cells in all chambers × 2 × $10^4$

Where "2" is the dilution factor calculated by: $\frac{\text{Volume of cells suspension}}{\text{Total volume (cells + dye)}}$

$10^4$ is used to obtain the no. of cells volume of 1 ml.

Subsequently, same no. of cells, with and without mRNA were transferred into 2 different flasks carrying 5 ml complete medium (described above), to give a final volume of 6 ml. The flasks were incubated at 37°C with 5% $CO_2$ supply.

## 3.13 MTT Cell Viability Assay:

Following a previous study human (Gonçalves et al., 2015), 5 x $10^4$ cells were seeded in 96 well plates, in triplicates from both control and transfected cells. Total volume of 100 µl cell suspension in each well was incubated for 48 hours at 37°C with 5% $CO_2$ supply. After incubation 10 µl MTT (tetrazolium) dye solution (5mg/ml) by Sigma Aldrich, prepared in PBS (pH 7.4) was added to each well and further incubated for 4 hours at 37°C with 5% $CO_2$ supply. Later, 100 µl DMSO was added to solubilize formazan crystals, to each well and incubated overnight at 37°C with 5% $CO_2$ supply. The absorbance of the solution was assessed in microplate (ELISA) reader by BIO RAD with a wavelength of 550 nm. The mean absorbance of both control and test samples were under the following formula to calculate the viability in percentage:

$$\text{Viability \%} = ((A_T - A_B) / (A_C - A_B)) \times 100$$

Where $A_C$ and $A_T$ are the absorbance values of transfected cells and non-transfected (control) cells. $A_B$ is the mean absorbance of wells without the cells.

## 3.14 Total RNA Extraction from PBMCs:

Three aliquots were taken from both the control and test (mRNA transfected) cell culture flasks of PBMCs at the interval of 6, 24 and 48 hours after transfection

(electroporation). Total RNA was extracted from the cells using TRIzol™ by Invitrogen™ under the light of user guide of the product. The total RNA was stored at -20°C until further use.

## 3.15 Evaluation of Interlukin-12 & Interferon gamma Expression by Real-Time Polymerase Chain Reaction:

**Primers Optimization with Conventional PCR:**

Primers of both IFN γ & IL-12 were obtained from Macrogen, Inc. The primers of IFN γ were designed for a previous study and kindly provided by the supervisor Dr. Sobia Manzoor, of the project. The sequence of IL-12 primers were taken from a website (*IL12B Human QPCR Primer Pair (NM_002187) – HP205923 | OriGene*, n.d.). The primers used for each of them are as follows:

| **Genes** | **Template DNA Primers** | |
|---|---|---|
| **IFN γ** (Macrogen, Inc) | **Forward Primers** | 5' TGACCAGAGCATCCAAAAGA 3' |
| | **Reverse Primers** | 5' CTCTTCGACCTCGAAACAGC 3' |
| **IL-12** (Macrogen, Inc) | **Forward Primers** | 5' GACATTCTGCGTTCAGGTCC 3' |
| | **Reverse Primers** | 5' CATTTTTGCGGCAGATGACC 3' |

Freshly isolated PBMCs from healthy blood were used to extract total RNA as described above. The RNA was used as template for the PCR. The RNA in 1 µg quantity was converted to cDNA with RevertAid RT Reverse Transcription kit

Thermo Scientific™ and Random Primers by Invitrogen™ according to the user manual.

The PCR was performed on a conventional thermo cycler by Applied Biosystems (model no. 2720). The amplification condition contained initial denaturation of 95°C for 5 minutes and 35 cycles of denaturation for 45 seconds, annealing for 58°C -62°C for 45 seconds, and extension at 72°C for 45 seconds with final extension at 72°C for 10 minutes.

**Real-Time Polymerase Chain Reaction:**

1 µg of total RNA, extracted at 3 different time intervals from both the control and test PBMC culture was converted into cDNA using RevertAid RT Reverse Transcription kit Thermo Scientific™ and Random Primers by Invitrogen™. The expression of interlukin-12 & interferon gamma was normalized with a housekeeping gene i.e. Glyceraldehyde 3-phosphate dehydrogenase GAPDH, by real-time polymerase chain reaction.

GAPDH- forward and reverse ready to use 10µM Primers from Thermo Scientific™.

The reaction was set in triplicates for each gene of test samples with a control. 5 µl of cDNA, 0.2 µM of each forward and reverse primers of the three genes and 4 µl of 5x Hot FIREPol® EvaGreen® qPCR mix plus ROX by Solis BioDyne were mixed with nuclease free water giving a final volume of 20 µl.

## 3.16 Relative Quantification of IL-12 & IFN γ with GAPDH:

The mRNA expression of IL-12 & IFN γ was normalized with a reference gene, GAPDH mRNA expression using the following formula:

$$2^{\Delta Ct}\,(\text{Test}) = \frac{2^{\,(Ct\,(IFN\,\gamma/\,IL\text{-}12)\,-\,Ct\,(GAPDH))}\ \text{Test}}{2^{\,(Ct\,(IFN\,\gamma/\,IL\text{-}12)\,-\,Ct\,(GAPDH))}\ \text{Control}}$$

Where CT is the threshold cycle.

## 3.17 Statistical Analysis:

Both the MTT cell viability assay and qRT-PCR results were interpreted through Prism GraphPad 8.0.1. The percentage of cell viability was assessed by One sample t test and the fold change value of mRNA expression of IL-12 & IFN γ were assessed by Bonferroni's multiple comparison test.

# Results

## 4.1 Cytotoxic T lymphocytes and B-cell epitopes:

The selected 6 cytotoxic T lymphocytes and 2 B-cell epitopes from the respective study (Islam et al., 2020) are non-homologous to human, conserved among all 4 serotypes of DENV (to prevent antibody dependent enhancement), most immunogenic, non-allergen and non-toxic. The epitopes are taken from the envelope protein of DENV. Their sequence are as follows:

| S.no. | T Cell Epitopes | S.no. | B Cell Epitopes |
|---|---|---|---|
| 1. | DTAWDFGSV | 1. | NPVVTKKEEPVNIEAEPP |
| 2. | RGARRMAIL | 2. | TWIGLNSKNTS |
| 3. | RRMAILGDT | | |
| 4. | GLDFNEMIL | | |
| 5. | KGSSIGKMF | | |
| 6. | VNIEAEPPF | | |

## 4.2 Verification of Conservancy of Antigens:

All the epitopes selected for vaccine construction showed 100% conservancy with the consensus protein sequences of all serovars of DENV protein BLAST®.

## 4.3 Computational Vaccine Construct:

After assembling the epitopes with the cleavable linkers, a 107 residues long peptide sequence was formed with a molecular weight of 10.981 KDa.

| | Whole Protein |
|---|---|
| Length | 107 aa |
| Molecular Weight | 10,981.43 Da |
| Extinction Coefficient (280 nm) | 11,000 $M^{-1}$ $cm^{-1}$ |
| Absorbance (280 nm, 0.1%) | 1.00 |
| Isoelectric Point (pI) | 9.95 |
| Charge at pH 7.0 | 2.84 |

*Figure 5.1. Properties of the antigenic protein. Antigen to be expressed by the mRNA is 107 amino acids long with 10.981 KDa weight.*

The addition of the 20 amino acids long peptide upstream of the antigen Igκ signal sequence made it upto128 amino acids long peptide.

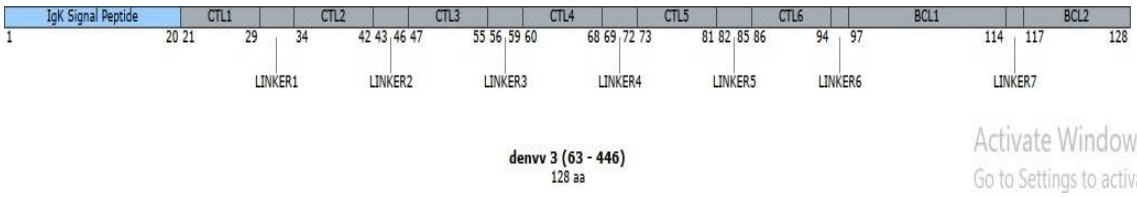


*Figure 4.2. DENV antigens with Igκ signal peptide. Addition of Igκ makes the antigen 128 amino acids long.*

The probability of Igκ signal peptide sequence cleavage by cellular signal peptidase was 0.7861. The cleavage site was found between the last residue of signal sequence, glycine and first residue of the DENV antigen, aspartic acid, at position 20 and 21 respectively.

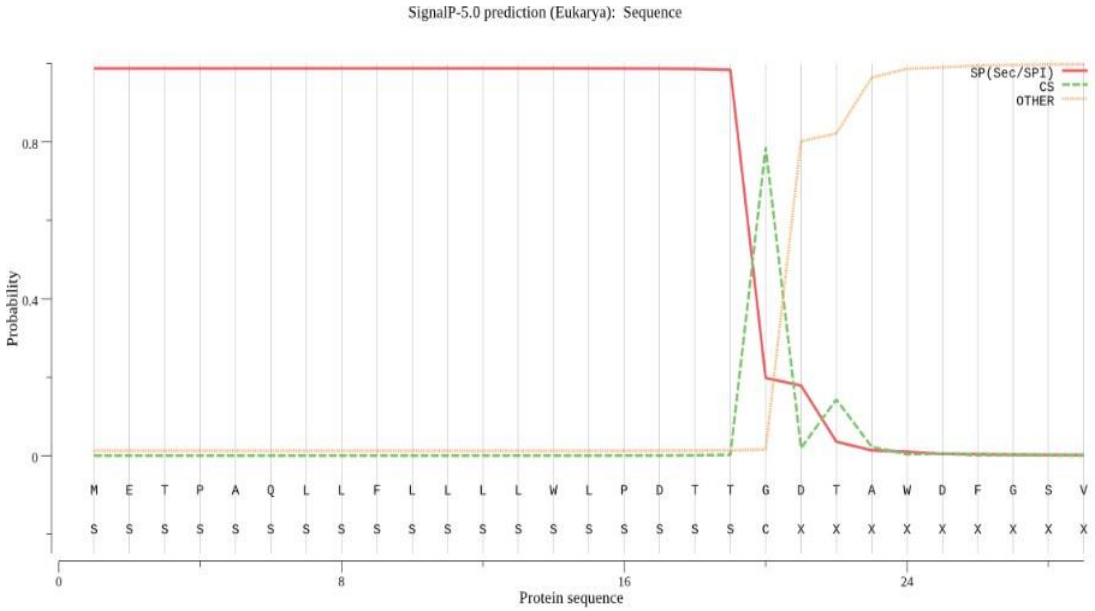


*Figure 5.3. Signal peptidase cleavage of Igκ signal sequence by Signal peptidase.*

The whole protein was then reverse translated to double strand DNA. On addition of 5' and 3' UTRs and T7 promoter a 556 basepairs (bp) long dsDNA was finalized to be used as template for in vitro transcription.

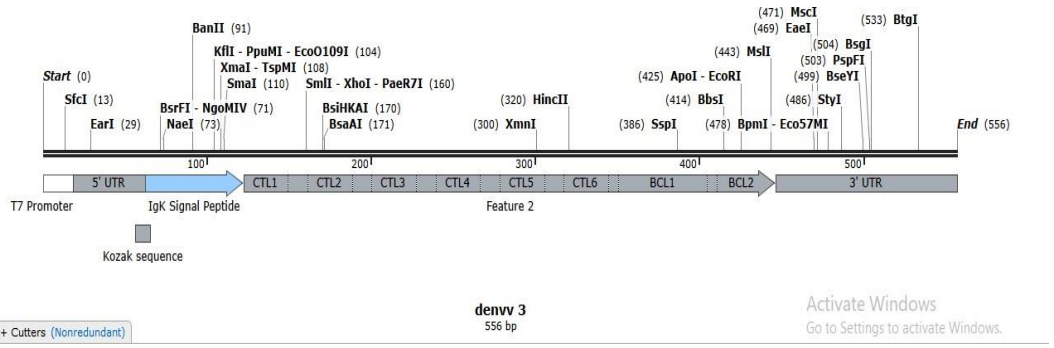


*Figure 5.4. Double Strand DNA Template of DENV Vaccine. The template DNA is composed of Igκ SP at 5' end of CTL epitopes linked with GGGS linkers and BCL epitopes with KK linkers and the two with KK linkers, flanked with 5' and 3' UTRs, & T7 promoter at 5' end.*

**Amplification of Template DNA by PCR:**

The primers of template DNA were annealed at 53°C and appeared as sharp and bright band between 500 to 600 bp of the DNA marker. Appearance of the bands of PCR products the size of it was same as the template that is 556 bp.

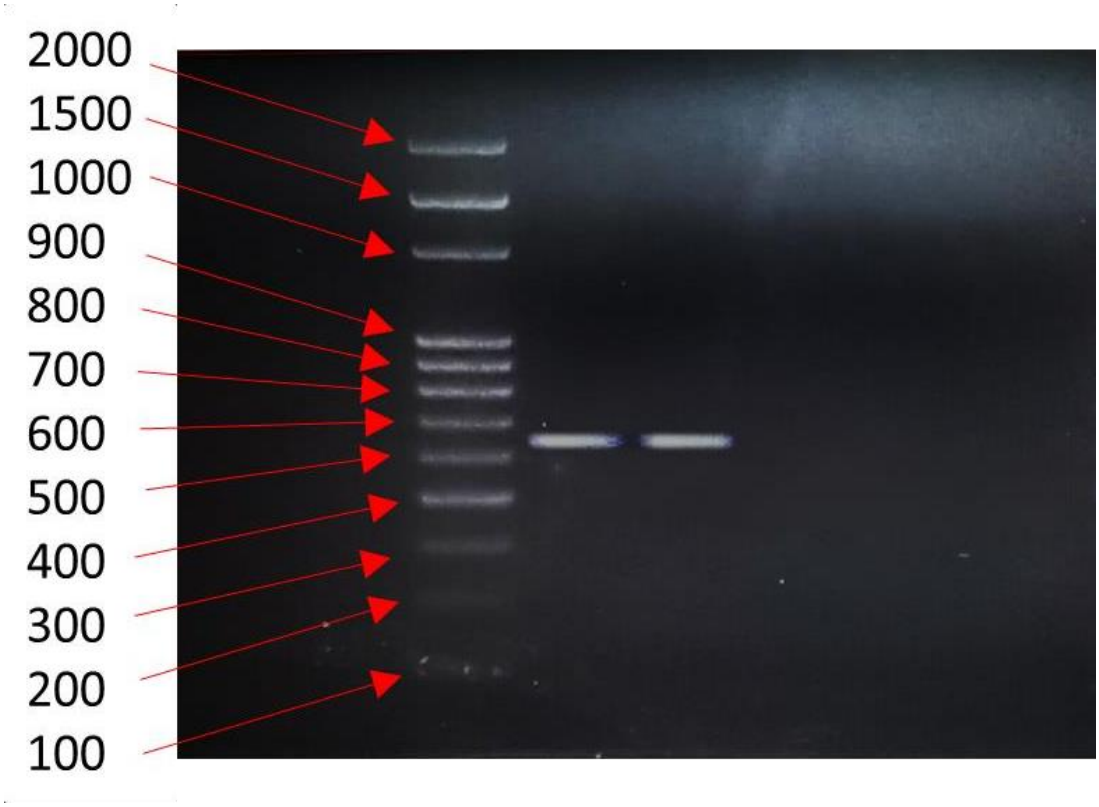


***Figure 5.4.*** *Gel Electrophoresis of Template DNA's PCR Product.* ***556bp long*** *Template DNA bands with 100 bp DNA marker on 1x TAE 2% agarose gel*

## 4.4 Concentration of mRNA Vaccine:

The synthetic dsDNA was in vitro transcribed with ARCA and later tailed with poly A. After purification of the IVT mRNA its nanodrop spectrophotometric analysis revealed the concentration be as follows:

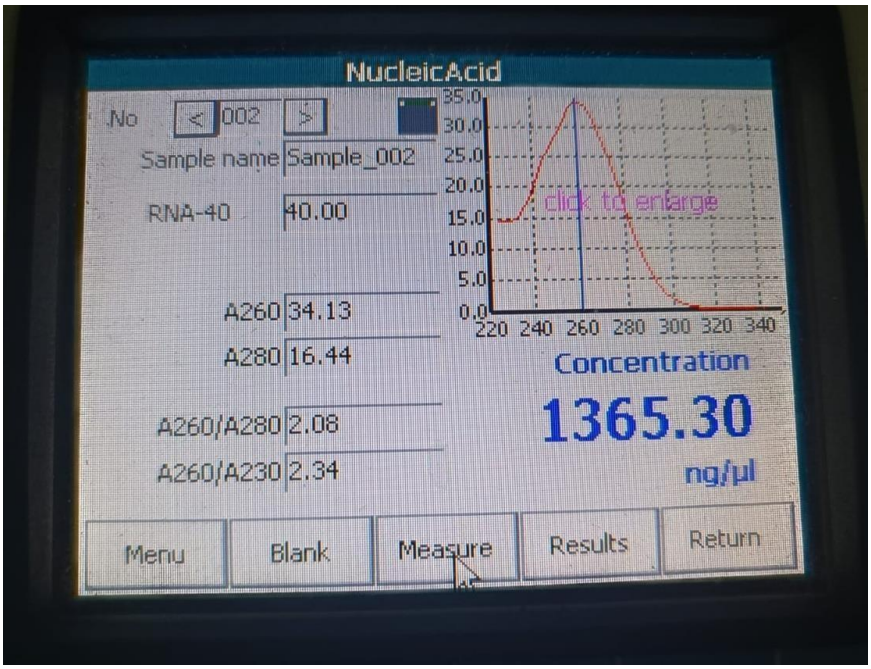


*Figure 5.5. Purity and concentration of IVT mRNA. The concentration is 1365.30 ng/µl and the absorbance is 2.08 and 2.34 at A260/280 and A260/230 respectively.*

According the spectrophotometric the in vitro transcription of the DNA template of the vaccine resulted in 68.265 µg of the mRNA. The mRNA also appeared to be pure.

## 4.5 Quality of IVT mRNA on Agarose Gel:

The denaturing agarose gel appeared with two bands for both the control and the mRNA vaccine. The untailed mRNA band appeared at 556 bp and the tailed one between 700 to 600 bp on scale of the 100 bp ladder. On the other hand, the positive control pTRI-Xef untailed mRNA showed up at and tailed at.

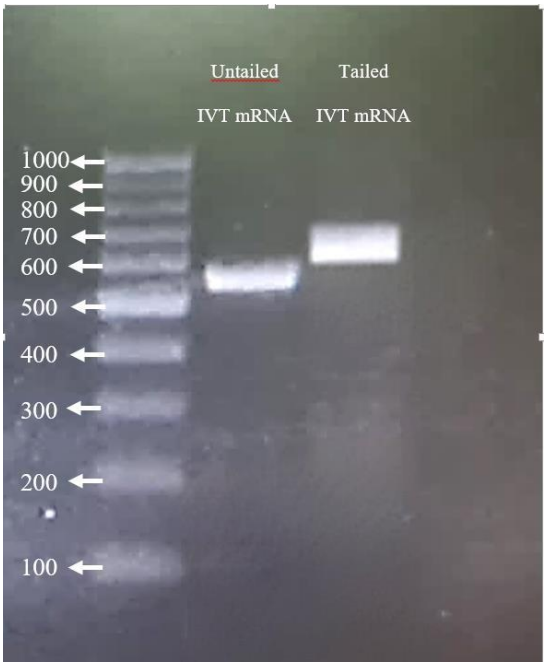


*Figure 5.6. DENV IVT untailed and tailed mRNA bands on denaturing agarose RNA Gel. 556 bp long untailed IVT mRNA & around 650 bp tailed IVT mRNA on 1x TBE 1.2% agarose denaturing gel.*

## 4.6 Isolation & Culture of PBMCs:

After density gradient centrifugation 4 distinct layers appeared in the tube. At LSM™ and plasma interface a thread like structure was collected using 1 ml Pasteur pipette.

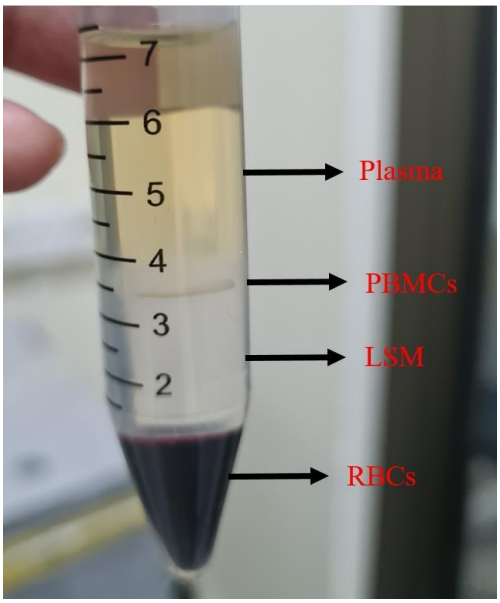


*Figure 5.7. Layers of blood after density gradient centrifugation with LSM™. Blood separation into plasma, PBMCs, LSM and RBCs.*

3.6 million PMBC isolated from 8 ml blood were cultured half as control and half as cells to be transfected. The cells appeared as rounded bodies under light microscope at 20x magnification, with no signs of RBCs appearance.

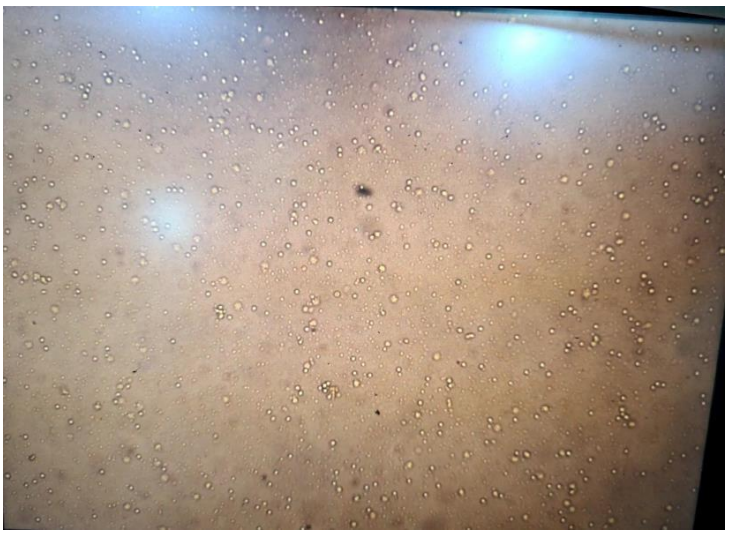

*Figure 5.8. Culture of isolated PBMCs after density gradient centrifugation. 20x magnified micrograph of PBMCs culture.*

## 4.7 MTT Cell Viability Assay:

After 48 hours of incubation for the MTT cell viability both the control and the transfected cells were observed under light microscope at 20x magnification. The transfected cells appeared almost fully confluent illustrating stimulation by antigens leading to proliferation of monocytes and lymphocytes.

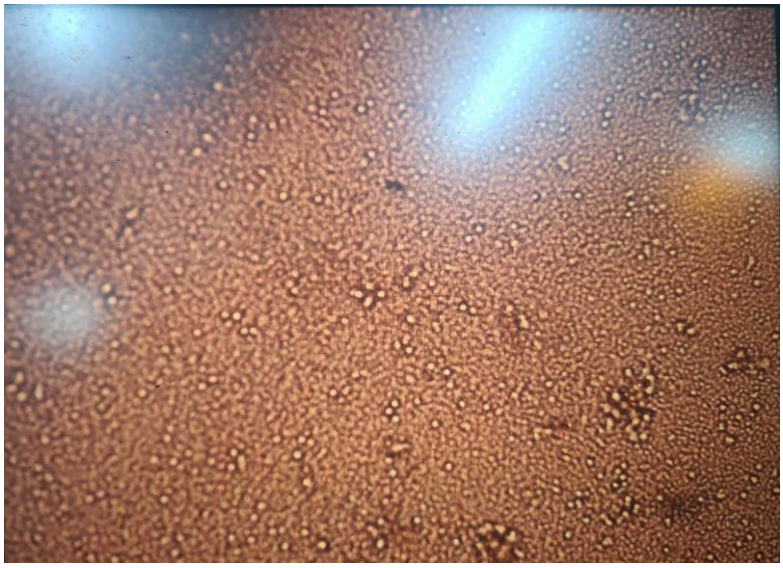

*Figure 5.9. Transfected PBMCs after 48 hours. Over grown and clustered PBMCs in double layer after antigen stimulation expressed by IVT mRNA under 20x magnification.*

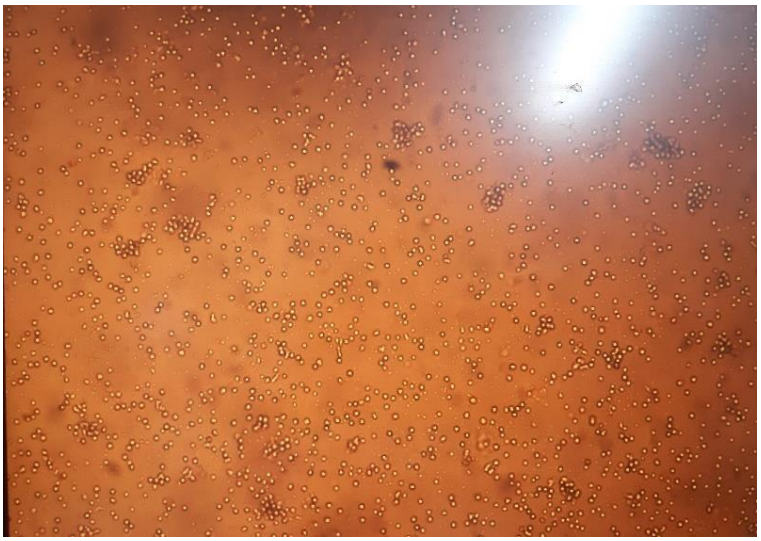

*Figure 5.10. Non-Transfected PBMCs after 48 hours.* ***Less confluent PBMCs without IVT mRNA under 20x magnification.***

According to the given formula, the percentage of the viable transfected cells calculated is 33.13% higher than that of control. The mean increase in proliferation of transfected PBMCs is highly significant with a p value of <0.001 and 95% CI 120.4-145.9.

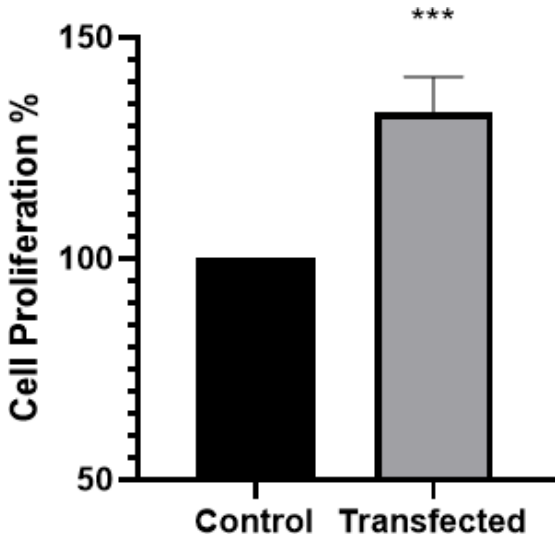


*Figure 5.11. Percentage of cell proliferation in control and transfected PBMCs.* ***Transfected PMBCs with IVT mRNA were significantly more viable then the controlled non transfected ones.***

**Evaluation of Interlukin-12 & Interferon gamma Expression by Real-Time Polymerase Chain Reaction:**

**Primer's optimization:**

The primers of all IL-12, IFN γ and GAPDH were annealed at 60°C. The amplified segment of the genes was appeared as sharp and bright bands. The band of IL-12 appeared between 100-50 bp illustrating a size of 98 bp. The band of IFN γ appeared between 300-250 bp illustrating a size of 236 bp. The band of GAPDH appeared just above 500 bp.

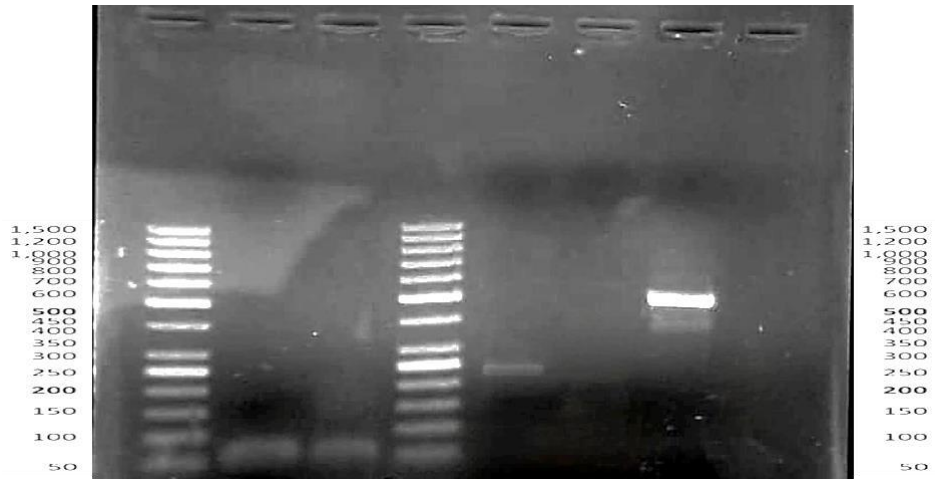


Figure 5.12 *Gel Electrophoresis of IL-12 IFN γ & GAPDH's PCR Product. 98 bp long IL-12, 239 bp long IFN γ and 500 plus bp long GAPDH bands on 1x TAE agarose gel.*

## 4.8 mRNA Expression Analysis of TH1 Cytokines:

Both the mRNA expressions of IL-12 and IFN γ were upregulated in transfected PBMCs as compared to that of control PBMCs. On comparing the mRNA levels of IL-12, increase in mean fold change was observed at all 4 (7.618-fold), 24 (5.726-fold) and 48 (2.667-fold) hours. However, the increase in fold change of mRNA level at 48 hours was not significant. The multiple comparison revealed p values of <0.001 at 4

and 24 hours and 0.063 at 48 hours; and 95% CI "-8.363 to -4.874" at 4, -6.470 to -2.982" at 24 & "-3.411 to 0.07741" at 48 hours.

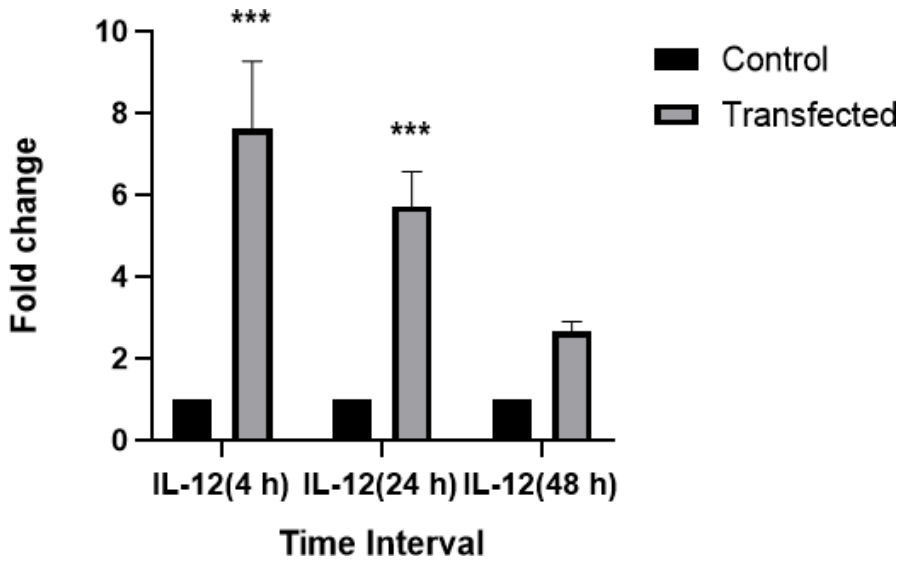


*Figure 5.13. Fold change in mRNA levels of IL-12 at 4, 24 and 48 hours in control and transfected PBMCs. Increased mRNA expression of IL-12 in PBMCs at 4, 24 and 48 hours, post transfection.*

IFN γ mRNA level was also upregulated at all the time interval of observation with increase in mean fold change at 4 (2.994-fold), 24 (7.619-fold) and 48 (20.61-fold) hours. The mRNA expression at 48 hours was significantly the highest among all. The multiple comparison revealed p values of >0.9999 at 4, 0.2045 at 24 hours and 0.0002 at 48 hours; and 95% CI "-11.24 to 7.451" at 4, "-15.80 to 2.561" at 24 & "-28.79 to -10.43" at 48 hours.

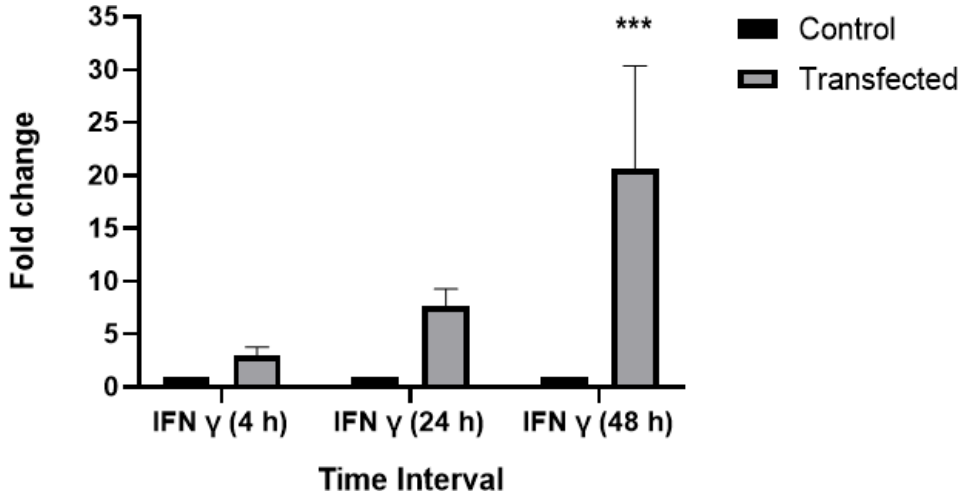


*Figure 5.14. Fold change in mRNA levels of IFN γ at 4, 24 and 48 hours in control and transfected PBMCs. Increased mRNA expression of IFN γ in PBMCs at 4, 24 and 48 hours, post transfection.*

Relating fold increase in both IL-12 and IFN γ mRNA levels, the highest significance was attributed IFN γ mRNA level at 48 hours (p value: <0.001; 95% CI -27.29 to -8.602).

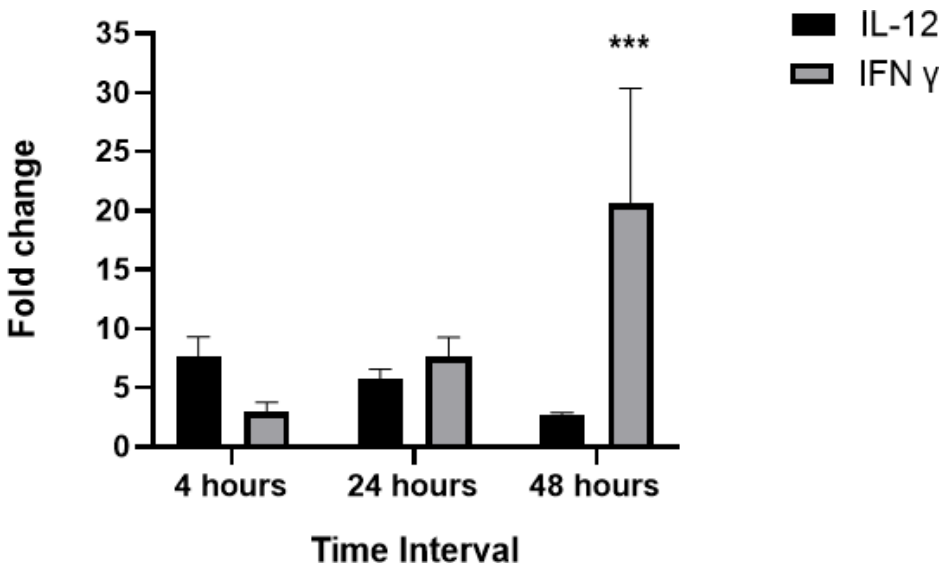


*Figure 5.15. Fold change in mRNA levels of IL-12 and IFN γ at 4, 24 and 48 hours in control and transfected PBMCs. Upregulation of IL-12 and IFN γ in PBMCs post transfection at transcriptional level.*

# Discussion

Control and prevention of a disease has always been considered than its treatment. Vaccine plays a key role in preventing and controlling an infectious disease. As in the case of viral diseases, due to the limited available anti-viral drugs and their wide range of side effects, immunization decrease the burden of outbreaks of viral diseases worldwide. However, the development of a vaccine is lengthy, expensive, and labour intensive not only in industries but in research laboratories as well. During the recent advancements in the field bioinformatics computer tools have astounded the process of designing a vaccine. The vaccines entered commercial market, developed by the approach of reverse vaccinology are not only against infectious diseases (Moncunill et al., 2020; Pack & Peters, 2022) but cancer and chronic diseases such as multiple sclerosis (PhD, 2022) and diabetes mellitus (Desai et al., n.d.).

Considering Dengue, a global threat, several attempts on reverse vaccinology for DENV have been reported. There is only one licensed anti-dengue vaccine available, Dengvaxia. The drawbacks of vaccine include less efficacy due to different serotypes, and its approval to use in just 20 countries for only seropositive people against DENV. There are 5 other anti-dengue vaccines at different stages of clinical trials. All of these 6 vaccine formulations are tetravalent, composed of membrane and envelope proteins (Wollner & Richner, 2021). There is still an unmet need of an effective vaccine for the eradication of fatal Dengue virus. To avoid time-intensive approach of developing a potent vaccine against all serotypes, (Islam et al., 2020) presented a multiple epitope for potential vaccine candidate against all the serotypes of DENV.

The epitopes retrieved from the immunoinformatic study have been utilized to construct an mRNA vaccine. The epitopes were from the envelope E protein, most immunogenic part of DENV. Those were selected due to high immunogenicity score and their conservancy among all the serotypes of DENV. As suggested in the immunoinformatic study, the CTL epitopes were linked with GGGS and BCL epitopes with KK. Both Glycine/Serine and Lysine residues linkers are flexible and allows to separation and movement of functional domains in a protein (X. Chen et al., 2013).

The purpose of selecting 6 potential CTL epitopes was to develop memory of these among T cells. Those memory T cells can proliferate upon infection with any serotype of DENV into CTLs. The CTLs would then stimulate immediate and strong immune reaction by releasing IFN ɣ, perforins and granzyme to kill infected cells and ceases the spread of DENV further (Kumar et al., 2021). B cell epitopes further enhanced the potential of the vaccine because these are the regions on the antigens to which specific antibodies can bind. Subsequent to an infection after immunization with DEN vaccine these antibodies will actively participate to the clear the infection immediately by opsonisation and enhanced phagocytosis of the virions (Galanis et al., 2021).

The final fusion protein as a vaccine candidate was further fused with signal peptide sequence of human immunoglobulin kappa (Igκ) following previous studies (Hajam et al., 2020; Meyer et al., 2018). According to the studies the SP of Igκ contributed to higher protein translocation as compared to the other signal peptides. Cleavage of SP of Igκ by cellular signal peptidase has probability of 0.7861 which is a high score. The cleaved SP drives the protein to the ER to follow the secretory pathways as soon as the mRNA is translated by the ribosomes.

The untranslated regions used to flank the double strand DNA sequence of the antigens and the SP has been reported in former studies (Richner et al., 2017) as it is provided in the vectors for in vitro transcription (NCBI Accession no. MZ362875.1, MW045215.1, MW045214.1, MZ362876.1, MZ362874.1, MZ362873.1 and MZ362872.1).

The promoter used for the in vitro transcription was of the phage T7 that has been utilized in several studies. It is an 18 bp long stretch DNA sequence upstream of the sequence that has to be transcribed with a "G" at 3' end that is the first nucleotide added to in vitro transcript. Although addition of two or even three Gs is linked with highly efficient in vitro transcription but one G is also enough and necessary with a slightly decreased transcription rate (Calvopina-Chavez et al., 2022).

In vitro transcription condition was optimized by incubating the reaction overnight. It enabled T7 RNA polymerase binding with continuous transcription in the presence of abundant rNTPs.

Stability and improved half-life of the mRNA was conferred by addition of cap and poly A tail. Cap was provided by means of ARCA during the transcription. In ARCA N-methyl guanosine ribose is either methylated or deoxygenated at 3' hydroxyl group. The use of ARCA is advantageous, as it is inexpensive and prevents reverse incorporation over enzymatic capping and cap analogues, respectively (Muttach et al., 2017).

Base analogs such as pseudo uridine, 5-methylcytidine, $N^6$-methyladenosine & others are used to further enhance the stability of the mRNA. Foreign mRNA being self-immunogenic and recognizable by innate immune's toll like receptors (TLR 3, 7 &8) results in deprivation and less yield of the protein it encodes. To reduce its vulnerability to the

immune system base analogs are the desirable addition in its modification (Ouranidis et al., 2021). Due to financial constraints the replacement of normal bases with base analogs couldn't be achieved in the development of DENV mRNA vaccine.

The assessment of the IVT mRNA quality and quantity revealed a bright thick band above 600 bp due to addition of tail with a concentration of 1365.30 ng/µl.

Tailing the IVT mRNA is one of the primitive and crucial step for the modification of mRNA. Tailed IVT mRNA (b/w 600-700 bp) appeared thicker and 50-100 bp above the untailed IVT mRNA (b/w 500-600 bp).

PBMCs were the choice to evaluate the immunogenicity of the IVT mRNA as a vaccine candidate. The in vitro PBMC culture replicates the natural environment in responding a vaccine, as PBMCs comprised of mostly lymphocytes and monocytes. The monocytes are differentiated to dendritic cells with an antigen stimulus and presents the antigens to the T lymphocytes, as APCs. Taking advantage of it, a mixed cell population was successfully cultured. The PBMCs appear as a thread like structure after the density gradient centrifugation at the LSM™ and plasma interface. The PBMCs culture appeared free of RBC and platelets contamination light microscope.

A square wave pulse of 5 ms at 500 V was slightly decrease to a final voltage of 590 V in control and 587 V in cells transfected with the IVT mRNA, respectively. The decrease is the consequence of resistance of the cells and the capacitance of the electroporator. An ideal electroporation is when the final voltage is similar to the voltage applied. In the present case both electroporation resulted in only 2% approximately which makes it closed to the ideal situation.

The initial indication of successful transfection after 48 hours, is the higher confluence of the transfected PBMCs than that of control PBMCs. The higher cell concentration was further proved by MTT cell viability assay. The percentage of proliferation transfected PBMCs was 32% significantly higher than the control ones. The high proliferation is attributed to the lymphocytes as lymphocytes are in majority among the population of PBMCs (Gonçalves et al., 2015). Rationally, they proliferated with stimulation by DENV antigens encoded in the IVT mRNA. Whereas the control cells lacked the antigen stimulus, so the concentration of the viable cells was less.

The immunogenicity of vaccines is tested by evaluation of antigen specific antibodies and IFN γ producing T lymphocytes by ELISA (enzyme-linked immunosorbent assay) and ELISPOT (Enzyme-linked immunosorbent spot) techniques respectively. This primitive and standard technique couldn't be implemented, again due to small budget and shortage of time. Evaluation of IFN γ producing T lymphocytes by ELISPOT also highlights the role of T helper 1 (TH1) immune response in immunization by vaccines. Thus, immunogenicity was determined by the assessment of mRNA levels of cytokines involved in the TH1 immune response, alternatively. When dendritic cells encounter endogenous antigens they secret IL-12 which is the major cytokine to TH1 immune response. It is a bridge to connect innate immunity with adaptive immunity by pleiotropic effect. Antigen stimulus by vaccines also induces the production of IL-12 which in turn activates naïve T cells to proliferate and differentiate into TH1 cells. Further it also promotes the production of IFN γ by antigen specific $CD8^+$ cells (Zheng et al., 2016). IFN γ is again a pleiotropic cytokine and acts on both innate and adaptive immune cells. It enhances proliferation of memory $CD8^+$ and their differentiation into cytotoxic T lymphocytes CTL, promotes

natural killer T cells and macrophages for improved phagocytosis (Jackson et al., 2011). Briefly both cytokines are the key components of TH1 cytokines which are produced abundantly in the course of vaccination.

The qRT-PCR results of mRNA expression of the two cytokines also turned in the favour of the IVT mRNA as potential vaccine candidate. Both the mRNA expressions of IL-12 and IFN γ were upregulated in transfected PBMCs as compared to that of control PBMCs. The increase in fold change of IL-12 mRNA expression in transfected PBMCs was highest at 4-hour interval with gradual decrease, down to 48 hours. On the contrary, the increase in fold change of IFN ɣ mRNA expression in transfected PBMCs was lowest at 4-hour interval with sharp increase, at 48 hours interval. The difference in the time period of upregulated genes could be innate immune response is more active during the earlier encounter of antigens up to 48 hours (Bedoui et al., 2016). Whereas adaptive immune response appears later than 48-96 hours post infection/vaccination. As a consequence, IL-12 being a dominant innate immune cytokine gradually decreased after 48 hours but IFN γ increased after that.

# Conclusion

The present study concludes that IVT mRNA containing multiple epitopes from envelope of DENV has a potential to be a virtuous vaccine candidate. The higher levels of TH1 response inducer, IL-12 and TH1 cytokine IFN γ mRNA proves the immunogenicity of the antigens expressed by the IVT mRNA.

The current research has some limitations which can be overcome by enduring the research to achieve its prospects. Beginning with evaluation of in vitro antigen expression by IVT mRNA either by western blotting or flow cytometry is crucial to determine the exact amount of antigens. Furthermore, for enhanced antigen yield different signal peptides can be incorporated other than Igκ SP e.g. SP of human immunoglobulin E (IgE), Japanese encephalitis virus (JEV) or tissue plasminogen activator which also have been reported to server the purpose (Richner et al., 2017; Wollner et al., 2021).

Following the standard preclinical study of a vaccine, evaluation of antigen specific antibodies and IFN γ producing T lymphocytes by ELISA and ELISPOT techniques, respectively is mandatory to supplement the research. The in vitro primary culture of PBMCs can be used to isolate B cells and IFN γ producing T lymphocytes by fluorescence activating cell sorting (FACS). Eventually, transfection of B and T cells, separately with IVT mRNA coding for DENV antigens would be implemented. (Tapia-Calle et al., 2019).

As the IVT mRNA constructed currently contains all the consensus conserved domains across all the DENV serotypes, conformation of eliminated or reduced risk of ADE should be assessed. Antibody dependent enhancement assay can be analysed in both in-vivo or in-vitro infected animal model or PBMCs, post immunization, respectively,

with all serovars of DENV. The same protocol would be useful in evaluating the impact of the vaccine on viral load by both qRT-PCR and plaque reduction neutralization assay.

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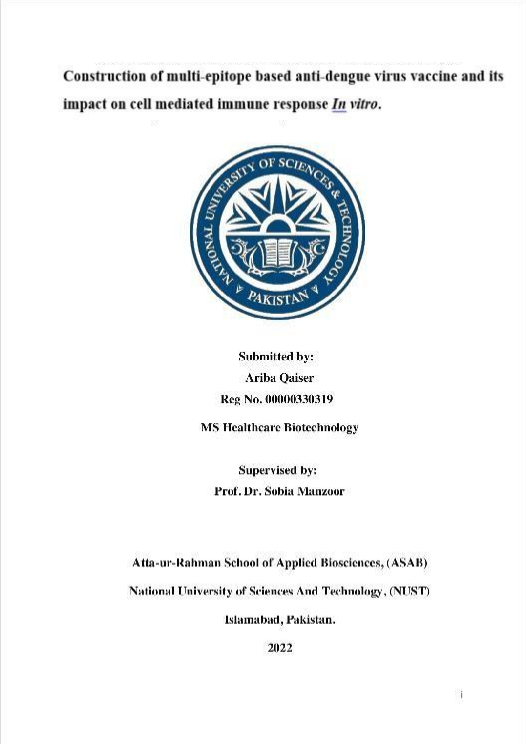

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2022

# Construction of multi-epitope based anti-dengue virus vaccine and its impact on cell mediated immune response *In vitro.*

*by* Ariba Qaiser

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