RECOMBINANT VIRAL PARTICLE PROTEIN EXPRESSION AGAINST DENGUE VIRUS SEROTYPE-1

M.Sc. Thesis

2024

Submitted To

Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

For Partial Fulfillment of the Master's Degree in Biotechnology

Submitted By

Smita Shrestha
Roll No.: BT 617/075
T.U. Regd. No.: 5-2-0282-0178-2013

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Date: 18 April 2024

Recommendation

This is to certify that the research work entitled "**RECOMBINANT VIRAL PARTICLE PROTEIN EXPRESSION AGAINST DENGUE VIRUS SEROTYPE-1"** has been carried out by **Ms. Smita Shrestha** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is her original findings. We hereby recommend this thesis for final evaluation.

……. ……..

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Certificate of Evaluation

This is to certify that the thesis entitled "**RECOMBINANT VIRAL PARTICLE PROTEIN EXPRESSION AGAINST DENGUE VIRUS SEROTYPE-1"** presented to evaluation committee by **Ms. Smita Shrestha** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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Acknowledgment

My longest never-ending thesis is finally here to an end. Pinning down this section gives me immense pleasure and I am full of smiles. This journey would never have been possible without the support and guidance of these names. Meeting them and learning valuable lab and life experience has been an absolute privilege.

Firstly, I would like to express my deepest gratitude to my supervisor **Prof. Dr. Krishna Das Manandhar** and head of Central department of Biotechnology, Tribhuvan University for granting me the opportunity to conduct thesis in his laboratory. Your supportive nature fostered a good working environment. Your insightful feedback, guidance, and patient encouragement helped me strive for the best in every aspect of this thesis.

I am especially grateful for my co-supervisor, **Dr. Rajindra Napit** for entrusting me with this project. Your profound expertise, constructive criticism, and candid feedback has been instrumental in shaping this thesis. I am forever grateful for dedicating your valuable time and effort in mentoring me. Your encouraging words were the source of motivation during challenging times.

I would like to express my sincere thanks to **Mr. Ajit Poudel** and **Ms. Roji Raut**, who have been an integral part of VLP project support team. Your immense guidance throughout the project has been invaluable. Your unwavering support and mentorship have been key for completion of this thesis. Your technical guidance and encouragement will forever be cherished. I cannot forget to mention the love and encouragement I received from **Prof. Dr. Rajani Malla** and **Dr. Jarina Joshi**. During the most chaotic period of my life, the gift of the holy Gita from Prof. Malla, has brought me peace. I aspire to pass on the soulful teachings of Prof. Malla and the honesty of Jarina ma'am to my fellow juniors.

I am indebted to my seniors **Dr. Eans Tara Tuladhar** and **Dr. Aunji Pradhan** for their mentorship, love, and generosity. You have always been there to help me with my lab work or any of my personal issues. I've enjoyed working in the lab during your

presence. The trip to Chandigarh with Aunji didi and lunch hour with Eans ma'am will always be a fond memory.

I am deeply appreciative of the **University Grant Commission** for awarding me with master's Thesis Research Grant. The grant has served as a significant source of motivation for my research endeavors. The national priority research grant provided by **Research coordination and development council (RDRC), Tribhuvan University** also played a significant role for continuation of the project.

My heartfelt thanks go out to my lab mates **Binod Khadka, Lata Karki, Mamata Tamang, Sangharsika Chaudhary, Monika Rana and Sulochana Khanal** for making the lab a fun place to work. I would also like to thank my classmates who assisted me during my graduate journey.

I would like to extend gratitude to all my teachers and staff members of the central department of Biotechnology, Tribhuvan university for their support and encouragement throughout my thesis period.

I am forever grateful to my friends and family for their unconditional love and unwavering support. The silly talks, laughter and emotional support brought my painstaking ride to ease. Lastly, I would like to thank a higher power that aligned all the dots together that made this journey possible.

Glossary Acronyms

ADE	Antibody dependent enhancement
APC	Antigen presenting cell
C	Capsid protein
CAG	Chicken beta-actin promoter
DHF	Dengue hemorrhagic fever
DENV1 – 4	Dengue serotype 1, 2, 3, 4
DSS	Dengue shock syndrome
DV1	Dengue Vector type-1 plasmid
DENV	Dengue virus
DNA	Deoxy-ribonucleic acid
E	Envelope protein
ELISA	Enzyme linked immunosorbent assay
M	Membrane protein
NS	Non-structural proteins
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PrM	Pre-membrane protein
RNA	Ribo nucleic acid
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
TEM	Transmission electron microscopy
UTR	Untranslated regions
VLP	Virus like particle

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ABSTRACT

The global expansion of Dengue due to increasing habitat range of Aedes mosquitoes underscore a dire need for the development of safe and effective vaccine. The failure of current vaccines to protect against severe Dengue of all serotypes highlights the necessity to explore alternate vaccine approaches. Dengue virus-like particle (VLP) offers a promising substitute to traditional live-attenuated vaccines. VLPs are generated during the course of natural infection. These entities lack genetic materials but mirror the structure of natural infectious viruses. The toll-like receptors recognize VLPs, prompting the production of neutralizing antibodies. We were able to transiently produce a non -infectious Dengue-1 VLP in a mammalian cell using plasmid DNA. Under the control of CAG promoter, the plasmid encodes prM and E structural proteins of the virus. Gene expression in post-transfected cells was assessed through RNA transcript analysis and microscopic evaluation of self-assembling VLP. In addition, transmission electron microscopy was utilized to examine the structural features of secreted VLPs in culture supernatant. Semi-quantitative amplification of promoter gene along with microscopy demonstrated that the transfected cells were able to transiently transcribe plasmid DNA to mRNA, further translating resulting in expression of E proteins that accumulated near the cell membrane. Electron microscopy identified the presence of electron-dense spherical particles measuring approx. 59 nm diameter, closely resembling the structural characteristics of mature wildtype Dengue-1 virus. Thus, the findings indicate that DENV-1 VLP can be produced using mammalian cells which provides an optimum environment for structural protein of virus to self-assemble into mature particles. These VLPs functioning as an antigen display platform needs to be further explored for vaccine development and as a serodiagnosis tool.

Keywords: Dengue, VLP, CAG promoter, transfection, PEG purification, VLP vaccines

Chapter I

INTRODUCTION

1.1 Dengue an overview

Dengue is an acute febrile disease, caused by Dengue virus (DENV). The virus belongs to the family *Flaviviridae*, of the genus *Flavivirus*, that consists of four genetically distinct, but closely related serotypes (DENV1, DENV2, DENV3, and DENV4). All four serotypes share 60-70% sequence homology and are equally capable of provoking the disease (Islam et al., 2020; Tuiskunen Bäck & Lundkvist, 2013). Globally, Dengue is the most common cause of arboviral disease; transmitted to humans by the bite of infected female mosquito of genus *Aedes aegypti* and less frequently by *Aedes albopictus* (Puschnik et al., 2013).

The four serotypes of DENV are responsible for wide range of clinical manifestation of the disease that ranges from asymptomatic cases, mild Dengue fever (also called as classical Dengue fever) to life threatening severe infection characterized as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (Rothman, 2004). After the incubation period of 3-15 days, the classical Dengue fever begins with abrupt onset of fever, headache, retro-orbital pain, myalgia, arthralgia, macular popular rashes, and leucopenia. The disease is usually self-limiting but sometimes progresses into hemorrhagic manifestation, plasma leakage, thrombocytopenia, and shock (Tuiskunen Bäck & Lundkvist, 2013).

In epidemic areas, multiple infections with different serotypes have been documented in a single individual. Infection with one serotype provides long term immunity of a year to the same serotype. A secondary Dengue with a different serotype increases the risk of sever Dengue due to interplay of partial antibodies developed during previous infection which enhances viral replication and cytokine storm. Secondary infection with a different DENV serotype increases the risk of severe forms of Dengue involving shock, severe bleeding, organ failure that requires urgent medical attention

(Urakami Akane, 2017). The fatality rate ranges from 0.3% to 2.6% between countries (Malavige et al., 2022).

While there are no definitive curative medications available for Dengue, the treatments are usually supportive in nature. Timely diagnosis of cases, recognition of warning signs for severe Dengue and proper clinical management plays crucial role to reduce the risk of Dengue fatalities. Absence of specific treatment for Dengue highlights the importance of Dengue prevention through vector management and vaccination. More than 90% of arboviral diseases including Dengue can be effectively controlled through vector population suppression (Ogunlade et al., 2023). However, the evolving nature of Aedes mosquitoes to resist insecticides and climatic barriers, emphasizes the necessity of actively immunizing the population through vaccination.

1.2 Epidemiology of Dengue virus

Dengue fever was first reported in 1779 as arthritic fever from Jakarta, Indonesia. Epidemics resulting from severe forms of Dengue DHF and DSS were only reported in 1950s from Southeast Asia. Dengue prevails mostly in tropical and subtropical regions of the world. Dengue infection gradually evolved and expanded in geographical distribution with time due to factors such as urbanization, deforestation, migration, climatic change that caused increased temperature, humidity, rainfall (Malavige et al., 2022). In addition, a usual than longer seasonal Dengue epidemic has been documented by several countries.

Global mosquito distribution map revealed *Aedes* mosquitoes are now found across all continents thus placing more than half of world's population at risk of Dengue infection. Dengue is now endemic in more than 100 countries. The disease is poised to expand even further in terms of geographical distribution as the habitat for the Dengue vector expands (Wilder-Smith, 2019). In the last two decades, WHO has reported over 18-fold increase in the number of Dengue cases. It has been estimated that more than 6.1 billion people will be affected by 2080 (Wu et al., 2022).

Dengue transmission is cyclic and large outbreaks are observed every 3-4 years. A drastic increase in cases was reported in 2019 from all around the world. An upsurge in Dengue cases was seen again globally in 2023. Cases were reported from countries

previously unaffected. Over 5 million cases and more than 50,000 deaths were documented from Africa, America, South-East Asia, Western Pacific, and Eastern Mediterranean regions. However, these numbers underestimate the true burden of Dengue as most of the primary infections do not require hospitalizations and goes unreported (WHO, 2023).

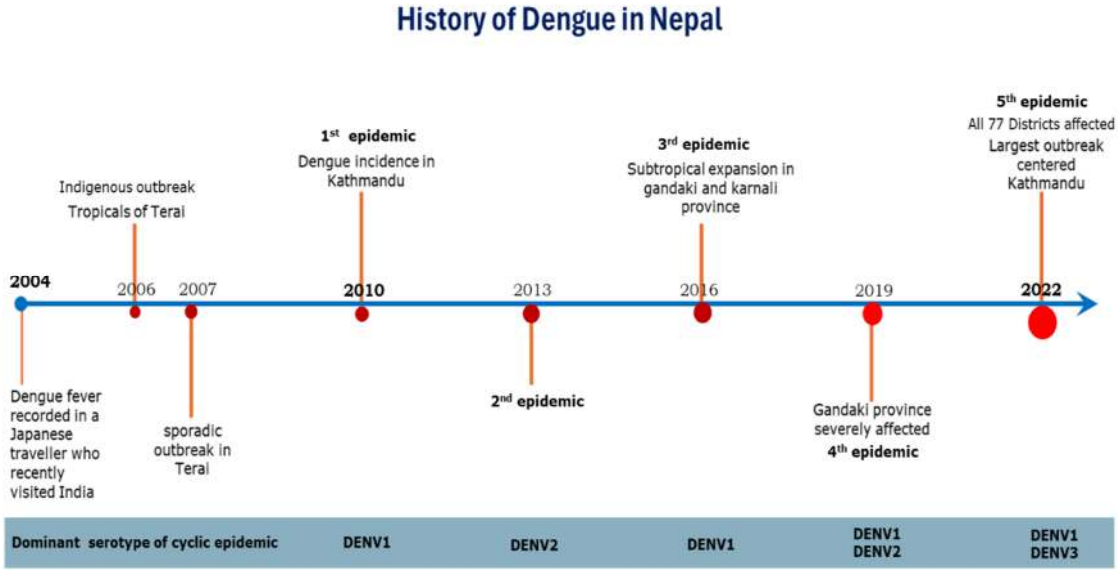

Figure 1: Timeline showing key events of Dengue outbreak in Nepal (data source: EDCD, 2023; Rijal et al., 2021).

Over the last 20-year, Dengue fever has emerged as the most important mosquito borne viral infection in Nepal. During this period, Dengue infection initially clustered in tropical of Terai, has now been reported from all geographical regions, despite climatic barriers. Dengue infection was first reported back in 2004 when a Japanese traveler visited Nepal after his recent tour to India. He was later diagnosed with DENV-2 infection on his journey back home (*Dengue - Nepal*, 2023). In 2003, India saw a huge spike of dengue cases with incidence of all four serotypes (Gupta et al., 2006). The following year, Dengue was reported in Nepal. In the pre-epidemic era, all four serotypes were also discovered in Nepal (Rauniyar et al., 2023; Rijal et al., 2021).

The epidemic outbreak of Dengue started in the second half of 2010. Since then, the country is known to have a periodic three-year cyclic outbreak. Till now the country has experienced five dengue epidemics with each outbreak getting larger in terms of cases, severity, and geographical expansion than the previous *(fig. 1)*. After the

introduction of all four serotypes, DENV-1 and DENV-2 began their cyclic dominance switching between them for four cyclic epidemic years till 2019. The 2016 epidemic was solely caused by DENV-2 (Pokharel et al., 2023). A switch in dominant serotype and disease severity was observed in 2022 outbreak with co-dominance of DENV-1 and DENV-3. The 2022 epidemic was largest in the 20-year long Dengue history of Nepal. The Kathmandu valley was severely hit. Dengue cases was reported from all climatic zones of Nepal including the Himalayan districts of Mustang (*EDCD*, 2023).

1.3 Dengue structure and genome

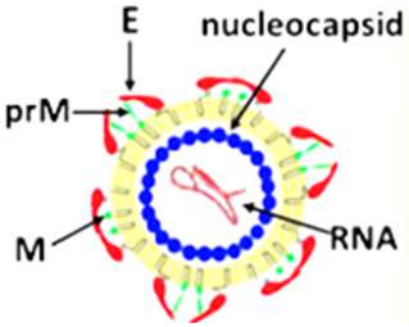

Figure 2. Dengue structural organization (Shang et al., 2012).

The genus Flavivirus includes well-known human pathogens: Yellow fever virus (YFV), Dengue fever virus (DENV), West nile virus (WNV), Japanese encephalitis virus (JEV) (Urakami Akane, 2017). Flavivirus are enveloped viruses with around 11 kb single stranded RNA genome that consists of a single open reading frame *(Fig. 2)*. The genome encodes a single long polyprotein, which is subsequently processed by viral and host protease to produce 10 distinct structural and non-structural proteins (*Fig. 3).*

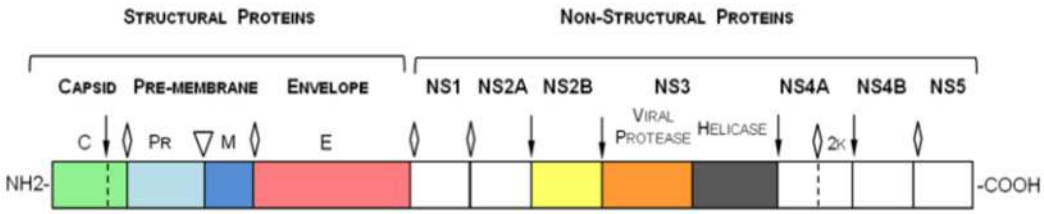

Figure 3. Dengue polyprotein (Boigard et al., 2018).

The 3 structural proteins (capsid, membrane, and envelope) are part of mature virus, and are not involved in replication of the genome, while the non-structural proteins

are required for replication, translation, and regulation (Liu et al., 2016). The E antigen is the major antigen responsible for eliciting antibody response, in addition, the protein plays a crucial role in virus-host interaction. The E protein is found to be conserved in flavivirus and requires co-expression of prM to achieve its native conformation; most likely prM functions as chaperon for E during viral assembly (Li et al., 2013).

1.4 Scenario of Dengue vaccines

There are several Dengue vaccine candidates in the developmental pipeline, including recombinant live attenuated vaccine, protein subunit vaccine, VLP vaccine, and DNA vaccine. But only two live attenuated vaccines have passed clinical trials and successfully obtained license *(Table: 1).*

Table 1. Overview of Dengue vaccines (Torres-Flores et al., 2022)

Vaccine	Manufacturer	Platform	Phase	Design	Efficacy	Comments
Dengvaxia®	Sanofi Pasteur	LAV	Licensed (2016)	YFV backbone/ DENV1-4	65.6 % over 9 years old	Increases hospitalization in seronegative vaccinees
Qdenga®	Takeda	LAV	licensed (2023)	Attenuated DENV2 backbone/ DENV 1,3,4	73.3-85.3 %	Well-tolerated in adolescents and children
LATV (TV003/005)	NIAD/Merck		phase III	DENV1,3,4 and rDENV2/4	not released	not released
V180	Merck/NIAID	Subunit	phase I	-	not released	not released
DENV-1-LVHC	-	LAV	phase I	-	not released	not released

The year 2016 marked as significant milestones as World Health Organization (WHO) granted approval for the world's first Dengue vaccine, Dengvaxia®. The vaccine was able to induce balanced immune response against all four serotypes with an overall efficacy of 60.3% during the first year of immunization. However, long-term safety studies revealed variable efficacy based on age as well as elevated risk of severe

Dengue and hospitalization among Dengue seronegative individuals and children under age nine (Urakami et al., 2017). A second tetravalent vaccine, Qdenga® was approved at the end of 2023 by WHO for use in countries with high incidence of Dengue. Phase 3 clinical trial data highlighted an increased risk of DENV 3 and DENV 4 infections among seronegative individuals, suggesting inadequate antibody production against one or more serotypes (Thoresen et al., 2023).

The development of safe vaccine against Dengue is a major challenge because of the presence of four closely related serotypes that co-circulates in endemic areas. The limitations of live attenuated vaccines to protect against all age group irrespective of Dengue serostatus opens opportunities to investigate alternative vaccine approaches.

1.5 Alternate vaccine approach

Virus particles that mimic the replication and antigenic structure of authentic virus while being devoid of the viral genome has been utilized as a safe alternative vaccine approach against many infectious diseases. The success of Virus like particles vaccines to prevent malaria, hepatitis B, Human papilloma infections highlights the significance of exploring VLP platform in the fight against Dengue.

1.5.1 Virus like particle

Virus like particles (VLPs) are naturally occurring entities that lack genetic materials but possess inherent capacity to self-assemble into viral structures in sole presence of structural proteins. They are also capable of triggering immune response as they morphologically resemble authentic infectious viruses (Urakami Akane, 2017). The natural existence of VLP was first identified in 1960 as empty capsid protein of Hepatitis B virus (HBV). In subsequent years, the relationship between host immune system and VLP was characterized and its wide range biomedical applications as vaccine, nanomaterial, targeted drug delivery, genetic therapy and cancer treatment was studied (Yan et al., 2015). Many VLPs based vaccines are now commercially available against infections of HBV (GlaxoSmithKline Engerix®, Recombivax HB®); Human Papilloma Virus (Cervarix®, Gardasil®, Gardasil9®). Lately, the first VLP based malaria vaccine MosqurixTM® has been approved by European regulators. Several

other VLP based vaccines against Influenza, Chikungunya, HIV, Zika, JEV are under preclinical and clinical development (Kushnir et al., 2012a; Mohsen et al., 2017).

In Flaviviruses, the structural proteins (C, prM, and E) are able to assemble into VLPs even in the absence of other non-structural proteins. These particles display similar fusion activity and maturation process as infectious virions. The morphological as well as physiochemical resemblance of VLPs to natural infectious virus provides them unique immunogenic advantage. Their surface epitopes are recognized by antigen presenting cells (APC), which subsequently elicit cellular and humoral immune response. Several VLP based Flavivirus vaccines against JE, Zika, WNV, chikungunya virus including Dengue are under clinical studies (Zhang et al., 2020).

VLP is an excellent platform to develop a safe, effective vaccine since they are immunogenic, non-infectious, non-replicating, and do not possess risk of mutation or recombination. Immunogenicity of VLP can be further enhanced by designing chimeric proteins that elicit heterologous antibodies. For example, the malaria vaccine, Mosqurix® was designed to enhance immunogenicity through use of Hepatitis B virus surface protein in addition to plasmodium protein (Shukla et al., 2019). The lack of genetic material required for replication makes them safer to use in young children and immuno-compromised individuals (Thoresen et al., 2023). These characteristics makes VLP based vaccines safer and more efficient in comparison to the traditional attenuated and subunit vaccine approaches.

In this study, we focused on the development of VLP for Dengue serotype 1 by using the HeLa cell line platform. A plasmid was designed consisting of DENV-1 epitopes to induce cells for transient production DENV-1 VLP. The cellular ability of production of VLPs was tracked through RNA transcript formation. Further, the study centers on purifying the VLP and explores its morphological characteristics.

1.6 Research Hypothesis

1.6.1 Null Hypothesis

The computationally designed DENV-1 VLP based plasmid construct might not lead to the expression of VLP in mammalian cell line.

1.6.2 Alternative Hypothesis

The designed DENV-1 VLP plasmid might lead to the expression of VLP in mammalian cell line which could be purified and morphologically characterized.

1.7 Research Objectives

1.7.1 General Objective

Computational construction of DENV-1 VLP based plasmid and study the production of VLP in mammalian cell line.

1.7.2 Specific Objectives

1. Identify major epitopic site of Dengue virus responsible for immune response.
2. Construct DENV-1 VLP plasmid containing the major epitopic regions.
3. Transiently express the synthesized construct in a mammalian cell line.
4. Purify the VLP formed within and released by the transiently transfected cells in the supernatant fraction.
5. Characterize the purified VLP through Transmission electron microscopy.
6. Assess the production of VLP through detection of transfected RNA transcript in the transfected cells using conventional PCR.

1.8 Rationale of the Study

Despite several efforts, a safe and effective vaccine against all serotype of Dengue has not been developed. A major challenge in Dengue vaccine development is Antibody Dependent Enhancement (ADE) mediated response, in which the non-neutralizing levels of anti-DENV antibody by previous infection enhances viral entry and cytokine

storm; thereby, increasing the severity when exposed to a different serotype. The shortcoming of licensed live attenuated vaccines to successfully elicit a balanced immune response against all serotype and overcome ADE emphasizes the need to investigate an alternative approach to generate vaccine against Dengue. Nepal, with the decade long history of Dengue epidemic, studies on the development of safe Dengue vaccine is of prime importance. Keeping this in mind, we focus our study to explore the potential of VLP as a vaccine platform for Dengue infection.

VLP offers several advantages as vaccines against emerging viruses. First, safety consideration in developing these vaccines is not necessary as they are not live viruses. Second, they can display major antigens and elicit strong humoral and cellular immune responses. The potency of VLPs in stimulating immune responses can be significantly enhanced relative to attenuated and subunit vaccines (Lua et al., 2014). Third, the lack of genetic material makes them risk free and safe to be used in immunocompromised individuals including children. (Liu et al., 2016).

Although, with the decade long knowledge of VLP potential, the production of Dengue VLP has not been satisfactory. CAG promoter is better known for the efficient production of transgene as compared to traditional CMV promoter (Dou et al., 2020). Therefore, in this study, we chose to use CAG promoter as a strong transcriptional regulator for the expression of DENV1 structural genes. In addition, we found discrepancy regarding the role of DENV1 signal peptide, some article indicated the presence of RE retention signal in prM gene that affects extracellular production of DENV1 VLP, while others suggested a stable interaction between prM and E domains leads to the extracellular production of the DENV1 VLP without the need to exchange the signal sequence (Zhang et al., 2011). The primary objective of this investigation is to assess the suitability of the CAG promoter for facilitating the extracellular secretion of DENV1 virus-like particles (VLPs). Furthermore, this study focuses on manufacturing VLPs in HeLa cells, a readily accessible mammalian cell line in Nepal. Its utilization is advantageous to produce glycosylated proteins closely mirroring those present in the host of the virus (Buffin et al., 2019).

This study aims to express a plasmid containing the structural genes of DENV1 under the control of the CAG promoter in HeLa cells via transient transfection. The primary emphasis of this research lies in evaluating gene expression levels and characterizing virus-like particles (VLPs) based on their morphological structures. Through this investigation, we aim to lay the groundwork for vaccine production tailored to the context of Nepal, thereby broadening the scope of VLP-based therapeutic interventions within the nation.

Chapter II

LITERATURE REVIEW

2.1 Dengue Overview

Dengue is the fastest spreading vector-borne viral disease affecting humans (Ramasamy et al., 2018). It is an acute febrile illness with a wide range of clinical symptoms and severity. In most cases, the disease is asymptomatic; while in some, symptoms are usually self-limiting non severe febrile illness, and classical Dengue fever. Whilst in few cases, the disease progress to life threatening Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) requiring medical intervention and urgent hospitalization (Tuiskunen Bäck & Lundkvist, 2013). The viral hemorrhagic fever is considered the most serious biological illness, caused by pathogens such as: Ebola virus, Yellow-fever virus, Lass virus, Hanta virus and others. Over 99% cases of hemorrhagic fever reported worldwide are related to Dengue hemorrhagic fever (Rothman, 2004) making Dengue virus one of the most serious threat to public health and to the global economy.

About 2-2.5% of DHF progresses to fatal Dengue shock syndrome (DSS). The mortality ranges between 1-20% depending on resources (Tuiskunen Bäck & Lundkvist, 2013). DHF is defined with four symptoms: fever, headache, thrombocytopenia (<1,00,000 platelets/mm^3), hemorrhagic manifestation, and plasma leakage in interstitial spaces (accumulation of fluids in peritoneal, plural, pericardial spaces, hypoalbuminemia, or hemoconcentration) (Ajlan et al., 2019). DSS is differentiated from DHF when cardiovascular system is compromised due to plasma leakage in interstitial spaces resulting in shock. Early symptoms of DSS includes rapid rise of haematocrit, intense abdominal pain, and persistent vomiting (Tuiskunen Bäck & Lundkvist, 2013).

Historically, Dengue prevention was largely limited to mosquito population control through the use of insecticides. Nevertheless, Aedes spp. notably *Aedes albopticus* and *Aedes agypti* have evolved resistance to commonly used insecticides such as DDT (dichlorodiphenyltrichloroethane), pyrethroids (Gómez et al., 2022). New vector control strategies such as deliberate introduction of *Wolbachia* endosymbiotic

bacterium to reduce mosquito population density and competence has resulted in significant reduction of Dengue incidence by around 95%. Despite the observed success, its efficacy drastically reduced to 80% in parts of the world with high temperature, seasonal fluctuation, and high Dengue endemicity (Ogunlade et al., 2023).

Vaccination is considered the safe and effective strategy to prevent viral diseases. The licensure of live attenuated tetravalent vaccines Dengvaxia®, and Qdenga® has given hope and opened new opportunities for the development of safe and efficacious vaccines. However, it is important to acknowledge that these live attenuated vaccines carry risk of severe Dengue in infants, and increased replication or genetic reversion in immunocompromised individuals. Safety and efficacy studies of both vaccines have revealed variable efficacy based on age and Dengue serostatus at the time of vaccination (Thoresen et al., 2023). Thus, it is crucial to explore new avenues for the development of safe vaccines that provides balanced immunity against all serotype, and targets people of all age group, including Dengue naïve individuals.

2.2 Dengue burden

Dengue incidence has increased globally across the tropical and subtropical regions. Today, Dengue is one of the most rapidly spreading mosquito-borne arboviral diseases. Annually, around 100 to 400 million cases are documented. The WHO has reported an over 18-fold increase in Dengue cases, soaring from 5,00,000 to 4.2 million between 2000 to 2022. Over half of the world's population are exposed to Dengue infection, that impacts 129 countries worldwide (*Half of World Population at Risk of Dengue Virus*, 2023).

Recent best estimates of Dengue infection suggest that 390 million cases are reported annually. Out of these cases, 96 million individuals experience symptomatic infections. Among them 2 million suffer from severe Dengue resulting in 21,000 deaths. The highest incidence of Dengue is observed in Asia, primarily affecting children aged 5 to 15 years. Dengue virus is endemic to 10 out of 11 member states of southeast Asia (*Dengue- Global Situation*, 2023).

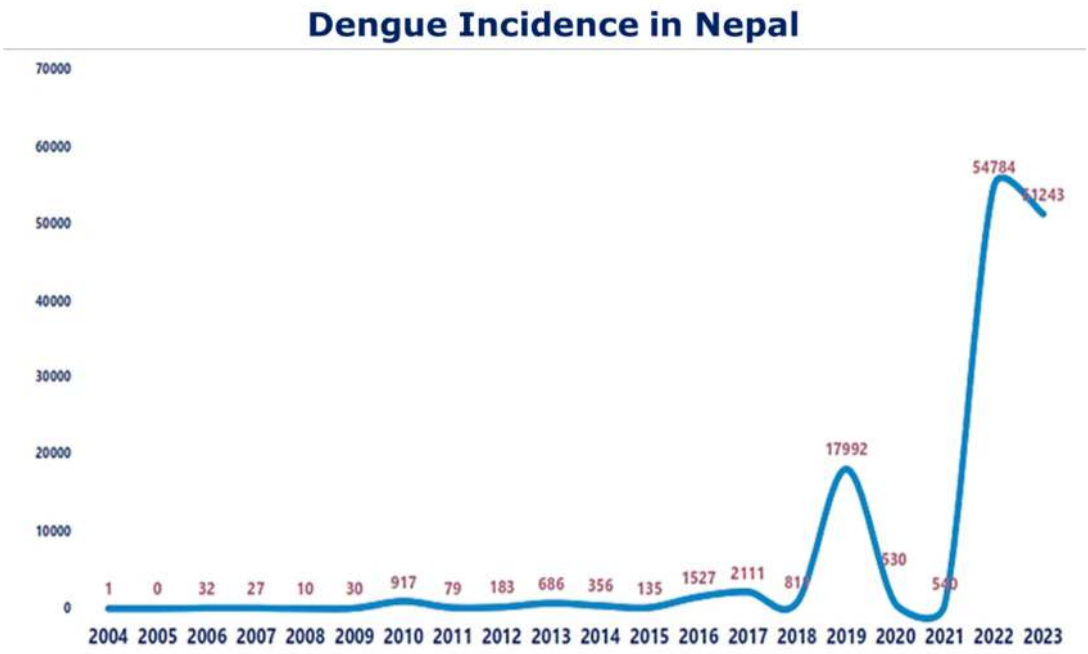

Figure 4. Trend of Dengue incidence in Nepal, 2004-2023 (EDCD, 2023).

Nepal has witnessed several epidemic outbursts of Dengue *(fig. 4)*. In the first epidemic year of 2010, a case load of 917 was recorded, which centered in the tropical region of Terai. The first incidence of Dengue in Kathmandu valley was also recorded the same year. The epidemic of 2019 documented a case load of 17,992 across all provinces. This figure was 140 times greater than the case load reported during the previous outbreak in 2016, which stood at 1,527 (Rijal et al., 2021). The year 2022 witnessed an unprecedented surge in number of cases with a total of 54,784 incidence that resulted in hospitalization of at least one member from every household in the Kathmandu valley (EDCD, 2022). Notable changes in the distribution of Dengue cases were observed during the year 2022 and 2023 (*Dengue Situation Update*, 2023). Cases initially concentrated in Kathmandu valley in 2022 shifted towards hill districts of Gandaki and Koshi province in 2023 (*Dengue- Global Situation*, 2023).

2.3 Transmission of the Dengue virus

The Dengue virus is transmitted to humans by the bite of infected female *Aedes* mosquitoes, predominantly *Aedes aegypti* and *Aedes albopictus*. These mosquitoes are prevalent in tropical and subtropical regions and are distributed around the world. Both species have been documented across Nepal, ranging from lowlands to highlands

(Dhimal et al., 2014). In recent decades, incidence of Dengue has increased by 30-fold due to expansion of *Aedes* habitat. Factors such as climate change, globalization, urbanization, and resistance to different insecticides have contributed to the geographical expansion of Dengue (Puschnik et al., 2013).

Competition between these two species leads to competitive displacement, yet they are able to co-exist in the same geographical areas. *Ae. aegypti,* with its origin in Africa is more prevalent in urban areas, while *Ae. albopictus* originating in Asia tends to dominant rural areas. These two species are successful invaders as they exhibit numerous characteristics that give them an edge over others. Their swift dissemination and ability to adapt in tropical, subtropical and temperate regions led to their global expansion, which consequently increased Dengue outbreaks (Gómez et al., 2022).

2.4 Dengue case classification

Before 2009, Dengue was classified into four major categories based on symptoms: non classical DF, classical DF, DHF, and DSS. This classical scheme was criticized for lack of correlation between the categories and lacks sensitivity to detect severe forms of Dengue that require specialized monitoring especially in clinical setting, led to new model for classifying Dengue (Ajlan et al., 2019).

The new classification model is based on the levels of Dengue severity. Here, Dengue is classified into two major categories: non-severe and severe Dengue fever *(Fig. 5).* The non-severe Dengue is further divided into two classes: Dengue without warning sign (D-W), and Dengue with warning signs (D+W). This new WHO classification model was devised by global expert consensus meeting at Geneva, Switzerland, 2008. It facilitated Dengue surveillance along with early detection of potential severe case load (Ajlan et al., 2019; WHO, 2009). The new WHO model is now used by experts as a gold standard to access the level of care required for proper management of Dengue.

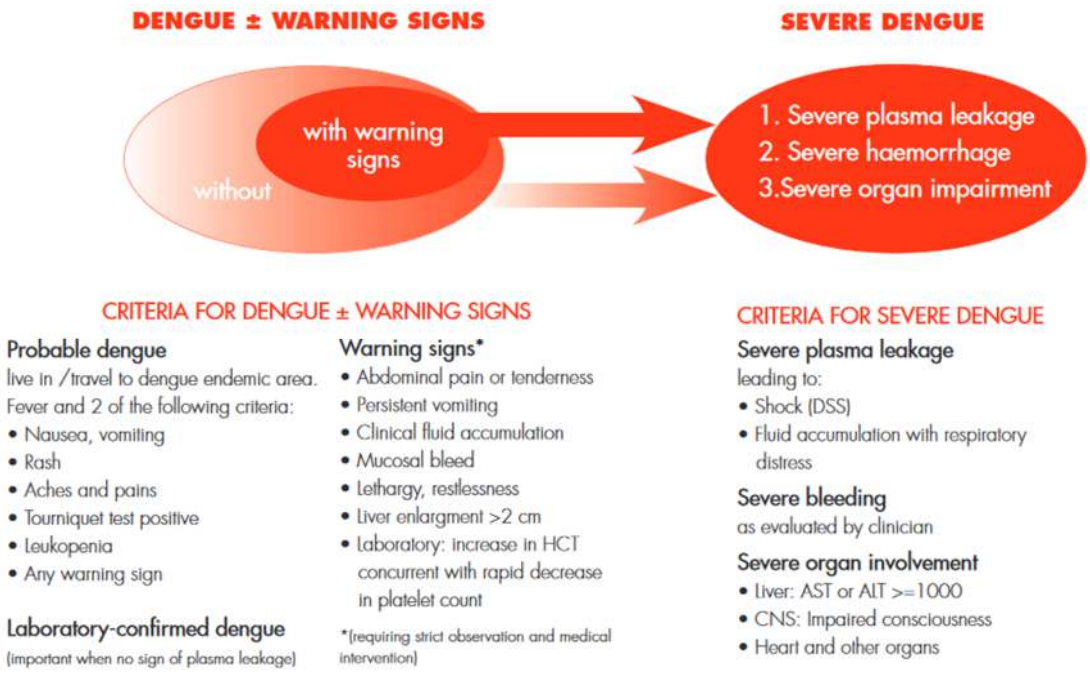

Figure 5. New World Health Organization classification of Dengue 2009 (WHO, 2009).

2.5 The Dengue virus

Dengue virus belongs to Flaviviridae family of small, enveloped viruses; within genus Flavivirus that carry single stranded RNA genome. The virus consists of four antigenically distinct serotypes designated as Dengue serotype-1 (DENV-1 to DENV-4) (Rothman, 2004). These serotypes share 60-70 % sequence homology, amid 25-40% difference in amino acid level within Envelope protein. Each serotype exhibits distinct genetic variation with 3% differences at amino acid level, giving rise to multiple genotype (Ramasamy et al., 2018).

2.6 Genome Organization

The genetic information of Dengue is present on 10.7 kb long, single stranded, positive-sense RNA. The genome has 10,700 nucleotides that encode a single polyprotein precursor of 3,411 amino acid residues *(Fig. 6)*. The genome consists of a single open reading frame flanked by 5' and 3' untranslated regions (UTR). The UTRs of all serotypes have conserved sequences with characteristic secondary structures. The 5' UTR of around 95-135 nucleotide long, contains type-1 cap, which is scanned to initiate viral RNA translation. The 3' UTR is of 114-650 nucleotide long that consist of a conserved stem-loop structure (Tuiskunen Bäck & Lundkvist, 2013).

Figure 6. Organization of Dengue genome and its resulting proteins (Rothman, 2004).

Inside the host cell, the precursor polyprotein is processed by viral and cellular proteases that generate three structural proteins; capsid (C), pre-membrane (prM) and envelope (E); and seven non-structural (NS) proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Galula et al., 2014; Rothman, 2004). The mature virus particle comprises solely of structural protein, whereas the NS proteins are specifically expressed within the infected cells and help in replication and translation of viral genome. It is important to note that the structural proteins do not play a role in replication of viral genome (Tuiskunen Bäck & Lundkvist, 2013).

2.7 Dengue structure:

The mature form of Dengue is a small spherical structure with a diameter of around 500 Å *(Fig. 7)*. The virus is covered with dense icosahedral structures of Envelope proteins. The central RNA genome is surrounded by a nucleocapsid core containing capsid protein (C). This core is enveloped by lipid bilayer derived from endoplasmic reticulum of the host cell. Outside of envelope is embedded in E glycoproteins arranged in herringbone pattern and M protein. (Shang et al., 2012; Tuiskunen Bäck & Lundkvist, 2013; Zhang et al., 2003).

The E glycoprotein exists as homodimer, which mediates two crucial functions in the viral lifecycle: viral attachment and fusion with host cell. Also, the protein harbors important epitopes that elicit neutralizing antibodies against the virus, making it an ideal target for therapeutic development.

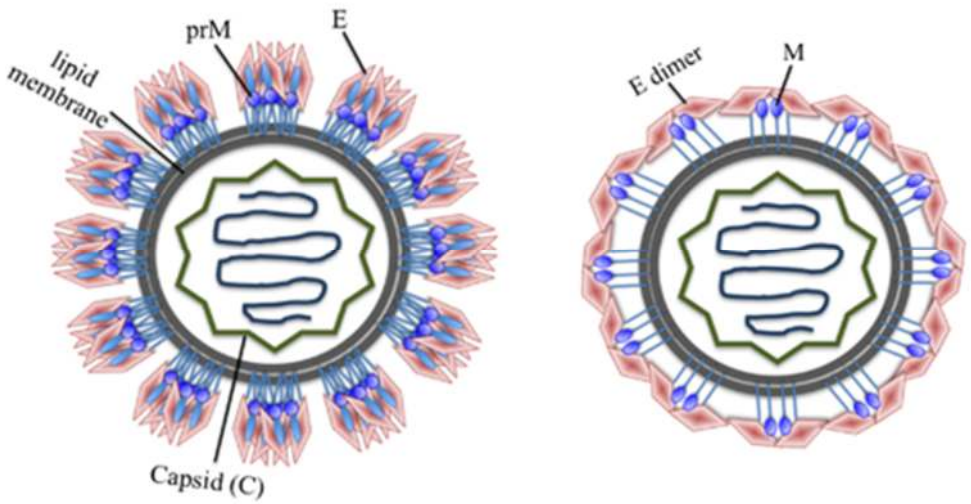

Figure7. Schematic representation of immature (left) and mature (right) Flavivirus particle (Zhang et al., 2020).

The protein is an elongated structure formed primarily with β-strands which folds into three structurally distinct components referred to as domain I, domain II and domain III that are connected with flexible linkers *(Fig. 8).* Domain III is an immunoglobulin-like structure responsible for interaction with cellular receptor. The Domain III is connected with Domain II through central Domain I. Domain II harbors two extended loops called as fusion loops, at its distal end needed to coordinate antiparallel arrangement and dimerization of E protein (Galula et al., 2014; Pierson & Kielian, 2013; Tuiskunen Bäck & Lundkvist, 2013).

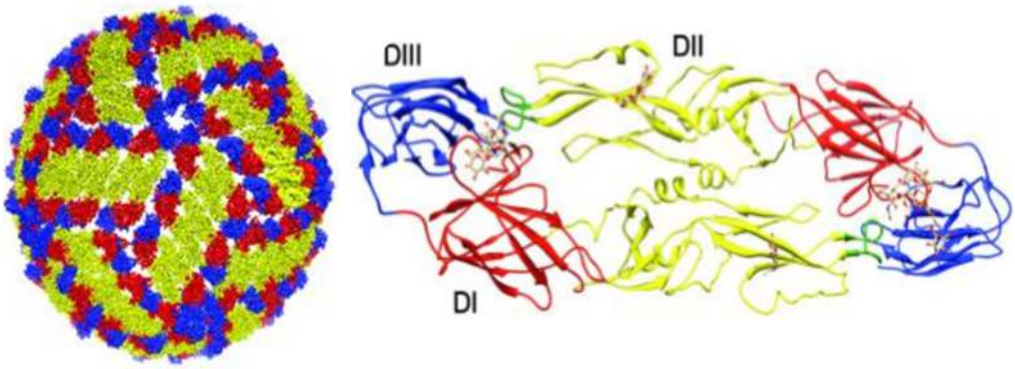

Figure 8. Arrangement of E protein on mature Dengue virus (left). Structure of E protein shown as ribbon diagram (right). Domains I, II, and III are represented in red, yellow, and blue respectively. DII fusion loops are colored green (Pierson & Kielian, 2013).

2.8 Dengue virus lifecycle

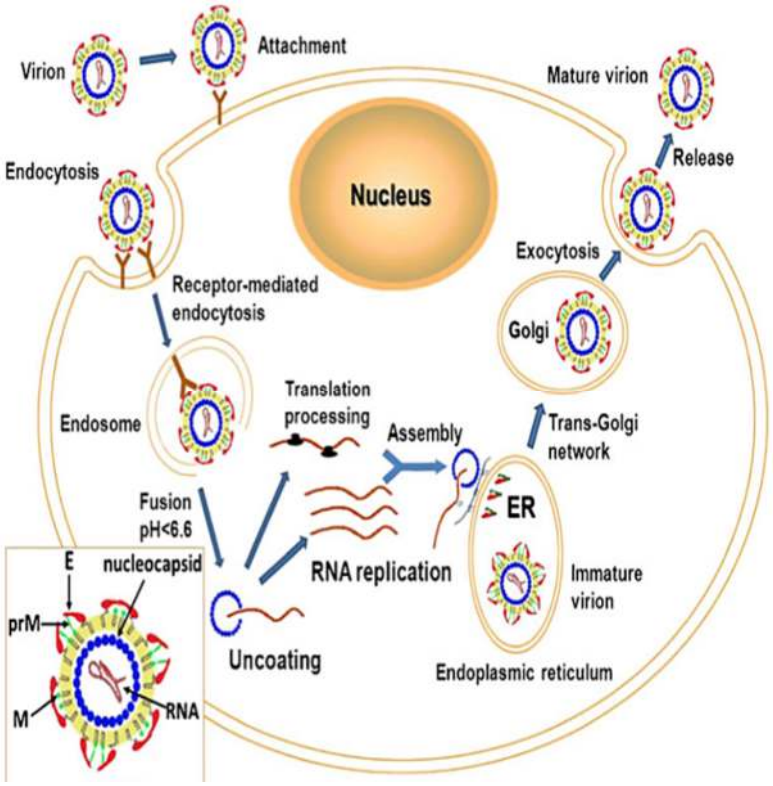

Figure 9. Dengue replication cycle (Shang et al., 2012).

The virus enters host cells through E protein mediated endocytosis. Major cellular receptors involved are C-type lectin-like molecule (CLEC5A), glycoproteins, dendritic cell specific adhesion molecules and mannose receptor. The fusion peptide present in domain III of E protein is responsible for receptor-binding activity; low pH of host induces trimerization of E protein which exposes normally unavailable hydrophobic fusion peptide for receptor binding, leading to internalization of virus (Tuiskunen Bäck & Lundkvist, 2013)

Upon internalization, acidic pH of endosome enables E protein to mediate membrane fusion of the virus and endosome *(fig. 9)*. This results in the release of viral nucleocapsid into cytoplasm, which in turn releases viral genome. The input positive-strand viral RNA is directly translated into a single polyprotein, which is cleaved into structural and NS protein. The input strand further synthesizes negative strand intermediate, which serves as a template for new positive strand RNA to translate higher level of viral proteins. The progeny viral proteins accumulate nearby endoplasmic reticulum (ER) by forming cytosol vesicle packets. The C-terminal region

of C, prM and E serves as signal sequences for post-translational processing of viral proteins inside ER membrane. The ER signal peptidase in conjugation with viral NS2B-NS3 protease, cleaves structural and NS proteins, to form distinct proteins. This immature virus particles are transported in Trans-Golgi network, where E proteins are glycosylated. Here, prM peptide is also processed into mature M protein by host furin protease. The maturation event of prM occurs late in viral post-translation because prM is known to protect E protein from pH induced reorganization to avoid premature fusion. Cleavage of prM is a prerequisite for acquisition of infectivity, as pr portion of prM functions as mechanical barrier to protect the fusion loop of E glycoprotein. Finally the mature virus releases through cellular budding (Shang et al., 2012; Tuiskunen Bäck & Lundkvist, 2013).

The formation of mature, partially mature, and immature viruses is a natural event. Viruses released from host cells are heterogenous. More than 90% of released virus retains an uncleaved prM, making the virion immature (Pierson & Kielian, 2013). Sometimes, the partial cleavage of prM can generate a third population of virion called as partially mature virus. The complete prM cleavage generates mature particles which are smooth surface with diameter of about 50 nm. During transport of spiky immature particles from ER to trans-golgi network, prM peptide undergoes furin cleavage that results Pr and M proteins. This cleavage also contributes to conformational rearrangement of E protein from trimer to dimer, which turns the virus into smooth mature particles before released from infected cell (Boigard et al., 2018). These genome containing particles: mature, partially mature and immature particles differs in size, surface morphology, infectivity and cleavage of prM protein (Junjhon et al., 2010).

2.9 Host immune response

After inoculation of virus by infected mosquito, the virus begins its multiplication in sub-dermal langerhans dendritic cells. These activated dendritic cells try to limit virus spread by eliciting IFN α/β and TNFα response together with pro-inflammatory response. Following initial dissemination from the local skin site, the virus disseminates to lymph nodes, systematic lymphatic tissues, and blood circulation. The

viremia spreads to secondary visceral organs where macrophages in spleen, liver and bone marrow are infected (Tuiskunen Bäck & Lundkvist, 2013).

Viremia is effectively controlled within 3-7 days by natural antibodies, complement, and potentially NK cells. Further the cellular immune system identifies infected cells and subsequently eliminates them through the action of cytotoxic T lymphocytes. The antibodies developed after primary infection provide life-long homotypic immunity. While, subsequent infection (secondary/tertiary) with a heterologous serotype is clinically severe, challenging and an important risk factor for developing DHF/DSS (Rothman, 2004).

2.10 The humoral immune response

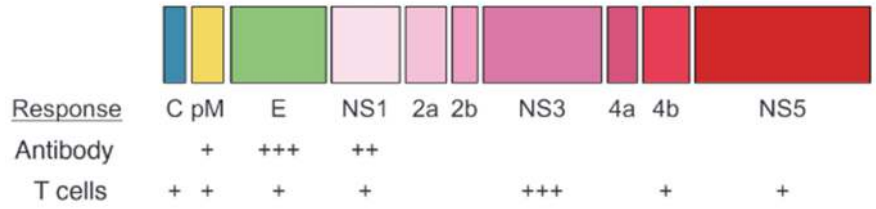

Figure 10. Major targets of immune response (Rothman, 2004).

The primary immunological mechanism that confers protection against Dengue is by neutralizing antibodies. Epitopes present on prM, E, and NS1 protein are responsible for eliciting major antibody response *(Fig 10).* A feeble antibody response against NS3 and NS5 has also been observed. The neutralizing antibodies, as the name suggests, are involved in neutralization of circulating virus through inhibition of viral attachment, internalization, and replication. These antibodies of humoral arm provide long-lasting immunity to a particular serotype, referred to as Homotypic immunity. But on subsequent infection, it also provides short-lived protection against different serotypes, called Heterotypic immunity. This heterotopic immunity is the principal cause of clinically severe Dengue, which occurs due to ADE response (Tuiskunen Bäck & Lundkvist, 2013).

The E glycoprotein present on the external surface of the virus is the dominant target for antibody response. Multiple epitopes reside within its three domains. Its compressed packaging and dimeric structure though, restricts most epitopes from

antibody attachment (Tuiskunen Bäck & Lundkvist, 2013). Antibodies developed specifically against E protein, neutralize viruses, and hinders virus binding to cells. These antibodies are more specific to particular serotype and shows variable degree of cross-reactivity across serotype (Rothman, 2004). Among serotypes, the receptor binding site of Domain III exhibits high degree variability in amino acid sequence, thus serotype specific antibody is particularly generated against E protein.

Secondary Dengue infection has been observed to be clinically severe and highly fatal. The major cause is attributed to Antibody dependent enhancement (ADE) response (Rothman, 2004). ADE refers to the phenomenon where non-neutralizing, cross reactive antibodies acquired during primary infection, or passively at birth binds to non-neutralizing epitopes of a virus, which facilitates virus entry into Fc-receptors bearing monocytic immune cells. ADE also occurs when antibodies are generated below the neutralization threshold that leads to uptake of virions into monocytes. This increased number of infected cells further induces increased inflammatory cytokines production leading to capillary leakage (WHO, 2009).

Apart from antibodies, complement system also gets activated as a key response to Dengue infection. Complement activation is the major factor that initiates vascular leakage. Cross-reactive antibodies produced against prM and E protein, react with plasminogen, that leads to increased vascular permeability, plasma leakage, and ultimately to fatal hypovolemic shock (Tuiskunen Bäck & Lundkvist, 2013). In addition, viral NS1 directly induce vascular leakage and dysfunctional endothelial cell (Cabezas et al., 2018).

2.11 The cellular immune response

In Dengue pathogenesis, the virus infects both CD4+ T-cells and CD8+ T-cells making Cellular immune response equally protective or harmfully reactive as in DENV specific antibodies response. The T cells are responsible for a wide range of effector functions such as proliferation, target cell lysis, and the production of cytokines. CD4+ T-cells are responsible for the production of IFNγ, TNFα, TNFβ, Interleukin (IL-2), and CC-chemokine ligand 4 (CCL4) that contributes to vascular permeability and leakage which ultimately leads to fatal hypovolemic shock (Rothman, 2004). In uncomplicated

infection, relatively more cytotoxic CD8+ T-cells are present resulting in lower levels of IFNγ and TNFα (Tuiskunen Bäck & Lundkvist, 2013). In addition, viral NS1 protein is also known to directly induce vascular leakage and endothelial cells dysfunction. NS1 acts as a principal regulator of complement pathway. It activates complement at endothelial cell surface to constrain the infection. Also degrades C4 to C4b which in turn protects virus from complement dependent neutralization (Cabezas et al., 2018).

2.12 Dengue vaccine

The imperative role of vaccination in mitigating the global impact of Dengue has been extensively acknowledged. Nevertheless, the creation of a Dengue vaccine that ensures both safety and efficacy has been exceptionally challenging. To mitigate this, different vaccine approaches has been explored including inactivated, live attenuated, recombinant subunit, viral vectored and nucleic acid vaccines. Some of them have surpassed developmental pipeline and is currently under safety and efficacy testing (Yauch & Shresta, 2014). Despite the exploration of various approaches, only live attenuated virus vaccine have successfully obtained licensure (Thomas, 2023).

2.12.1 Challenges

The major challenges for Dengue vaccine development are **Protection against all serotype without predisposing ADE.** In order to ensure safety, an effective Dengue vaccine should exhibit functional tetravalency, thereby providing simultaneous protection against all four serotypes of the Dengue virus. Incorporating all four serotypes in recombinant formulation retains immunogenicity for all serotypes has faced difficulties. This requires the use of multi-dose immunization regimens which makes vaccination expensive and compliance even harder (Rothman, 2004).

The second issue is the lack of well-defined immune system mechanism involved in Dengue protection. Major protection against Dengue infection or vaccination is provided by neutralizing antibodies than cellular T response (Yauch & Shresta, 2014). It has been observed in non-human primates, a quantitative level of neutralizing antibody threshold is required to protect against the disease. These neutralizing antibodies are also correlated with vaccine efficacy. A sub-immunogenic titer has been allied with ADE response (Thomas & Endy, n.d.). Therefore, theoretically the ability to

control ADE response through a fixed antibody titer should directly lower the likelihood of DHF and DSS. However, the precise antibody titer and neither the quality of neutralizing antibody that needs binding with specific epitopes to provide protective immunity has not been clearly defined (WHO, 2009).

2.13 Dengue vaccination approaches

Several studies have been employed to develop Dengue vaccine such as Live attenuated, subunit, chimeric and DNA vaccine (Yauch & Shresta, 2014; S. Zhang et al., 2011).

2.13.1 Live attenuated vaccine

While numerous approaches for Dengue approaches are being explored, till now only live attenuated vaccine has been exclusively approved licensure (Thomas, 2023). However, Live attenuated vaccines come with a risk of eliciting severe Dengue to Dengue naïve (seronegative) individuals at the time of vaccination. In these individuals live attenuated vaccine imitates as primary infection, making them more susceptible to severe Dengue when they encounter natural infection later on (Shukla et al., 2019).

2.13.1.1 Dengvaxia®

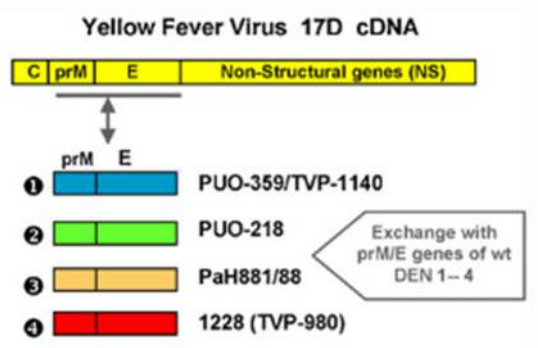

Figure 11. Genomic construction of Dengvaxia vaccine (Guy et al., 2011).

Dengvaxia (CYD-TDV), a live attenuated vaccine developed by Sanofi Pasteur is the first Dengue vaccine licensed and approved by WHO (Wilder-Smith, 2019). Dengvaxia is a chimeric tetravalent vaccine, designed using the backbone of yellow fever virus vaccine (YF-17D). The structural genes of yellow fever virus (YFV) are replaced with structural genes (prM and E) of each Dengue serotype fused together as shown in *(fig*

11). Virus replication and attenuation is generated through non-structural genes of YFV (Guy et al., 2011; Li et al., 2013).

Long term safety data revealed a potential risk of severe Dengue among individuals who had no prior exposure to Dengue virus at the time they received the first vaccine dose. Increased rate of hospitalization was seen in youngest and non-immune vaccine recipients in comparison to their unvaccinated counterparts.

The immune response to Dengvaxia is analogous to natural secondary Dengue infection in which attenuated vaccine acts as primary Dengue infection and the subsequent secondary infection with wild type Dengue heightens the risk of severity (Wilder-Smith, 2019). The increased Dengue severity was hypothesized to be due to ADE in Dengue naïve vaccine recipients when they encountered their first natural Dengue infection. Also, Imbalanced homotypic and heterotypic immunity among four serotypes, and lack of Dengue non-structural protein prevented formation of protective cellular antibody including NS1 antibodies (Thomas, 2023).

Thus, considering young recipients (below 9 years) and serostatus negative individual as a potential risk factor, WHO modified its original recommendation to be used only in Dengue immune individuals 9 to 45 years of age living in endemic areas (Wilder-Smith, 2019).

2.13.1.2 QDENGA® (TAK-003)

QDENGA is a live attenuated tetravalent vaccine developed by Takeda. The vaccine has gained approval in Brazil and some European countries. On October 2023, the WHO strategic advisory group of experts (SAGE) recommended the use of QDENGA as vaccine (WHO, 2023). A two-dose regimen is administered subcutaneously within a three-month interval (Wilder-Smith, 2019).

The vaccine consists of attenuated DEN-2 virus (DEN2-PDk-53) as the genetic backbone into which prM and E genes of DEN-1, DEN-3 and DEN-4 are lodged to create a tetravalent formulation. The DENV-2 virus strain (PDK-53) originally isolated form a patient in Thailand, was derived by 53 serial passages in primary dog kidney cells. Unlike Dengvaxia, the presence of conserved NS proteins within the Dengue backbone

was associated with generation of T cell mediated response, along with cross-protective humoral response mediated by NS1 antibodies (Osorio et al., 2016).

In phase-three clinical trials, the vaccine exhibited satisfactory safety profile in both Dengue naïve and Dengue exposed individuals. The vaccine also showed excellent tolerance level among individuals aged 4-60 years. Although varying efficacy was seen in individual serotypes, efficacy was lowest in DEN-3 and DEN-4. Severe forms of Dengue was reported in DEN-3 and DEN-4 seronegative individuals (Patel et al., 2022; Thoresen et al., 2023).

2.14 Virus like particle

Virus like particle (VLP) based vaccines are feasible alternatives to live attenuated vaccine. VLPs along with infectious virus are released during natural viral infection (CHANG et al., 2006). These entities are composed of viral structural proteins that mimic the overall morphology and confirmation of native viruses but lacks viral genome (Shang et al., 2012; Urakami et al., 2017). These empty viruses, lacking viral genome are easy to produce and purify which therefore, formed the basis of safe and efficacious subunit vaccine against hepatitis B virus, human papillomavirus and recently malaria (Metz et al., 2018; Shukla et al., 2019).

A single or multiple structural proteins have the innate ability to come together and form a structure that is morphologically identical in size and confirmation to the native virus. These particles hence retains native epitopic regions and higher order protein structure, making them immunogenic and more effective than conventional attenuated and subunit vaccines (Metz et al., 2018). They can stimulate both cellular and humoral immune response. The display of antigenic epitopes leads to effective B cell activation. Their size and structure favor easy uptake by antigen presenting cells, particularly dendritic cells. The MHC molecules internalizes, processes and displays antigen leading to cytokine production, stimulation and activation of CD4 T helper cells and cytotoxic CD8 T cells (Kushnir et al., 2012b; Lua et al., 2014).

Apart from tailored vaccine approach for presentation of specific antigens, VLP technology has opened new avenues for additional medical uses. VLPs are being utilized as a tool for diagnosis, biosensing/imaging, and disease monitoring. They are

often applied as a delivery system for nucleic acid, and specific anti-cancer drugs to targeted cells. VLPs are also employed as drug cargo to cross the blood-brain barrier for diseases such as Alzheimer's. Additionally, their particulate nature and intracellular transport capacity is being explored in synthetic biology as nanoparticle (Effio & Hubbuch, 2015; Mellid-Carballal et al., 2023; Shang et al., 2012).

2.15 VLP approaches

VLPs can be produced in multiple expression systems such as bacteria, yeast, insect, mammalian, and plant cells. *Escherichia. coli* is most used in bacterial expression system where the desired genes are cloned in commercial vectors for higher protein production. However, the application of bacterial system is restricted because of their inability to introduce post-translational glycosylation on eukaryotic proteins, and possible risk of contamination from endotoxin. More complex VLP consisting of several proteins and lipid layers requires eukaryotic systems. Yeast and mammalian cell systems are more commonly used for production of eukaryotic proteins (Mohsen et al., 2017). Based on the structure of parent virus, VLPs can be classified into two major groups (Effio & Hubbuch, 2015)

2.15.1 Enveloped VLPs

Enveloped VLPs obtain their lipid membrane from the infected host cell during their assembly and budding. These enveloped VLPs are less structurally uniform compared to their non-enveloped counterparts. They are therefore poorly characterized. In addition, enveloped VLPs are known to be more sensitive to external environment (Nooraei et al., 2021). Influenza, Human immunodeficiency virus (HIV), Ebola and Chikungunya enveloped VLPs has been well documented (Lua et al., 2014).

2.15.2 Non-enveloped VLPs

One of the simplest forms of non-enveloped VLP is an HPV VLP consisting of a single capsid protein. The core capsid region provides morphological stability to non-enveloped VLPs. They can be produced in both eukaryotic and prokaryotic expression system. They can also be generated in cell-free medium. Multiple capsids can also be

generated in one cell. Poliovirus, rotavirus VLPs are examples of non-enveloped VLPs (Nooraei et al., 2021).

2.16 Commercially available VLP vaccines

The immunogenic characteristics of VLP has paved the way for approval of numerous vaccines for combating infectious diseases. A few of them are described herewith.

2.16.1 Human Papilloma virus

Several VLP based vaccine produced in different expression system have been licensed to combat Human Papilloma virus (HPV), the leading cause of cervical cancer and genital warts. Their effectiveness led WHO to approve the use of Gardasil®, Cervaris®, Gardasil® 9, and Cecolin® as vaccine. In the 1990s, it was discovered that the component of capsid protein (L1 major) was able to self-assemble into structure identical to wild HPV and simultaneously trigger immune response. These vaccines have been shown to provide excellent long-lasting protection against HPV (Mohsen & Bachmann, 2022).

Gardasil® produced in *Saccharomyces cerevisiae*, is a tetravalent vaccine composed of recombinant L1 protein of four HPV types (6, 11, 16, and 18). Gardasil® 9 approved in 2017 is a recent variation containing protein of nine HPV types (6, 11, 16, 18, 31, 33, 45, 52, and 58). Both vaccines administered intramuscularly are formulated with aluminum hydroxyphosphate sulfate as adjuvant (Mohsen et al., 2017; Mohsen & Bachmann, 2022).

Cervaris® a bivalent vaccine against HPV types (16 and 18) consists of recombinant baculovirus and L1 proteins assembled in *Trichoplusia ni* insect cell. To increase production and reduce the cost of vaccination, Cecolin® was produced using *Escherichia coli* expression system in China. Recently in 2020, WHO has qualified Cecolin® to be used against HPV types (16 and 18) (Mellid-Carballal et al., 2023).

2.16.2 Malaria

Mosquirix® (RTS/AS01) is the first vaccine developed against Malarial parasite targeting the pre-erythrocytic stage. On September 20222, the vaccine was prequalified by WHO to combat Malaria in regions with higher *Plasmodium falciparum*

incidence. It comprises 20% circumsporozoite protein (CSP), which is a part of parasite capsid protein fused Into HBV VLP. This chimeric VLP assembled in yeast is formulated with AS01 adjuvant. The large scale study of the vaccine is encouraging, but the vaccine has shown extensive variability in efficacy and possible safety concern in young female recipients (Mellid-Carballal et al., 2023; Mohsen & Bachmann, 2022).

A second generation VLP based vaccine, R21 is under phase-II clinical trials. As opposed to Mosquirix, here the whole CSP protein is directly fused with HBS vlp, thus increasing the CSP antigen concentration. Also, a more potent Matrix-M and Abisto-100 adjuvant is used. A higher level of B and T cell response was accounted in mice (Mohsen et al., 2017).

2.17 Dengue VLP

Dengue VLPs as vaccine candidates are still in early phase pre-clinical studies. Dengue VLP was first produced in 1997 by the co-expression of three structural proteins in yeast *Pichia pastoris*. This spherical particle showed immunogenicity and was able to elicit neutralizing antibodies in rabbit (Sugrue et al., 1997; Zhang et al., 2020). In previous studies, VLP expression plasmid were often used as a DNA vaccine (Zhang et al., 2011). Many strategies such as utilization of vector with cytomegalovirus early promoter, optimization of codon sequence, intradermal route of vaccine administration were used to improve the expression of plasmid DNA (Ketloy et al., 2017). Since then, Dengue VLP plasmid has been engineered with wide variety of structural and non-structural genes that has been expressed in insect, yeast, and mammalian cells to enhance cellular secretion and immunogenicity (Zhang et al., 2020).

Dengue VLP of each serotype was successfully produced in mammalian HEK 293 cells (Metz et al., 2018). A chimeric tetravalent Dengue VLP was developed by fusion of envelope protein domain III of all four serotype with Hepatitis B surface antigen was able to induce neutralizing antibodies against all four serotype (Ramasamy et al., 2018). The mouse immunization study, revealed monovalent and tetravalent formulation (constituting four type of VLP at equal amount) exhibited a greater IgG serum titer in comparison to the wild type individual and tetravalent viruses infection

(Zhang et al., 2011). A separate study conducted comparison of antibody titers generated by tetravalent VLP and monovalent VLP which revealed that tetravalent immunization led to elevated antibody titers (Liu et al., 2014). An envelope modified tetravalent VLP produced in 293F cell was able to elicit neutralizing antibodies against all four serotypes for upto 1 year in non-human primates. Furthermore, during the time period ADE activity was not detected against any serotype (Thoresen et al., 2023).

Previous study indicated an important role of signal peptide in correct processing of E protein which ultimately led to extracellular secretion of VLP. The transmembrane domain of E protein consist of strong ER retention signal, replacement of which eased extracellular secretion of DEN 2, 3 and 4 VLP but has no effect on the extracellular secretion of DEN 1 VLP (Zhang et al., 2011). In the previous study the expression plasmid containing entire prM and E sequence of DEN-1 was able to successfully express DEN-1 VLP in mammalian cell line 293T without replacement of carboxy terminal containing ER retention signal.

2.18 Downstream processing of VLP

During the production of VLP using a recombinant system, a multitude of impurities are generated. These impurities are categorized into two major groups: **process related contaminants** and **product related contaminants.** Host cell impurities including cells, cell debris, host cell protein (HCPs), nucleic acid, proteases, endotoxin, polysaccharides, and lipids are primarily responsible for process related contamination. On the other hand, product related contaminants originates from media components and its supplements such as stabilizers, excipients, anti-foam reagents (Effio & Hubbuch, 2015).

The elimination of these impurities is an essential step in the downstream process of VLP production. The specific downstream process is influenced by various factors including the structure of VLP (size, molecular weight and presence of envelope), its accumulation site (intra or extracellular), type of culture media used (serum supplemented or free) and the specific production method (transfection and transduction) used (Roldão et al., 2010).

2.18.1 Clarification

The first phase of VLP purification involves separation of solid and liquid components in cell culture broth. Depending on specific protein, VLPs can be generated either intracellularly or extracellularly. When VLPs are not released naturally into the extracellular medium a further cell lysis step becomes crucial before clarification. Clarification involves removal of cells, cells debris, and other insoluble aggregates from media fraction in the form of pellet. It is usually performed by low-speed centrifugation and filtration steps (Effio & Hubbuch, 2015).

2.18.2 Capturing and concentration of VLP

The purpose of this step is to increase the concentration of VLP to host cell impurities. Most of the contaminants are removed at this step. The VLP diluted in media can be concentrated with sedimentation, centrifugation, ultrafiltration, precipitation, and chromatography are employed. Depending on the type of VLP various combinations of methods can be used.

2.18.2.1 Precipitation

Numerous VLPs such as cowpea mosaic VLP, noro-VLP, chikungunya VLP have been concentrated with polyethylene glycol (PEG) of varying molecular weight (4000 to 8000 Da). It involves precipitation of target component with high molecular density particles such as PEG/ammonium sulfate, followed by redissolution in suitable buffer (Effio & Hubbuch, 2015). PEG, a non- toxic, water soluble synthetic polymer is widely used as a fractional precipitating agent for purification of proteins obtained from wide sources. The use of hydrophilic polymer modifies protein called PEGylation; prolongs protein half-life, reduces self-aggregation, and increases water solubility. However, modification of physical property of PEG-conjugated-protein, including increased size, reduced immunogenicity and co-precipitation of larger molecules limits its use (Plesner et al., 2011).

The phase separation method is utilized to isolate protein from PEG-6000. Addition of some salts (sodium or potassium chloride) to PEG solution results in formation of two liquid phases. Protein passively partitioned into salt-rich lower phase. The relative

volume of two phases depends on the amount of salt added and time required for phases to separate (Ingham & Busby, 1980). After phase separation, centrifugation can be employed to separate protein from salts and residual PEG. PEG-6000 was successfully used for precipitation and purification of Influenzas virus, retroviruses and SARS-CoV-2 (Aboud et al., 1982; Napit et al., 2023; Petrova et al., 2020)

2.18.3 Intermediate purification / concentration

This step is employed during the downstream processing to reduce stream volume and further concentrate VLPS. Subsequent purification after capturing is typically carried out through a subsequent chromatographic separation. A common two-step process for reducing bulk volume involves combining precipitation with PEG and Ion exchange chromatography. Various groups have achieved successful purification of VLPs derived from insect cells, plant cell, and mammalian cells using a similar method (Effio & Hubbuch, 2015; Vicente et al., 2011).

2.18.4 Polishing and formulation

Polishing is the final and the most critical step for the downstream process. All product related and process related contaminants need to be removed for a clinical grade material. Final purification of VLP is mostly done by Size-exclusion chromatography, Ultrafiltration, diafiltration and sterile filtration. Affinity exchange chromatography is ideal to separate host cell related contaminants, while Size exclusion chromatography becomes a better alternative when difference of protein size is significant compared to electrostatic properties. In regard to scalability, tangential flow filtrations such as Ultrafiltration and diafiltration are more scalable than size exclusion chromatography (Vicente et al., 2011).

2.19 VLP characterization

Thorough characterization of purified VLP plays a crucial role in VLP vaccine development. VLP characterization is an essential quality control check point to ensure potency, safety, and maintain batch to batch consistency. Poorly characterized VLP can lead to design-redesign of VLP, misinterpretation of immunological data, and hamper manufacturing process. Proper characterization of VLP employs biochemical,

biophysical, and immunological techniques that allows assessment of the overall structural and functional aspects of the particle (Lua et al., 2014). In this study, methods employed for the characterization of VLP have been outlined below.

2.19.1 VLP Composition analysis

The composition of VLPs widely varies depending on the parent virus, type of proteins used, and the presence of envelope and the host expression system. Impurities such as media components, debris, host nucleic acid and exosomes can modify VLP and impact its application. Hence, it is imperative to identify the composition of VLP in order to properly characterize them. Mass spectroscopy, SDS-PAGE, Western blotting, and reverse- phase HPLC are frequently used to access the biochemical properties of VLP (R. Kumar et al., 2020).

2.19.1.1 SDS-PAGE

SDS-PAGE is widely used to access the molecular weight and purity of VLP. The identification of individual protein components is further confirmed using Western blotting (Kumar et al., 2020). SDS-PAGE involves denaturation of proteins with sodium dodecyl sulfate (SDS), and the use of polyacrylamide gel to sieve proteins in presence of an electric field (PAGE). Here, the proteins are separatee based on their molecular weight and surface charge. SDS denatures, unfolds protein structures, and minimizes protein charge difference by adding net negative charge based on their size. Thus, the detergent ensures protein migrates as anion towards cathode, a positively charged electrode in an electric field.

Gel matrix is formed by copolymerization of Acrylamide and a cross-linking agent (bis-acrylamide) in presence of catalyst TEMED (N, N, N, N-tetramethylethylenediamine) and ammonium persulfate. Depending on the molecular weight of the protein to be separated, pore size of the gel is tailored by changing the ratio of acrylamide and bis-acrylamide. The pore size of the polyacrylamide gel is inversely proportional to the concentration of acrylamide. Higher percentage of acrylamide better separates low molecular weight protein and vice versa.

Laemmli SDS-PAGE is the most widely used discontinuous system where proteins are separated through use of two separate gels: differing in acrylamide concentration, and pH range. This flat slab gel consists of upper stacking gel and lower resolving gel. The stacking gel has low acrylamide concentration (4%) with larger pore size, and buffer with low pH (high ionic strength) facilitates concentration of protein into a starting zone before entering the resolving gel. The lower resolving gel has higher acrylamide concentrations (10%) and higher pH (low ionic strength) resulting in smaller pore size that reduces migration rate but allows sharper protein separation.

Visualization of protein bands is performed by incubating the gel with staining solution. Coomassie or silver stains. Coomassie dye interacts with amino acids and stains them blue while silver selectively reacts with protein carboxyl and sulfhydryl moieties. Silver stain, although being more sensitive, makes protein oxidized and therefore has limited use in downstream application. Coomassie stain although being less sensitive is quantitative and can be used for downstream applications such as Western-Blotting (Al-Tubuly, 2000; Matsumoto et al., 2019).

2.19.2 Morphological analysis

The bioactivity of VLP depends not only on the property of monomeric building block protein but also on their correct assembly and interaction. Particle shape and size is known to affect affinity, avidity, and overall potency of VLP (De Sá Magalhães et al., 2022). Thus, ensuring accurate morphology is essential for VLP characterization. Several analytical techniques such as Transmission electron microscopy (TEM), dynamic light scattering, analytical ultracentrifugation, size-exclusion chromatography, and nanoparticle tracking analysis (NTA) are available to assess the morphology of VLP (Kumar et al., 2020).

2.19.2.1 TEM

Transmission electron microscopy (TEM) is widely employed to analyze the ultrastructure of nanoparticles such as VLP. TEM provides high resolution image at nanoscale (up to 0.19nm) to study morphological structure, chemical composition, and their bonding. The technique allows interaction of electrons as they pass through the thin specimen to derive range of signal that allows observation of internal

structures at atomic level. This transmission of electron beam allows imaging at higher magnification and resolution than light microscopy (Mammadov et al., 2012).

In TEM imaging, samples are usually stained with heavy metal to increase the contrast of biological samples under electron beam. Staining of either the sample (positive staining) or the background (negative staining) can be performed. Negative staining is widely preferred for protein samples because is easy, quick and does not demand specialized equipment to generate images with high level of contrast. Depending on specimen properties, radioactive and non-radioactive metals such as uranyl acetate, phosphotungstic acid are employed. Uranyl acetate (UA) is the most widely used stain for imaging biological samples (De Sá Magalhães et al., 2022).

In negative stain TEM, image contrast is achieved through heavy metal stain solutions, by embedding with particle of interest. Thus, the stain scatters more electron than the particles, resulting in image where the particles appear bright against the dark background (Hauser et al., 2020). The samples are allowed to adsorb into carbon film grid where samples are quickly air dried allowing heavy metal salts to form a thin film around the particle of interest. The resulting grid is imaged on electron microscope (Baxa, 2018).

2.19.3 Functional analysis

Functional characterization includes assays that evaluate antigenicity and immunogenicity of VLP. This is crucial for ensuring appropriate downstream application and determining their potency. Additionally, it aids in identifying any changes in epitopes that may occur during production, purification, and storage. Both animal based and invitro assays such as Enzyme-linked immunosorbent assay (ELISA), virus neutralization assay, surface plasmon resonance, and invitro relative potency (IVRP) are employed for this purpose (Kumar et al., 2020; Zhao et al., 2013).

2.19.3.1 ELISA

Enzyme linked immunosorbent assay (ELISA) is the quantitative assay especially used to monitor VLP manufacturing process instead of plaque assay or tissue culture infectious dose (TCID50) test because of its non-multiplicative nature. Several ELISA

methods have been developed to accurately quantify Dengue VLP antigens (Tsai et al., 2020). In addition several VLP based ELISA has also been developed for serological test in epidemiological surveillance for diseases such as SARS-CoV-2, enterovirus (Kumar et al., 2023; Lim & Cardosa, 2019).

ELISA is a quantitative method that detects antigen and its concentration by using highly specific antigen-antibody interactions through color change. The assay makes use of catalytic property of enzyme linked conjugate and enzyme substrate to identify low concentrations of molecules such as peptides/proteins, hormones, vitamins, and drugs in biological fluids (Aydin, 2015).

Basically, an antigen is immobilized directly or indirectly via capture antibody (primary antibody) onto a solid surface such as microplate. This immobilized antigen is then detected through use of detection antibody (secondary antibody) conjugated with enzyme. Upon addition of substrate, the enzyme undergoes catalytic reaction with the substrate, causing a noticeable change in color. The intensity of color change is directly proportional to the concentration of the antigen (Aydin, 2015).

Chapter III

MATERIALS AND METHODS

3.1 Research Design

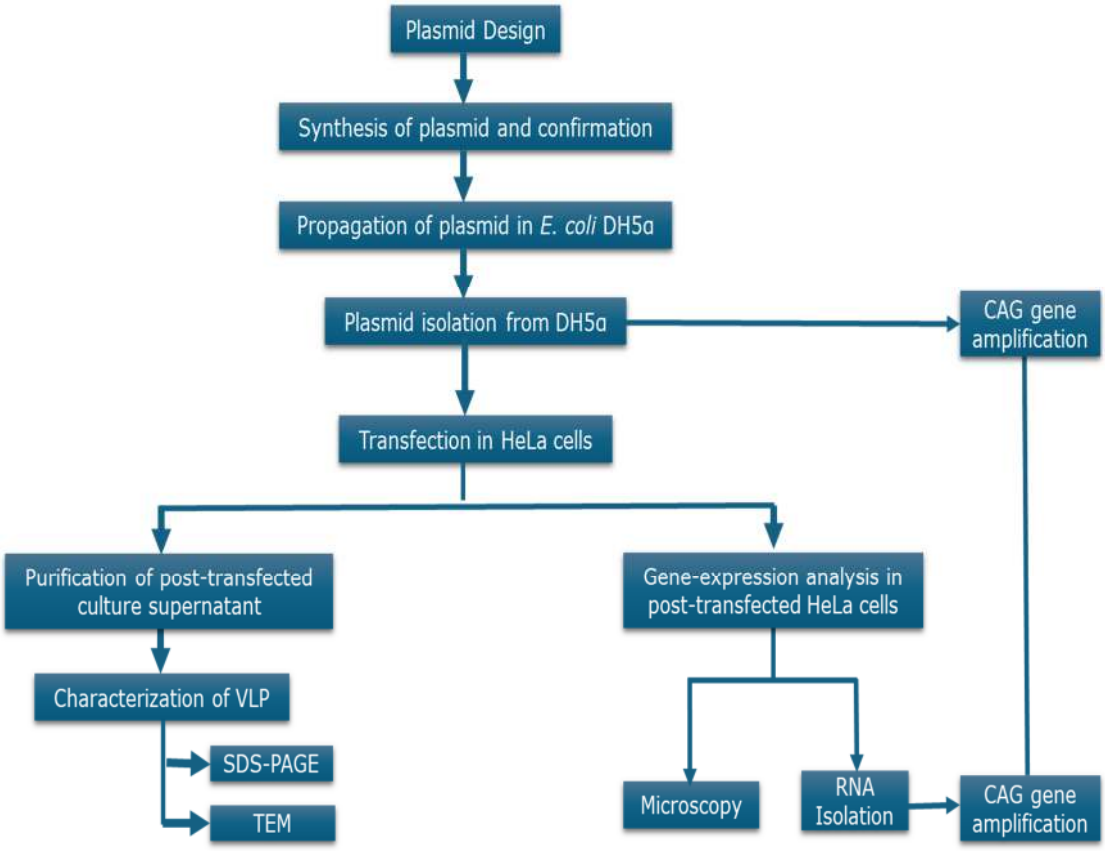

Figure 12. Schematic representation of research design.

3.2 Design and synthesis of DV1 plasmid

The Dengue-1 VLP construct is comprised of two major proteins: prM and E. The prM protein is encoded by prM gene (NCBI accession no. NP_733807.2) that encodes 166 amino acids. Similarly, the E protein, encoded by E gene (NCBI accession no. NP_059433.1) codes for 495 amino acids. These gene sequences were codon-optimized for better expression in HeLa cell line. The sequences were edited using the sequence viewer platform, AliView. The plasmid was designed with an online platform, Benchling.

The codon optimized sequence of prM and E gene was inserted to multiple cloning site (MCS) between EcoRI and BglII restriction site of pCAGGS vector (GeneBank accession no. LT727518). The structure protein of Dengue-1 was cloned into pCAGGS backbone, which was then custom-synthesized and made available by NovoPro synthesis. The prM and E gene were flanked by 5' CAG promoter and 3' transcription terminator respectively on pCAGGS vector. This plasmid was named Dengue Vector type 1 (DV1).

3.3 Transformation and selection of transformants

The plasmid DV1 was transformed by the heat-shock method into competent *Escherichia coli* DH5α. The competent DH5α cells were prepared following Hanahan procedure. The transformed colonies were selected on Nutrient agar (NA) plates, containing Ampicillin (100 µg/ml). Single isolated colonies were selected and inoculated into 10 ml Luria broth (LB) containing Ampicillin (100 µg/ml) for preservation along with plasmid amplification and isolation. These colonies were named as E1, E2…E16, E17, E18 respectively.

3.4 Plasmid isolation

Plasmid DNA was isolated using the Alkaline-lysis method from an overnight culture of transformants grown on LB media containing Ampicillin, which were incubated at 37° with constant agitation. The pellets of isolated plasmid were reconstituted in Tris-EDTA (TE) buffer. This purified plasmid was subjected to agarose gel electrophoresis. Different forms of plasmid were visualized by UV transilluminator.

3.5 Identification of transformed plasmid

The identification of transformed plasmid containing correct coding cassette of prM and E gene was detected using semi-quantitative PCR and sequencing.

3.5.1 Semi-quantitative PCR

The gene encoding CAG promoter was amplified by conventional PCR using the forward primer CAGGS F (5'-TAATCAATTACGGGGTCATTAGTTCATAGC-3') and reverse primer CAGGS R (5'-TCCCATAAGGTCATGTACTGGGCATAATGC-3'). Amplicons were generated in 25µl reaction containing 2µl DNA template, 2.5µl each 10µM forward

and reverse primers, 0.5µl of nuclease-free water, 12.5µl of 2X master mix, and 5µl of 5X Q solution (Qiagen cat. #206145). The cycling condition consisted of 35 cycles of denaturation (95°C for 30s), annealing (55°C for 1 min) and extension (72°C for 45s), with a final extension at (72°C for 3 min). Amplicons were run through agarose gel (1.5%) electrophoresis and visualized by UV transilluminator.

3.6 Transient expression of DV1 plasmid in HeLa cells

HeLa cells were transiently transfected with DV1 plasmid to evaluate the gene expression. 0.3X10^6 cells/well were seeded in 6 well cell culture plates to achieve 70% confluency. After 16h of seeding, these cells were transfected with 5µg of DNA using Lipofectamine 2000 (cat. #11668030), following the manufacturer's recommendation. After a brief incubation, Lipofectamine was mixed with plasmid. This lipo-DNA mixture was shortly incubated for 30 min, and then added to each well containing 70% confluent cells. The cells were then incubated at 37°C with 5% CO_2. 5hr post-transfection, cells were supplied with maintenance media containing 10% FBS and further incubated at 37°C for 72hr with 5% CO_2 to facilitate VLP production. Cells and culture supernatant were harvested separately 72hr post transfection for VLP purification and characterization.

3.7 Harvest and purification of culture supernatant

Post-72hr the media fraction containing secreted VLP were collected. This supernatant was clarified by short centrifugation for 30 minutes to pellet down cells debris. The clarified supernatant was then concentrated using Polyethylene glycol (PEG-6000 and 2% NaCl) in equal volume. The mixture was incubated at 4°C on a constant shaking platform. After 48hr, VLP was pelleted down using high-speed centrifugation platform, Optima XPN-100 ultracentrifuge (Beckman Coulter) at 10,000 X g for 40 min at 4°C. The pellets obtained were then resuspended in 200µl of SM buffer.

3.8 Characterization of DV1 VLP

The VLP isolated from supernatant fraction was further analyzed by SDS-PAGE and TEM. The protein concentration was measured using the Folin-Lowery procedure. The

size and Integrity of VLP was examined through TEM. Molecular weight of the protein was determined by gel analysis of SDS-PAGE stained with Coomassie blue.

3.8.1 SDS-PAGE

Protein concentration was assessed by the Folin-Lowery method. The purity of VLPs was assessed by visualizing the protein band on SDS-PAGE. The protein samples containing an equal amount of sample loading buffer were loaded onto 5% stacking gel and separated on 12% resolving gel via electrophoresis set at appropriate voltage and time for the respective gel. After electrophoresis the separated proteins were visualized by incubating the gel overnight in Coomassie blue followed by overnight destaining of the gel. The separated protein bands were visualized within the gel after blue background was completely cleared by destaining solution.

3.8.2 Electron microscopy

The morphology of DV1 VLPs were visualized by Transmission Electron Microscope (TEM) analysis. The purified VLP and control supernatant were fixed in 2% paraformaldehyde. Small droplets of fixed samples were placed in carbon coated copper grids. The grids were then stained with 2% saturated Uranyl Acetate and visualized at the TEM facility, AIIMS, Delhi. The micrographs were recorded by Talos (HR-TEM) operating at 200 kV. The diameter of the particles was measured by ImageJ software.

3.9 Harvest of transfected cells

Following post-72hr transfection, cells fraction was separated from the medium fraction through low-speed centrifugation. The protein aggregates formed by the cells were visualized by inverted microscope using trypan blue stain. These cell pellets were further preserved for gene expression studies.

3.10 Confirmation of transient Transcription in *HeLa*:

The transient formation of RNA transcript required for protein synthesis was detected in post-transfected cells. From these cells RNA was isolated which were converted to cDNA, followed by amplification of CAG gene through semi-quantitative PCR.

3.10.1 Semi-quantitative PCR

From the harvested cell, RNA extraction was performed by kit-based method. The cells suspended in PBS were lysed by boiling lysis method through brief incubation at 95°C. From the lysate RNA was extracted using Qiagen RNA extraction kit (cat. #52904) as per the manufacturer's instructions.

The RNA was converted to cDNA using iScript cDNA synthesis kit (BIO-RAD cat. #1708891). A 20 µl reaction mixture was prepared with 5µl RNA, 10 µl nuclease free water, 4 µl of 5X master mix and 1 µl RT. The reaction was primed at 25°C for 5 min, reverse transcribed at 46°C for 2o min, and terminated at 95°C for 1min. The resulting cDNA was utilized as a template for semi-quantitative amplification of the CAG promoter gene, following the procedure described earlier in the section **3.5.1.**

Chapter IV

RESULTS

4.1 Aim 1. Synthesis of DV-1 expression plasmid

4.1.1 Construction of expression plasmid

The full-length sequence of DENV1 ectodomain prM and E protein was downloaded from NCBI database. The design of recombinant plasmid includes origin of replication (*ori*), ampicillin resistance gene, CAG promoter region along with transcription enhancer, multiple cloning site containing insert of DENV1 complete prM and E gene, and Beta-globin poly (A) transcription termination signal. The transcription of inserted Dengue-1 genes (prM and E gene) in pCAGGS plasmid was controlled by CAG early gene promoter/enhancer. The mRNA thus formed was terminated by Beta-globin transcription terminator and stabilized by poly (A) signal (Chang et al., 2000). The final length of the construct was 6,767 bp as shown in *(fig. 13).*

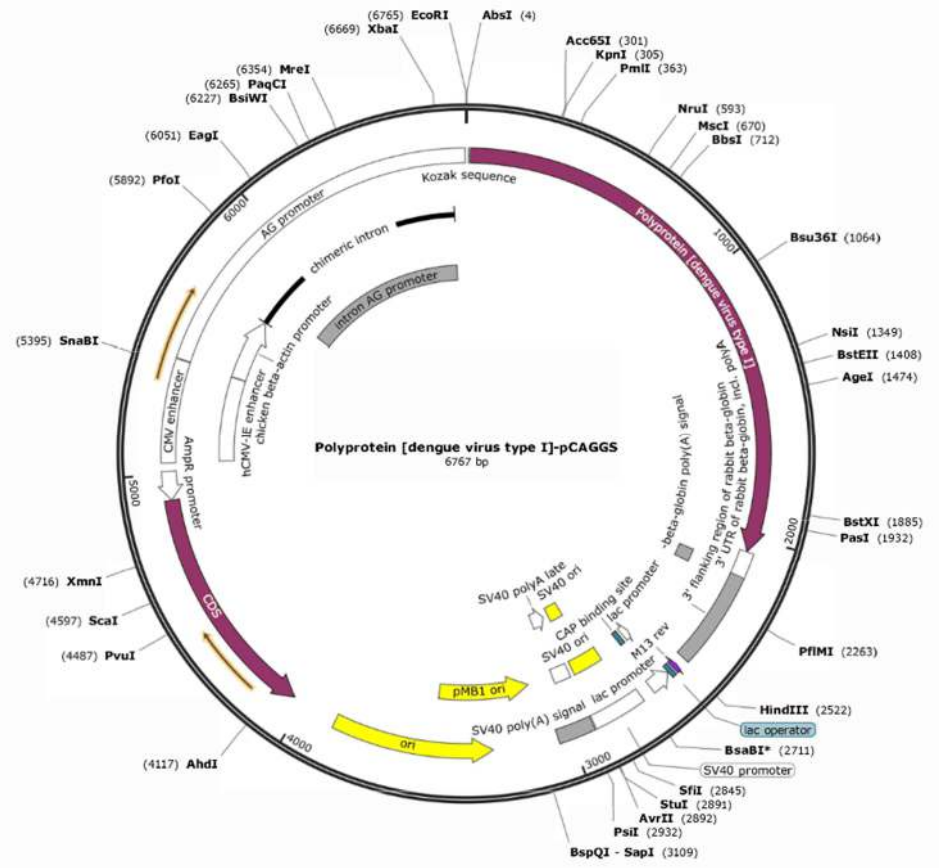

Figure 13. Schematic representation of DV1 plasmid. The Dengue-1 prM/E gene was integrated into pCAGGS vector.

4.1.2 Synthesis of the designed plasmid

Plasmid with inserted gene of interest of Dengue-1 was synthesized by NovoPro synthesis. The confirmation of the insert and quality of the plasmid was measured using Next generation sequencing, and restriction digestion analysis.

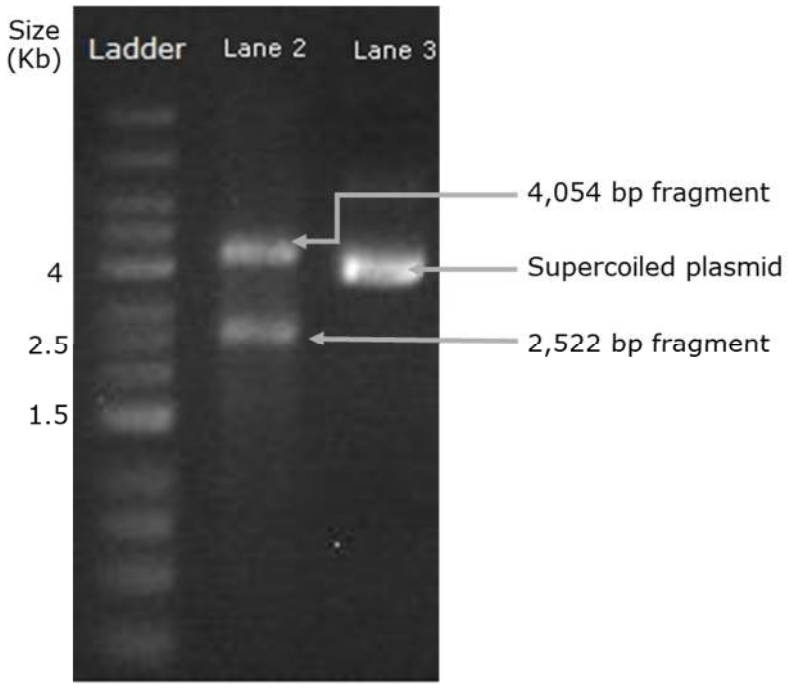

Figure 14. Gel electrophoresis image of plasmid DNA restriction digest: lane 1- a 1kb DNA marker; lane 2-plasmid after double digestion with ECoRI-HindIII to produce 4054 bp and 2522 bp fragments; lane 3-supercoiled plasmid.

The successful addition of insert was confirmed by restriction enzyme digestion. The double digestion was performed using EcoRI and HindIII which produced two fragments visible in gel electrophoresis image as shown in lane 2 of *(Fig 14).* The linearized pCAGGS plasmid is represented by the larger 4000 bp fragment following digestion, while the smaller 2500 bp fragment represents the Dengue insert drop out. The single recognition site of EcoRI present at 4054 bp of plasmid resulted in larger fragment, and HindIII recognition site at 2522 bp led to smaller fragment at 2500 bp. These two bands validate that the 2064 bp Dengue insert was ligated in the plasmid.

4.1.3 Plasmid transformation in DH5α and selection of transformants

Propagation of DV1 plasmid was achieved through transformation in *E. coli* DH5α. The transformants generated by the Heat-shock method were selected on LB plates containing Ampicillin. Thus, after 18hr of incubation bacterial colonies transformed

by the plasmid containing our gene of interest was only able to grow in culture plates as shown in *(fig. 15)*.

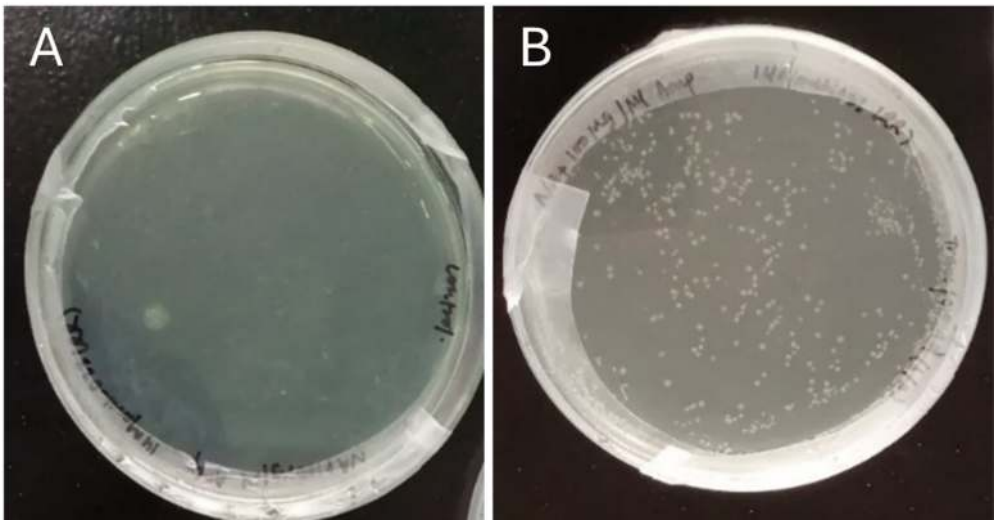

Figure 15. Screening of transformed colonies on agar plates containing Ampicillin: A. negative control as competent DH5α, B. transformed DH5α colonies.

4.1.4 Plasmid isolation

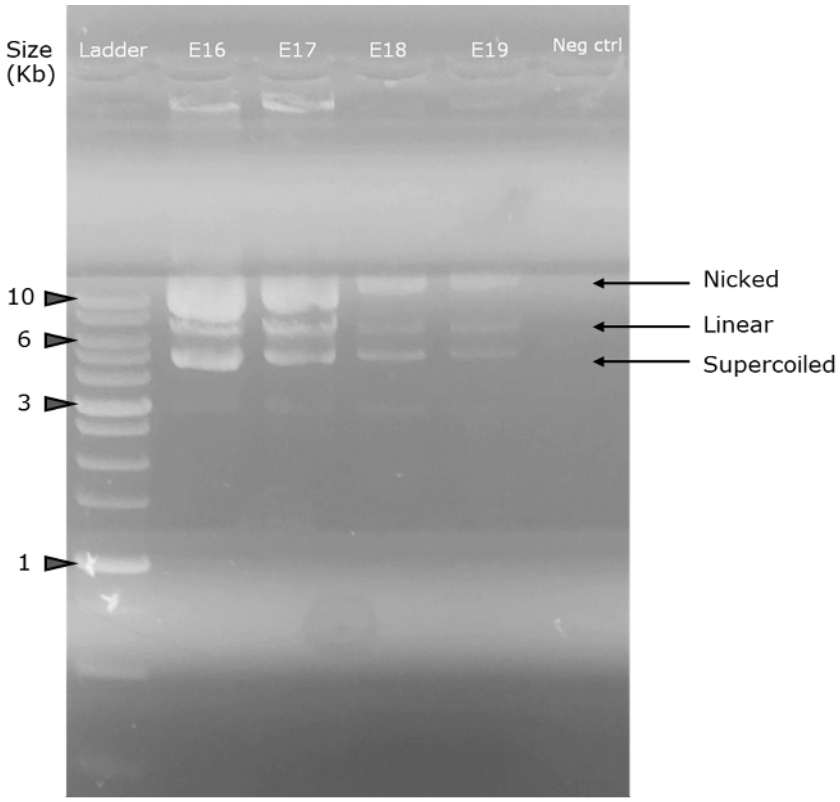

Figure 16. Agarose (0.8%) gel of plasmid DNA isolated from transformed DH5α: Lane 1, DNA ladder (1 Kb); lane 2 and 3, E16 and E17 transformant with intense 3 plasmid forms labelled: linear form at ~7kb; lane 4 and 5, E18 transformant with faint plasmid bands; lane 6, negative transformation control.

These transformed colonies containing DV1 plasmid were extracted following the standard Alkaline Lysis Method. Their yield and size were analyzed through gel electrophoresis. Three forms of plasmids were observed with intense band at 5kb, ~7kb and above 10kb corresponding to supercoiled, linear, and nicked open circular plasmids as shown in *(fig. 16).* The supercoiled forms were especially more intense in E16 and E17 isolates. Notably, no bands were visible in transformation negative control. The size of the linear form of transformed plasmid corresponds with the designed Insilco form.

4.1.5 Confirmation of transformed plasmid

The presence of DV1 plasmid in transformants was examined by semi-quantitative PCR as shown in *(fig 17)*. To ascertain the extracted plasmid can drive protein expression, these plasmids were examined for the presence of CAG promoter. The plasmid isolates E16 and E17 which aligned in size with the designed Insilco form, were examined for the presence of gene cassette containing CAG promoter. A positive amplification of 301 bp CAG gene implied that the selected plasmid construct can direct proper intracellular expression of VLP protein in host mammalian cells. No amplification was observed in plasmids extracted from untransformed DH5α (transformation negative control).

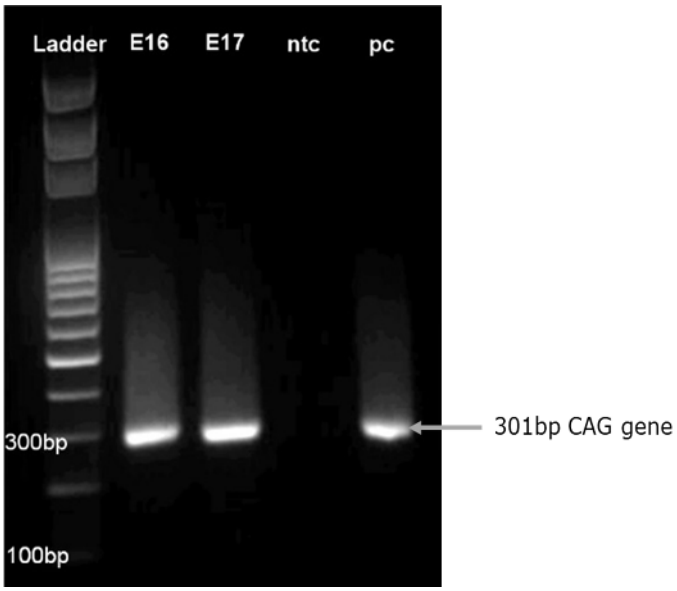

Figure 17. Agarose (1.5%) gel of CAG promoter gene amplification by conventional PCR: lane 1, DNA marker (100 bp); lane 2, E16 plasmid; lane 3, E17 plasmid; lane 4, plasmid of transformation control (ntc); lane 5, plasmid positive control (pc).

Following the verification of CAG gene in plasmids E16 and E17, they were pooled together for transfection.

4.2 Aim 2. Expression of DV1 plasmid in HeLa cells

The verified plasmids were employed for transient expression of Dengue-1 VLP in HeLa cells. 72 hour-post transfection, HeLa cells were evaluated for changes in cell viability and morphology. The visible confluency of Hela cells following transfection (test) dropped to approx. 50% *(fig 18. B)*, compared to untransformed HeLa cells (negative control) as shown in *(fig 18. A)*.

Trypan blue staining was performed to analyze the apparent morphological changes in both test and negative control cells *(fig 18. C, D)*. Notably, the test cells exhibited visible protein assembly near the cell membrane, unlike the control cells where no such formations were observed. The experiment was duplicated, and the results were consistent across batches. In summary, transfection had visible impacts on the viability and morphology of HeLa cells.

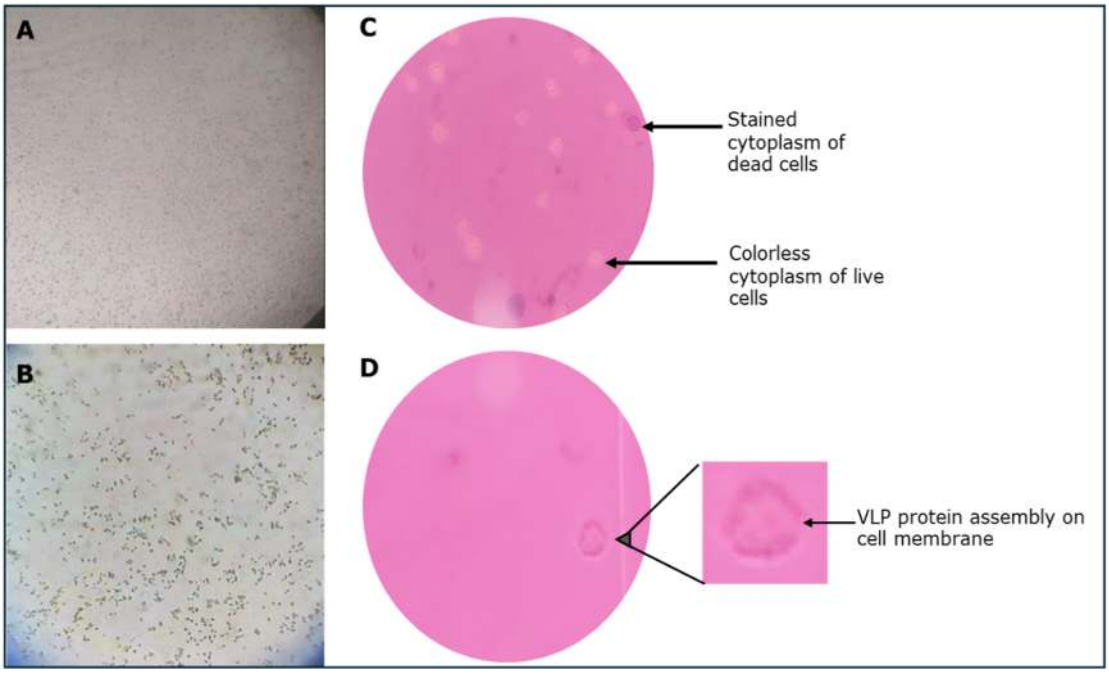

Figure 18. Microscopic analysis of HeLa cells. Confluency of Hela cells 72 hr-post transfection: A) negative control cells; B) test cells. Morphology of these cells subjected to trypan-blue stain at 40X microscopy: C) control HeLa cells containing stained live and dead cells; D) transfected test cells with VLP protein assembly.

4.3 Aim 3. Purification of VLP

The supernatant fraction containing the extracellular VLPs was clarified with low-speed centrifugation to remove host and culture related impurities, followed by PEG-based precipitation. The Purified VLP fraction was subjected to SDS-PAGE, followed by analysis through Coomassi blue staining *(Fig. 19)*. A highly concentrated DENV protein band was observed at expected ~60 KDa, consistent with the estimated molecular weight of Dengue-1 E protein. Impurities related to host cells were observed as faint bands in the SDS-PAGE gel. The test was duplicated, and uniform bands were observed across batches.

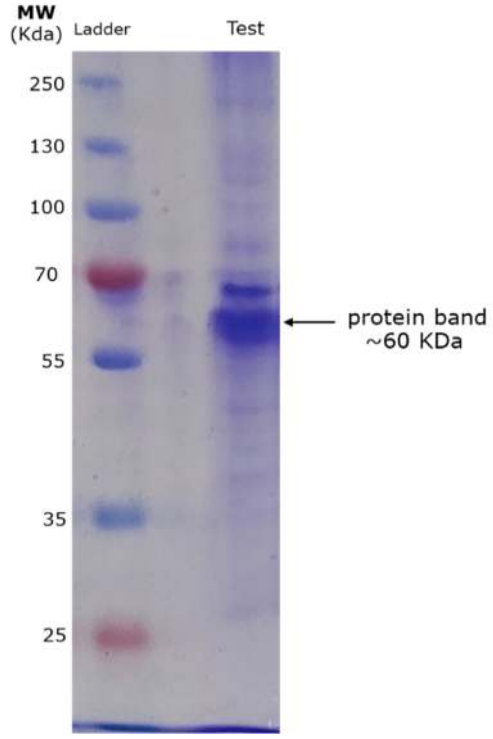

Figure 19. Estimation of molecular weight of purified VLP protein through SDS-PAGE. Lane 1- molecular weight marker; Lane 2- PEG purified culture supernatant of test. A highly concentrated DENV-1 VLP protein band observed at 60 KDa.

4.4 Aim 4. Morphological characterization of the VLP

4.4.1 TEM analysis

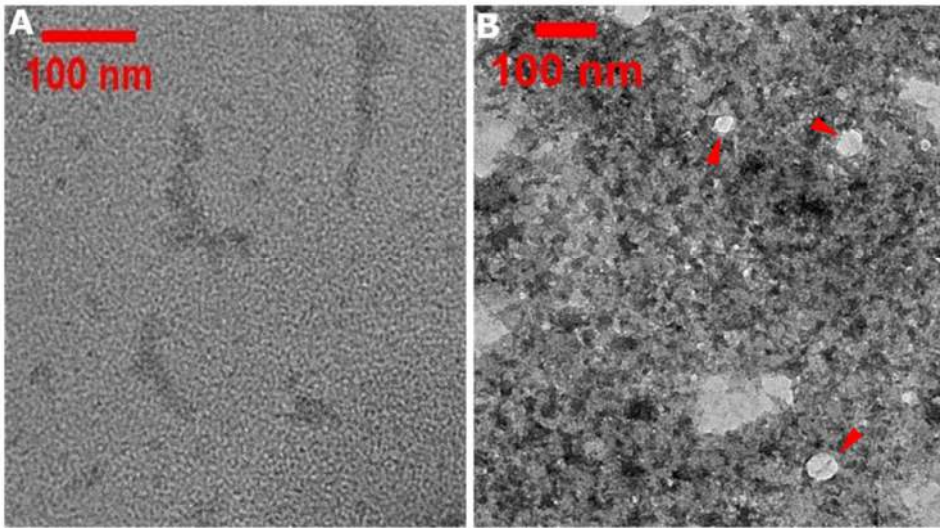

Figure 20. Electron micrographs of purified culture supernatant of negative control and test at 100 nm scale bar performed for the characterization of particle shape and size. A) TEM image of negative control; B) TEM image of test. A few spherical dense particles of size app. 55nm resembling the size of DENV-1 observed in test supernatant indicated as red arrowhead.

TEM analysis was performed on the purified culture supernatant of both non-transformed cells (negative control) and transformed cells (test) for the characterization of particle shape and size. A noticeable variation in the size of particle diameter between negative control and test samples was observed. In the test supernatant, a few spherical electron dense particles of size approximately 55 nm, resembling the size of native dengue virus were observed *(fig. 24. B).* However, particles of this diameter were completely absent from the negative control supernatant *(fig. 20 A).*

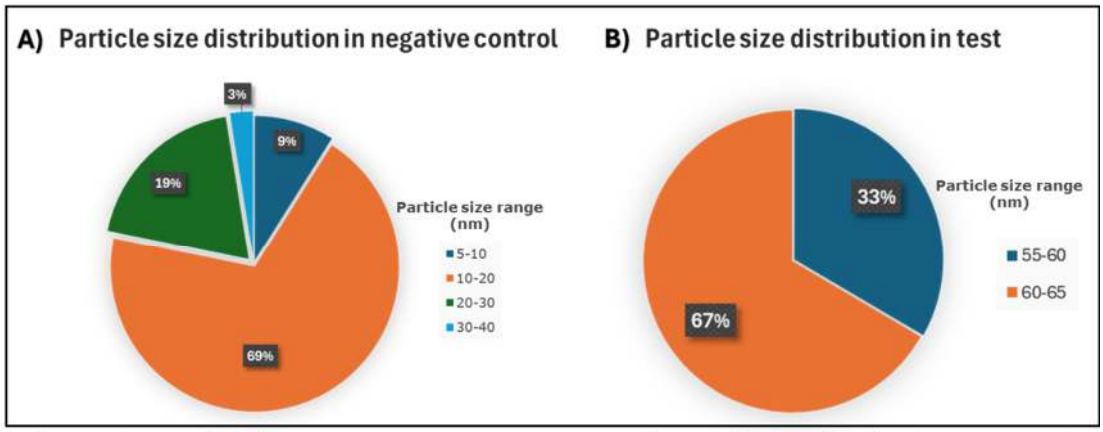

Figure 21. Graphical representation of TEM image: A) Particle size distribution in negative control; B) Particle size distribution in test supernatant.

In the negative control, the particles size was much smaller, ranging from 10-30 nm with the average particle distribution at 17 nm *(fig. 21 A).* On the other hand, the particle size distribution in the test supernatant ranged between 55-62 nm, with an average particle distribution at around 59 nm *(fig. 21 B).*

4.5 Aim 5. Assess expression of plasmid through RNA transcript analysis

Following RNA isolation from post transfected control and test HeLa cells. These cells were detected by semi quantitative PCR for the presence of transient mRNA containing DV1 cassette. Here, as well CAG promoter gene was amplified for the detection of DV1 cassette. The 301 bp band observed in transfected HeLa also corresponds to the size of CAG gene in plasmid DNA *(Fig. 22)*. Notably, the bands were visible only in RNA isolates of test cells. The same band was observed in plasmid utilized for transfection. No bands were observed in RNA isolates of control Hela and extraction control. The test was duplicated, and the results were consistent.

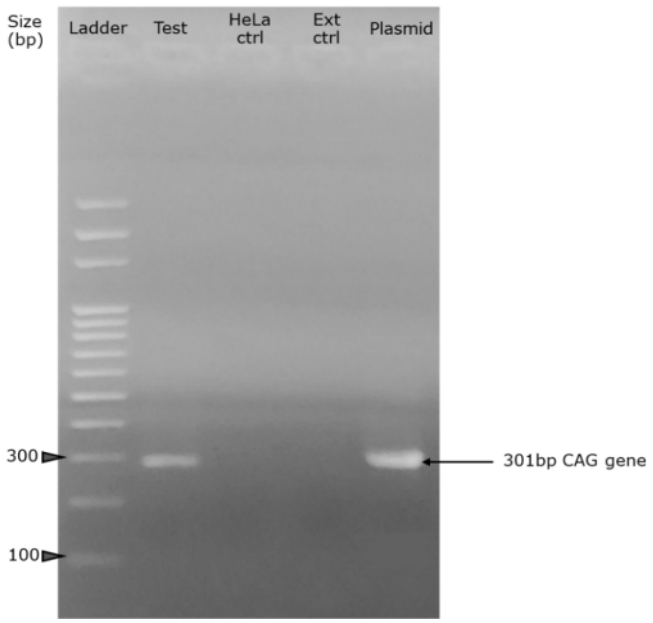

Figure 22. Agarose (1.5%) gel of CAG promoter gene amplification: lane 1, DNA ladder (100 bp); lane 2, transfected Hela; lane 3, transfection control; lane 4, extraction control; lane 5, plasmid used for transfection.

Chapter V

DISCUSSION

The increasing prevalence of Dengue and its gradual expansion into previously unaffected regions, even in face of climatic barriers underscores the pressing need for research on a secure and reliable Dengue vaccine. The inefficiency of traditional live attenuated vaccines stresses the importance of investigating alternative vaccine approach. The VLP containing the prM and E protein have been demonstrated by several group as a potential dengue vaccine candidate. This study explores the feasibility of producing VLP that has a potential to be used as an alternative vaccine approach. Here, we developed a simple and effective way to produce DENV-1 VLP in HeLa cells. This VLP could serve as a valuable resource for investigating maturation pathway of the virus and its immunological cascade in animal model. Additionally, the VLP could be utilized for the development of diagnostic tools like ELISA.

The study presents a simple method to produce monovalent VLP of DENV-1 serotype and examine its characteristics through TEM. Successful expression of DENV-1 VLP proteins was achieved in HeLa cells by transient infection with plasmid DNA containing DENV-1 structural proteins (prM and E). This process followed the fundamental principles of the central dogma of life, where the viral protein Information encoded in the plasmid DNA was transcribed to mRNA, a single stranded copy of gene, which then facilitated translation into functional proteins. Microscopy with trypan-blue staining detected assembly of VLP proteins on the cell membrane of HeLa transfected with DENV-1 engineered plasmid. Furthermore, transmission electron microscopy confirmed the expression of DENV-1 VLP with particle diameter of 55-62 nm *(Fig. 21 B)* in the purified culture supernatant. SDS-PAGE revealed protein bands of ~60 KDa, corresponding to the size of DENV-1 virus Envelope protein *(Fig. 19).* These findings, along with the detection of RNA transcript in transfected cells, correlates to the expression of the designed transgene. These expressed proteins self-assembled into

an average particle diameter of approx. 59 nm VLP, which corresponds to the VLP studied by other research groups.

The use of plasmid vector to express Dengue structural proteins, resulting in the formation of VLP with the correct conformational change, represents an alternative strategy for the development of Dengue vaccines. We achieved this through the design of a recombinant mammalian expression plasmid which contain an optimized transcriptional and translational element. This facilitated expression and extracellular release of DENV-1 VLP from HeLa cells. This approach was adapted from the previous studies on DENV-2 virus, where a recombinant plasmid was able to enhance the secretion of DENV-2 prM and E proteins in COS-1 cells (Chang et al., 2003; Zhang et al., 2011).

Efforts to develop DENV VLP in mammalian cells resulted in various degrees of success with production factor being a major limitation (Chang et al., 2003). To address this, the present study utilized an artificial expression vector, pCAGGS, which contained enhanced transcriptional and translational elements to increase the gene yield *(Fig. 13)*. The promoter of expression vector plays a crucial role in amplifying the expression of a gene of interest. The artificial chicken beta-actin (CAG) promoter composed of cytomegalovirus (CMV) enhancer combined with the chicken beta-actin promoter is known to enhance translation efficiency (Dou et al., 2020). Furthermore, the RNA termination signal, another key transcriptional element, plays an important role in transcription. Also, Bovine growth hormone poly (A) termination signal is known to enhance translation efficiency (Chang et al., 2003; Dou et al., 2020).

Promoters are DNA elements that initiate transcription of specific genes and are the key factors in determining the strength of transcription. In mammalian cells, CAG promoter exhibits the highest level of mRNA transcription. There exists a linear relationship between the expression levels of mRNA and protein. This indicates the importance of transcription regulatory elements in the expression vector (Dou et al., 2020). In our study, the RNA transcript formed in transfected cell was analyzed for the presence of CAG promoter gene by semi-quantitative PCR *(Fig. 22)*. The 301 bp CAG gene present in expression plasmid was also detected in RNA isolates of transfected

cells *(Fig. 17)*. This result indicates that the RNA transcript was successfully generated in the transfected HeLa cells.

In this study, the VLP was purified using the PEG-based precipitation method. The ~60 KDa VLP protein band corresponding to Envelop protein was clearly visible on SDS-PAGE gel, while the 20 KDa band corresponding to M protein was not visible (Fig. 19). The absence of M protein band could be either due to incomplete processing of prM or the protein level was too low for the detection. Similar findings were reported in studies of DENV-2 VLP (Chang et al., 2003). Additionally, minor bands of impurities related to host cell proteins were observed on SDS-PAGE gel. This was expected as the release of VLP involves acquisition of envelope derived from the host cell, leading to the presence of host encoded proteins in culture supernatant. Similar problems were encountered with influenza VLP produced in MDCK and Vero cells (Buffin et al., 2019). While the PEG-based method effectively eliminated majority of host and culture related contaminants, additional refinement of the purification process is necessary.

Table 2. Tabulation of VLP Particle diameter

Particle diameter (nm)	Particle range (nm)	Percentage
55.4	55-60	33
61.1	60-65	67
61.7	65-70	0

Under TEM, the purified VLP closely exhibited similar size and particle morphology to Dengue VLP as reported in previous studies (Zhang et al., 2011). The particle size ranged from 55-62 nm *(Fig. 21 B)*. These 55 nm particles are consistent with the size of mature flaviviruses. The variation in sizes could be attributed to the lack of core structures (Charoensri et al., 2014). A direct comparison of the morphology of the VLP with the natural DENV1 virus was not conducted. However, VLPs are known to be smaller in size than the natural viruses of approximately 55 nm. The DENV VLP typically range from 45-55 nm with size differences between serotypes. The variation in size is attributed to the maturation differences between serotypes (Metz et al., 2018). In our

study, the presence of VLP in test supernatant was analyzed by comparing it with the supernatant of HeLa cells (used as negative control). Both underwent the same culture conditions and purification process, except for plasmid infection. Unlike other studies that compared size of engineered VLP with specific mutated virions (Shen et al., 2018). The diameter of the VLP of our study as shown in *(Table 2)* was found to be comparable to mature dengue-1 virion as reported in other studies (Metz et al., 2018). Moreover, HeLa cells were found to provide suitable maturation environment for Dengue viral proteins, allowing them to self-assemble into mature VLP structures. The observed particles on the negative control exhibit the typical features of extracellular vesicles *(Fig. 21 A)*. These vesicles have well defined structure with smooth edges. They are generated either through exocytosis or budding from the plasma membrane and are involved in intracellular communication and are responsible for cellular exchange of proteins, lipids and genetic material (Reiter et al., 2019).

To produce Dengue-1 VLPs in HeLa cell, expression plasmid was constructed containing the entire sequence of prM and E region of DENV-1 virus. Depending on the degree of cleavage and release of pr portion to form mature M protein, heterogenous population of immature, partially immature and mature dengue particles are released by host cell. As VLPs undergoes same maturation pathway as wild-type DENV; VLP produced by infected cells generates similar heterogenous population (Shen et al., 2024). The furin cleavage site present in prM protein was not changed in our study. This could have possibly generated heterogenous VLP population of different diameter as shown in *(Table 2)*. However, the extent of VLP maturation cannot be concluded in the study since the epitopes displayed by the VLP were not examined through western blot or ELISA. This major limitation of the study was because of the challenges in supply chain and the restricted timeframe allocated for the research.

The study demonstrated the expression of DENV-1 prM and E proteins by utilizing pCAGGS expression vector containing CAG promoter, resulted in successful production of VLP in mammalian cell line. The proteins expressed in HeLa cells accumulated in cell membrane which eventually bud off from the plasma membrane

as self–assembled particles with diameter of ~59 nm. Additionally, a method for transient VLP production was established, setting the stage to produce VLPs for the remaining three serotypes in future.

Chapter VI

CONCLUSION

The structural protein based VLP are recognized as versatile particle for their applications in vaccines, targeted drug delivery, and serodiagnosis. We attempted to synthesize Dengue serotype-1 VLP that morphologically resembles natural mature Dengue virus. Additionally, the study also established a feasible method for transient production of VLP which could be employed in studies of other flaviviruses. This is the first of its kind research on the development of vaccine technology based on Nepal, against the disease that substantially impact public health and economy of the nation on a yearly basis.

The study aimed to produce VLP based on two structural proteins (prM and E) of the Dengue serotype-1 on the commonly used HeLa cell line. RNA isolated from transfected cells indicates transient formation of RNA transcript with the designed plasmid DNA. Assembly of VLP near cell membrane as observed in microscopic images of transfected cells could have resulted from translation of this RNA. Synthesis of the VLP was further confirmed by TEM images that depicted spherical particles resembling mature Dengue viruses. The study was able to generate simple DENV VLP without the hassle of modifying Dengue native sequence. This study forms the basis for future research on the development of monovalent DENV2-DENV4 VLP, along with tetravalent Dengue VLP.

In conclusion, the study outlines a viable and cost-efficient technique to produce DENV-1 VLP on mammalian cells utilizing structural proteins. The effective processing of proteins within the expression system was evident from the morphological characteristics of the particles, which closely mimicked the structural feature of mature Dengue virus. Although, the immunological aspect has not been studied, this strategy holds potential for the development of vaccines against emerging diseases.

LIMITATIONS OF THE STUDY

1. The morphology of the VLP was not directly compared with the natural Dengue virus.
2. The epitopes displayed by the VLP through was not studied.
3. PEG-based purification was not completely successful in eliminating host related protein contaminants.
4. HeLa cells were the only readily available cell line during the time of study.

RECOMENTAIONS / FUTURE PERSPECTIVES

1. VLP purification needs to be further optimized with other methods such as size exclusion chromatography.
2. The epitopes displayed by VLP need to be studied with ELISA or western blot. Additionally, the E antigen level present in supernatant needs to be quantified.
3. The immunogenicity induced by the purified VLP needs to be evaluated in a suitable animal model. Several lines of studies have suggested VLPs are able to elicit neutralizing antibody titers even higher than the natural viral infection which can eliminate the risk of ADE.
4. The purified VLP can be explored for the development of ELISA, a serodiagnostic tool to detect serum antibodies produced against Dengue.

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APPENDICES

Appendix 1: Protocol

VLP production

1. Seed cells to 80% confluency at the time of transfection in a 6 well plate by plating $0.3X10^6$ cells in 2ml of complete DMEM atleast16 hr prior to transfection.
2. Prepare transfection complex for each well of test plate as follows:
    a. Dilute 2.5 ug/well plasmid in 150 ul of reduced serum medium (Opti-MEM™).
    b. Dilute 10 ul Lipofectamine 2000 in 150 ul of reduced serum medium.
    c. Incubate tubes for 5 min at RT.
    d. Combine diluted Lipofectamine with diluted plasmid.
3. Incubate for 20 min at RT.
4. Wash the cells and dispense 700 ul of reduced serum medium in each well. Add 300ul of the above complex to each well of the test plate. For control plates dispense 1000 ul of reduced serum medium in each well.
5. Incubate the plates at 37°C for 5 hr.
6. Remove lipo-plasmid mixture. Wash the cells and dispense 2 ml of complete media in each well of Test and control plates.
7. Incubate at 37°C with 5% CO_2 for 72 hr.
8. Harvest both cell and culture supernatant after 72 hr.

Purification of culture supernatant

1. From each well collect supernatant of both test and control in a falcon tube
2. Clarify supernatant by low-speed centrifugation at 3000 rpm for 30 minutes.

3. Mix purified 15% PEG-6000 with equal amount of supernatant collected from above.
4. Incubate the tubes at 4°C for 48hr with constant agitation.
5. Pellet down the pegylated protein with low-speed centrifugation at 4000rpm for 40minutes. Repeat the centrifugation step twice.
6. Resuspend pellet in 100 ul of SM buffer.

SDS-PAGE

1. Prepare 12% resolving gel stacked with 5% stacking gel.
2. Prepare 20 ul each of test and control samples with equal amount of loading buffer. Boil the mixture at 95°C for 5 minutes.
3. Load the above sample onto the wells in addition to protein ladder.
4. Set appropriate volt and time for stacking and resolving gel until the dye reaches the bottom of the gel.
5. After washing the gel, perform Coomassie blue staining. Shake the gel on a horizontal rotator for 48 hr.
6. Destain the gel and incubate the gel on the same shaker overnight.
7. Capture the picture of gel with a white background.

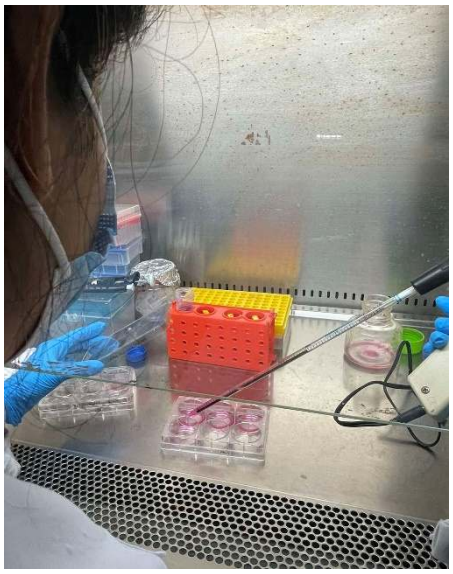
Cells harvest

1. After harvesting culture supernatant, wash cell with PBS
2. Trypsinize and dislodge cells from each well.
3. Pellet cells at low-speed centrifugation and resuspend pellet with 1 ml PBS.
4. Mix above cell suspension and 0.4% trypan blue in equal ratio to make 20ul final volume.
5. Load the cell-dye mixture in each chamber of the hemocytometer. Examine morphology of both test and control cells immediately under inverted microscopy.

RNA extraction from post transfected cells

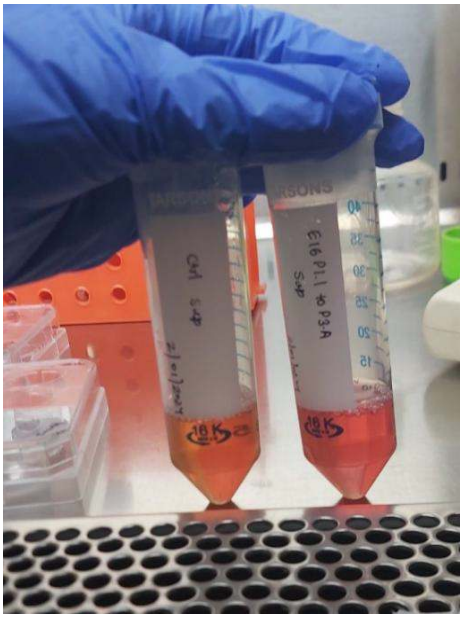
1. Lyse the cells suspended in PBS by heating at 95°C for 5 minutes.
2. Remove cellular debris by low-speed centrifugation.
3. Perform RNA extraction with Qiagen kit following manufactures instruction.
4. Assess the purity of extract with Nanodrop.

Appendix 2: Lab photographs



Harvesting of post-transfection culture supernatant

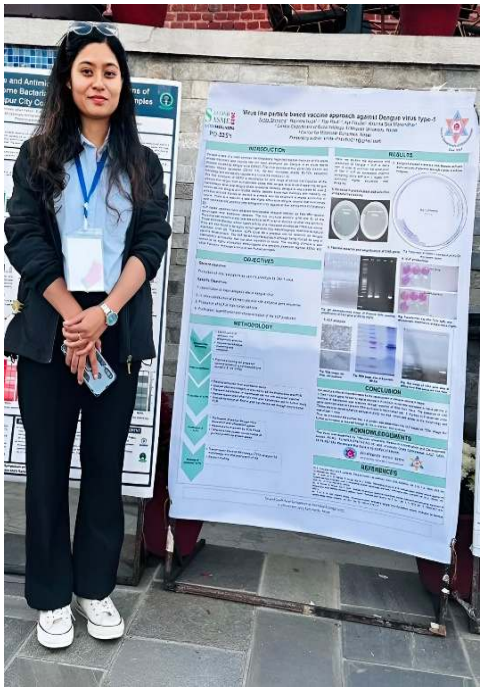
Purification of supernatant through high-speed centrifugation (Beckman coulter ultracentrifuge)



Harvested culture supernatant from test and control plates.

Harvesting of post-transfected cells.

Photographs



Poster presentation at South-Asian Symposium on Microbial Ecology (SASME-2023)

Conference presentation at CREID 2023, NIH, Maryland