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Cloning and Expression of Dengue Virus 2 NS3 Protein, Containing Helicase Domain

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ABSTRACT
Dengue is an intercontinental public health threat for human race transmitted by Aedes aegypti mosquitoes that endangers an estimated 2.5-3 billion people (approximately 40%-50% of the world's population) and represents a rapidly growing public health challenge. Dengue is an icosahedral, enveloped virus with a single stranded positive sense RNA genome. Dengue Virus (DENV) is a member of the flaviviridae family and has 4 antigenically distinct serotypes (DENV1-4). DENV causes a wide spectrum of clinical manifestations, from DF to dengue hemorrhagic fever (DHF), which may progress to dengue shock syndrome (DSS). The positive-sense flavivirus RNA genome of 11 kb forms a single open reading frame that is translated into a polyprotein precursor of ca. 370 kDa consisting of the structural proteins C, prM, and E and seven nonstructural proteins, nonstructural protein 1(NS1), NS2B, NS3, NS4A, NS4B, and NS5. The dengue non-structural 3 (NS3) is a multifunction protein, containing a serine-protease, located at the N-terminal portion, and Helicase, NTPase and RTPase domains present in the C-terminal region. Cleavage of the polyprotein is mediated by the seine protease N-terminal domain of NS3, with a hydrophilic segment of 40 residues from the trans- membrane NS2B protein acting as a cofactor necessary for this activity. In this context, the current study has been conducted to express, purify and characterize DENV2 full length NS3 protein along with NS2BH in E.Coli cells under native condition. For this, hydrophilic region of NS2B was amplified and fused with the amplified full length NS3. The resultant product was cloned into pQE30 expression host and expressed in BL21 cells. The purification showed a band at ~70 kD. However, there was some degradation in the purified protein. This could be due to the proteolytic activity of NS3. In the current study full length DENV2 NS3 protein was expressed in E.Coli cells along with NS2BH as cofactor. The purified NS2BH-NS3 protein needs to be characterized for its activity such as, protease, helicase and NTPase. This protein can be used to screen and identify novel inhibitors against helicase activity of NS3 and the selected inhibitor can be used as an antiviral compound against dengue.
KEYWORDS –Helicase, Non- Structural Protein, RNA virus, Viral Replication

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INTRODUCTION

Dengue is a globally important arboviral infection transmitted by Aedes mosquitoes that endangers an estimated 2.5 billion people and represents a rapidly growing public health problem [1, 2]. There are between 50 and 100 million infections each year, with approximately 500,000 cases admitted to hospital with severe and potentially life-threatening disease [3, 4, 5]. Dengue is an icosahedral, enveloped virus with a single stranded positive sense genome. It is a member of the flaviviridae family and has 4 antigenically distinct serotypes (DENV1-4) [6, 7]. After infection of a susceptible host, an acute, self-limiting febrile systemic syndrome ensues. Resolution of infection occurs within 4-7 days and is associated with a robust innate and adaptive immune response [1]. Infection with any of the DENV serotypes may be asymptomatic in the majority of cases or

may result in a wide spectrum of clinical symptoms, ranging from a mild flu-like syndrome (known as dengue fever [DF]) to the most severe forms of the disease, which are characterized by coagulopathy, increased vascular fragility, and permeability (dengue hemorrhagic fever [DHF]) [8]. The latter may progress to hypovolemic shock (dengue shock syndrome [DSS]). In Asia the risk of developing severe disease is greater in DENV-infected children (15 years) than in adults [9, 10, 11, 12]. In contrast, in the Americas mainly the adult populations is affected, resulting in mild disease, although an increasing trend o f cases progressing toward DHF/DSS has also been observed in adults there [13, 14, 15,16]. DF is manifested as an incapacitating disease in older children adolescents, and adults. It is characterized by the rapid onset of fever in combination with severe headache, retro-orbital pain, myalgia, arthralgia, gastrointestinal discomfort, and usually rash. Minor hemorrhagic manifestations may occur in the form of petechiae, epistaxis, and gingival bleeding. Leucopenia is a common finding, whereas thrombocytopenia may occasionally be observed in DF, especially in those with hemorrhagic signs [17, 18].

DENGUE VIRUS REPLICATION AND LIFE CYCLE

The intracellular life cycles of the flaviviruses are very similar. Infection with one of the arthropod-borne flaviviruses begins when the vector takes a blood meal and the virus is introduced into the host. The virus binds to and enters a permissive host cell via receptor-mediated endocytosis. Upon internalization and acidification of the endosome, fusion of viral and vesicular membranes allows entry of the nucleocapsid into the cytoplasm and genome uncoating.

Translation of the input strand takes place; then the virus switches from translation to synthesis of a negative-strand intermediate, which serves as a template for the production of multiple copies of positive-strand viral RNA (vRNA). Successive rounds of translation produce high levels of viral proteins:- the structural protein capsid or core (C), pre membrane (prM), and envelope (E) proteins, along with vRNA, are assembled into progeny virions, which are transported through the Golgi compartment and secreted[100]. While much that is assumed about DENV has been characterized for related flaviviruses such as yellow fever virus, West Nile Virus, apanese encephalitis virus, and tick-borne encephalitis virus.

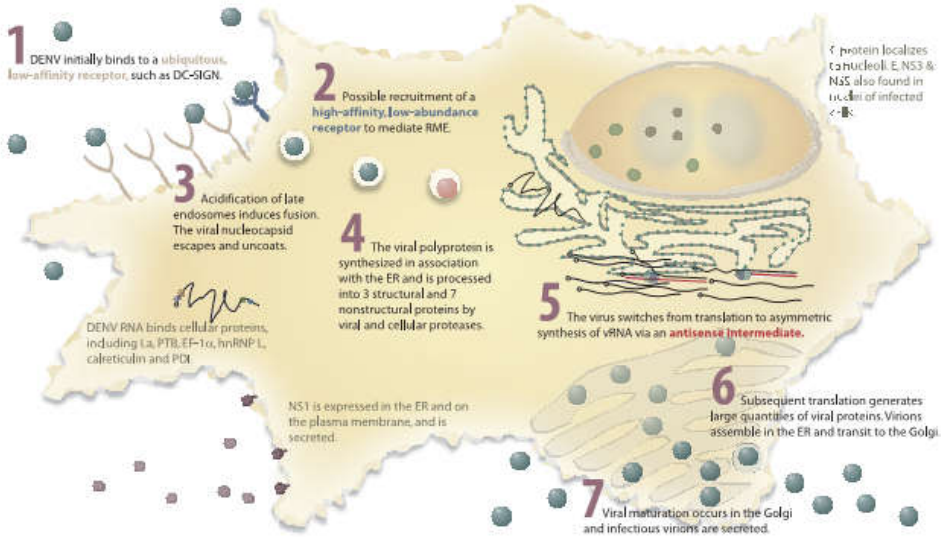

Figure 1.1 :- Intracellular life cycle of dengue virus. DENV binds (step1) and enters (step 2) cells via an uncharacterized receptor by RME. Endosomal acidification(step 3) results in an irreversible trimerization of the viral E protein, exposing the fusion domain. After being uncoated, the vRNA is translated (step 4) at ER- derived membranes, where it is processed into three structural and seven nonstructural NS proteins. After the viral replication complex is synthesized, vRNA translation switches off the RNA synthesis (step r5) begins. Subsequently, successive rounds of translation (step 7) and exist via the host secretory pathway. Viral protein C, E, NS3 and NS5 have been observed in the nuclei of the infected cells [91, 92, 93, 95] the vRNA association specifically with a number of cellular proteins

[96, 97, 98, 99] however, the biological significance is unknown. L-SIGN, liver/lymph node-specific ICAM-3 grabbing nonintegrin; PTB, polyprimidine tract binding protein; EF- 1, elongation factor 1; hn RNP L, heterogeneous nuclear ribonucleoprotein L; protein disulfide isomerase

GENOME ORGANIZATION

The positive-sense flavivirus RNA genome of 11 kb forms a single open reading frame that is translated into a polyprotein precursor of ca. 370 kDa consisting of the structural proteins C, prM, and E and seven nonstructural proteins, nonstructural protein 1(NS1), NS2B, NS3, NS4A, NS4B, and NS5 [19].

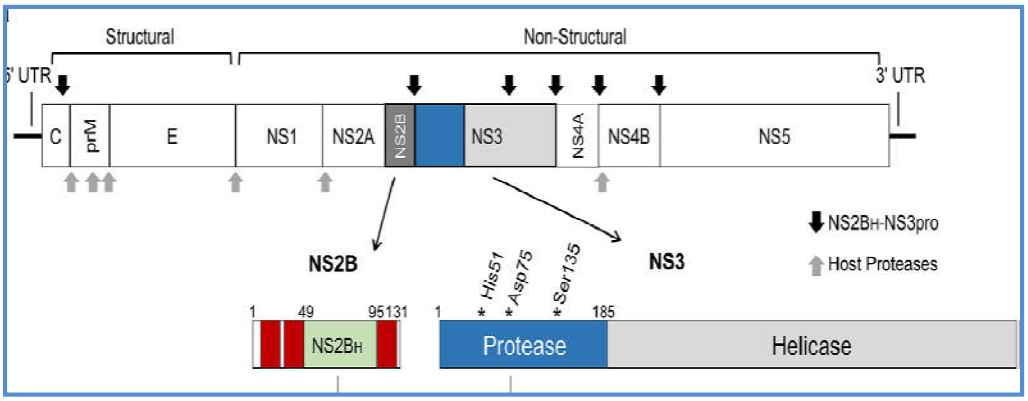

Figure 1.2:- Dengue virus precursor polyprotein with cleavage site of viral **NS2B-NS3pro** and host proteases [111]

As NS3pro alone is insoluble and self-aggregates, introduction of linker domain enabled highly efficient purification of a soluble, catalytically active protease complex [111].NS2BH actively participate in the formation of the S2 and S3 sub-pocket in the protease active site
NS5 is a large multifunctional protein with a C-terminal RNA-dependent-RNA polymerase domain that is required for viral replication [20] and an N-terminal methyltransferase domain required for RNA capping [21]. The functions of the remaining nonstructural proteins, NS1, NS2A, NS4A and NS4B, are less well understood. A number of functional studies, however, have shown that these proteins are involved in dengue pathogenesis and immune response in humans. Individual expression of NS2A, NS4A, or NS4B proteins can enhance replication of an interferon (IFN)-sensitive virus and down-regulate the expression of
IFN-β- stimulated reporter genes, suggesting that these proteins contribute to inhibition of the IFN-mediated viral defense system [22].
NS5 also inhibits expression of IFN-stimulated genes. NS1 (a secreted protein) contributes to immune evasion at least in part by interfering with the complement system [23, 24]. Although viral replication and maturation occur in the cytoplasm, endoplasmic reticulum, and Golgi apparatus [25], the capsid and NS5 proteins are also detected in the nucleus [26, 27].

Table 1.1: - Functions of non-structural proteins [28].

NS proteins	Function
NS1	Plays role in viral RNA replication complex, act as soluble components fixing antigen.
NS2A	Forms part of RNA replication complex
NS2B	Binds to NS3 protein as a cofactor
NS3	Serine-protease, RNA helicase and RTPase /NTPase
NS4A	Possibly induces membrane alteration. Involves in viral replication
NS4B	Blocks IFNα/β induced signal transduction
NS5	Methyltransferase (Mtase), RNA dependent RNA polymerase.

Non-Structural Protein 3

During viral maturation, this polyprotein is cleaved by host cell proteases in the endoplasmic reticulum and by the NS3 protein in the cytoplasm [19]. Cleavage of the polyprotein is mediated by the seine protease N-terminal domain of NS3, with a hydrophilic

segment of 40 residues from the trans- membrane NS2B protein acting as a cofactor necessary for this activity. The domain required for ATPase/helicase and nucleoside 5-triphosphatase activity is located at the c -terminus of NS3. Thus, the NS3 protein is a target of prime importance for antiviral therapy [29, 30, 31]. Several nonstructural proteins encoded by the viral genome form a membrane-bound RNA replication complex, possibly with the participation of some host factors. The atomic structures of several individual components of this replication complex are known, including the NS3 protease domain (NS3pro) in the absence of the NS2B cofactor [32], the active NS3 protease domain (NS2B40NS3pro) [33, 34], the ATPase/helicase domain (NS3 helicase domain [NS3hel]) [35, 36] , the NS5 methyltransferase, and the RNA-dependent RNA polymerase catalytic domain [37, 38, 39]. Using full-length NS3 enzymes linked to 40 residues of the NS2B cofactor, elegant studies recently demonstrated that the NS3 protein undergoes auto cleavage at two sites located at the NS2B-NS3 junction and within the helicase C-terminal region, respectively [40].

The dengue non-structural 3 (NS3) is a multifunction protein, containing a serine-protease, located at the N-terminal portion, and helicase, NTPase and RTPase domains present in the C-terminal region. This protein is considered the main target for CD4+ and CD8+ T cell responses during dengue infection, which may be involve in protection [41, 42, 43, 44]. However, few studies have been undertaken evaluating the use of this protein as a protective antigen dengue, as well as other flavivirus [45, 46]. The helicase domain unwinds RNA during viral RNA replication and energy for reaction is provided by the NTPase activity [47, 48]. In addition, the RTPase activity catalyzes the cleavage of the γ-β phosphoric anhydride bond of 59-triphosphorylated rna, important for rna 59-capping [48].

The development of an effective vaccine against dengue became a priority for the World Health Organization (WHO) due to the increasing incidence of severe cases of the disease and its large geographical extension in the world. However, one of the main obstacles for developing such vaccine is the requirement of activating a protective immune response against all four DENV serotypes, without the risk of inducing severe disease [49, 50, 51]. The dengue infection elicits different immune responses towards the viral proteins. Antibodies are generated mainly against the virus surface E protein and the secreted NS1 protein [52, 53, 54], while the majority of T-epitopes are concentrated within the NS3 protein, the main target for CD4+ and CD8+T cell response [55, 56, 57, 58]. Vaccines against flavivirus are generally based on the E protein, which contains most of the epitopes that elicit neutralizing antibodies. This protein may also induce non-neutralizing antibodies [49, 57] involved in the phenomenon of antibody-dependent enhancement (ADE) of DENV infection [59, 60, 61], which can be associated to the occurrence of increased numbers of DHF in secondary infections. Alternatively, some reports suggest the use of non-structural proteins for dengue vaccines to overcome such problem [62, 63, 64, 19, 65].

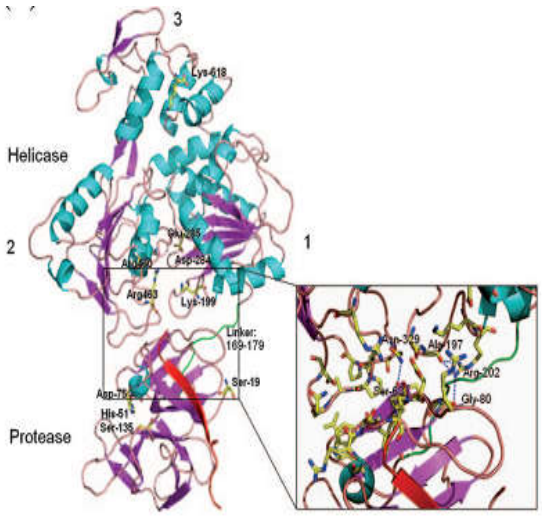

Figure 1.3:- Ribbon representation of the NS2B-NS3 structure. Secondary structure elements are colored in cyan (a-helix) and magenta (β-strand). The three subdomains of NS3hel are numbered. NS2B, which forms a β-strand, is red. The region linking the

protease and helicase (residues 169 to 179) is green. Key residues for NS3 enzymatic activities are shown as sticks and labeled. N-terminal residues are also labeled. A close-up view of the interface between the helicase and protease domains is also shown [19].
The NS1 is also highly immunogenic and may generate antibodies with complement fixing activity, probably triggering the lyses of infected cells which present this protein on its surface [61, 63, 65]. Nevertheless, antibodies against the NS1 may also cross-react with human proteins, which can be associated to some pathological effects of the dengue infection [66, 67, 68]. In contrast, there are only few studies evaluating the use o the NS3 protein as a protective antigen against DENV, as well as other virus from the Flaviviridae family. Immunication with the NS3 from flavivirus induced only marginal protection in different animal models [69, 70,71]. Studies with the NS3 from DENV reported that mice inoculated with monoclonal antibodies against this protein showed an increase survival time after virus challenge, although most of animals died in the end of the experiment [72]. Further studies suggested that the combination of NS3 with other flavivirus proteins may have a synergetic effect, leading to the Increase of protection rates against virus challenge [73, 74]. On the other hand, other reports suggested the involvement of an immune response against the NS3 in the pathogenesis of DHF [75, 76, 77]. Therefore, in the present work we are evaluating the expression of NS3 and Helicase activity of DENV2. Different plasmids were constructed encoding the full-length NS3 of DENV2 or only its functional domains (helicase), fused or not to a signal peptide in order to secret the recombinant protein to extracellular medium.

MECHANISM OF ACTION

Upon primary infection with DENV, there is an incubation period averaging 4-7 days. In this time the virus replicates in the dendritic cells in close proximity to the bite, also infecting macrophages and lymphocytes, and finally into the bloodstream. Dendritic cells (DCs) are antigen-presenting cells that are integral to inducing an immune response. Wu et al. (2000) have demonstrated that the dengue virus preferentially targets DCs, specifically monocyte-derived DCs (resembling interstitial DCs) and human skin Langerhans cells (LCs). By inoculating human skin explants with DENV in vitro, they demonstrated 60-80% expressed DENV antigens, and only immature cells were permissive to infection. The cells infected consisted of a mixture of LCs and interstitial DCs [78, 79]. Cell surface heparin sulfates are also involved in cell surface attachment of dengue virus to mammalian cells. Heparin sulfates are repeating disaccharides of uronic and L-iduronic acids (derived from glucosamine), and are variably O- glycosylated [80]. Despite these efforts, the attachment and viral entry into the cell remains poorly characterized.

Table 1.2: Dengue serotype association to severity

Dengue Serotype	Association to Dengue disease
DENV-1	Primary infection results in frequently more severe disease when compared to DENV-2 or DENV-4
DENV-2	Secondary infection associated with more severe disease(Twice as likely to result in DHF than DENV-4)
DENV-3	Primary infection results in frequently more severe disease when compared to DENV-2 or DENV-4 .Asecondary infection is twice as likely to result in DHF than DENV-4
DENV-4	Least associated with severe dengue disease

Co- receptors for viral entry into dendritic cells have been identified and analyzed. One of the receptors is a C-type lectin, CD209/DC-SIGN, and is thought to blind to the viral E protein, aiding in entry into the dendritic cells [81, 82]. Another finding identified the mannose receptor present in macrophages [83]. Kwan et al. (2008) presented evidence that drmal macrophages serve as the first innate immune cell response, with capabilities of protecting against the dengue virus after a mosquito bite. Additionally, the C-type lectin domain family 5 A (CLEC5A) can serve as a pattern recognition receptor for macrophages interacting with dengue viruses to stimulate proinflammatory cytokines release [84].
Dengue infection is an immune pathological disease in which an immune response may aggravate DENV infection and cause damage to the host. This is thought to be caused by antibody-dependent enhancement (ADE), and was proposed as an underlying pathogenic

mechanism of DHF/DSS as early as 1960 [85]. It is thought this occurs because of pre-existing sub-neutralizing antibodies and the DENV form complexes that blind to Fc-γ receptor bearing cells, which leads to increased viral uptake and replication. This increase in viral replication directly increases the amount of virus in the blood, contributing to DHF/DSS. A strong association between severe secondary infections and host genetics, as well as virulence of different virus strains may also play a role in DHF pathogenesis [86, 49, 42, 87, 88]. Although ADE is a widely accepted theory, there is still much to be proven regarding the direct cause of enhancement. The helicase domain of NS3 (NS3Hel, residues, 180-618) has seven structural motifs reminiscent of superfamily 2 helicases [104]. It has three subdomain with significant sequence identity and structural similarity to other flavivirus helicases [105, 106] subdomain I and II are also structurally similar to the corresponding domains in the hepatitis C virus, suggesting a common functional mechanism [107].

VIRUS INTERACTION

Transmission of DENV is dependent on the vector mosquito *Aedes aegypti*, and to a lesser extent *Aedes albopictus*. The spread of DENVs mirrors the vectors geographical distribution underlining why mosquito density is an important parameter for predicting DENV epidemics [89]. The female mosquitoes lay their eggs in artificial water containers such as tires, cans, and jars. Due to water requirements for breeding, mosquito densities peak during wet season, with the direct consequence of rising numbers of dengue cases. The *Aedes Aegypti* mosquito is well adapted to an urban environment and is a highly competitive vector due to its anthropophilic nature. It thrives in close proximity to humans and is an intermittent feeder implying a high frequency of multiple host contacts during a single gonotrophic cycle. Thus, the female mosquito can infect multiple persons in order to complete a single blood meal. Protective clothing and mosquito repellent sprays are essential to avoid DENV transmission since the *Aedes* mosquitoes are active during the day, minimizing the use of mosquito net.

In general, *Aedes aegypti* is less susceptible to infection by DENV than *Aedes albopictus*, which could act as a selection mechanism for more virulent strains of DENV; the lower susceptibility would require a higher viral load in the human host in order to infect the mosquito. High viral titers in humans have been seen to be correlated to severe DHF/DSS. On the contrary, the secondary vector *Aedes albopictus* could transmit DENV strains that do not replicate to such high titers resulting in less clinically overt or severe disease. This scenario proposes that *Aedes albopictus* could function as a maintenance vector involved in the silent transmission of DENV during inter-epidemic periods. However, the susceptibility of the mosquito vector and transmission dynamics are also dependent of DENV strains, but the mechanisms underlying the inter-specific and inter-strain differences in vector susceptibility to DENV infection remains to be determined.

Once ingested by the mosquito, the DENV establishes a productive infection in the mosquito mid gut, where from the virus disseminates and replicates in other tissues. In order to be transmitted to a human (or non-human primate [NHP]) host during the next blood meal, the DENV must ultimately infect the salivary glands and be shed in the saliva. Vector competence is genetically determined and genetic traits influencing both mid gut infection and escape barriers have been mapped to various loci on the *Aedes aegypti* chromosomes [90].

In this context, the current experiment was proposed to clone, express, purify and characterize Dengue 2 NS3 full-length containing Helicase protein in E.Coli cells. The protein will be used to screen antiviral compounds targeting helicase activity of the protein.

MATERIAL AND METHODS

Chemicals and reagents

1. Tris acetate EDTA (Appendix)
2. LB Broth (Appendix)
3. LB Agar (Appendix)
4. Kanamycin (Cat no- K 4378, Sigma) (Appendix-4)
5. Ampicillin(Catalogue number A0166, Sigma) (Appendix-5)
6. Trackit 1 Kb plus Ladder (Cat no-10488-085,Invitrogen)

7. Ethidium Bromide (Cat no-1510,Invitrogen)
8. Agarose (Cat no-a9538,Sigma)
9. Pure Link Viral RNA/DNA mini kit (Cat no-12280-050,Invitrogen)
10. Mini Elute Gel Extration Kit (Cat no-28604, Qiagen)
11. Mini Elute Pcr purification Kit (Cat no-28004, Qiagen)
12. LauriaBertani Agar(Cat no-M1151,Himedia)
13. LauriaBertani Broth(Cat no- M1245,Himedia)
14. EcoRI (Cat no-15202-013, Invitrogen)
15. Platinum PCR SuperMix(Cat no-11306-016,Invitrogen)
16. RNase A(Cat no-1007885,Qiagen)
17. Ethanol (Cat no-XK-13-011-00009,Changshu yangyuan chemical
18. pQE-30 vector Qiagen
19. SDS (Sigma Aldrich)
20. Tris base (SRL)
21. Glycine (SRL)
22. TEMED (Hi- media)
23. Denv2 replicon template(Gift from Dr. Barry Falgout , FDA, USA.)
24. Platinum PCR SuperMix(Cat no-11306-016,Invitrogen)
25. BamHI&HindIII (NEB)
26. pQE-30 vector Qiagen
27. T_4ligaase (Invitrogen)
28. P_1 buffer (Appendix 10)
29. P_2 buffer (Appendix 11)
30. P_3 buffer (Appendix 12)
31. Protein storage buffer(Appendix 7)
32. 200Mm imidazole
33. RNase A(Cat no-1007885,Qiagen)
34. APS (Sigma- Aldrich)
35. Acrylamide (GeNei)
36. B- mercaptoethanol(SRL)
37. Bromophenol blue (SRL)
38. Coomasie brilliant blue R250 and G250 (SRL)

Construction of DENV2 NS3 FULL

Design the primer for of DENV2 NS3 FULL

NS2B hydrophobic region(NS2BH),4276-4407 nt region in NS2BH is selected for PCR using forward primer1(5'-GATTTGGAACTGGAGAGAGAGCCGCC-3') Reverse primer1(5'-TTGGCGCGCTGTTCTTCCTCTTCGTTTTTTATCGAC-3').The NS3 helicase domain spans 4522-5076 nt, encoding 185 amino acid from the N-terminus, is amplified using forward primer2 (5'-TTGGCGCGCTGGAGTATTGTGGGATGTCCCTTCACC3') and reverse primer2 (5'-CGCGGATCCGCCGATTTGGAACTGGAGAGAGCCGCC-3'). The component of PCR given in the following table(table 1). DENV2 Full length clone was used as template.

TABLE 2.1:- PCR Reaction

S.I	COMPONENT	VOLUME (µl)
1	Platinum super mix	6.25
2	Template DNA	3
3	Forward primer	0.5
4	Reverse primer	0.5
5	Nuclease free water	2.25
6	Total volume	12.5

TABLE 2.2:- Thermal cycler program for PCR

Denaturation	95®C	5 min	
Subsequent denaturation	95®C	30 sec	
Annealing	55®C	30 sec	35X
Extension	72®C	60 sec	
Final extension	72®C	10 min	
Hold	4®C	∞	

PCR condition and Analysis of PCR Products:
Initial denaturation for 5 min at 95℃(1cycle); subsequentcycles (~35) consist of denaturation for 30s at 95℃, annealing for 30s at 60℃, and extension for 60s at72℃; the final extension for 10 min at 72℃; keep at 4℃ until it is used at a subsequent step.PCR product were analysed by (1%) agarose gel (Appendix 6) electrophoresis; .0.5 g of (1%) agarose was added into 50mL of 1x TAE buffer (Appendix 1) and heated in microwave oven to dissolve and make a clear solution ,kept it for gradually cool down, and added 2.0 µL syber safe (Invitrogen) before the solution is solidified. Poured theagarose solution into a gel electrophoresis tray. Let it kept for 30 min. Add 10µl loading dye (6x)(invitrogen) into the 50µL PCR products. Gently mixed it and loaded into the wells and run the electrophoresis at 65V mA for 60 minutes.(BioRad Gel Electrphoresis system).The (1%) agarose gelwas observed under the uv light.(BioRadVersaDoc)

Gel Extraction & Purification
PCR amplified product was eluted from the gel using Mini Elute Gel Extraction Kit (Qiagen) and purified as per the manufacture's instruction. The purified samples were analysed on 1% TAE agarose gel (Appendix 6)

PCR product ligation
The gene of interest (NS3 FULL) was ligated to PQE vector. For this, the PQE vector was digested using restriction enzyme, BamHI and HindIII. Then the digested (BamH1 and HindIII) PCR product was ligated to the vector. Ligation was carried out by following components as described below (Table 2.3).

Table 2.3: -Ligation reaction.

Components	volume
10X T4 DNA Ligase Buffer	2 µl
Vector DNA	2 µl
Insert DNA	6 µl
Nuclease-free water	9 µl
T4 DNA Ligase	1 µl
Final volume	20 µl

Competent cell preparation
A single colony of DH5-α cells or BL-21 were picked from a plate and inoculated into 5ml LB broth medium and incubated the culture overnight at 37°C in the shaking incubator at 250rpm . One ml from the overnight culture is inoculated into 100 ml of fresh LB broth and incubated it in the shaking incubator at 37°C at 250rpm until the OD (Optical Density)reached to 0.4 at 600nm . Then the culture was placed on ice for 10 minutes. After this the culture was transferred into two pre-chilled 50ml Tarson tubes and centrifuged at 2700xg for 10 minutes at 4°C, the supernatant was removed and leaving the pellet. The pellet was resuspended with 1.6ml of pre-cooled 100mM $MgCl_2$-$CaCl_2$ by swirling on ice gently and incubated on ice for 30 minutes, then centrifuged at 2700xg for 10 minutes and removed the supernatant and leaving the pellet.
Then the pellet was resuspended with 1.6ml of pre-cooled 100mM $CaCl_2$ by swirling on ice gently and incubated for 20 minutes. The two solutions were combined into one tube and added with 0.5ml ice-cold Glycerol and swirled to mix properly. Aliquoted 100µl of cells pipetted into 1.5ml eppendorff centrifuge tubes and stored at -80°C.

Transformation of NS3 full into DH5-α
The Competent DH5α cell thawed on ice gently mixed it by taping. 100µl DH5α was added into 1.5 eppendorf,kept on ice for 5 minutes. Ten microliter of the above ligated mixture was added to the cell and mixed gently, kept on ice for 30 minutes. the cells were treated to heat shock at 42ºC for 45 seconds without shaking, placed in ice for 5 minutes.900 µl LB broth(Appendix 3) was added to the cell,incubated for 1-2 hours at 37ºC on 125rpm on shaking incubator(Orbitek).Then the culture was centrifuged at 5000 rpm for 3 minutes,discarded the supernatant and resuspended the pellet with 600µl of fresh LB media(Appendix 3)and spreaded into LB-ampicillin plate and kept in 37ºc for 12 hours.Clear colonies were appeared in the plate, indicating the positive transformation. The colonies were screened using PCR as per the protocol mentioned above.

Plasmid isolation

The positive colony was picked from the culture plate and inoculated into 5ml LB broth and incubated for overnight at37°C. The over–night culture taken into 2ml eppendorf microcentrifuge tubes and centrifuged at 7000 rpm for 10 minutes at 4°C and then discarded the supernatant and leaving the pellet. The pellet was resuspended with 250µl of resuspension buffer (P1 buffer), and the cell was lysed by adding 250µl Lysis buffer (P2 buffer) and Then the solution was neutralized with 3M Potassium acetate buffer (P3 buffer), mixed gently by inverting the tubes and kept on ice for 5 minutes. Then centrifuged at maximum speed (14,000 rpm) for 10-15 minutes at 4°C and carefully collected the supernatant. 700µl of ice cold isopropanol added to the supernatant and let the plasmid to precipitate to 15-20 minute on ice and centrifuged at maximum speed for 15 minute and collected the pellet. Then, 500µl of ice cold 70% ethanol was added to the pellet and mix well and centrifuged at 14,000rpm speed for 15 minute at 4°C. Removed the supernatant carefully and the pellet was subjected to air dry. Then the pellet was dissolved in 40µl of 1X TE- buffer and stored in -20°C.Plasmid were analyzed by (1%) agarose gel (Appendix 6) electrophoresis.

Restriction digestion

The plasmid is digested with BamHI and HindIII(New England Biolabs) with the reaction component following table 2.4.as per manufacture's instruction. Restricted fragment were analysed by (1%) agarose gel electrophoresis.

Table 2.4: Restriction Digestion Reaction

Components	Volume (10 µl)
Buffer D	3µl
Nuclease free BSA	1µl
HindIII	0.5µl
BamHI	0.5µl
Plasmid	3µl
Distilled water	2µl
Total reaction volume	10 µl

Transformation NS3 full into BL-21

The Competent BL-21 cell thawed on ice,gently mixed it by taping. 100µl BL-21 added into 1.5 eppendorftube,kept on ice for 5 minutes. 5µl of plasmid DNA was added to the cell and mixed gently and kept on ice for 30 minutes. The cells were treated to heat shock at 42°C for 45 seconds without shaking, placed in ice for 5 minutes.900 µl LB broth (Appendix1) was added to the cell,incubated for 1-2 hours at 37°C on 125rpm on shaking incubator (Orbitek).Then the culture was centrifuged at 5000 rpm for 3 minutes ,discarded the supernatant and resuspended the pellet with 600µl of fresh LB media (Appendix1) and spreaded into LB-ampicillin plate (Appendix 1) and kept in 37°c for 12 hours. Clear colonies were appeared on the plate,indicating the positive transformation.

IPTG induction and expression of protein

The plasmid was transformed into BL-21 bacterial expression system. A single transformed colony was inoculated into 5ml of LB media containing ampicillin (Appendix 1) and kept in 37°C incubator for overnight.1ml of the culture transferred to 100 ml of LB media containing ampicillin and incubated at 37°C until the OD reached to 0.6 at 600nm.Then 1M IPTG was added to the cell culture andkept in 37°C for 3 hours. The induced culture was collected every 1 hour interval (2ml) for three times and centrifuged at 4000 rpm for 10 minutes which Showed the pattern of expression by SDS PAGE analysis. The pellet was resuspended with 50µl of protein storage buffer(Appendix 1). The pellet was freeze thawed on ice for 3 times and was sonicated for 10 minutes,centrifuged at 14000rpm for 10 minutes.

Transferred the supernatant into 1.5ml Ni-NTA beads and incubated at the Ni-NTA column for overnight.Washed the protein using protein storage buffer 10 times and eluted the protein using 200mM imidazole (Appendix 1). Proteins were analyzed by SDS-PAGE method.

Purification of protein by Ni-NTA.

After 3hrs of expression, the culture was centrifuged. Resuspended the pellet with 50µl of protein storage buffer. Pellet was freeze thawed on ice for 3 times and was sonicated for 10 minutes at 15 pulse on and 45 pulse off and 30% amplitude,centrifuged at 12000rpm for 15 minutes.

Transferred the supernatant into 1.5ml Ni-NTA beads and incubated at the Ni-NTA column for overnight.Washed the protein using protein storage buffer 10 times and eluted the protein using 200mM imidazole.Protein were quantified by BCA assay and analyzed by SDS-PAGE.

Estimation of protein concentration by BCA assay

The BSA standard were prepared,working reagent were prepared by mixing 50 part of BCA reagent A with 1 part of BCA reagent B(50:1). 25µl of unknown sample is loaded into the microplate well.200µl of working reagent added ito each well.covered the plate and incubated for 30 minutes at 37°C. Kept the plate for cool to Room Temperature.Measured the absorbance at 562 nm on a Multimode plate reader (Perkin Elmer).

SDS-PAGE analysis.

SDS-PAGE is the most widely used technique to separate the protein. In the SDS-PAGE, the detergent SDS and heating step determine that the electrophoretic mobility of a single kind of protein is only affected by its molecular weight.SDS gel in single electrophoresis can be divided into stacking gel and resolving gel.Stacking gel(5%) is poured on the top of the resolving gel(10%) and gel comb is inserted in to the stacking gel. The casting frames were set on the casting stands.The stacking and resolving gel prepared as given below.

Table 2.5:- Resolving gel and stacking gel composition.

Components	Resolving gel (10%) ml	Stacking gel (5%) ml
Distilled water	4	3.4
30% acrylamide/bis	3.3	0.83
1.5 M Tris, pH 8.9	2.5	-
1.0 Tris, pH 6.8	-	o.63
10% SDS	0.1	0.05
10% APS	0.1	0.05
TEMED	0.013	0.09

Appropriate amount of resolving gel solution was poured onto the gap between the glass plate. To make the top of the resolving gel be horizontal, filled with water into the gap, waited for 20-30 minutes to solidify. Discarded the water and poured the stacking gel. Inserted the well forming comb without trapping the air under the teeth and kept it for 20 minutes to solidify. After the completion of solidification took the glass plates out of the casting frame and placed them in the cell buffer dam. Poured the running buffer inner chamber and kept until the buffer surface reached to the required level in the outer chamber and take out the comb. Sample were heated in a water bath at 95ºC for 5-8 minutes. Loaded the protein marker on first lane and cooled sample were loaded in to the well immersed in 1x tris glycine electrophoresis buffer, pH 8.3 (Appendix 1) and electrophoresis was carried out at stacking gel for 75V and resolving gel for 95V until the tracking dye reaches anode tank buffer at the bottom.(BioRad Mini-Protean Gel electrophoresis system).

RESULTS

PCR NS2BH-NS3 Amplification

NS2B hydrophilic region(NS2BH),4276-4407 nt region in NS2BH is selected for PCR using forward primer1(5'-GATTTGGAACTGGAGAGAGAGCCGCC-3') Reverse primer1(5'-TTGGCGCGCTGTTCTTCCTCTTCGTTTTTTATCGAC-3'). The NS3 helicase domain spans 4522-5076 nt, encoding 185 amino acid from the N-terminus, is amplified using forward primer2 (5'-TTGGCGCGCTGGAGTATTGTGGGATGTCCCTTCACC3') and reverse primer2 (5'-CGCGGATCCGCCGATTTGGAACTGGAGAGAGCCGCC -3')

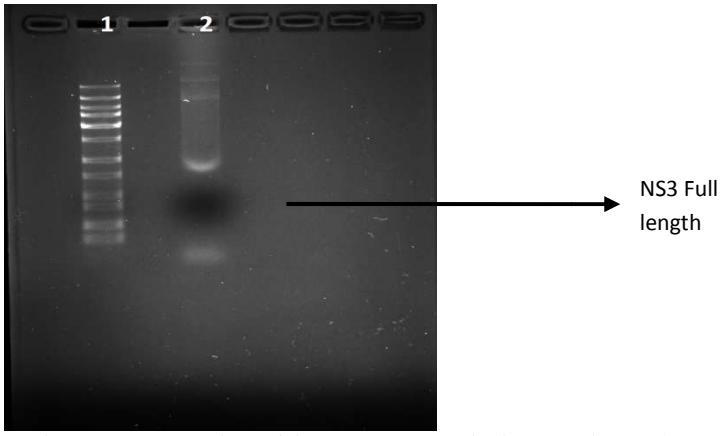

Figure 3.1: PCR amplified product were analysed by agarose gel electrophoresis. Lane 1- molecular marker,lane-2 NS2BH-NS3 full

Transformation of NS3-Full length into DH5-α

The above PCR product were ligated with predigested (BamHI and HindIII) pQE-30 vector and transformed into DH5α competent cells.

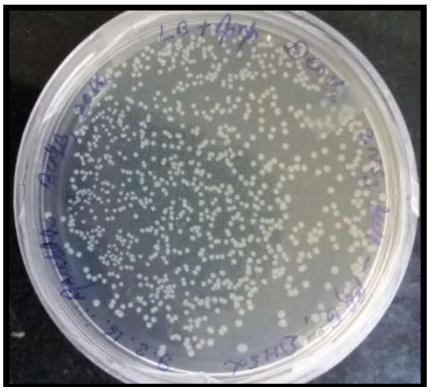

Figure 3.2 The PCR product were ligated with pQE-30 vector and transformed into DH5α competent cells.

Plasmid isolation

Plasmid Isolation from the above DH5α colony.

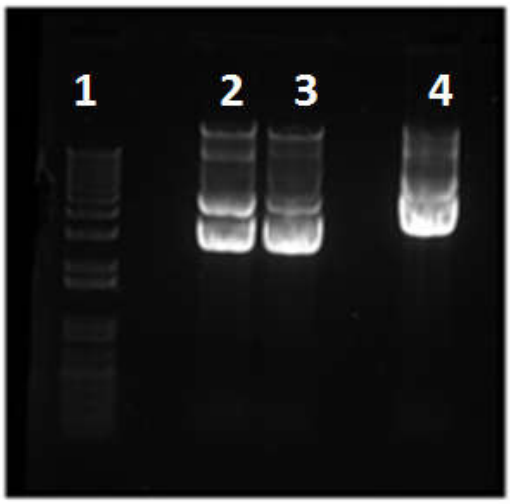

Figure 3.3: Plasmid were isolated from DH5-α and analysed by (1%) agarose gel electrophoresis. Lane-1 molecular marker and lane-2 pQE-30-NS2BH-NS3-full plasmid. Lane 3 – sample 2, lane 4- sample 3

Restriction digestion

Ligation were confirmed by digesting the plasmid with BamHI and HindIII

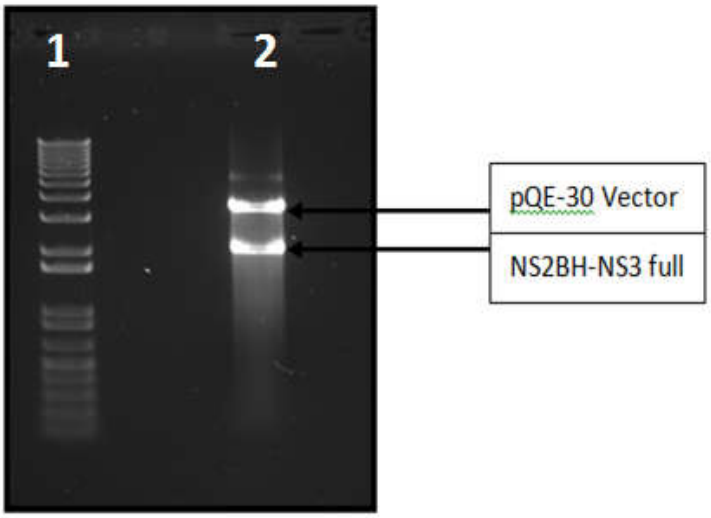

Figure 3.4: Ligation was confirmed by digesting the plasmid with BamHI and HindIII.Product were analysed by (1%) agarose gel electrophoresis. Lane-1 molecular marker and lane-2 showing plasmid digested into pqe vector and NS3 full length separately.

Transformation of NS3 full length into BL-21

The Isolated plasmidpQE-30 NS3 full (Figure 3.3)were transformed into Bacterial expression host (BL-21).

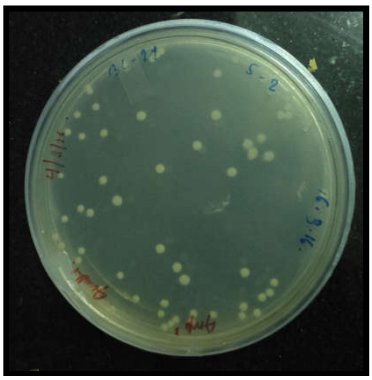

Figure 3.5. The Isolated plasmidpQE-30-NS3 full length were transformed into Bacterial expression host (BL-21).

IPTG induction and expression

BL-21 transformed colony containing pQE-30-NS3 full length were induced with IPTG. A single transformed colony was inoculated into 5ml of LB media containing ampicillin (Appendix 1) and kept in 37°C incubator for overnight.1ml of the culture transferred to 100 ml of LB media containing ampicillin and incubated at 37°C until the OD reached to 0.6 at 600nm.Then 1M IPTG was added to the cell culture and kept in 37°C for 3 hours. The induced culture was collected every 1 hour interval (2ml) for three times and centrifuged at 4000 rpm for 10 minutes which Showed the pattern of expression by SDS PAGE analysis. The pellet was resuspended with 50µl of protein storage buffer (Appendix 1). The pellet was freeze thawed on ice for 3 times and was sonicated for 10 minutes, centrifuged at 14000rpm for 10 minutes. Transferred the supernatant into 1.5ml Ni-NTA beads and incubated at the Ni-NTA column for overnight

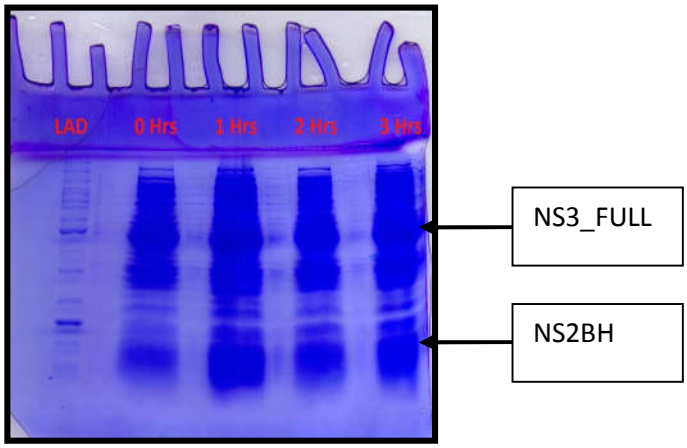

Figure 3.6 :BL-21 transformed colony were induced with IPTG. In every 1 hour interval 2ml of the culture was pellet to show the pattern of expression. Expression was analysed by SDS-PAGE. Lane-1 molecular marker,lane-2 0 hour,lane-3 one hour,lane-4 two hour and lane-5 three hour.

Purification of protein by Ni-NTA

Ni-NTA purification system designed for purification 6xHis-tagged recombinant protein. Ni-NTA resin exhibits high affinity and selectivity for 6xHis tagged recombinant protein. Protein was purified under native condition. Protein bound to the resin is eluted by using imidazole.

Protein estimation by BCA assay

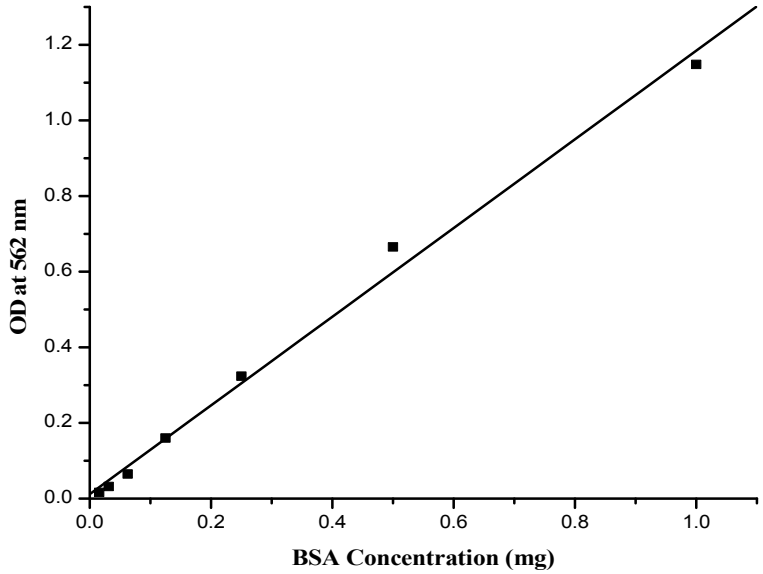

Figure 3.7A. Determination of protein concentration. Standard curve of bovine serum albumin (BSA) for estimation of protein. Protein was estimated using 100 µg/ml of BSA according to Bicinchoninic Acid protein microassay method.

Table 3A: Estimation of unknown sample

S.I	SAMPLE	O.D AT 562nm	concentration
1	Unbound	3.416	3.2368
2	Wash 1	2.818	2.6470
3	Wash 2	1.577	1.4216
4	Wash 3	0.8395	0.6969
5	Wash 4	0.5429	0.4044
6	Wash 5	0.3628	0.2262
7	Wash 6	0.2587	0.1240
8	Elute 1	0.498	0.3569

9	Elute 2	0.408	0.2744
10	Elute 3	0.231	0.0983
11	Elute 4	0.152	0.0187
12	Elute 5	0.078	0.0530
13	Elute 6	0.037	0.0946
14	Elute 7	0.0158	0.1151
15	Elute 8	0.0322	0.0993
16	Elute 9	0.0092	0.124
17	Elute 10	0.0033	0.1019
18	Elute 11	0.087	0.1220

SDS-PAGE analysis

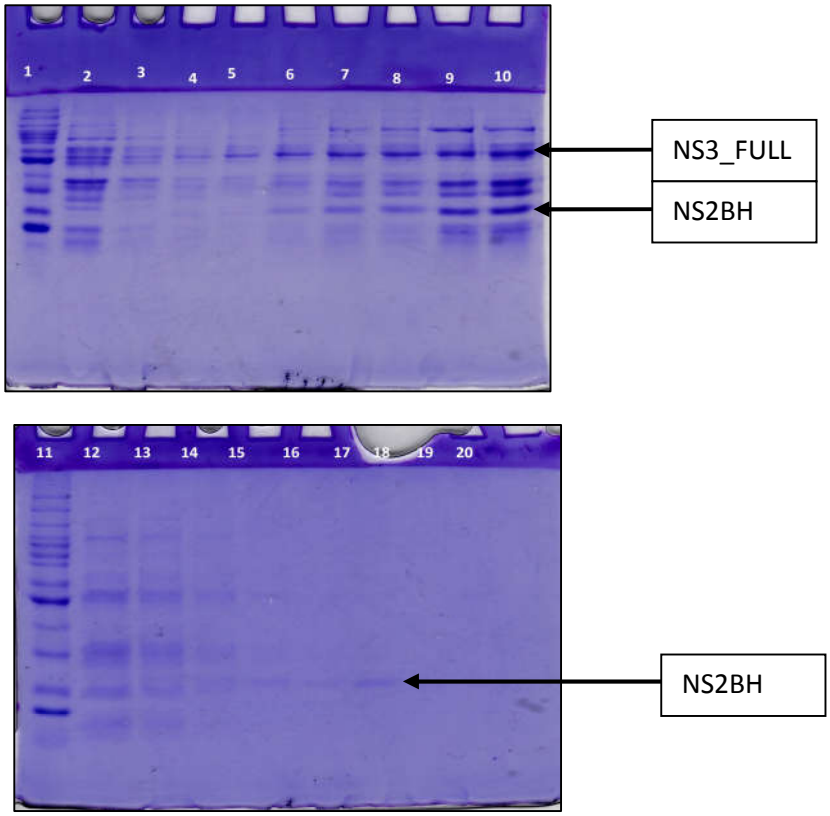

Figure 3.7B: SDS-PAGE analysis of Ni-NTA purified protein: Recombinant NS2BH-NS3 full precursor polypeptide were expressed and purified from E.coli (BL-21) cell by using Ni-NTA affinity column chromatography. Purified protein were separated by SDS-PAGE. Lane 1 &lane 10 is molecular marker, lane 2-8 washed fraction from the Ni-NTA using protein storage buffer and lane 9-20 eluted fragment from the Ni-NTA using 200 mM imidazole.

DISCUSSION

Dengue is a globally important arboviral infection transmitted by *Aedes* mosquitoes that endangers an estimated 2.5 billion people and represents a rapidly growing public health problem [1,2]. Dengue is an icosahedral, enveloped virus with a single stranded positive sense RNA genome [6]. The positive-sense flavivirus RNA genome length is >= 11 kb forms a single open reading frame that is translated into a polyprotein. It consists of the three structural proteins C, prM, and E and seven nonstructural proteins, nonstructural protein 1(NS1), NS2B, NS3, NS4A, NS4B, and NS5 [19]. During viral maturation, this polyprotein is cleaved by host cell proteases in the endoplasmic reticulum and by the NS3 protein in the cytoplasm [19]. Cleavage of the polyprotein is mediated by the seine protease N-terminal domain of NS3, with a hydrophilic segment of 40 residues from the trans- membrane NS2B protein acting as a cofactor necessary for this proteolytic activity. The domain required for

ATPase/helicase and nucleoside 5-triphosphatase activity is located at the c -terminus of NS3 [29,30]. The nonstructural proteins encoded by the viral genome form a membrane-bound RNA replication complex, possibly with the participation of some host factors. Using full-length NS3 enzymes linked to 40 residues of the NS2B cofactor, recent studies demonstrated that the NS3 protein undergoes auto cleavage at two sites located at the NS2B-NS3 junction and within the helicase C-terminal region, respectively [40]. The dengue non-structural 3 (NS3) is a multifunction protein, containing a serine-protease, located at the N-terminal portion, and helicase, NTPase and RTPase domains present in the C-terminal region [41, 42, 43, 44]. The helicase domain unwinds RNA during viral RNA replication and energy for reaction is provided by the NTPase activity [47, 48]. Thus, the NS3 protein is a important target for antiviral therapy [29, 30, 31].

There are several reports, which highlight the relevance of NS3 protease, and NS3 helicase domain interaction. The N-terminal protease domain residue next to the entrance of the ATPase active site between helicase subdomain 1 and 2. They involve the inter domain (linker) region at approximately residues 169 to 179 and two loops that encircles the entrance to the ATP binding pocket [19]. These interactions are necessary for the replication of dengue virus genome and particle formation. Also, NS2B – NS3 protein creates a hub for effective genome replication inside the infected cells [29]. The NS2B hydrophilic region (residues 49-95) is connected to the NS3 protease domain via a nine amino acid (Gly4-Ser-Gly4) linker. The C-terminal part of the central region of NS2B (aa 68-96) forms a β- hairpin that contribute to shape the hydrophobic pockets in the substrate binding sites of NS3pro. Thus, this region of NS2B directly interacts with either the substrate or substrate based-inhibitors, supporting a direct catalytic role for NS2B.

There are some reports showing the identification of inhibitors against NS3 helicase activity. Kinetic studies proved that the mechanism of inhibition is uncompetitive. Moreover, it was demonstrated that *ivermectin* is capable of inhibiting in vitro dengue. Recently, a high throughput screening of 200,000 compounds library identified the *benzoxazole ST-610* as dengue helicase inhibitor [103]. Therefore, NS2B-NS3 is an important target for antiviral drug development, especially targeting helicase activity.

In this context, the current study has been conducted to express, purify and characterize DENV2 full length NS3 protein along with NS2BH in E.Coli cells under native condition. For this, hydrophilic region of NS2B was amplified and fused with the amplified full length NS3. The resultant product was cloned into pQE30 expression host and expressed in BL21 cells. The purification showed a band at ~70 kD. However, there was some degradation in the purified protein. This could be due to the proteolytic activity of NS3.

In summary, in the current study full length DENV2 NS3 protein was expressed in E. Coli cells along with NS2BH as cofactor. The purified NS2BH-NS3 protein needs to be characterized for its activity such as, protease, helicase and NTPase. This protein can be used to screen and identify novel inhibitors against helicase activity of NS3 and the selected inhibitors can be used as an antiviral compound against dengue.

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APPENDIX

Appendix-1

Tris acetate EDTA (TAE) preparation:

50 X stock solution:

242 gm Tris base, 57.1 ml glacial acetic acid and 100 ml of 500 mM EDTA (pH 8.0) solution, bring the final volume up to 1000 ml. This stock solution can be diluted 50 times with water to make a 1 X working solution. This 1 X solution will contain 40 mM Tris, 20 mM acetic acid and 1mM EDTA.

Appendix-2

Primer dilutions: Theprimers (in lyophilized form) were received from IDT, USA. The primers were re suspended in MilliQ water. The purity of primers were analysed on 2% TAE agarose gel by loading 3 µl of sample. In the subsequent reactions 10 µM concentrations of each primer was used for amplification.

Appendix-3

Preparation of LB (Luria-bertani) broth: Weigh 2 gm of LB powder (containing Yeast extract: 5 gm, Tryptone: 10 g, NaCl: 10 g) and add to Erlenmeyer flask containing 800 ml of MilliQ water. Make up the volume to 1liter. Autoclave at 121 degree centigrade for 15 minutes. Store the solution at 4 °C.

Appendix-4

Kanamycin (50 µg/ml) solution: Weigh 50 µgm of Kanamycin powder (Catalogue number K 4378, Sigma) and dissolve in 1 ml of MilliQ water, filter sterilize by 0.2 micron syringe filter (Millipore) .Store the solution at 4 °C.

Appendix-5

Ampicilin (100 µg/ml) solution: Weigh 100 µgm of ampicillin sodium salt powder (Catalogue number A0166, Sigma) and dissolve in 1 ml of Milli Q water, filter sterilize by 0.2 micron syringe filter (Millipore). Store the solution at 4°C.

Appendix-6

Preparation of formaldehyde denaturing gel: Add 1 gm of agarose to 85 ml of water in a flask and dissolve the agarose in a microwave. Add 10 ml of 10 × MOPS buffer to the agarose solution, and then allow it in a flask to cool to 55 °C. Add 5.4 ml of 37 % formaldehyde solution to the agarose solution, mix them, quickly pour the agarose into a gel mold and set a comb in a fume hood. Cover the gel with 1 × MOPS buffer until use. Formaldehyde is supplied as a 37-40 % W/V (12.3 M) solution that contains a stabilizer such as methanol (15 %). The 37 % formaldehyde solution is used for mixing with RNA loading buffer .

Appendix-7

Vector details: The PQE 30 Vector (Qiagen) was used to clone PCR products.

Appendix-8

200Mm imidazole

136.16 mg of imidazole in 10 ml of distilled water

Appendix-9

1x tris glycine electrophoresis buffer p^H 8.3

25Mm Tris base-3.03g

190Mm glycine-14.26g
o.1% SDS-1g

Appendix-10

P_1 buffer

50 mM Tris HCl (p^H.8)
10 mM EDTA
RNAase A 10µg/ml

Appendix-11

P_2 buffer

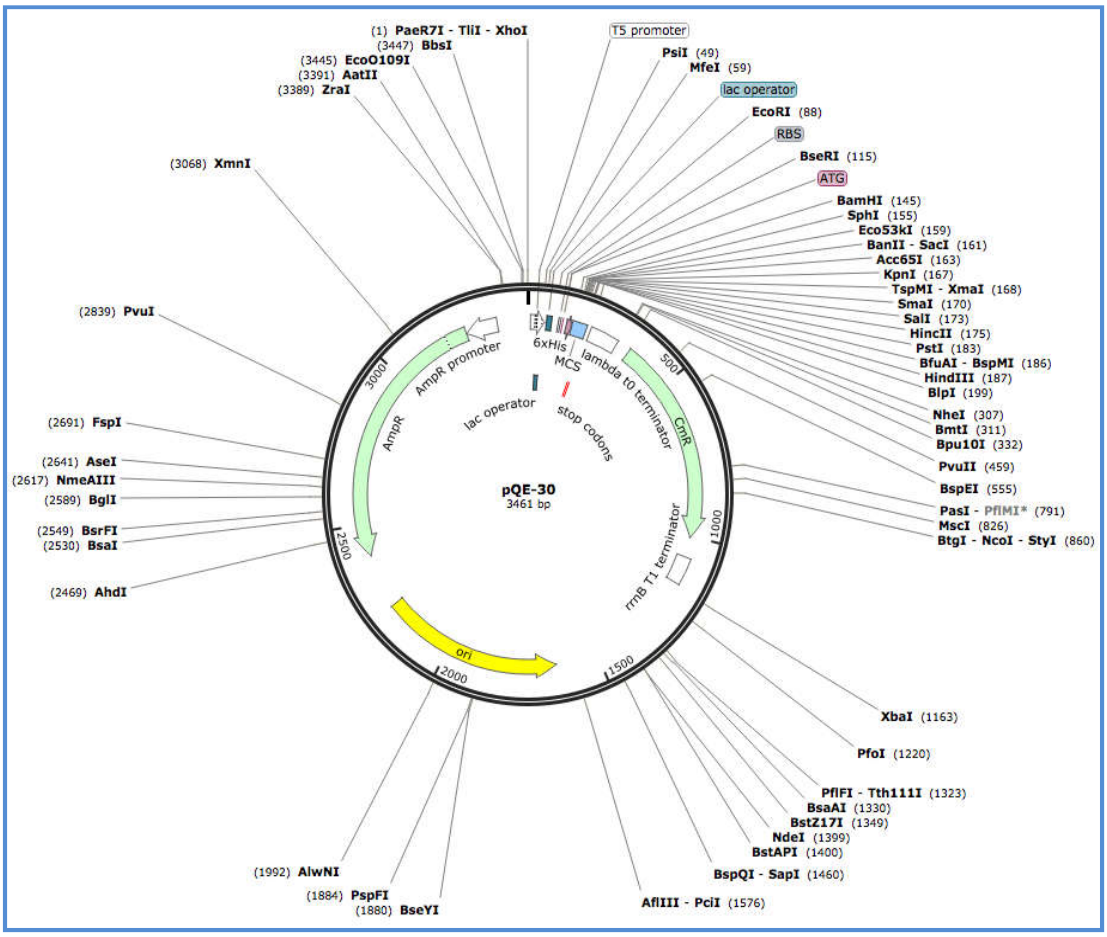
200 mM NaOH
1% SDS

Appendix-12

P_3 buffer

3 M potassium acetate, p^H adjusted to 5.5 with glacial acetic acid.

Appendix-13



Vector details: The PQE 30 Vector (Qiagen) was used to clone PCR products.