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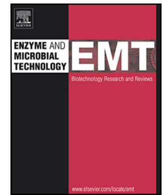

Development of robust *in vitro* RNA-dependent RNA polymerase assay as a possible platform for antiviral drug testing against dengue

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ABSTRACT

NS5 is the largest and most conserved protein among the four dengue virus (DENV) serotypes. It has been the target of interest for antiviral drug development due to its major role in replication. NS5 consists of two domains, the N-terminal methyltransferase domain and C-terminal catalytic RNA-dependent RNA polymerase (RdRp) domain. It is an unstable protein and is prone to inactivation upon prolonged incubation at room temperature, thus affecting the inhibitor screening assays. In the current study, we expressed and purified DENV RdRp alone in *Esherichia coli* (*E. coli*) cells. The N-terminally His-tagged construct of DENV RdRp was transformed into *E. coli* expression strain BL-21 (DE3) pLysS cells. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM. The induced cultures were then grown for 20 h at 18 °C and cells were harvested by centrifugation at 6000 x g for 15 min at 4 °C. The recombinant protein was purified using HisTrap affinity column (Ni-NTA) and then the sample was subjected to size exclusion chromatography, which successfully removed the degradation product obtained during the previous purification step. The *in vitro* polymerase activity of RdRp was successfully demonstrated using homopolymeric polycytidylic acid (poly(rC)) RNA template. This study describes the high level production of enzymatically active DENV RdRp protein which can be used to develop assays for testing large number of compounds in a high-throughput manner. RdRp has the *de novo* initiation activity and the *in vitro* polymerase assays for the protein provide a platform for highly robust and efficient antiviral compound screening systems.

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1. Introduction

Dengue is currently considered as one of the most important mosquito-borne diseases worldwide. According to recent estimates, the global incidence of dengue infections has increased from 50 to 100 million to almost 400 million cases per year [1]. Despite the enormous efforts towards the development of dengue vaccine, there is still no licensed vaccine available. This is primarily due to the complex pathology of the disease and immune mediated responses against the four distinct serotypes [2]. Presently, many promising vaccine candidates are in pre-clinical and clinical development stages including live attenuated, inactivated, recombinant subunit, DNA, and viral vectored vaccines [3]. The development of therapies for DENV requires both an understanding of the viral life cycle and rational strategies for identifying antiviral inhibitors. Both viral and host proteins essential for viral replication cycle are potential targets for antiviral drug development [4].

Dengue related infections are caused by the dengue viruses, which belongs to the family *Flaviviridae* and include small enveloped viruses with a diameter of about 50 nm [5]. Dengue virus contains an 11 kb positive-sense, single-stranded RNA genome. The genome consists of a single open reading frame which encodes three structural proteins (capsid C, pre-membrane/membrane (prM/M), and envelope (E) protein), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [6]. The structural proteins form the viral particle and the non-structural proteins participate in the replication of the RNA genome, virion assembly and invasion of innate immune response [7]. NS5, with a molecular weight of around 104 kDa is the largest of dengue virus proteins containing 900 amino acids. NS5 is the most conserved protein of the dengue proteome as it shares a minimum of 67% amino acid sequence across all four dengue serotypes [8,9]. NS5 is essential for RNA replication and performs enzymatic activities required for capping and synthesis of RNA genome of virus. It consists of two domains with distinct functions, the N-terminal methyl transferase (MTase) and the C-terminal RNA-dependent RNA

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polymerase (RdRp) catalytic domain [9,10]. The tertiary structure of RdRp consists of palm, thumb and finger subdomains. The catalytic site contains conserved aspartic residues and coordinated magnesium cations and nucleotide incorporation occurs through two metal ion catalytic mechanism [9].

The MTase activity of NS5 is responsible for both guanine N-7 and ribose 2-O methylations [11]. The replication of flaviviral RNA occurs through the formation of replicative intermediates synthesized by the RdRp, an essential component of a replication complex [12]. Recombinant NS5 full-length protein along with C-terminal domain alone have been shown to possess RdRp activity *in-vitro*, which was also evaluated in a study with NS5 protein of DENV-1 expressed in *E. coli* as a glutathione-S-transferase (GST) fusion. The *in vitro* demonstration of polymerase activity in recombinant NS5 protein has made it possible to study and identify the host and virus factors involved in the dengue virus genome replication [13,14]. Flavivirus RdRp initiates the RNA synthesis *via de novo* initiation mechanism in which first nucleotide serves as a primer to provide the 3′-hydroxyl group which differs from a primer (an oligonucleotide, a protein linked to nucleotides, or intra-molecular self-priming) dependent mechanism used by other viruses, such as poliovirus and SARS-CoV [15,16].

Owing to the central role of polymerase enzymes in viral life cycles, RdRps have been the target of considerable interest for antiviral drug development [17]. RdRp is an unstable protein and is prone to inactivation upon prolonged incubation at room temperature (RT) thereby affecting the inhibitor screening assays. It has been observed that addition of linker residues to the N-terminal end of the RdRp domain stabilizes the protein and enhances its *de novo* polymerase initiation activity and thermostability [18]. Mutation studies of the linker residues have indicated their importance for viral replication, consequently, accelerating the development of antivirals against dengue. The 74 kDa RdRp domain of DENV-2 has been expressed in *E. coli* and purified with a high quantity of the protein. It was observed that the protein was active with considerably similar enzymatic characteristics as its full-length counterpart [13]. Previous studies with NS5 RdRp of DENV-3 have described high quantity production of the protein with >95% purity, yielding 3 mg protein from 1 L of culture [9]. In the current study, expression and purification of the RdRp domain of DENV-2 NS5 protein from *E. coli* was carried out and the polymerase activity of the recombinant protein was successfully demonstrated using an *in vitro* transcribed RNA. Based on its multifunctional role in viral replication, NS5 has been identified as an interesting drug target for the development of therapies against dengue. The aim of this investigation was to demonstrate the *in vitro* polymerase activity of the purified recombinant RdRp (NS5) protein. This assay will provide a platform for screening and development of antiviral compounds specifically targeting the polymerase protein.

2. Materials and methods

2.1. Construct for DENV-2 RdRp (NS5) protein

The genomic construct of RdRp domain of NS5 protein for expression in *E. coli* was kindly provided by Dr. Richard J Kuhn (Department of Biological Sciences, Purdue University). The region encoding the catalytic RdRp domain (amino acid residues, 273–900) was amplified using the genomic clone of DENV-2 strain 16681 (Genbank Accession No. U87411) by PCR. The amplicon was then cloned into the expression vector pET30a to allow expression of recombinant protein carrying an N-terminal TEV protease cleavable His6 affinity tag in *E. coli* cells. The pET30a expression vector used in this construct had been modified for ligation-independent cloning (LIC) by Dr. Etti Harms, high-throughput facility (HTP) [19].

2.2. Expression of DENV-2 RdRp in E. coli

The N-terminally His-tagged RdRp domain of DENV-2 NS5 were transformed into *E. coli* expression strain BL-21 (DE3)pLysS cells. The transformed cells were first grown in small scale culture of 5 mL to test for the expression and solubility of DENV-2 NS5. Large scale culture was then grown in 1 L LB medium containing 50 μg/mL of Kanamycin (Kan) and 35 μg/mL of Chloramphenicol (Cam) overnight at 37 °C, till an OD_{600} of 0.7–0.8 was reached. Protein expression was induced using 0.4 mM IPTG. The induced cultures were then grown for 20 h at 18 °C and cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C. Samples were analyzed for expression on 10% SDS-PAGE gel (Bio-Rad Laboratories) and visualized by coomassie staining according to Laemmli method [20].

2.3. Purification of DENV-2 RdRp

The harvested pellets were resuspended in 50 mL of lysis buffer containing Buffer A (20 mM sodium phosphate pH 7.0, 0.5 M NaCl, 50 mM L-Arginine, 50 mM L-Glutamic acid, 5 mM Imidazole) and 1 mM PMSF, 5 mM β-Mercaptoethanol (ME), 0.01 mg/mL each of DNaseI and RNaseA. The Samples were lysed by sonication on ice at amplitude of 60% using sonicator machine (Hielscher Ultrasonics GmbH, USA). The lysates were centrifuged at 25,000*g* for 45 min at 4 °C. The supernatants were clarified by filtration through 0.45 μm filter unit and the clarified supernatants were loaded onto a Ni-NTA agarose beads (Qiagen) column pre-equilibrated with Buffer A, and incubated for 1 h at 4 °C. Column was washed with 40 mL wash1 buffer (Buffer A + 30 mM imidazole + 1 M NaCl) and then 20 mL of wash 2 buffer (Buffer A + 50 mM imidazole + 1 M NaCl). The protein was eluted with 20 mL of 100 mM and 500 mM imidazole in buffer A. Finally, the column was washed with 1 M imidazole to remove any bound protein and then with excess of deionized water.

Samples were collected at various stages during purification, analyzed on a 10% SDS-PAGE gel and visualized by coomassie staining. The fractions containing the purified protein were pooled after buffer exchange (50 mM Tris buffer pH 8.0, 500 mM NaCl, 5 mM βME, 5% glycerol) and were concentrated using a 50 kDa centrifugal concentrator. The concentrated protein was applied onto a Superdex 200 10/300 HL size-exclusion column (GE Healthcare, USA), pre-equilibrated with the exchange buffer. Fractions containing the purified recombinant RdRp protein were pooled and the protein was concentrated using a 50-kDa vivaspin concentrator (GE Healthcare, USA). The final concentration of protein was assessed by measuring absorbance at 280 nm using nanodrop (Thermo Scientific, USA).

2.4. In vitro RdRp polymerase assay

The purified protein (1 μM) diluted in RdRp buffer containing 50 mM HEPES, 10 mM KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$ and 10 mM DTT, was then tested for *in vitro* RdRp activity assessed by liquid scintillation counter (Beckman LS6500, USA). Following optimization, the polymerase assays were carried out with RdRp protein using poly(rC) RNA as template (Amersham Biosciences, USA) and monitored by filter-binding and scintillation counting as previously described [13]. The final RdRp assays were performed in RdRp buffer containing 1 μM NS5, 0.1 mg/mL poly(rC) RNA, 20 U RNase OUT, 0.2 μg Actinomycin D in a volume of 50 μL. Reactions were initiated by addition of 5 μM GTP, 0.25 μi [^{3}H]-GTP pre-incubated at 30 °C. The reactions were incubated at 30 °C for 1 h, terminated by addition of 10 mM EDTA and spotted onto DE-81 filter discs (Whatman). The filter discs were air dried, washed thrice with 0.3 M ammonium formate (pH 8.0) and once with ethanol and air dried

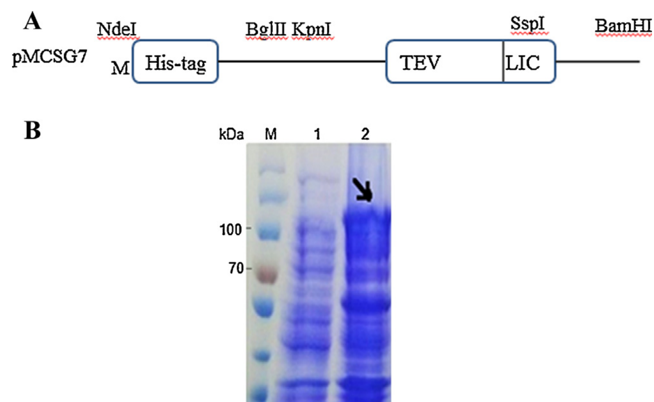

Fig. 1. Expression of recombinant RdRp (NS5) protein cloned into pMCSG7 (A) Schematic illustration of the leader sequence encoding regions of LIC vector modified from the commercial vector pET-30Xa/LIC (Novagen) to generate pMCSG7. (B) SGS-PAGE analysis of RdRp protein expressed in BL-21 (DE3)pLysS cells using IPTG induction. The clarified supernatant from un-induced (Lane 1) and induced (Lane 2) samples were subjected to 10% SDS-PAGE gel, the gel was stained with coomassie brilliant blue. Lane M shows the pre-stained protein ladder (PageRuler Plus) in kilo daltons. The arrow represents the position of RdRp (approx. 100 kDa).

again. Liquid scintillation fluid was added to the filter discs and incorporation was measured in counts per min (cpm).

3. Results

3.1. Expression of RdRp protein of DENV-2

The NS5 protein RdRp domain plasmid construct was transformed into *E. coli* expression strain BL-21 (DE3) pLysS cells because these cells allow high efficiency protein expression of the cloned genes under the control of T7 promoter. RdRp protein was successfully expressed in small scale (50 mL) cultures of *E. coli* expression strain (Fig. 1). It was observed that the IPTG induction results in the expression of the predicted ~100 kDa protein. Large scale (1 L) culture was then grown overnight at 37 °C until OD_{600} of 0.7–0.8 was reached. Induction was done at 18 °C for 20 h using 0.4 mM IPTG. Induced culture was centrifuged at 6000*g* for 15 min at 4 °C

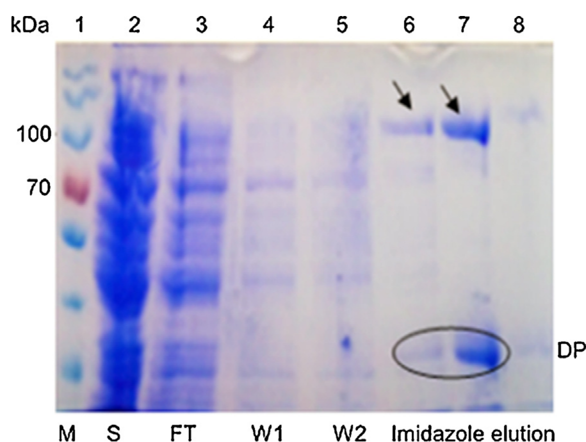

Fig. 2. Purification of recombinant RdRp protein from *Escherichia coli.* Fractions of sample eluted from HisTrap affinity column (Ni-NTA) by imidazole were subjected to 10% SDS-PAGE gel and stained with coomassie blue. Lane 1, pre-stained protein marker (PageRuler Plus, 10–250 kDa); Lane 2, Supernatant sample (S) before running through column; Lane 3, Flow through (FT) sample; Lane 4 and 5, fractions obtained after washing column with wash 1 and wash 2 buffers, respectively; Lane 6 and 7, fractions eluted after treatment with 100 mM and 500 mM Imidazole; Lane 8, fraction obtained after column was washed with 1 mM imidazole. Arrows represent positions of the N-terminally His6-tagged recombinant RdRp. Gel shows a ~30 kDa degradation product (DP) co-purified along with RdRp.

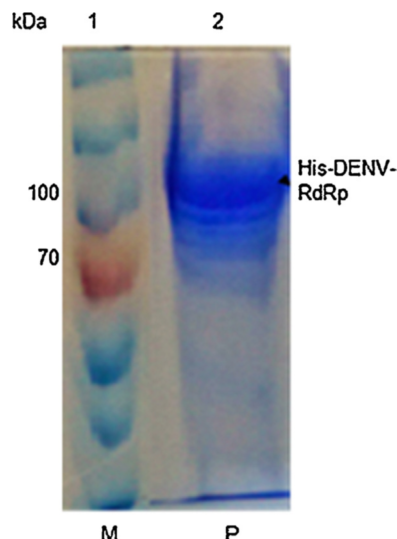

Fig. 3. SDS-PAGE gel (10%) analysis of the purified RdRp protein obtained after running through size-exclusion column. Lane 1, prestained protein marker (PageRuler Plus); Lane 2, purified recombinant RdRp protein. The degradation product was successfully separated from the protein by size-exclusion chromatography.

to pellet down the cells containing the expressed protein. Cells were then lysed with lysis buffer containing lysozyme and sonicated at 60% amplitude and centrifuged again to pellet down the lysed cells. The supernatant containing the expressed protein was collected for further purification.

3.2. Purification of RdRp

The supernatant from previous step was then subjected to immobilized metal affinity chromatography (IMAC) using 5 mL HisTrap Ni-NTA columns. Most of the protein was eluted at the end of the imidazole gradient *i.e.* 100 mM and 500 mM as observed from the SDS-PAGE gel results (Fig. 2). The addition of RNase A and DNase I in the lysis buffer helped get rid of contaminating nucleic acids. After this first step purification (IMAC), along with other contaminating proteins, a small degradation product of about 30 kDa was consistently observed. However, significant amount of the RdRp protein was recovered after affinity chromatography. The fractions containing the purified RdRp protein were pooled and concentrated to final volume of 0.5 mL followed by another step of purification, size exclusion chromatography.

After running through the size exclusion column, the fractions containing the purified protein were pooled and concentrated to a final volume of 0.5 mL. It was observed from the gel filtration profile (chromatogram) that most of the RdRp protein was obtained as a single peak. SDS-PAGE analysis of the purified protein also revealed that gel filtration helped get rid of the contaminating proteins as well as separated the degradation product from the RdRp protein (Fig. 3). DENV-2 RdRp domain of the NS5 protein was successfully purified using the two-step purification scheme with considerable yield (Table 1). We obtained a very good yield of 6.5 mg/L of the purified protein after the gel filtration.

Table 1
Protein yields for recombinant His-DENV-RdRp.

His-DENV- RdRp	Yield (mg/L of culture)
Yield Post IMAC	14
Final yield	6.5

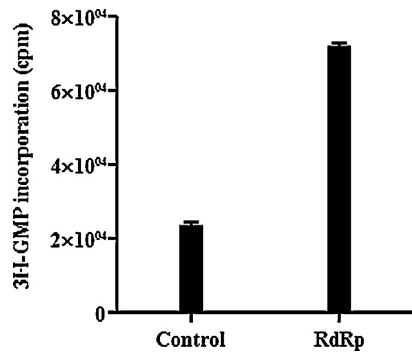

Fig. 4. *In vitro* polymerase activity of recombinant RdRp. The activity was assayed by incorporation of radiolabel ^{3}H-GTP onto a poly(rC) template by RdRp, measured by liquid scintillation counting. Control represents the reaction in the absence of RdRp (background radioactivity). The background counts were subtracted from the final counts for the protein. Data reflect means ± SD (n = 3 replicates). Results are representative of three independent experiments.

3.3. In vitro *polymerase assays for DENV-2 RdRp (NS5)*

Using a filter binding assay, we successfully demonstrated the polymerase activity of recombinant RdRp domain of NS5 in the presence of poly(rC) RNA template. To test the RdRp activity of the purified protein, the assay was first performed with 1 μM RdRp in the reaction mixture. The reaction was initiated by addition of 10 μM GTP, 0.25 μCi [^{3}H]-GTP and terminated by addition of 10 mM EDTA and reaction mixtures were spotted onto DE-81 filter discs, washed and used in liquid scintillation counting. Polymerase reactions were assayed by radionucleotide (^{3}H-GMP) incorporation which was monitored by filter binding and liquid scintillation counting. Fig. 4 represents the *in vitro* polymerase activity of RdRp domain of NS5. It was observed from the scintillation counting results that the ^{3}H GMP incorporation of purified protein was significantly higher in comparison to the control which confirms that our protein is enzymatically active.

Having identified the RNA polymerase activity of the purified RdRp domain of NS5 protein, we then optimized the RdRp assay conditions including protein concentration, GTP concentration and incubation time. Optimization experiment was first conducted using different concentrations of the protein i.e 0.2 μM, 0.5 μM and 1 μM using 1 μM poly(rC) RNA template at 30 °C for 1 h with RdRp buffer (pH 7.0). Maximum activity was observed with 1 μM protein (Fig. 5A). The optimal GTP concentration for maximal activity was determined to be 5 μM (Fig. 5B) and with further increasing concentration, activity was decreased. RdRp activity was also dependent on the incubation time of the reaction mixture and maximum activity was observed post–60 min of incubation (Fig. 5C). It was observed that further increase in incubation time decreased the RdRp activity as detected by radiolabel (^{3}H-GMP) incorporation.

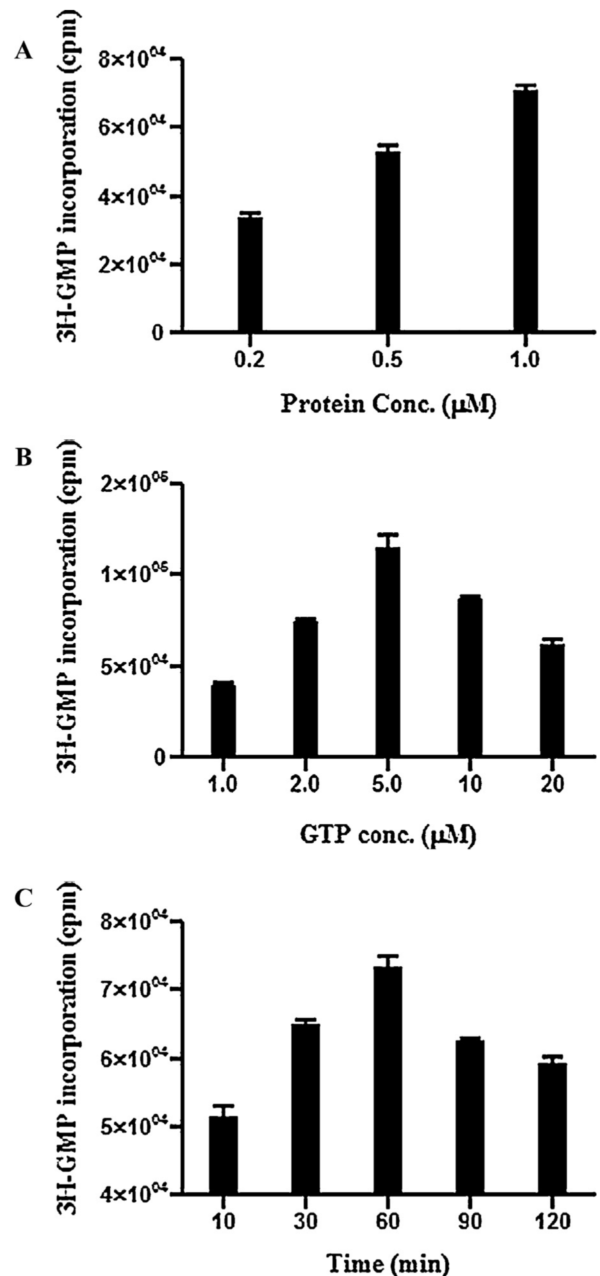

Fig. 5. Optimization of DENV RdRp assays conditions; (A) Protein concentration (B) GTP concentration (C) incubation time. RdRp assays were performed with poly(rC) RNA template under the indicated conditions. Reactions were initiated by addition of 0.25 μCi [^{3}H]-GTP and terminated by addition of 10 mM EDTA. Reaction mixtures were spotted onto DE-81 filter discs, washed and subjected to liquid scintillation counting. Data shown is the mean ± standard error from three independent experiments.

4. Discussion

The aim of this study was to express and purify the RdRp domain of NS5 protein of DENV-2 and evaluate its enzymatic activity using the *in vitro* polymerase assays. We successfully expressed RdRp domain of NS5 protein in high expression strain of *E. coli* using cloned DNA fragments of dengue-2 virus [21]. It was observed that utilizing BL21 (DE3) pLysS cells for expression increased the overall yield of the protein. The protein was purified to homogeneity by successive chromatographic techniques, immobilized metal affinity chromatography and size exclusion chromatography. The two-step purification procedures helped get rid of the degradation product continuously obtained during initial expression and purification steps. Similar degradation products of ~35 kDa size have also been reported during studies with Japanese encephalitis virus (JEV) and were later identified to be cleavage products of NS5 [22]. It was also observed that RdRp was very sensitive to temperature and a very short exposure to increased temperature lead to a decline in the polymerase activity. Previous studies regarding induced mutation within NS5 RdRp have shown improved thermostability of the protein [18]. It is essential to further optimize the screening procedure and efforts are being made in order to obtain a thermostable protein. In this study, a decline in the activity was observed when protein was incubated for longer than 60 min during *in vitro* RdRp assay. The reduction in the activity suggests that the reaction was completed within 60 min of incubation and the protein starts to

degrade at longer time points. Therefore, all the subsequent optimization reactions were incubated for 60 min.

The polymerase activity of the recombinant protein was successfully demonstrated in the absence of viral or host cofactors, suggesting that the protein was enzymatically active. These results indicated that the RdRp domain of DENV-2 NS5 protein alone was able to synthesize RNA *de novo* using homopolymeric poly(rC) RNA template without the help of other viral/cellular proteins, as previously indicated by studies with other flaviviruses such as DENV-1, Kunjin virus and Japanese encephalitis virus [14,23,22]. Previous studies have also reported polymerase activity of recombinant NS5 (RdRp) protein in the presence of poly(rC) RNA template [24,13]. Therefore, optimization studies were carried out in the presence of poly(rC) RNA template in primer-independent setup. Literature suggests that DENV NS5 has the ability to initiate RNA synthesis in the presence of high concentrations of GTP which is the second nucleotide to be incorporated during negative sense RNA synthesis [25]. Structural studies with Bovine viral diarrhea virus (BVDV) polymerase indicated the existence of GTP binding site in the polymerase and during *de novo* initiation GTP binds to the site serving as a primer by providing the 3′-OH for the formation of first phosphodiester bond [26]. These studies also indicated the importance of the use of poly(rC) RNA template during the in *vitro* polymerase assays. The DENV-2 RdRp protein purified during the course of this study showed optimal polymerase activity in the presence of 1 μM protein, 5 μM GTP, divalent cations $-Mn^{2+}$ and Mg^{2+}, and incubation time of 1 h at 30 °C.

NS5, being the largest and most conserved protein among the four dengue serotypes, is an interesting target for the antiviral drug development. Recent studies on enzymatic activities of NS5, coupled with structure–function and interaction studies reveal the multifunctional role of the protein in viral replication [17]. The high-level production and purification of recombinant versions of either the full-length NS5 or the two individual NS5 domains has also led to detail enzymatic studies on NS5 and the determination of structures of the two NS5 domains [10]. Research on flaviviruses has led to the characterization of an increasing number of virus-encoded proteins and enzymes, including envelope and capsid proteins, polymerases, helicases and proteases. Chemical libraries containing molecules of natural and synthetic origins can now be screened against these novel pathways and targets. This study provides an optimized method for the production of an active DENV RdRp (NS5) protein which will further help establish high-throughput compound screening assays directed against NS5. In future, mutation studies of NS5 protein can prove to be helpful in enhancing thermostability and *de novo* RNA polymerization activities of the protein.

Acknowledgments

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