

Optimization of Dengue-3 recombinant NS1 protein expression in *E. coli* and in vitro refolding for diagnostic applications

T. N. Athmaram · Shweta Saraswat · Princi Misra ·
Saurabh Shrivastava · Anil K. Singh · Shailendra K. Verma ·
N. Gopalan · Prativa K. Behara · P. V. L. Rao

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Abstract Dengue non-structural protein (NS1) is known to be protective antigen and also has immense application for serodiagnosis. Several serodiagnostic assays available for dengue viral infection are dependent on tissue culture-grown viral proteins. This task is unsafe, laborious, more expensive that makes it unsuitable for routine large-scale production. Although bacterial expression is relatively simple and easy for recombinant protein expression, it is more challenging to make NS1 protein with native structural and immunological features using bacterial expression system. We have successfully developed a method leading to the purification and refolding of recombinant dengue virus type 3 (DENV3) NS1. The gene encoding NS1 was amplified and cloned in pET28a (+) vector. In order to increase the purity of the recombinant NS1, the

transgene was engineered to carry 6× Histidine tags at both N and C-terminal ends. The recombinant construct (pET-NS1) was transformed into *E. coli* Rosetta-gami cells and the expression conditions viz IPTG concentration, media type, temperature, and harvest time were optimized. The size of the expressed protein was found to be ~45 kDa and the authenticity of the expressed protein was confirmed using anti-His and anti-NS1 monoclonal antibodies. The NS1 protein was purified under denaturing conditions, to attain the native conformation, NS1 protein was in vitro refolded and dialyzed. The refolded NS1 protein was detected by commercial Immuno chromatographic strip and NS1 specific monoclonal antibodies. IgM antibody capture ELISA was performed using refolded recombinant NS1 protein which recognized the IgM antibodies in dengue-positive samples of acute phase of infection. Our result suggests that rNS1 protein has immense diagnostic potential and can be used in developing point of care diagnostic assays.

T. N. Athmaram (✉) · S. Saraswat · P. Misra ·
S. Shrivastava · A. K. Singh
Bio-process Scale Up Facility, Defence Research
and Development Establishment, Ministry of Defence
(Government of India), Gwalior, MP 474 002, India
e-mail: athmabiotech@gmail.com

S. K. Verma
Division of Microbiology, Defence Research and Development
Establishment, Ministry of Defence (Government of India),
Gwalior, MP 474 002, India

N. Gopalan
Vector Management Division, Defence Research and
Development Establishment, Ministry of Defence
(Government of India), Gwalior, MP 474 002, India

P. K. Behara
ISPAT General Hospital, Rourkela, Odisha 769 005, India

P. V. L. Rao
DRDO-BU Centre for Life Sciences, Bharathiar University
Campus, Coimbatore 641 046, India

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Introduction

Dengue virus is a member of the *Flaviviridae* with a single-stranded positive-sense RNA genome of about 11 kb [1]. The mosquito-borne dengue viruses cause dengue fever, a severe flu-like illness. The viral RNA genome encodes a polyprotein that cleaves into 10 known proteins (C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by viral encoded or host proteases. Among these proteins, NS1 is viral non-structural protein that is detected in sera of infected individuals and also in vitro infected cells [2].

There are four distinct but antigenically related serotypes of dengue viruses. Dengue fever and its more serious forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) and circulatory failure are becoming important public health problems. As per WHO report (2011), more than 2.5 billion people live in areas where there are risks of exposure to dengue virus infection and the WHO currently estimates that globally, 50 million people are infected with dengue virus every year [3]. Controlling dengue infections is challenging because it requires not only effective control of vectors responsible for transmitting the virus but also accurate and rapid diagnosis. To date, accurate and timely diagnosis of early detection with dengue virus remains a problem for management of dengue infected patients in many parts of the world, especially in developing countries where limited resources are available. Primary infection with dengue virus results in a self-limiting disease characterized by mild-to-high fever lasting 3–7 days, severe headache with pain behind the eyes, muscle and joint pain, rash, and vomiting [3]. Secondary infection is the more common form of the disease in many parts of Southeast Asia and South America. Early diagnosis of DSS is particularly important, as patients may die within 12–24 h if appropriate treatment is not administered. Primary dengue virus infection is characterized by elevations in specific NS1 antigen levels during 0–9 days after the onset of symptoms; this generally persists up to 15 days. Earlier diagnosis of Dengue reduces risk of complication such as DHF or DSS, especially in countries where dengue is endemic.

Several laboratory methods, such as virus isolation, genomic RNA, antigen, and antibody detection methods are available to diagnose dengue infections. However, methods such as virus isolation and genomic RNA detection (PCR) need a specialized laboratory and well-trained laboratory personnel. Recently, Dengue virus (DENV) nonstructural (NS1) antigen has gained a lot of interest as a new biomarker for early diagnosis of DENV infection. The NS1 protein is approximately 43–48 kDa protein with two *N*-linked glycosylation sites. It exists in multiple forms viz, monomer, dimer, and hexamer in infected mosquito and mammalian cells [4–9]. Soluble NS1 can be detected in the culture media and acute sera from dengue virus infected patients. Dengue NS1 antigen, produced in both membrane-associated and secretion forms, is abundant in the serum of patients during the early stages of DENV infection [5]. Several studies conducted revealed the importance of dengue NS1 antigen as a biomarker; these antigens can be detected before the formation of antibodies [10–12]. NS1 antigen is detectable in blood from the first day after the onset of fever up to day 9; once the clinical phase of the disease is over, it is still detectable even when viral RNA is negative by RT-PCR and in the presence of IgM antibodies

[10]. Currently, NS1 antigen-capture ELISA and rapid NS1 antigen commercial kits for detection of NS1 antigen have been developed and evaluated. Studies revealed the detection rate of NS1 antigen is higher in acute primary dengue than in acute secondary dengue infection [13, 14]. The use of NS1 antigen has been suggested for early diagnosis of dengue infection after the onset of fever [12, 15, 16]. Detection of dengue NS1 antigen represents a new approach for the diagnosis of acute dengue infection. Among ELISA based tests, IgG-ELISA is very nonspecific and exhibits broad cross-reactivity among flaviviruses, hence, IgM antibody capture ELISA is preferred over IgG-ELISA for serodiagnosis and seroepidemiological survey of dengue virus during the past few years [17]. However, the diagnostic antigens used in ELISA are prepared from dengue viruses-infected mouse brain or cultured cells and are extracted by acetone-sucrose gradient that are laborious and unsafe and also result in differences in batch quality [17]. There exist several reports on the use of heterologous systems like vaccinia virus, baculovirus, yeast and *E. coli* [18–21] for expressing NS1 protein. However, no systematic studies have been carried out on Dengue virus of Indian origin for NS1 protein similarity, epitope analysis and possibility of using single rNS1 protein that can recognize IgM antibodies of other serotypes. With this background, the current study was focused on making a properly folded recombinant NS1 (rNS1) expressed in *E. coli* (Rosetta-gami) cells that is known to form disulfide bridges within the expressed recombinant protein for increased stability. The expressed rNS1 was evaluated for its diagnostic potential using known Dengue positive sera samples of acute phase infection.

Materials and methods

Dengue NS1 gene analysis

The NS1 gene of different Indian Dengue virus serotypes (Dengue virus serotypes 1, 2, 3, and 4 with NCBI accession numbers AFN54943, ACQ44493, AY770511, and AEX09561, respectively) were analyzed for its amino acid sequence similarity among four Indian isolates with ClustalW multiple alignment tool using DNA star software (DNA STAR, Inc., Madison, USA). The prediction of linear B-cell epitopes based on seven different parameters viz, hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity, and antigenic property was done using Bcepred tool with a threshold of 1.9, 2, 1.9, 2.4, 2.3, 1.8, and 1.9, respectively [22]. *N*-linked glycosylation of NS1 protein was studied using NetNGlyc 1.0 server of Technical University of Denmark.

Preparation of DEN-3 virus RNA

Monolayer cell cultures of C6/36 cells in Eagle's MEM (Sigma, USA) supplemented with 5 % fetal calf serum (Sigma, USA) were infected with Dengue-3 virus [strain GWL-25 (Indian isolate)] obtained from Division of Virology, DRDE, Gwalior. After being incubated at 32 °C for 72 h, infected cell culture fluid was harvested, and the viral RNA was extracted using Qiagen viral RNA extraction kit (Qiagen, Germany) as per the manufacturer's protocol.

Reverse transcription-polymerase chain reaction (RT-PCR)

Dengue-3 NS1 primers used in the present study were: D3NS1 sense primer—5' TGAATTCGACATGGGGTGTGTCATAAAC 3' corresponding to NS1 gene from nucleotides 2,414–2,434 (NCBI accession no AY 770511) with an added upstream *EcoRI* restriction site (underlined); D3NS1 antisense primer—5' TGCGGCCCGCCGCTGAGACTAAAG 3' corresponding to the sequence complementary to the NS1 gene from nucleotides 3,448–3,435 (NCBI accession no AY 770511) with a downstream *NotI* restriction site (underlined). Dengue 3 virus total RNA was reverse-transcribed in a total of 20 µl of reaction solution that included 5 units of MMLV reverse transcriptase, 1 unit of RNase inhibitor (Sigma, USA), 4 µl of 5× RT buffer (Fermentas, USA), and 1 µM antisense primer at 42 °C for 1 h. PCR was performed in 50 µl of a mixture containing 1 unit of *Pfu* DNA polymerase (Fermentas, USA), 1× PCR buffer, 0.1 mM dNTP mix, 1.5 mM MgCl₂, and 1 mM sense and antisense primers using ABI thermal cycler (Applied Biosystems, USA). The PCR reaction conditions consisted forty cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 50 s, and extension at 72 °C for 60 s, with the final cycle at 72 °C for 10 min.

Cloning of NS1 gene into pET28(a) vector

The full-length NS1 gene from Dengue virus serotype 3 was cloned into pET28(a) vector (Novagen, USA) under the control of T7 RNA polymerase promoter at *EcoRI* and *NotI* sites. The resultant recombinant plasmid pETNS1 (Fig. 3) had the transgene fused with 6× Histidine-fusion tag both at N-terminal and C-terminal ends. After transformation into *E. coli* (Rosetta-gami), the recombinant clones were screened on LB agar plates containing 50 µg/ml of kanamycin and incubated at 37 °C for 18 h. The inserted NS1 gene was verified by restriction digestion with *EcoRI* and *NotI* enzymes and nucleotide sequencing using ABI DNA sequencer. DNA sequences were analyzed using DNASTAR software. The *E. coli* (Rosetta-gami) clones

containing correct nucleotide sequences of the transgene were examined for gene expression along with appropriate negative controls.

Expression profile and localization of recombinant protein

Single positive clone known to carry NS1 gene was grown in LB broth and the logarithmic phase cultures were induced with different concentration of IPTG (0.1, 0.25, 0.5, and 1 mM) and checked after a period of 3 h. Following induction, cell pellets were lysed in sample loading buffer and analyzed by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described earlier [23]. In order to identify the localization of recombinant protein, the cell suspension was sonicated and the resultant cell lysate was centrifuged at 10,000×g for 30 min at 4 °C. The clear supernatant and remaining pellet were collected separately and then analyzed on 10 % SDS-PAGE.

Media optimization

After confirmation of the expression of recombinant protein and determination of optimum IPTG concentration, the glycerol stock of the positive clone was inoculated into seven different media viz. Luria–Bertani broth, Super broth, Terrific broth modified 1, Terrific broth modified 2, Terrific broth modified 3, Terrific broth modified 4, and SOB medium. The composition of each medium is as given in Table 1. All these media were individually tested for better expression of the recombinant rNS1 protein at shake flask level. About 1 % (v/v) of overnight grown culture (37 °C, 150 rpm) was added to 200 ml of each of the five media containing 50 µg/ml Kanamycin in separate 1 liter flasks and incubated at 37 °C, 200 rpm in shaker-incubator (New Brunswick Scientific, USA). Cultures were analyzed for optical density at a wavelength of 600 nm (OD₆₀₀) using spectrophotometer (Thermo Electron corp., USA) once in every 1 h. Protein expression was induced with 1 mM IPTG at exponential phase (OD₆₀₀ ~ 0.7) for 5 h at 37 °C.

Biomass and total protein estimation

To measure biomass and protein concentration, 2 ml of culture broth was collected separately in triplicates into pre-weighed eppendorf tubes. The cultures were centrifuged in microcentrifuge (Eppendorf, USA) at 10,000 rpm for 15 min. Supernatant was discarded, and the pellets were washed with sterile PBS. Protein content of the cell biomass obtained from each medium was determined using Bradford method [24]. The cell pellets from the second

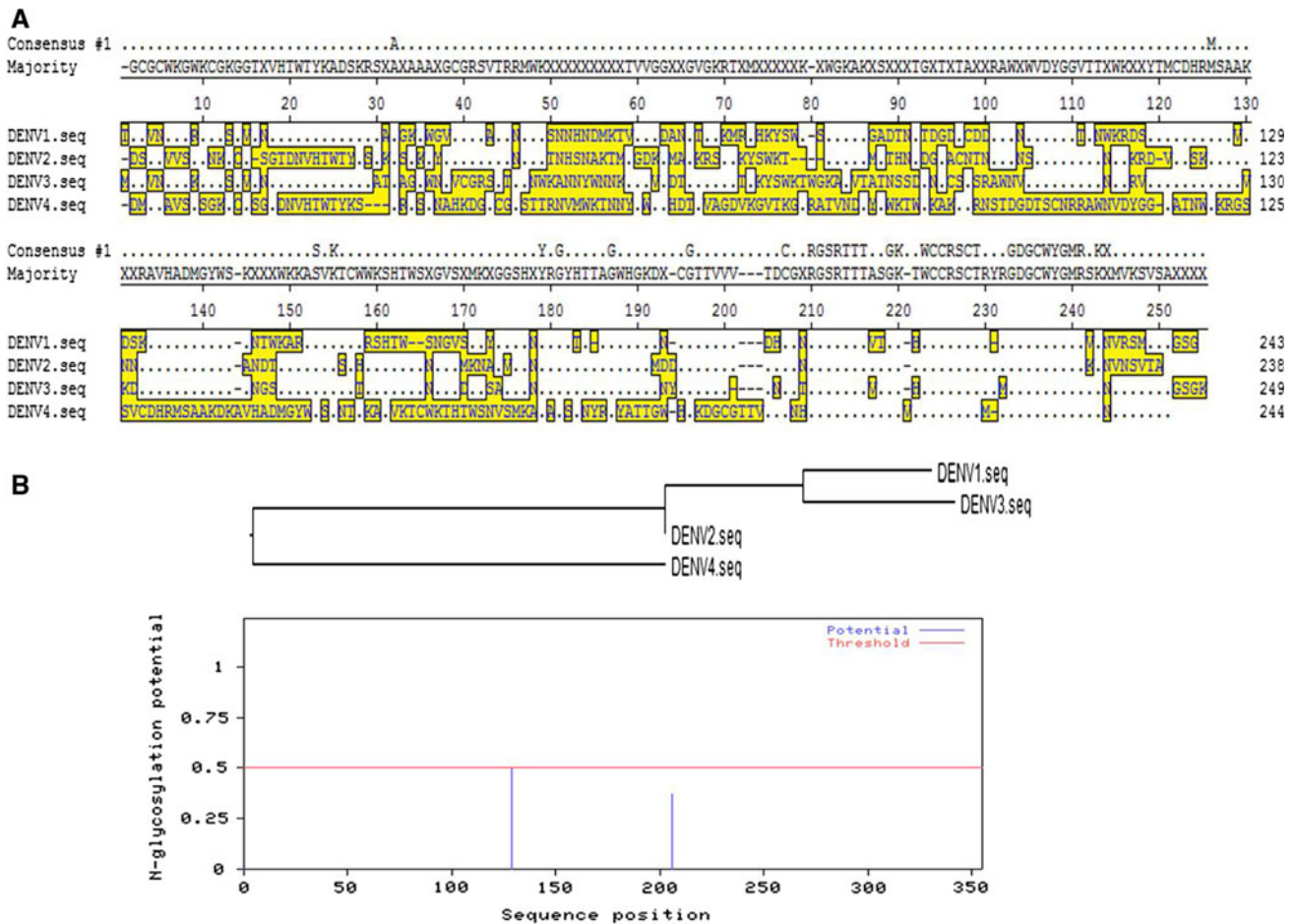


Fig. 1 a Clustal W multiple alignment of NS1 protein of dengue serotypes 1, 2, 3, and 4 (Indian isolates) *sequence shaded in yellow color indicates the consensus sequence. b* Prediction of N-Linked glycosylation of NS1 protein: The N-linked glycosylation was predicted using NetNGlyc 1.0 server. The *peaks* represents the glycosylation points on the whole dengue-3 NS1 protein

Table 1 Media composition of different *E. coli* media used for optimizing Dengue recombinant NS1 protein

S. no.	Medium	Composition (per liter)
1	LB broth	Tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g
2	SOB broth	Tryptone, 20 g; yeast extract, 5 g; NaCl, 0.5 g; MgSO ₄ , 2.4 g; KCl, 1.86 g
3	Super broth	Soy hydrolysate, 12 g; yeast extract, 24 g; dipotassium phosphate, 11.4 g; monopotassium phosphate, 1.7 g; glycerol, 5 ml
4	Terrific broth-1	Pancreatic digest of casein, 12 g; yeast extract, 24 g; dipotassium phosphate, 9.4 g; monopotassium phosphate, 2.2 g; glycerol, 4 ml
5	Terrific broth-2	Terrific broth 1 + M9 salts (2×); yeast extract (2 %); MgSO ₄ , 1.2 g; glycerol, 8 ml [M9 salts (5×): disodium phosphate (anhydrous), 33.9 g; monopotassium phosphate, 15.6 g; sodium chloride, 2.5 g; ammonium chloride, 5 g; H ₂ O, 1 l]
6	Terrific broth-3	Terrific broth1 + M9 salts (2×); yeast extract (2 %); trace metal solution, 1 ml; glycerol, 4 ml (trace metal solution: 50 μMFeCl ₃ ·6H ₂ O, 20 μMCaCl ₂ , 10μMMnCl ₂ ·4H ₂ O, 10μM ZnSO ₄ ·7H ₂ O, 2 μMCoCl ₂ ·6H ₂ O, 2 μMCuCl ₂ ·2H ₂ O, 2 μMNiCl ₂ ·6H ₂ O, 2 μM Na ₂ MoO ₄ ·2H ₂ O, 2 μM, Na ₂ SeO ₃ ·5H ₂ O, 2 μM H ₃ BO ₃ , H ₂ O 36 ml)
7	Terrific broth-4	Terrific broth 1 + M9 salts (2×); yeast extract (2 %); MgSO ₄ , 1.2 g; trace metal solution, 1 ml; glycerol, 4 ml

batch were weighed on a balance to obtain the wet mass weight. To obtain dry mass weight, same tubes were left overnight at 100 °C in incubator. The tubes were then

immediately transferred to desiccators containing calcium oxide (CaO) and then consistent dry weights were recorded.

Downstream processing of cell pellet for purification

Solubilization of inclusion bodies

Recombinant NS1 protein was purified from the induced cell culture pellet grown in TB-3 medium (1 liter). The induced culture was centrifuged at $5,000\times g$ for 20 min at 4 °C. The cell pellet was suspended in 20 ml of cell lysis buffer (10 mM Tris–Cl pH 8.0, 10 mM EDTA, 100 mM NaCl, and 100 µg/ml lysozyme). The cell suspension was sonicated for 10 min on ice (10 min with 10 s on and 10 s off cycles) using microprobe set at 40 % frequency of sonicator. The resulting cell lysate was centrifuged at $13,000\times g$ for 30 min at 4 °C. Supernatant was discarded, and the inclusion bodies (IB) pellet was further resuspended in 20 ml IB wash buffer (10 mM Tris–Cl pH 6.0, 5 mM EDTA, 200 mM NaCl, 1 M urea) by continuous stirring for 2 h at 4 °C. The IB solution was centrifuged at $13,000\times g$ for 30 min at 4 °C. The pellet obtained was further resuspended in 15 ml of IB solubilization buffer (10 mM Tris–Cl pH 8.0, 100 mM NaCl, 100 mM NaH_2PO_4 , and 8 M urea) by continuous stirring for overnight at 4 °C. The resultant solution was centrifuged at $13,000\times g$ for 30 min at 4 °C. The supernatant obtained was filtered through 0.45 µm filter and used for the purification of rNS1.

Immobilized metal affinity chromatography purification of rNS1

The recombinant protein was purified exploiting both its N-terminal and its C-terminal 6× His tag by immobilized metal affinity chromatography (IMAC) using Ni–NTA super flow chelating agarose column (Qiagen, Germany). Initially, the column was equilibrated with 5 bed volumes of IB solubilization buffer (pH 8.0). The filtered supernatant obtained from the previous step was loaded onto the Ni–NTA column, allowed to bind for 30 min on a rotary shaker. The unbound protein was removed from the column through gravitational flow. The column was washed with 15 bed volumes of wash buffer (10 mM Tris–Cl pH 5.5, 100 mM NaCl, 100 mM NaH_2PO_4 , and 8 M urea), and finally, the protein bound to the column was eluted by passing 10 bed volumes of elution buffer (10 mM Tris pH 4.0, 100 mM NaCl, 100 mM NaH_2PO_4 , and 8 M urea). Each elutes were collected in 1 ml fractions and analyzed on 10 % SDS-PAGE. The protein concentration was estimated by Bradford method [24]. The fractions containing rNS1 protein were pooled together and subsequently used for refolding.

Refolding of rNS1 protein

The pooled rNS1 protein after purification was taken into 10 kDa molecular weight cut off (MWCO) dialysis bag

(Millipore, USA) after adjusting the concentration to 100 µg/ml. The protein was refolded for 3 days at 4 °C in 500 ml refolding buffer containing 50 mM Tris (pH 8.0), 0.4 M L-arginine, 1 mM Glutathione reduced (GSH), and 0.1 mM glutathione oxidized (GSSG) with three changes of refolding buffer once every day as described earlier [25]. Any aggregates formed were removed by centrifugation. Finally, dialysis was done overnight against PBS (pH 7) at 4 °C.

Western blot analysis using anti-Dengue NS1 and anti-HIS monoclonal antibodies

The purified protein was subjected to western blotting in duplicates. In brief, for the western blot analysis, the purified protein along with molecular weight marker was electrophoretically transferred onto polyvinyl difluoride (PVDF) membrane (Millipore, USA) using semidry transfer unit (Biorad, USA). The membranes were blocked overnight at 4 °C in 3 % BSA solution prepared in phosphate-buffered saline (PBS). The blots were washed with PBS-T [(PBS containing 0.05 % Tween-20(v/v))] thrice for 5 min and incubated either with anti-His HRP conjugated monoclonal antibody or with three different anti-Dengue NS1 monoclonal antibodies (Thermo Scientific, USA Catalog number MA1-71253 to MA1-71255) for 1 h at 37 °C. The blots were then thoroughly washed thrice with PBS-T for 5 min and developed with DAB- H_2O_2 chromogen-substrate mixture. The blots that were reacted with anti-Dengue NS1 monoclonal antibodies were further incubated with anti-mouse HRP conjugate (Sigma, USA) for 1 h at 37 °C before color development as mentioned above.

Confirmation of refolded NS1 protein through Immunochromatographic lateral flow test

The refolded NS1 protein was applied onto Dengue NS1 Ag + Ab Combo (Dengue Duo) Immuno chromatography test strip (Standard Diagnostics, Inc., Germany) to check the reactivity with NS1 specific captured antibodies. The results were interpreted as per the recommendations of the manufacturer.

Evaluation of diagnostic potential of rNS1 protein through IgM antibody capture ELISA

In order to evaluate the diagnostic potential of the rNS1, the purified and properly refolded protein was diluted to a final concentration of 1 µg/ml in 0.01 M carbonate buffer (pH 9.6) and further coated onto Nunc maxisorp plates (100 µl/well) by incubating at 37 °C for overnight; wells were washed with $1\times$ PBS and then blocked with 3 % BSA

for 2 h at 37 °C. The blocked wells were washed twice with 1× PBST and stored at 4 °C until further use. A total of fourteen acute phase sera samples that were found positive for Dengue IgM antibodies and NS1 antigen via Dengue Duo ICT strip (Standard Diagnostics, Inc) which were received from ISPAT General Hospital, Raurkela, India were diluted (1:300) and reacted with the antigen coated wells at 37 °C for 1 h. After incubation, the wells were washed thrice with PBST and incubated with anti-human IgM HRP conjugate antibody (1:10,000 dilution) for 1 h at 37 °C. The wells were washed thrice with PBST and color was developed with 100 µl of TMB-H₂O₂ liquid chromogen-substrate solution (Sigma, USA). The reaction was stopped with the addition of 50 µl 1 N H₂SO₄, and the absorbance was read at 450 nm in an ELISA reader (Bio-tek, USA). The OD values that were greater than that of negative control plus 2SD were considered to be positive.

Results and discussion

Dengue NS1 gene analysis

ClustalW multiple alignment of NS1 protein sequences of four Dengue serotypes of Indian origin showed a sequence similarity up to 57 %. Phylogenetic analysis of four Dengue Indian serotypes revealed that serotypes 1 and 3 are closely related and forms a single clade along with serotype 2. Whereas serotype 4 was distinct from the remaining three serotypes that formed a separate clade (Fig. 1a). The *N*-linked glycosylation study of NS1 protein sequence revealed the presence of two *N*-linked glycosylation points at Asn 130 and Asn 207 (Fig. 1b). As NS1 protein showed significant homology across the four serotypes at amino acid level, serotype-3 was selected for recombinant expression of NS1 protein. The linear B-cell epitope prediction on Dengue 3 NS1 protein based on different parameters viz, hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic property revealed more than eleven B-cell specific epitopes that were uniformly distributed all throughout the protein (Fig. 2). The protein regions having values greater than threshold value and also qualifying at least more than four parameters were considered to be potential epitopes. In order to have increased sensitivity with more number of linear epitopes, the whole NS1 gene was selected for recombinant expression rather than partial sequences.

Amplification, cloning and selection of DEN-3 full-length NS1 clone

Using the RT-PCR described above, the full-length NS1 gene (1,056 bp) was amplified corresponding to the

nucleotide 2,414–3,470 of Dengue-3 genome. The amplified full-length NS1 gene was cloned into pET28(a) vector under the control of T7 RNA polymerase promoter at *EcoRI* and *NotI* sites. The present cloning strategy allowed the recombinant construct to have 6× His tag on both N-terminal and C-terminal of the transgene (Fig. 3). The recombinant plasmid DNA (pETNS1) from *E. coli* Rosetta-gami positive transformant used in the present study gave a release of 1.05 Kb upon digestion with *EcoRI* and *NotI* enzymes. The nucleotide sequences obtained from the sequencing reactions also showed homology (>99 %) with that of Dengue-3 GWL-25 strain retrieved from NCBI GenBank indicating the authenticity of the cloned gene (data not shown).

Expression profile and localization of recombinant protein

After induction of positive *E. coli* Rosetta-gami culture with different concentrations of IPTG (0.1, 0.25, 0.5, and 1 mM), a thick band with molecular mass of approximately 45 kDa was observed in case 1 mM IPTG concentration (Fig. 4). No such protein was detected in cell extracts of non-induced culture. Majority of the recombinant protein expressed was detected in the insoluble fraction of the cell extract. Attempts to change bacterial culture conditions like temperature, harvest time, and IPTG concentrations did not yield any significant amount of protein in soluble fraction (data not shown).

Media optimization

For selecting an optimum medium to obtain the maximum yield of expressed rNS1 protein, seven different types of media viz Luria–Bertani broth, SOB broth, Super broth, Terrific broth modified 1, Terrific broth modified 2, Terrific broth modified 3, and Terrific broth modified 4 were tested. Cultures were analyzed for optical density at a wavelength of 600 nm (OD₆₀₀) once in every 1 h. Protein expressed in case of each medium after induction with 1 mM IPTG is shown in Fig. 5. The rNS1 protein intensity appeared to be better in case of both Terrific broth modified 2 (Fig. 5e) and Terrific broth modified 3 (Fig. 5f) medium without any degradation. Interestingly, the proteins derived from the cultures grown in the above two media had comparatively less background proteins. The other media types used in the present study also expressed the recombinant protein but there was some degradation of the expressed rNS1 resulting in a cleaved protein fragment that was running slightly below the expected rNS1 (Fig. 5a–d, g). In light of our present observation, we presume that the type of *E. coli* medium used earlier by Amorima et al. [26] might have

Sequence	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Hydrophilicity	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Flexibility	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Accessibility	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Turns	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Exposed Surface	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Polarity	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Antigenic Propensity	<u>1MGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGA WENGVCGIRSTTRMENLLWKQIANELNYILWENNIKLTVVVGDTI</u>
Sequence	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Hydrophilicity	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Flexibility	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Accessibility	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Turns	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Exposed Surface	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Polarity	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Antigenic Propensity	<u>TIGVLEQGRKRLTPQPMELKYSWKTWGKAKIVTAETQNSSFIIDGPNTPPCPSASRAWNVWEVEDYGFVFTTNIWKLREVYTQMCDHRLMSAAVKDERAV</u>
Sequence	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Hydrophilicity	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Flexibility	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Accessibility	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Turns	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Exposed Surface	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Polarity	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Antigenic Propensity	<u>.VHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWSNGVLESDMIIPKSLAGPISQHNYPGYHTQTAGPWHLGKLELDFNYCEGTTVVITENCGTRG</u>
Sequence	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>
Hydrophilicity	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>
Flexibility	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>
Accessibility	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>
Turns	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>
Exposed Surface	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>
Polarity	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>
Antigenic Propensity	<u>GPSLRTTTSVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSAGSGK³⁵⁵</u>

Fig. 2 B-cell epitope prediction. The blue and underlined amino acid stretch indicate the potential linear B-cell epitopes as predicted using Bcepred tool based on parameters viz, hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity, and antigenic property

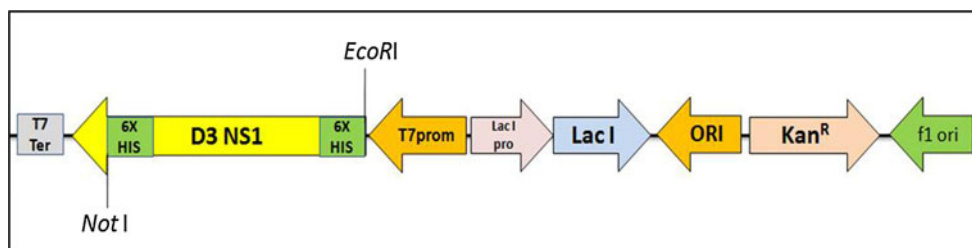


Fig. 3 Schematic representation of pETNS1 recombinant plasmid DNA. The expression cassette of pETNS1 consists of Dengue 3 NS1 gene cloned under T7 RNA polymerase promoter and T7 terminator.

The Dengue-3 NS1 gene is cloned at *EcoRI* and *NotI* in frame with 6X-His fusion tag at both N-terminal and C-terminal ends. The plasmid has Kanamycin resistance gene (*Kan^R*) for selection

played a crucial role in obtaining a low molecular weight (~32 kDa) NS1 protein along with the intact protein. The present finding of SDS-PAGE analysis for qualitative determination of rNS1 is significant and further hints the importance of selecting a suitable medium for culturing the

bacteria in which the expressed recombinant protein is more stable. Hence, to the best of our knowledge, this is the first report on successful expression of intact Dengue rNS1 as a single band without any degradation using an optimized *E. coli* culture conditions.

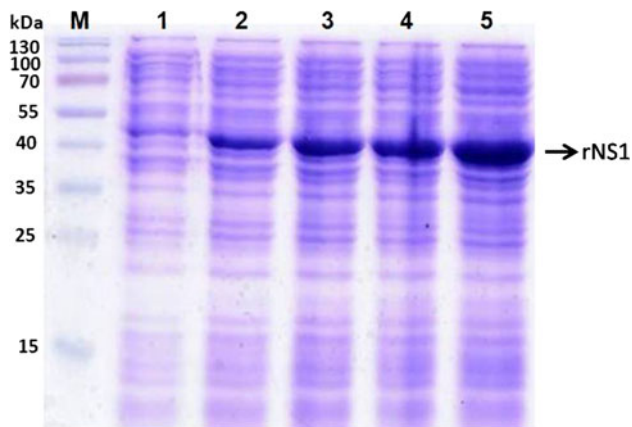


Fig. 4 SDS-PAGE profile of pETNS1 transformed recombinant *E. coli* Rosetta-gami culture with different IPTG concentrations lane *M* pre-stained protein marker, lane 1 pETNS1 transformed recombinant *E. coli* Rosetta-gami culture pre-induced (negative control), lane 2, 3, 4, and 5 pETNS1 transformed recombinant *E. coli* Rosetta-gami culture induced with 0.1, 0.25, 0.5, and 1 mM IPTG, respectively

Biomass and total protein estimation

As seen in Fig. 6a, the wet weight of the biomass obtained from Terrific broth-modified medium-3 (TB-3) was more compared to the other six media types. An average of 110 mg/l wet weight was obtained at the time of harvest in case of TB-3 medium whereas LB broth had lowest wet weight of 62 mg/l at the time of harvest after 9 h of total growth that corresponds to 5 h of post induction. As expected, similar observations were also noticed in case of the average dry weight of the biomass that resulted from different media types (Fig. 6b). A maximum dry weight up to 32 mg/l was obtained in case of TB-3 medium at the time of harvest. The highest optical density (3.75) at 600 nm was found in case of TB-3 medium as seen in Fig. 6c. A highest total protein concentration of 4.4 mg/ml was obtained from the culture grown in TB-3 medium (Fig. 6d). From all the above four parameters tested, it is evident that TB-3 medium is more suitable for the optimum expression and stability of rNS1 protein.

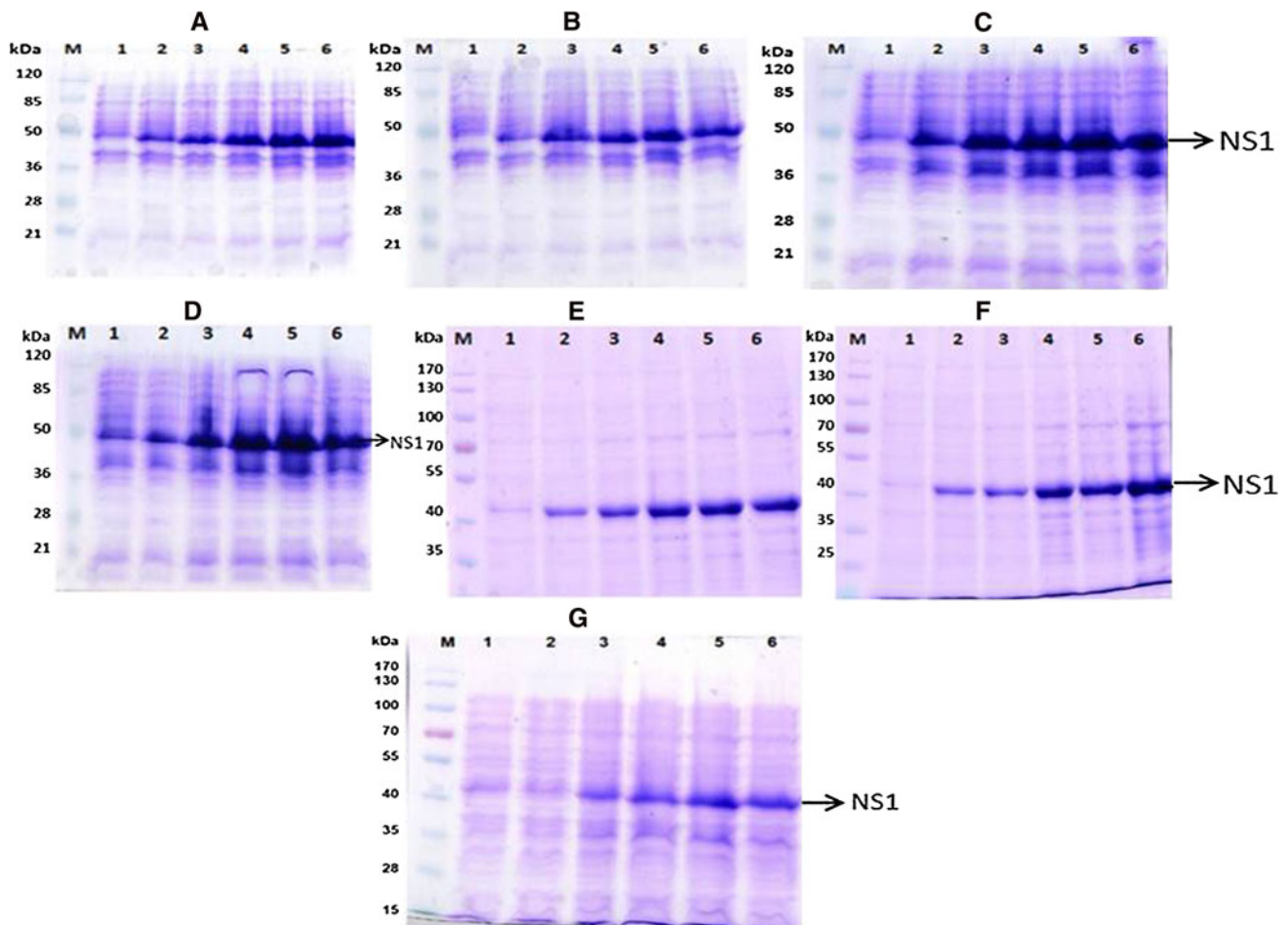


Fig. 5 SDS-PAGE profile of pETNS1 transformed recombinant *E. coli* Rosetta-gami culture in different media (a LB broth, b SOB broth, c super broth, d Terrific broth-1, e Terrific broth-2, f Terrific broth-3, and g Terrific broth-4). Lane *M* pre-stained protein marker,

lane 1 pETNS1 transformed recombinant *E. coli* Rosetta-gami culture pre-induced (negative control), lane 2, 3, 4, 5, and 6 pETNS1 transformed recombinant *E. coli* Rosetta-gami culture induced with 1 mM IPTG after 1, 2, 3, 4, and 5 h, respectively

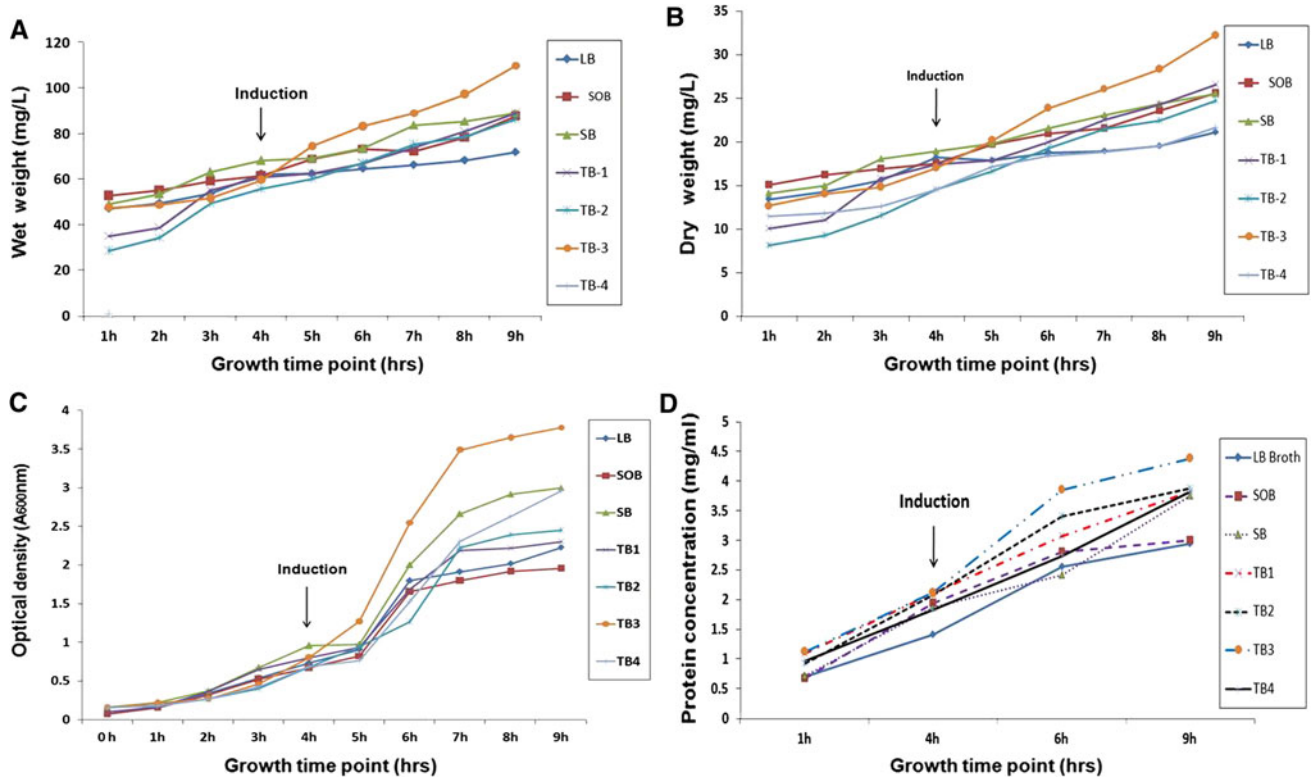


Fig. 6 Optimization of growth medium for pETNS1 transformed recombinant *E. coli* Rosetta-gami cells. **a** Wet weight profile in different media, **b** dry weight profile in different media, **c** optical

density profile at 600 nm in different media, and **d** protein concentration profile in different media

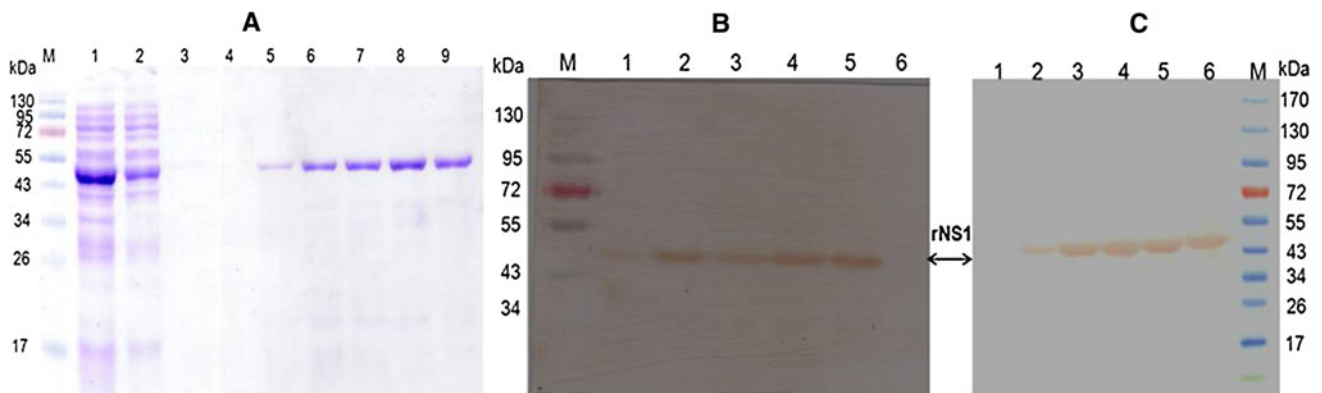


Fig. 7 SDS-PAGE and western blot analysis of purified rNS1 protein. **a** SDS-PAGE profile of affinity purified rNS1 protein. Lane *M* pre-stained protein marker, lane 1 solubilized inclusion bodies from the induced cell lysate, lane 2 flow through fraction collected after affinity binding, lanes 3, 4 washed fractions, lanes 5–9 eluate fractions. **b** Western blot analysis of affinity purified rNS1 protein

using anti-NS1 monoclonal antibodies (Thermo Scientific, USA, Catalog no: MA1-71255). Lane *M* pre-stained protein marker, lane 1–5 eluted fractions, lane 6 washed fraction. **c** Western blot analysis of affinity purified rNS1 protein using anti-His monoclonal antibodies. Lane 1 washed fraction, lane 2–6 eluted fractions, lane *M* pre-stained protein marker

Immobilized metal affinity purification of rNS1

The recombinant protein from the inclusion bodies was purified using both N-terminal and C-terminal 6× His tag via immobilised metal affinity purification. The final eluted fractions showed a single band on 10 % SDS-PAGE gel as seen in Fig. 7a without any visible impurities.

Western blot analysis using anti-Dengue NS1 and anti-His monoclonal antibodies

The expressed protein after affinity purification reacted with Dengue 1–4 group specific anti-NS1 monoclonal antibodies (Fig. 7b) and anti-His (Fig. 7c) monoclonal antibodies. No cross-reacting protein was detected as

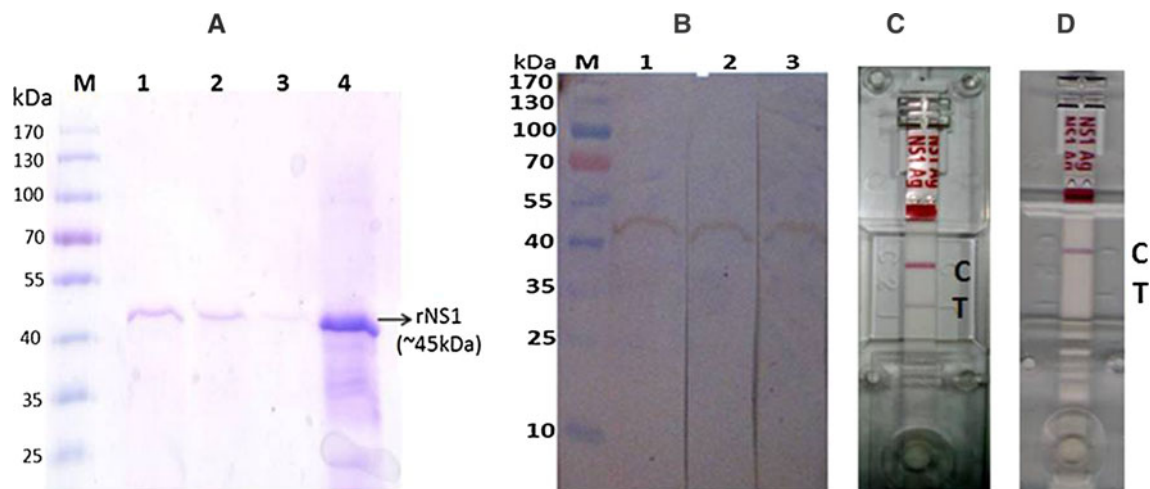


Fig. 8 Characterization of refolded rNS1 protein. **a** SDS-PAGE profile of affinity purified refolded rNS1 protein. Lane *M* pre-stained protein marker, lane 1, 2, 3 refolded rNS1 protein fractions, lane 4 refolded rNS1 protein after concentration. **b** Western blot analysis of affinity purified rNS1 protein using anti-NS1 monoclonal antibodies (Thermo Scientific, USA). Lane *M* pre-stained protein marker, lane 1,

2, 3 refolded rNS1 protein reacted with different anti-NS1 monoclonal antibodies against Dengue serotype 2 (catalog no MA1-71253), Dengue serotype 2 (MA1-71254), and Dengue serotype 1-4 (MA1-71255), respectively. **c** Reaction of refolded rNS1 via Dengue Duo ICT. **d** Reaction of protein extract of uninduced recombinant *E. coli* Rosetta-gami culture

Table 2 Comparative evaluation of rNS1-based IgM ELISA and commercial Dengue Duo test

Sample ID	Age/sex	Fever history (days)	Platelet count	<i>E. coli</i> expressed rNS1	Dengue Duo (standard diag)	
					rNSI IgM	IgM
3	15/M	4	96,000	–	–	+
5	24/M	6	32,000	+	+	+
7	26/M	5	73,000	+	+	+
9	37/F	4	62,000	+	+	+
13	1/M	5	34,000	+	+	+
20	30/M	3	27,000	+	+	+
22	55/F	6	100,000	+	+	+
24	59/M	6	34,000	+	+	+
28	50/M	5	44,000	+	+	+
32	36/F	4	62,000	+	+	+
47	45/M	3	30,000	+	+	+
52	32/M	4	17,000	+	+	+
57	5/F	3	169,000	+	+	+
63	36/F	6	23,000	+	+	+
Healthy control	24/M	–	NT	–	–	–

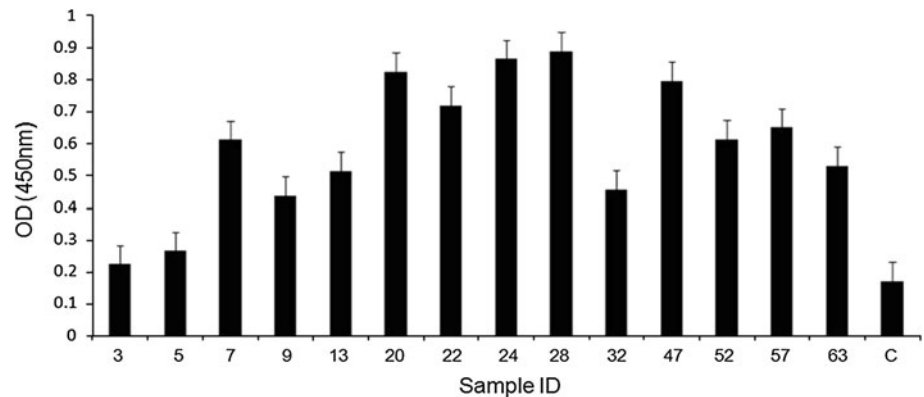
impurities in the washed fractions (Fig. 7b, c, lane 6 and 1, respectively) suggesting optimum purification of the rNS1 protein. The use of double Histidine-fusion in the present study has facilitated the stringent washing procedure with lower pH that helps in removing nonspecific proteins that are often co-purified during the elution step. The western blot results using anti-NS1 and anti-His monoclonal antibodies suggested that the addition of two histidine tags has

no detrimental effect on the immunoreactivity of the expressed rNS1 protein.

Refolding of rNS1 protein and its characterization

In order to attain the native conformation of denatured rNS1, successful refolding was achieved with a protein concentration of 100 µg/ml in the refolding buffer with the

Fig. 9 rNS1 protein based IgM antibody capture ELISA using known positive Dengue clinical sera samples



conditions as described earlier [25]. The refolded protein was dialyzed finally with PBS. Samples of the purified protein had a single band of approximate molecular mass of 45 kDa (Fig. 8a). The purified and refolded recombinant NS1 protein reached an amount of approximately 4.2 mg of protein per liter of bacterial culture. No dimeric or cleaved protein of lower molecular mass as reported by Amorima et al. [26] was observed in the present study. As earlier study [26] reports the presence of 32 kDa protein along with 43 kDa protein that has been attributed to the cleaved product of complete NS1 protein, our study suggests that the protein expressed from *E. coli* is more stable and do not undergo any proteolytic cleavage. Although no dimeric or tetrameric forms of NS1 were observed in the present study even after refolding, the rNS1 was recognized by three different commercial monoclonal antibodies raised against different epitopes of the NS1 protein of heterologous serotypes as seen in Fig. 8b. It is important to note that the refolded Dengue-3 rNS1 is being recognized by two different monoclonal antibodies that were raised against Dengue-2 NS1 (Fig. 8b, lanes 1 and 2) and also Dengue 1–4 group specific monoclonal antibody (Fig. 8b, lane 3) suggesting the cross-reactivity of our Dengue-3 NS1 with other serotypes. The refolded protein also gave a precipitin line with commercial Dengue Duo NS1 antigen detection ICT strip (Fig. 8c). No such precipitin line was observed from the protein extract of uninduced bacterial culture (Fig. 8d). This confirms the authenticity of the refolded protein for its immunoreactivity.

Evaluation of diagnostic potential of rNS1 protein through IgM antibody capture ELISA

Selected clinical samples (14 nos.) with a history of fever (3–6 days) and decreased platelet count as depicted in Table 2 were used in the present study. All these samples were also found to be positive both for NS1 antigen and NS1 IgM antibodies as determined by the commercially available Dengue Duo ICT kit. Out of fourteen known

positive clinical sera samples tested using our rNS1-based ELISA, thirteen samples were found to be positive with 92.8 % concordance (Fig. 9). In early stage of dengue infection, IgM antibodies are induced before the appearance of IgG. Thus, the use of antigenic rNS1 as diagnostic candidate may enhance the detection rate of dengue in patients with primary infection. Our results concerning the prevalence of anti-NS1 Ab in sera of primary dengue infection corresponds to the findings of Haung et al. [17] where Dengue 2 NS1 has been used in detecting all four serotypes of Dengue infection. This study has also experimentally demonstrated that Dengue-2 NS1 cross reacts with sera samples of other dengue serotypes, and NS1 is relatively conserved flaviviral protein. The above finding combined with our bio-informatics based study on B-cell epitope prediction and Dengue NS1 protein homology across all four Indian isolates further strengthens our argument that rNS1 protein of Dengue-3 also has the epitopes which are common to all four dengue virus serotypes as reported earlier [27]. Our preliminary findings suggest the possible use of in vitro refolded Dengue-3 rNS1 protein as a diagnostic candidate for early detection of Dengue infection. Further research using large number of Dengue clinical samples with known serotypes and cross-reactivity of rNS1 with the other flavivirus infection, combination of other immuno-dominant dengue recombinant NS1 fragments of other serotypes are underway for improving the sensitivity and specificity of rNS1.

In addition to the diagnostic potential, NS1 protein also has immense prophylactic value. Previous studies have indicated that individuals upon natural Dengue infection show high levels of antibodies directed against NS1 and envelope protein. Animals vaccinated with purified NS1 of DEN-2 [28] or yellow fever virus [29, 30] provided protection against challenges by the respective virus. This suggests that immunization with NS1 is of importance as it can overcome the antibody dependent enhancement of infection (ADE) related problems associated with the administration of structural proteins like envelope and pre-

membrane. However, vaccination using rNS1 protein for its protective efficacy against homologous and heterologous virus challenge is yet to be studied.

In summary, the *E. coli* derived recombinant NS1 protein produced in this study was found to be a useful diagnostic antigen that is easy to prepare and handle. The optimized expression and purification methods developed in the present study are suitable for inexpensive and bulk production rNS1 protein either for diagnostic or prophylactic applications with low batch variations.

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References

1. E.G. Westaway, M.A. Brinton, S.Y. Gaidamovich, M.C. Horzinek, A. Igarashi, L. Kaariainen, D.K. Lvov, J.S. Porterfield, P.K. Russell, D.W. Trent, *Intervirology* **24**, 183–192 (1985)
2. S. Noisakran, T. Dechtawewat, P. Rinkaewkan, C. Puttikhunt, A. Kanjanahaluethai, W. Kasinrerak, N. Sittisombut, M. Prida, *J. Virol. Methods* **142**, 67–80 (2007)
3. A.H. Yara, V. Dogra, N. Arora, B.K. Tyagi, D. Nandan, D.S. Shepard, WHO, *Dengue Bulletin* 35 (2011), http://www.searo.who.int/LinkFiles/Dengue_Bulletin_vol-35.pdf. Accessed 11 Nov 2012
4. G. Winkler, V.B. Randolph, G.R. Cleaves, T.E. Ryan, V. Stollar, *Virol. J.* **1**, 187–196 (1988)
5. P.R. Young, P.A. Hilditch, C. Bletchly, W. Halloran, *J. Clin. Microbiol.* **38**, 1053–1057 (2000)
6. M. Flamand, F. Megret, M. Mathieu, J. Lepault, F.A. Rey, V. Deubel, *J. Virol.* **70**, 6104–6110 (1999)
7. M.G. Jacobs, P.J. Robinson, C. Bletchly, J.M. Mackenzie, P.R. Young, *FASEB J.* **14**, 1603–1610 (2000)
8. H. Leblois, P.R. Young, *J. Gen. Virol.* **76**, 979–984 (1995)
9. P.W. Mason, *Virol. J.* **169**, 354–364 (1989)
10. S. Alcon, A. Talarmin, M. Debruyne, A. Falconar, V. Deubel, M. Flamand, *J. Clin. Microbiol.* **40**, 376–381 (2002)
11. P. Dussart, B. Labeau, G. Lagathu, P. Louis, M.R. Nunes, S.G. Rodrigues, C. Storck-Herrmann, R. Cesaire, J. Morvan, M. Flamand, L. Baril, *Clin. Vaccine Immunol.* **13**, 1185–1189 (2006)
12. A.H. Ramirez, Z. Moroz, G. Comach, J. Zambrano, L. Bravo, B. Pinto, S. Vielma, J. Cardier, F. Liprandi, *Diagn. Microbiol. Infect. Dis.* **65**, 247–253 (2009)
13. S.D. Blacksell, M.P. Mammen, S. Thongpaseuth, R.V. Gibbons, R.G. Jarman, K. Jenjaroen, A. Nisalok, R. Phetsouvanh, P.N. Newton, N.P.J. Day, *Diagn. Microbiol. Infect. Dis.* **60**, 43–49 (2008)
14. S. Zainah, A.H. Wahab, M. Mariam, M.K. Fauziah, A.H. Khairul, I. Rosalina, A. Sairulakhma, S.S. Kadimon, M.S. Jais, K.B. Chua, *J. Virol. Methods* **155**, 157–160 (2009)
15. W. Chaiyaratana, A. Chuansumrit, V. Pongthanapisith, K. Tangnararatchakit, S. Lertwongrath, S. Yoksan, *Diagn. Microbiol. Infect. Dis.* **64**, 83–84 (2009)
16. W.J.H. McBride, *Diagn. Microbiol. Infect. Dis.* **64**, 31–36 (2009)
17. J.L. Huang, J.H. Huang, R.H. Shyu, C.W. Teng, Y.L. Lin, M.D. Kuo, C.W. Yao, M.F. Shiao, *J. Med. Virol.* **65**, 553–560 (2001)
18. B. Zhao, G. Prince, R. Horswood, K. Eckels, P. Summers, R. Chanock, C.J. Lai, *J. Virol.* **61**(12), 4019–4022 (1987)
19. X. Qu, W. Chert, T. Maguire, F. Austin, *J. Gen. Virol.* **74**, 89–97 (1993)
20. J.M. Zhou, Y.X. Tang, D.Y. Fang, J.J. Zhou, Y. Liang, H.Y. Guo, L.F. Jiang, *Virus Genes* **33**, 27–32 (2006)
21. R.G. Kathryn, N.J. Moreland, C. Rued, S.G. Vasudevan, *Protein Expr. Purif.* **82**, 20–25 (2012)
22. S. Saha, G.P.S. Raghava, LNCS, vol 3239 (Springer, 2004), pp. 197–204
23. U.K. Laemmli, *Nature* **227**, 680–685 (1970)
24. M.M. Bradford, *Anal. Biochem.* **72**, 248–254 (1976)
25. D. Das, S. Mongkolaungkoon, M.R. Suresh, *Protein Expr. Purif.* **66**, 66–72 (2009)
26. J.H. Amorima, B.F. Porchiaa, A. Balan, R.C Cavalcantea, S.M. da Costac, A.M. de Alvesc, L.C. de Souza Ferreiraa, *J. Virol. Methods* **167**, 186–192 (2010)
27. A.K.I. Falconar, P.R. Young, *J. Gen. Virol.* **72**, 961–965 (1991)
28. J.L. Schlesinger, M.W. Brandriss, E.E. Walsh, *J. Gen. Virol.* **68**, 853–857 (1987)
29. J.L. Schlesinger, M.W. Brandriss, C.B. Cropp, T.P. Monath, *J. Virol.* **60**, 1153–1155 (1986)
30. J.L. Schlesinger, M.W. Brandriss, E.E. Walsh, *J. Immunol.* **135**, 2805–2809 (1985)