

Heterologous expression of envelope Protein (Domain III) of Dengue Virus Type 2 for Serodiagnosis

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Abstract

Dengue is re-emerging as one of the most important public health problems. Since there is no specific therapy available, the timely and rapid diagnosis plays a vital role in patient management. Thus, there is a need to develop an alternate antigen to replace the whole virus antigen in diagnostic tests, which has several drawbacks. In this study, domain III of envelope gene of dengue virus type 2 was cloned in pQE 30UA expression vector and expressed in *E. coli* strain M15 (pREP4). The recombinant dengue-2 domain III protein was purified from inclusion bodies by affinity chromatography. The reactivity of this protein was checked by Western blot using patient serum sample. This protein was evaluated by an in-house ELISA employing a panel of human serum samples and the results were compared with Pan-Bio IgM capture ELISA. These results revealed that the product could be used as diagnostic reagent in dengue diagnosis.

Keywords: *Dengue virus, ELISA, rD2 DIII protein, affinity purification, Western blot.*

Introduction

Dengue virus infection is now recognized as one of the most important mosquito borne human infection of 21st century. The global incidences of the dengue infection have increased enormously and an estimated 50-100 million cases of dengue infections are now reported annually from more than 100 tropical and sub tropical countries of the world (WHO, 2002). Dengue is caused by four antigenically distinct viruses designated as dengue virus type 1 - 4, belonging to genus *Flavivirus* of family *Flaviviridae*. Dengue is endemic in India and dengue 2 & 3 serotypes have been reported in various outbreaks in the past (Dar *et al.*, 1996; Parida *et al.*, 2002; Dash *et al.*, 2005-06). However, the large and severe outbreaks were caused by dengue virus type-2. All the four serotypes of dengue

viruses are primarily transmitted by *Stegomyia (Aedes) aegypti*.

Definitive diagnosis of dengue infection depends on the identification of the virus, virus-encoded antigens, viral genomic RNA or the virus-induced antibodies (Gubler, 1998). Viral RNA can be detected with a high degree of sensitivity, using reverse transcription coupled with polymerase chain reaction (RT-PCR). The shortcoming common to all these methods is the narrow window period (5~days), available for successful detection, which coincides with the febrile period during which patients are viremic (Vaughn *et al.*, 2000). This precludes diagnostic tests based on the identification of the virus or its RNA genome because of the short duration of viremia. Thus, in a majority of cases the only feasible diagnostic test

would have to be based on the identification of anti-dengue antibodies.

Numerous commercial kits are available using whole virus cell culture antigen (Groen *et al.*, 2000) in enzyme-linked immunosorbent assay (ELISA) format. The most commonly used diagnostic test is IgM-capture enzyme-linked immunosorbent assay (Groen *et al.*, 2000) performed with a single specimen. Viral antigen, commonly prepared in cell culture or in suckling mouse brain, is often the limiting reagent in developing countries. Utilization of recombinant proteins, expressed using baculovirus can be produced more easily, and present little or no health hazard, are an attractive alternative (Cuzzubbo *et al.*, 2001). The simplified production of recombinant dengue virus antigen in *E. coli* avoids problems associated with the quality and standardization of conventional dengue viral antigen preparations (Huang *et al.* 2001; AnandaRao *et al.*, 2005; Tripathi *et al.*, 2007).

Most of the recombinant DNA-based strategies focus on the envelope (E) proteins of the dengue viruses which is related with humoral immunity. This protein is about 500 amino acid (aa) residues long, with the N terminal ~ 80% constituting the ectodomain, exposed on the virion surface, and the C-terminal ~20% constituting the transmembrane hydrophobic domain anchors the molecule on the lipid bilayer surrounding the virion. It is a multifunctional protein with key roles in several aspects of virus biology,

notably host cell surface receptor binding (Lindenbach and Rice, 2001). A domain of the E molecule, designated as domain III, is particularly important as it contains multiple serotype specific conformation-dependent, neutralizing epitopes and the host cell receptor recognition site (Chen *et al.*, 1996). Further, chimeric proteins containing domain III have been demonstrated to be immunogenic, capable of inducing neutralizing antibodies in experimental animals (Jaiswal *et al.*, 2004; Pattnaik *et al.*, 2006). Therefore, in the current study, we focused on the expression and purification of domain III of the E protein of dengue-2 virus in *E. coli* rather than the full-length of E molecule. In addition, evaluation of recombinant dengue virus type 2 envelope domain III (rD2 DIII) protein also has been carried out by ELISA for dengue diagnosis.

Materials and Methods

Expression vector and reagents

Escherichia coli host strain M 15[pREP4], plasmid vector pQE 30UA, Ni-NTA super flow resin, Viral RNA Mini Kit were obtained from Qiagen, Germany. Luria bertani agar and Luria bertani broth were obtained from DIFCO Laboratories, USA. Dengue virus type- 2 NIV strain (P23085) was obtained from National Institute of Virology, Pune, India. Anti-human IgG-horseradish peroxidase (HRPO), anti-human IgM-HRPO and antibiotics were obtained from Sigma, USA. Access quick one-step RT-PCR kit was obtained from Promega, USA. Prestained Protein ladder and restriction enzymes were purchased from MBI, Fermentas(USA).

Reverse Transcription- PCR (RT-PCR)

The presence of dengue-2 specific RNA in the standard virus was identified employing dengue 2 specific primers designed by targeting domain III of E gene using Lasergene version 5.07/5.52 (DNASTAR, USA) and were custom synthesized from M/s Operon, Germany. Viral RNA was extracted from sample using QIAamp Viral RNA Mini Kit in accordance with the manufacturer's

instructions and used as template in RT-PCR. The RT-PCR amplification of RNA was carried out using the Access quick one-step RT-PCR kit in 50 µl reaction volume containing 2X RT-PCR master mix, 2.5 U of AMV-RT and dengue-2 virus specific primer pair targeting domain III fragment of E gene. The following oligonucleotide primers were designed on the basis of available genome sequence of Dengue-2 NIV strain (P23085) to amplify the domain III of E gene (nt. 1792-2175) with the forward primer *D2-F* 5'-AGGATCCAGAATGGACAACTACA-3' and the reverse primer *D2-R'* 5'-GCCAAGCTTCTTCGCTCCTCT-3' in a thermal cycler (Bio-Rad, USA). The thermal profile of RT-PCR reaction was: RT step at 45°C for 45 minutes and initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension step of 72°C for 10 minutes. After electrophoresis the PCR product was purified from 1.5% low melting point agarose gel using the QIAquick gel extraction kit (Qiagen) according to manufacturer's protocol. The purified PCR product was then used for ligation in pQE-30 UA expression vector.

Construction of recombinant pQE30 UA-Dengue-2 DIII

A DNA fragment (~ 0.384 kb) encoding 155 amino acid long carboxy terminal portion of dengue 2 envelope protein corresponding to domain III was ligated in pQE-30UA expression vector. The ligated product was transformed into *E. coli* M 15[pREP4] cells and the transformants were selected on LB agar plates in presence of kanamycin (50 µg/ml) and ampicillin (100 µg/ml). The resultant colonies were screened by plasmid PCR which revealed a 384 bp amplicon on 1.5% agarose gel. One of these clones was selected for further studies after sequencing by automatic sequencer ABI 310 sequencer (Applied Biosystems, USA).

Screening for expression

The transformed *E. coli*, developed as above was inoculated into 10 ml test tube culture and

allowed to grow at 37 °C in a shaker at 200 rpm. Cultures in log phase (OD600 ~0.8) were induced for 4 h with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, cells were lysed in 1X sample lysis buffer and analyzed by 15 % SDS-PAGE as described earlier by Pattnaik *et al.*, (2006). Gels were stained with Coomassie brilliant blue R-250.

Preparation of Inclusion bodies (IBs)

Transformed *E. coli* M15 [pREP 4] cells (preserved in glycerol at -80°C) were inoculated into 20 ml of LB medium containing 50 g kanamycin/ml and 100µg/ml of ampicillin. The culture was grown overnight at 37°C, 200 rpm and inoculated at the rate of 1% in 1 litre of LB medium containing antibiotics in a 4 litre Haffkine flask. It was then incubated at 37°C for 4 h at 150 rpm. When the OD600 of the culture reached ~0.8, it was induced with 1 mM IPTG and further grown at 37°C for 4 h. Aliquots of the induced and un-induced cultures were analysed by SDS-PAGE prior to the purification. The induced culture was centrifuged at 6000 rpm for 15 minutes at 4°C (Sorvall, USA). The cell pellet was suspended in cell wash buffer (1:40 w/v) (pH 8.0) containing 10 mM Tris, 100 mM NaCl, 10 mM EDTA to a homogenous suspension and centrifuged again to obtain pellet. The cell pellet was resuspended in 80 ml of cell lysis buffer (pH 8.0) containing 10 mM Tris, 100 mM NaCl, 10 mM EDTA and 100 µg/ml lysozyme supplemented with 1.0 mM PMSF (phenyl methyl sulphonyl fluoride). The cell suspension was disrupted for 10 minutes on ice (9 seconds on/ 9 seconds off) using microprobe set at 40% frequency of ultrasonic disintegrator (Sonic, USA). The resulting cell lysate was centrifuged at 10,000 rpm for 40 minutes at 4°C. The clear supernatant was discarded. The IB pellet was re-suspended in 100 ml of IB wash buffer (pH 6.0) containing 10 mM Tris, 200 mM NaCl, 5 mM EDTA, 1M Urea, 1% Triton-X100 and the suspension was centrifuged at 10,000 rpm at 4°C for 40 minutes. The supernatant was aliquoted and stored for subsequent analysis. The purified IB pellet was used for purification of rD2 DIII protein.

Solubilization of Inclusion Bodies

IB Solubilization buffer (40ml, pH 8.0) containing 10 mM Tris, 100 mM NaCl, 100 mM NaH₂PO₄ and 8 M Urea was added to the purified IB pellet and kept on stirring for overnight at room temperature. The solubilized IB was centrifuged at 10,000 rpm at 4°C for 40 minutes to remove any insoluble material or debris. The clear supernatant was used for affinity chromatography for the purification of rD2-DIII.

Purification and characterization of r D2 DIII protein

The recombinant protein was purified based on its C-terminal 6X His tag by affinity chromatography using a Ni-NTA resin (Qiagen, Germany). The column was packed with 5 ml of Ni-NTA resin and washed thoroughly with deionized water to remove traces of ethanol and equilibrated with 50 ml of Ni-NTA buffer, (pH 7.5). The clear supernatant was loaded to the column and the flow through was reloaded 4-5 times. The column was washed by passing 100 ml of Ni-NTA buffer (pH 6.0). The protein was eluted with 20 ml of Ni-NTA buffer (pH 4.2). The elutes were then analysed by SDS-PAGE using 15% polyacrylamide gel. The fractions of purified protein were pooled and concentrated using Amicon ultrafiltration cell with 10 kDa cut off membrane. The final protein concentration was estimated by BCA kit (Pierce).

Ni-NTA purified protein was transferred electrophoretically to the nitrocellulose membrane (Millipore, USA). After overnight blocking with 2 % bovine serum albumin (BSA) in PBS at 4°C, the nitrocellulose membrane was incubated with anti-dengue antibody (1:500) in PBS for one hour. The membrane was washed three times with PBS containing 0.1% Tween-20 (PBS-T) and incubated with anti-mouse IgG horseradish peroxidase conjugate (1: 6000) for 1 h at RT. The membrane was washed as above and the protein bands were visualized by incubating with diaminobenzidine substrate for 5

minutes at RT. Further the reactivity of this protein was also checked by dipstick ELISA (Tripathi *et al.*, 2007).

rD2 DIII protein as a diagnostic reagent

A panel of 40 defined dengue infected serum samples from various outbreaks (Parida *et al.*, 2002; Dash *et al.*, 2006) was included in this study. These 40 serum samples were first tested by IgM capture ELISA test kit (Pan-Bio, Australia) for detection of IgM antibodies of dengue virus according to manufacturer's protocol. Further, all these samples were also tested with rD2 DIII protein by an in-house developed indirect ELISA. The purified rD2 DIII protein was diluted to 6 µg/ml in 0.1M carbonate-bicarbonate buffer, (pH 9.6) and used for coating 96-well micro titer plates (100 µl/well) at 37°C for 1 h. The coated wells were washed once with PBS and blocked with 2% BSA in PBS overnight at 4°C. The wells were washed again with PBS+0.1%T and then incubated for 1 h at 37 °C with 100 µl dengue patient sera (1:100 dilution in 1X PBS+0.05% Tween-20) for detection of IgM antibodies. Wells were again washed as above and incubated with anti-human IgM-HRPO conjugate (1:2000 in PBS + 2% BSA). The wells were washed and incubated with 100 µl OPD substrate for 15 minutes at 37 °C. The enzyme reaction was terminated with 100 µl of 1M H₂SO₄ and the absorbance recorded at 490 nm. Apart from above, we have also tested this antigen with JE infected and healthy serum samples for checking cross-reactivity.

Results

Plasmid construction for producing rD2 DIII protein

A 384 bp DNA fragment encoding rD2 DIII protein was amplified by RT-PCR using D2 DIII specific primers and electrophoresed in 1.5% agarose gel. This amplified product was cloned into the pQE-30 UA expression vector and the recombinants were screened by plasmid PCR using the same set of primers. This vector was generated by fusing domain

III-encoding sequences in-frame with the translation initiation codon and the 6x His tag of pQE30-UA, under the control of IPTG-inducible phage T5 promoter. Figure -1. This construct is predicted to encode a 155 amino acid recombinant protein with a molecular weight of ~17 kDa.

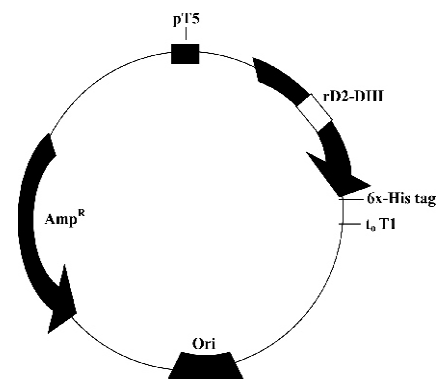


Figure 1. Map of the pQE-30UA. The rD2-DIII gene was inserted into the multiple cloning sites (MCS) that have an overhang on each 3' end of pQE30UA (this allows the direct ligation of PCR product which has A overhang at 3' end) in-frame with vector provided initiator codon and the hexa-histidine tag (6x-His tag). Other abbreviations are as follows: pT5, phage T5 promoter; T1, transcriptional terminator; Ori and AmpR, plasmid replication origin and ampicillin resistance marker, respectively.

Expression of the rD2 DIII protein

This construct was transformed into *E. coli* M15 (pREP4) host cells, which provided phage T5 RNA polymerase for expression of heterologous genes. Small scale cultures of the positive clones, which was selected on the basis of PCR, were subjected to IPTG induction to identify clones capable of expressing the predicted ~17 kDa recombinant protein. When the polypeptide profiles of un-induced and IPTG-induced *E. coli* cultures was compared, it was evident that IPTG induction results in the expression of 17 kDa protein. Out of the *E. coli* M15 (pREP4) clones identified, one was selected which expressed the recombinant protein maximally. Plasmid DNA was isolated and verified to be correct by sequencing (data not shown). This clone was used in further experiments. Expression was optimized using different concentrations of IPTG (0.2-1.5 mM) and induction duration (1-4 h). IPTG (1mM) with 4 h induction time gave optimum

protein yield (data not shown). Therefore, in all subsequent experiments induction was carried out with 1mM IPTG for 4 h.

Purification and characterization of recombinant protein

To devise an appropriate purification strategy, we examined the relative distribution of the expressed recombinant protein in the soluble and insoluble fractions. For this purpose, we checked the presence of the protein in supernatant as well as in pellet after sonication and centrifugation. When the cells were lysed under native conditions, virtually all the expressed recombinant protein was associated with the pellet fraction, demonstrating that the rD2 DIII protein is

insoluble. As the rD2 DIII protein is predominantly found in the insoluble fraction, we attempted to purify the rD2 DIII protein under denaturing conditions. IBs were harvested and purified from the induced and lysed cell mass and solubilized in buffers containing 8M Urea and purified by Ni-NTA chromatography under denaturing conditions. The protein was eluted with the pH gradient. SDS-PAGE analysis of gel showed it to be ~95% pure (Fig. 2 a). The yield of purified rD2 DIII protein was 12.5 mg/l in shake flask culture (Table 1).

After SDS-PAGE, the proteins were electrophoretically transferred onto the nitrocellulose membrane. Western blot analysis using anti-dengue antibody detected rD2 DIII protein at 17 kDa (Fig. 2b). These results showed that rD2 DIII protein

recognized anti-dengue antibody specifically. Further, this protein was also tested with dengue positive and negative human serum samples by dipstick ELISA (Fig. 2c).

In-house ELISA for detection of anti-dengue IgM antibodies

rD2 DIII protein was used in indirect ELISA for detection of anti-dengue antibodies in patient serum samples. We have tested 40 serum samples out of which 19 serum samples were found to be positive and 21 negative for IgM antibodies by indirect ELISA. The specificity of the indirect ELISA in comparison with Pan-Bio capture ELISA was found 100 %. The overall accordance of the results of the indirect ELISA with Pan-bio IgM capture ELISA was 95 %. Out of 40 serum samples, 21 samples were detected to be IgM positive by PanBio Dengue IgM capture ELISA. The indirect ELISA using rD2 DIII antigen picked up 19 positive samples but missed two samples, indicating the sensitivity to be 90.5%. The OD at 490 nm for these two samples were 0.224 and 0.234 respectively (Table 2). The cut-off value of (0.250) was determined based upon the average OD values of triplicate of negative sample. Further, 10 serum samples from patients with confirmed JE infection (Parida *et al.*, 2006) and 10 healthy serum samples were also tested to establish the specificity of the rD2 DIII antigen. None of these sera samples were found positive with rD2 DIII

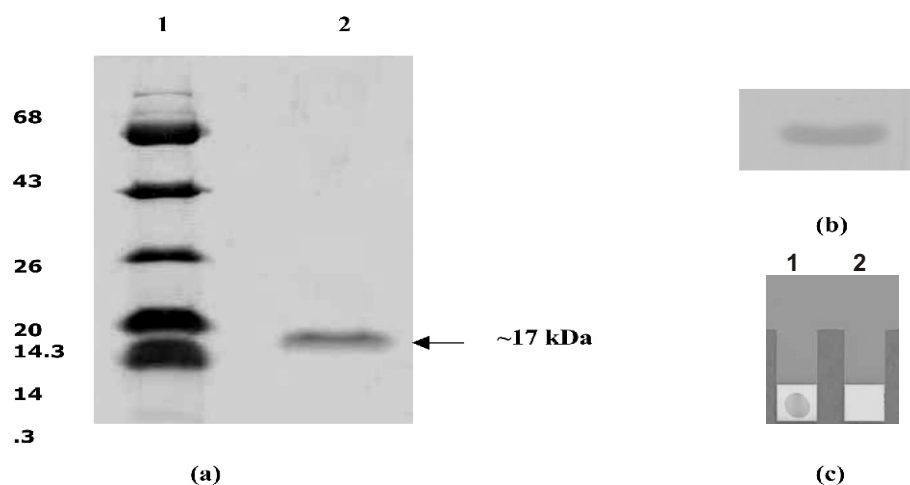


Figure 2. (a) SDS-PAGE analysis of rD2 DIII protein after Ni-NTA affinity purification. Lane 1, Molecular Weight Marker (kDa); lane 2, Purified rD2 DIII protein. (b) Western blot analysis of the purified rD2 DIII protein with IgG positive human serum sample. (c) Result of Dipstick ELISA with rD2 DIII protein. Lane 1, IgG positive and lane 2, negative serum samples.

Table 1. Summary of rD2 DIII purification

Purification step	Total protein (mg/l) a	Purity (%)b	Recovery (%)c
Crude lysate	724	0	100
Affinity chromatography	12	95	40

a. Protein content was determined by the BCA method.

b. Purity was assessed by SDS-PAGE analysis.

c. The amount of rD2 DIII protein in the crude lysate (which was estimated to be ~30 mg by densitometric analysis) this was designated as 100%.

Table 2: Range of OD₄₉₀ values in the in-house Indirect ELISA

Range of OD value	No. of samples
0.000-0.149	15
0.150-0.249	6
0.250-0.399	3
0.400-0.549	5
0.550-0.700	9
≥ 0.7	2
Total	40

ELISA OD₄₉₀ values of ≥ 0.25 were considered positive at a serum dilution of 1:100.

prote in thereby establishing the specificity of this protein for dengue diagnosis.

Discussion

The widespread use of whole virus preparations as antigen is associated with biohazards. Since the viruses are usually produced in tissue culture or suckling mouse brain, the high cost of production makes these kits unaffordable, particularly in the developing countries. The whole virus antigens invariably pick up antibodies against other *Flaviviruses* such as West Nile, Yellow fever and Dengue viruses, leading to ambiguity in diagnosis. Thus, it is apparent that an ideal antigen for use in a dengue diagnostic test must not only be free from the virus associated biohazard risk, but also be inexpensive and possess a high degree of specificity to facilitate unequivocal diagnosis of dengue infection. The present work is based on the premise that the use of a recombinant antigen, designed to be dengue-specific and expressed in an *E. coli* could effectively address the issues of biohazard risk, cost, and specificity. In an effort to develop dengue diagnostics indigenously, nitrocellulose (NC) membrane based indirect dipstick ELISA using purified viral native protein antigen has been successfully evaluated for detection of anti-dengue virus antibody in patient's serum (Parida *et al.*, 2001 and 2002; Dash *et al.*, 2005-06).

In this study an effort has been made to produce recombinant protein that could be of diagnostic use and be an attractive alternative of native viral preparations. E protein is the major structural component and the most immunogenic of all the dengue viral proteins, eliciting the first and longest-lasting antibodies. Immunodominant epitopes on the E protein are well-documented (AnandaRao *et al.*, 2005). We focused on domain-III of envelope protein of the dengue-2 virus as the highly immunodominant antigen known to primarily elicit the neutralizing antibodies (Jaiswal *et al.*, 2004). We designed the primers for the targeted protein for expression in *E. coli*, encoding the domain III of E protein of dengue virus. This gene designated as rD2 DIII is predicted to encode a ~17 kDa recombinant protein containing 6X-histidine tags at the C-terminal end. During characterization of this protein we found a specific reactivity towards anti-dengue antibodies.

The indirect ELISA is simple, rapid and easy-to-use. In comparison with Panbio kit used in this study, indirect ELISA using rD2-DIII protein showed > 90% accordance. The sensitivity and the specificity of the indirect ELISA were found to be 90.5 % and 100 % respectively. The common problem with dengue serological assays lies in detection of circulating cross-reactive antibodies against other members of *Flaviviruses*. This cross reactivity could be found to be significantly reduced, while identifying dengue infection, with the indirect ELISA employing pre-coated rD2 DIII protein. In this study, when the assay was carried out with a panel of defined sera samples from 10 healthy individuals and 10 JE patient sera from Gorakhpur Outbreak 2005 (Parida *et al.*, 2006), none of them were found to be positive by indirect ELISA. Indirect ELISA is also cheaper than other assays (using native viral antigen) owing to its use of the recombinant antigen expressed in *E. coli*. This demonstrated that the rD2 DIII protein expressed in the present study has a potential to be used as a diagnostic reagent as it overcomes the drawbacks associated with the use of whole virus.

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