Expression and purification of a full-length recombinant NS1 protein from a dengue 2 serotype viral isolate

✍ Gilda Lemos, Isabel Guillén, Julio R Fernández, Tamara Díaz, Amanda B Colarte, María E Fernández de Cossío

Centro de Ingeniería Genética y Biotecnología, CIGB
Ave. 31 e/ 158 y 190, Cubanacán, Playa, CP 11600, La Habana, Cuba
✍ gilda.lemos@cigb.edu.cu

RESEARCH

ABSTRACT

Dengue is an emerging disease that poses a threat to one-third of the global human population and produces over 50 million reported cases in tropical and sub-tropical regions every year. An accurate diagnosis of dengue infection is essential for timely management of the disease. NS1 is a 46- to 50-kilodalton highly conserved dengue virus glycoprotein that can be detected during the febrile phase of dengue virus (DENV) infection in both primary and secondary cases. This protein is a specific marker of DENV infection, and a sensitive test for NS1 would, if used together with IgM detection, provide an excellent diagnostic approach. Although the NS1 protein can be isolated from mammalian cell tissue cultures infected with DENV, this procedure is unsafe, laborious, and expensive and has very low yields, making it unsuitable for a large amount of antigen production. In this work, and with the objective of carrying out immunization experiments in mice, we cloned the full-length NS1 region from DENV serotype 2 (rNS1) in the vector pET28a with a 6xHis tag at the N-terminus. The protein was expressed in the *Escherichia coli* strain Rosetta as inclusion bodies, at the expected size of approximately 46 kDa, and further purified by metal-chelating affinity chromatography (IMAC) under denaturing conditions. Human sera from dengue positive cases showed reactivity to the recombinant NS1 protein by ELISA and Western blot. The unfolded rNS1 was directly used as immunogen. The polyclonal antibodies elicited in immunized mice with the recombinant antigen recognized the natural NS1 antigen from serotype 1 (sNS1).

Keywords: dengue virus, NS1, diagnosis, recombinant protein

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RESUMEN

Expresión y purificación de la proteína completa NS1 del virus dengue serotipo 2 a partir de un aislamiento. El diagnóstico certero de la infección por el virus dengue (DENV) es esencial para su tratamiento oportuno. La proteína NS1 (46-50 kDa) es una glicoproteína del DENV de secuencia altamente conservada en los cuatro serotipos del virus, que se puede detectar durante la fase febril del dengue en pacientes infectados por primera o segunda vez, como marcador específico de la infección. Por ello, un test basado en la proteína NS1, pudiera facilitar su diagnóstico cuando se emplea junto con la detección de la inmunoglobulina M (IgM) contra el DENV. Entre las principales dificultades para su obtención está el aislamiento de la proteína NS1 de cultivos celulares de mamíferos, lo cual no es seguro, es laborioso y caro, y con bajos rendimientos que impiden su escalado. En este trabajo se clonó la secuencia completa de la proteína NS1 (rNS1) del DENV serotipo 2 en el vector pET28a, fusionado con una cola de histidina 6xHis en el extremo N-terminal. Esta proteína se obtuvo de forma recombinante en *Escherichia coli*, cepa Rosetta, como cuerpos de inclusión, con aproximadamente 46 kDa, y se purificó por cromatografía de afinidad de quelatos metálicos (IMAC) en condiciones desnaturalizantes. Sueros humanos de pacientes positivos al dengue mostraron reactividad contra la rNS1 en ensayos de ELISA y Western blot. La proteína rNS1 desnaturalizada se administró directamente como inmunógeno en ratones Balb/C, cuya respuesta de anticuerpos policlonales detectó a la proteína NS1 natural del DENV serotipo 1 en ensayos de inmunoblot.

Palabras clave: virus dengue, NS1, diagnóstico, proteína recombinante

Introduction

Dengue is an arthropod-borne viral disease and has been a major cause of morbidity and mortality in recent decades. Dengue virus (DENV) is considered one of the most important emerging viruses, posing a threat to one-third of the global human population, with over 50 millions of cases reported in tropical and sub-tropical regions every year [1]. Most infections are asymptomatic, and symptomatic cases exhibit a wide range of clinical manifestations, being the most common outcome an acute febrile illness similar to influenza (dengue fever, DF). However, in a minority of cases, this progresses to spontaneous hemorrhaging (dengue hemorrhagic fever, DHF) and, most seriously, to dengue shock syndrome (DSS), characterized by circulatory failure. There are perhaps 500 000 cases of DHF/DSS each year, with case-fatality rates as high as 5 % depending on the availability of treatment [2].

Dengue is caused by one to four dengue serotypes (DENV type 1 through 4), of the genus flavivirus (family Flaviviridae).The viral agent is a single-stranded, positive-sense, RNA virus with a genome of approximately 11 kb.

Co- and posttranslational processing gives rise to three structural and seven nonstructural proteins: C, prM, E, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. Antigenic diversity of the DENV is important, since the lack of long-term cross-immunity among the four virus types allows for multiple sequential infections [3].

✍ Corresponding author

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NS1 is a 46- to 50-kilodalton highly conserved glycoprotein that is expressed in both membrane-associated (mNS1) and secreted (sNS1) forms [4, 5] and possesses both group-specific and type-specific determinants [6, 7]. NS1 is an atypical viral glycoprotein because it does not form part of the virion structure but is expressed on the surface of infected cells. While the function of NS1 is yet to be fully defined, preliminary evidence has shown it to be involved in viral RNA replication [8, 9].

More effective and rapid diagnosis can contribute to the control of dengue and DHF through more accurate public health notification. Many reports suggested the use of NS1 detection for early diagnosis of dengue infection in primary and secondary cases, period in which dengue antibodies are still undetectable [10-12], since high concentrations of the NS1 protein varying from 0.04 to 2 µg/mL in acute-phase serum samples to only 0.04 µg/mL or even less in convalescent phase serum [10] were found in blood samples of patients obtained during the early acute phase of both primary and secondary DENV infections and for up to 9 days after the onset of symptoms. Levels of NS1 antigen remain detectable even in some cases when viral RNA is negative by reverse transcriptase-PCR.

Early detection of NS1 antigen during the febrile stage of the disease combined with IgM detection should expand the time span during which a rapid test could detect a dengue infection and make it a sensitive diagnostic approach.

Methods for isolating the natural NS1 antigen from mammalian cells infected with DENV yield only low amounts of protein, since secreted antigen concentration ranges from 5 to 10 µg/mL culture supernatant [13]. Therefore, using recombinant NS1 protein for immunizing mice for hybridoma production, or as a diagnostic antigen for dengue viral infection, is a more suitable approach. Several works have reported the successful use of heterologous expression systems such as baculovirus [14] and *Pichia pastoris* [15] for the expression of the NS1. The *Escherichia coli* bacterial expression system has been also widely exploited for NS1 expression, although it always involves refolding procedures, facilitating disulfide bond formation and thus renaturation of the protein [8, 16-19].

In this work, we cloned and expressed the full length NS1 region from serotype 2 in *E. coli* and the denatured antigen was directly used as immunogen. The antibodies obtained in immunized mice will recognize only linear epitopes in the natural secreted antigen, but several reports have shown the immune-dominancy and cross-reactivity of linear epitopes present in NS1 protein from all four DENV serotypes [7, 13, 20, 21]. The polyclonal antiserum from animals immunized with the denatured recombinant NS1 protein from serotype 2 (rNS1) was found to specifically recognize the natural NS1 antigen from serotype 1, thus demonstrating the possibility of generating antibodies that recognize epitopes in the native NS1 antigen from a different serotype using the unfolded antigen.

Materials and methods

Human serum samples

A panel of sera from 11 dengue patients (5 samples from the acute disease stage and 6 samples from convalescent-phase of the disease) and 14 healthy human sera were used in the study. Dengue positive serum samples used in this study were confirmed from dengue infected patients collected during an epidemic DENV-4 outbreak in Havana city in 2006. DENV infections were defined as febrile illness associated with the detection of virus specific IgM (UMELISA® IgG and IgM antibody detection kits, TecnoSuma®, Havana, Cuba). Serum samples were collected between days 3 and 30 after the onset of symptoms. Convalescent-phase sera refer to specimens collected during days 7 to 30 days since onset of fever. Healthy human sera sample refers to sera collected from healthy blood donors.

Viral RNA isolation and purification from Dengue Virus Mammalian Cell Culture

Supernatant from Vero cells (10 mL) infected with 10^6 pfu/mL of SB8553 DENV-2 viral strain (kindly provided by Dr MJ Cardosa, University Sarawak, Malaysia) was harvested 144 hh post-inoculation without media changing. Viral particles were concentrated by centrifugation at 20 000 × g for 1 h at 4 °C after clarification using a solution of 4 % PEG8000 plus 0.5 M NaCl for 4 hours at 4 °C**.** RNA was purified from precipitated viral particles using the Ambion Tri-Reagent procedure for suspension cells. Briefly, the pellet was resuspended in 5 mL of Tri-Reagent (Sigma-Aldrich, St. Louis, USA) by vigorous vortex mixing and pipetting. After 5-min incubation at 20 °C, each sample was transferred to a 15-mL polypropylene centrifuge tube and 1.2 mL of chloroform was added. Samples were vigorously vortexed for 30 s, incubated at room temperature for 5 min and centrifuged at 12 000 × *g* for 5 min. The top aqueous layer, containing the RNA, was precipitated by adding 0.5 volumes (approximately 2.5 mL) of isopropanol for 5 min at 20 °C, followed by centrifuging at 5000 × *g* for 5 min. The resulting white pellets were washed with cold 75 % ethanol, and each pellet was then resuspended in 300 µL of RNase-free water.

Cloning of dengue NS1 full-length viral protein

cDNA was obtained from 2 µg of total RNA by using an RT-PCR Kit from Promega M-MLV procedure (Part# 9PIM170, USA). For PCR procedure, the forward primer 5´-GC**GGATCC**ATGAATTCAC GCAG-CACCTC-3´ and the reverse 5´-GCCTC**GAGCT** G GCTGTGACCAAGGAGT-3´, with *Bam*H I and *Xho* I sites included (bold letters) were used. The PCR-amplified region was inserted in the pGEM®-T Easy Vector (Promega, USA) and the *Bam*H I-*Xho* I-NS1 region was further inserted in the pET28a (+) expression vector (Novagen, Darmstadt, Germany), generating the recombinant plasmid pET28a-NS1, which was transformed subsequently in chemically competent *E. coli* DH5α cells. Plasmid DNA samples from recombinant bacterial colonies were analyzed by digestion with *Bam*H I and *Xho* I, PCR and DNA sequencing (Macrogen, Korea) coupled to BLAST (Basic Local Alignment Search Tool, NCBI), performed using the M13F-pUC/SP6 and T7 promoter/T7 terminator primers for vectors pGEM®-T Easy Vector and pET28a (+), respectively. The expressed protein was predicted to have an isotopically averaged molecular weight of approximately 46.4 kDa,

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corresponding to 380 amino acids of the NS1 protein and 34 amino acids encoded by the expression vector, including the N-terminal 6xHis-tag.

Expression and purification of the rNS1 antigen

Expression experiments of the recombinant protein were performed by the induction of the pET System *E. coli* host Strains: BL21 (DE3), Tuner (DE3) and Rosetta (DE3), cultured in 300 mL LB broth (containing 50 μg/mL kanamycin) in a 1000 mL conical flask and cultured at 37 °C in a shaker set at 200 rpm and induced by adding IPTG to a final concentration of 1 mM at 0.5-1.0 optical density (O.D.) at 600 nm. Cultures were harvested 3-4 hours after induction and *E. coli* cells were obtained by centrifugation at 3 000 × *g* for 30 min at 4 °C. After discarding the supernatant, 1 g of wet biomass was resuspended in 10 mL of TE 1× (10 mM Tris-HCl pH 8.0 and 5 mM EDTA) and disrupted by three passes in French press at a pressure of 100 bars at 4 °C. The obtained lysate was centrifuged at 20 000 × *g* for 20 min at 4 °C. Insoluble proteins were solubilized in 10 mL of Buffer A: 10 mM Tris-HCl, 100 mM NaH_2PO_4, 10 mM β-mercaptoethanol and 8 M urea, for 1 h at 4 °C, obtaining the soluble proteins fractions by centrifugation at 20 000 × *g* for 20 min at 4 °C and NS1 6xHis-tagged protein was further purified by affinity chromatography using 3 mL of Ni^{2+}-nitrilotriacetic acid (Ni-NTA) resin (Qiagen, Germany). Washed and elution steps were performed using 10 and 250 mM Imidazole in buffer A. Protein concentration was determined by the bicinchoninic acid assay (BCA, Pierce/ThermoFisher, Rockford, USA) and the purified antigen aliquots were stored at -20 °C until use. Protein expression level and purity were assessed by densitometric analysis of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of monoclonal antibody 15F3-1

Ascites fluid containing 15F3-1, an anti-NS1 Mab (ATCC HB-47), was extracted from mice and diluted 1:4 in phosphate buffer saline (PBS), purifying the antibody by chromatography on a Protein A-Sepharose column. Purified 15F3-1 Mab was further coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham / GE Healthcare, USA), according to the manufacturer's procedure.

Purification of NS1 natural antigen of dengue serotype 1 from Vero cell culture supernatant

The natural NS1 antigen from dengue serotype 1 was purified by affinity chromatography on 15F3-1-Sepharose using a method described by Young *et al*. [10]. Briefly, 350 mL of supernatant from Vero cells infected previously with 10^6 pfu/mL of DENV-1 West Pac 74 (NIBSC) and harvested after a period of 144 hours post-inoculation without changing the medium were clarified as described above and passed through 15F3-1-Sepharose, equilibrated with TNE buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl and 5 mM EDTA). After washing the column with TNE buffer, sNS1 was eluted in TNE containing 40 mM diethylamine. Protein concentration was determined by BCA (Pierce/ThermoFisher) and purified antigen aliquots were stored at -20 °C.

Mouse immunizations

Balb/c (Bc-H-2d) female mice (aged 6-8 weeks), 16-18 g of weight, purchased from Cenpalab (Havana, Cuba) were inoculated using two different procedures. Group 1 (5 animals) was inoculated subcutaneously using 100 μL of the immunogenic preparation (20 μg of rNS1 per dose) using Freund's complete adjuvant (Sigma-Aldrich, USA) in the first dose and incomplete adjuvant in subsequent doses. For Group 2 (5 animals), the first two doses were performed as for Group 1 but subsequently the animals were immunized intraperitoneally (i.p.) with 200 μL of the immunogen preparation in PBS. Both groups were immunized at 15-day intervals up to a total of 5 immunizations.

ELISA procedures

Costar 3591 plates were coated with 100 μL of 0.25 μg per well of rNS1 or 2 μg per well of natural DENV-1 sNS1 antigen in Coating Buffer (0.1 M carbonate/bicarbonate buffer pH 9.6). The plates were incubated for 1 hour at 37 °C. After washing three times with distilled water and 0.05 % Tween-20, the coated plates were blocked with 200 μL per well of blocking solution (PBS, 5 % skim milk powder, 0.05 % Tween-20) for one hour at 37 °C. Blocking solution was discarded by tapping the plate and 100 μL of test sample, at desired dilution in PBS, 0.5 % skim milk powder, 0.05 % Tween-20, were added per well and incubated for 1 hour at 37 °C. Bound specific antiserum was detected using 100 μL per well of a secondary antibody (1/10000 dilution of mouse (Fc specific) goat peroxidase-conjugate (Cat. # A2554, Sigma-Aldrich, USA, for mouse samples) and anti-human IgG goat peroxidase-conjugated (Cat. # A0170, Sigma-Aldrich, USA) for human samples, diluted in PBS-T solution (2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 8.1 mM Na_2HPO_4 and 0.05 % Tween-20). After washing, the color reaction was developed using 5 mg of *o*-Phenylenediamine dihydrochloride (OPD, Cat #P6912, Sigma-Aldrich, USA) as chromogen and 5 μL of 30 % hydrogen peroxide as substrate (Caledon, Canada). The reaction was stopped with a solution of 2 M H_2SO_4. Absorbance (O.D.) was read at 492 nm using a UMELISA® reader (PR-521, Tecnosuma Internacional, Cuba). The cut-off O.D. for testing the seropositivity of each sample was defined as an adjusted O.D. $_{492\ nm}$ of the mean plus 2 standard deviations of the negative control sera. For mouse serum samples, a non-immune mouse serum was used as a negative control and a pool of sera from mice immunized with the recombinant antigen was used as a positive control. For human ELISA assays, a serum pool from healthy individual was used as a negative control and serum sample from a dengue convalescent confirmed by serum IgM assay was used as a positive control.

Immunoblot analysis

Purified rNS1 expressed in *E. coli* was analyzed for its reactivity to DENV-specific antibodies present in

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mouse and human sera by Western blot. Briefly, samples from cell extracts and purified 6xHis-rNS1 were separated by 12.5 % SDS-PAGE [22], and either stained with Coomassie brilliant blue R250 (Sigma-Aldrich) or electro-transferred using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, USA) to a nitrocellulose membrane (Amersham/GE Healthcare, USA) for 30 min in transfer buffer (48 mM Tris pH 9.0-9.4, 39 mM Glycine, 20 % methanol and 1.3 mM SDS). The membrane was blocked for 1 hour at 37 ºC with blocking buffer (PBS-T and 5 % skim milk powder, BDH, UK). Human serum samples were diluted 1:20 with sample buffer (PBS, 0.25 % non-fat skim milk powder and 0.05 % Tween-20) and incubated with the nitrocellulose membranes for one hour at 37 ºC. After incubation, the membranes were washed three times, 5 min each, with PBS and 0.05 % Tween-20 solution. After washing, the membrane was allowed to react with a 1:1000 dilution of an anti-Human IgG (Fc specific)-peroxidase antibody produced in goat (Cat. # A0170, Sigma-Aldrich, USA) for detecting human antibodies or an Anti-mouse (Fc specific) -peroxidase antibody at the same dilution (Sigma-Aldrich, USA), for detecting mouse antibodies, for 1 h at 37 ºC. After a washing step, immunoreactivity was detected using 3, 3'-Diaminobenzidine tetra hydrochloride hydrate (DAB, Cat. # D5637, Sigma-Aldrich, USA) in 10 mL PBS and 0.05 % (v/v) H_2O_2. The reaction was stopped by washing several times with distilled water.

Commercially available recombinant protein NS1 from serotype 2 ($N_{terminal}$-rNS1; Cat #. 00342-V, Virogen, Boston, MA) was used as a control protein in immunoblotting assays.

Statistical analysis

The statistical significance of immunological differences among human serum samples and the analysis of immunized mouse groups were performed by unpaired Student's t-test. A P value of less than 0.05 was considered statistically significant.

Results

Cloning, expression and purification of DENV-2 rNS1 protein

The NS1 full-length gene was cloned with the 6xHis tag at the N-terminus in vector pET28a (+). Blast analysis of the cloned fragment (1139 bp) showed that the sequence matched to 99 % the sequence available for NS1 from a serotype 2 Malaysian isolate at GenBank (GenBank accession number FN429892.1).

In the expression experiments carried out with different *E. coli* strains, the Tuner and the Rosetta strains exhibited the best results (Figure 1). The rNS1 protein was expressed at the expected size of approximately 46 kDa, co-migrating with an *E. coli* protein as visualized in SDS-PAGE. A Western blot probed with mouse anti-NS1 polyclonal antibody showed some smaller sized bands in both strains. Therefore, further expression experiments of the rNS1 protein were carried out with the Rosetta strain. The best yields of wet biomass and antigen expression were obtained inducing the culture with IPTG when O.D. $_{600\ nm}$ was 1.0 and harvesting 4 hours after induction (results not shown).

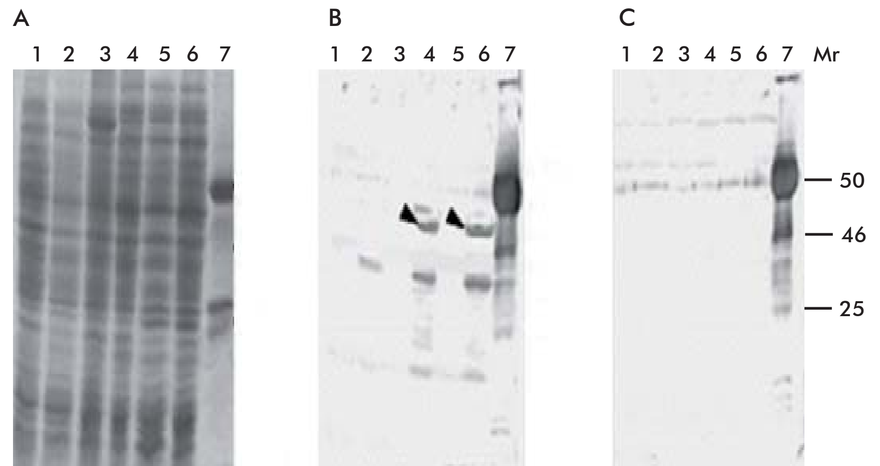

Figure 1. Expression analysis of the recombinant dengue virus NS1 (rNS1) protein in different *Escherichia coli* strains. A) SDS-PAGE. B) Western blot. Lanes 1 and 2: BL21 (DE3) *E. coli* strain. Lanes 3 and 4: Tuner (DE3) strain. Lanes 5 and 6: Rosetta (DE3) strain. Lane 7: monoclonal antibody used as molecular weight marker (kDa). Lanes 1, 3, 5: whole cell extracts from non-induced cultures. Lanes 2, 4 and 6: cultures induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG). B) Western blot probed with mouse anti-NS1 polyclonal antibody. C) Western blot probed with mouse polyclonal sera. Arrowheads indicate the rNS1 protein.

The rNS1 expression level in Rosetta *E. coli* strain was approximately 5 % by densitometric analysis of the SDS-PAGE.

The rNS1 protein, predominantly expressed in *E. coli* as inclusion bodies, was isolated from bacterial shake flask culture and purified by IMAC chromatography under denaturing conditions (Figure 2). During

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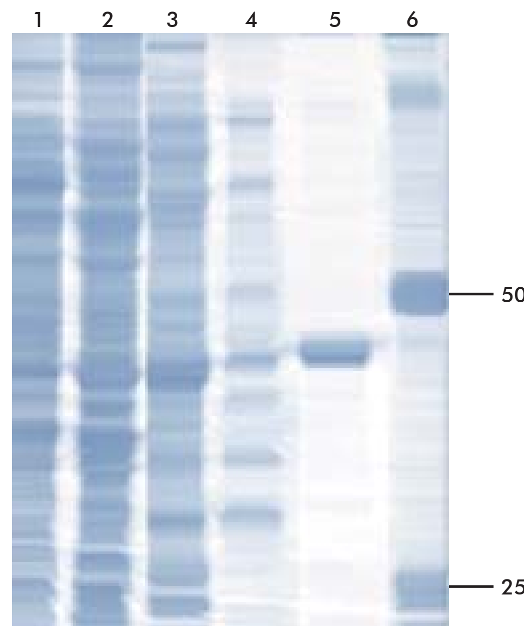

Figure 2. SDS-PAGE analysis of recombinant dengue virus NS1 protein purification steps by nickel affinity chromatography (IMAC) under denaturing conditions. Lane 1: non-induced *Escherichia coli* culture containing recombinant plasmid pET28a-NS1. Lane 2: *E. coli* induced for 4 hours with isopropyl-β-D-thiogalactoside (IPTG). Lane 3: flow-through. Lane 4: wash with 10 mM imidazole. Lane 5: elution with 250 mM imidazole. Lane 6: molecular weight marker (kDa).

the initial purification process by Ni-NTA columns, more than 20 % of the NS1 protein eluted during the 10 mM imidazole wash step. Then, for better protein recovery, 10 mM beta-mercaptoethanol was added to solubilization and chromatography purification buffers. IMAC purification under denaturing conditions yielded significant amount of more than 87 % pure rNS1 protein, with a single band as judged by SDS-PAGE (Figure 2). The rNS1 produced by *E. coli* Rosetta (DE3) was 13.1 mg/L. Purification steps showed a typical yield of 60-70 % of starting denatured inclusion bodies (Table).

Purification of DENV-1 sNS1 from Vero cell supernatant

Secreted DENV-1 NS1 protein was successfully purified from dengue-infected Vero cells culture supernatants by immunoaffinity chromatography, secreted NS1 usually reaches concentrations ranging from 5 to 10 µg/mL [23]. In this work, we obtained approximately 7 µg of antigen per milliliter of culture medium supernatant harvested at 144 h post-inoculation.

Immunological characterization of DENV-2 rNS1

The reactivity of human sera against rNS1 was established by ELISA and Western blot. The ELISA test results are shown in figure 3. From dengue-positive samples, only the six convalescent-stage cases exhibited reactivity against the antigen, a statistically significant difference compared to the reactivity exhibited by samples from the acute-stage of the disease ($p = 0.0002$) or from healthy donors ($p < 0.0001$). No significant difference in reactivity was observed between acute cases and healthy blood donors ($p = 0.38$). A serum sample from a 12 days convalescent severe DHF female case showed the highest reactivity. Western blot analysis of rNS1 (Figure 4) showed that human dengue convalescent patient sera bound the obtained rNS1 at least as well as it did with Virogen serotype 2 NS1, but in both cases, smaller sized bands were also recognized by positive human sera.

Mouse immunization results

Mice immunized with the recombinant antigen showed high reactivity against rNS1 in ELISA, but reactivity depended on inoculation route. After the fourth dose (Figure 5) Group 2 (mice inoculated by i.p. route) exhibited significantly higher levels of anti-rNS1 antibodies than mice immunized subcutaneously ($p = 0.0009$), prompting a switch to i.p. administration for both groups.

After the fifth and last dose, when comparing reactivity against the natural or recombinant antigens in ELISA, the immunized mice sera from Group 2 exhibited good reactivity against natural DENV-1 sNS1 (Figure 6), although their titers were significantly lower compared with those against DENV-2 rNS1 ($p < 0.0001$).

Discussion

Previous reports have described different heterologous protein expression systems, such as baculovirus, yeast expression systems and vaccinia virus, for producing secreted and glycosylated recombinant

Table. Purification steps` parameters of the recombinant dengue virus rNS1 protein from a 1-L culture of *Escherichia coli*

Purification step	Total proteins (mg)	rNS1 (mg)	Yield (%)
Insoluble fraction	327	16.3	100
Immobilized nickel affinity chromatography elution	15	13.1	66.9

DENV NS1 protein [24-29]. Several reports have also proposed achieving NS1 expression using bacterial cells [11, 16-19] since prokaryotic systems are easy to manipulate and express high levels of recombinant proteins. The inconvenience of these methods is that the expressed antigen lacks posttranslational

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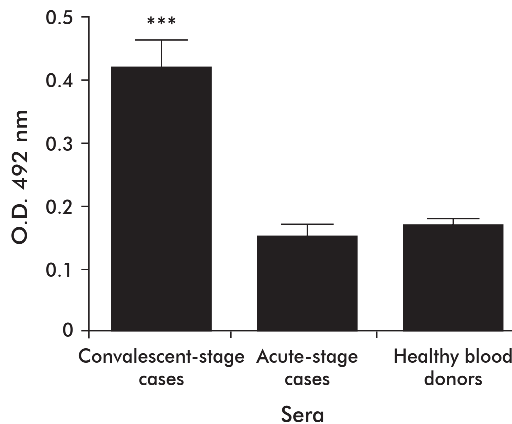

Figure 3. Comparison of human sera reactivity against rNS1 in ELISA. Human sera were diluted 1:5 in phosphate buffered saline containing 0.5 % skim milk powder and 0.05 % Tween-20. *** Very highly statistically significant differences of convalescent cases compared to acute-stage dengue cases ($p = 0.0002$) and healthy blood donors ($p < 0.0001$). No significant differences were observed in reactivity between acute dengue cases and healthy blood donors ($p = 0.38$). Error bars stand for the standard error of the mean (SEM).

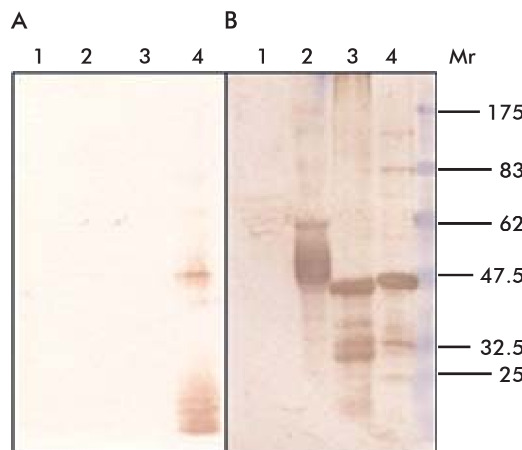

Figure 4. Western blot analysis of human sera reactivity against the dengue virus (DENV) serotype 1 NS1 native protein and DENV serotype 2 recombinant NS1 (rNS1). Human sera sample were diluted 1:20. A) Healthy human sera sample. B) Serum from a 12-days convalescent dengue hemorrhagic fever. Lane 1: Vero cell culture supernatant containing native DENV-1. Lane 2: affinity-purified NS1 from serotype 1. Lane 3: rNS1. Lane 4: Commercial Nt- rNS1 DENV2 positive protein control (Virogen). Mr: molecular weight marker (kDa).

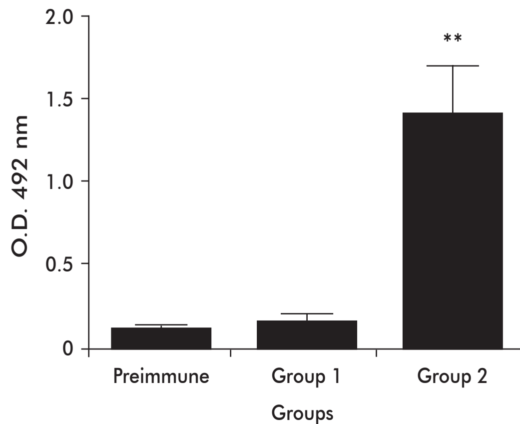

Figure 5. Comparison of Balb/C mouse sera reactivity against the recombinant NS1 protein from dengue virus serotype 2 (rNS1) after the fourth dose. Mouse sera were diluted 1:12 800 in phosphate buffered saline (PBS) containing 0.5 % skim milk powder and 0.05 % Tween-20. Group 1 (5 animals) included mice inoculated subcutaneously using 100 μL of the immunogenic preparation (20 μg of recombinant NS1 antigen per dose). Group 2 mice (5 animals) received the first two doses as performed for Group 1, but subsequently animals were immunized intraperitoneally with 200 μL of the immunogen prepared in PBS. Both groups were immunized at a 15-days interval. There were highly significant statistical differences (**) from preimmune mice ($p = 0.0016$) and Group 1 ($p = 0.0021$). No significant differences in reactivity were observed between preimmune mice and Group 1 animals ($p = 0.1325$). Error bars stand for the standard error of the mean (SEM).

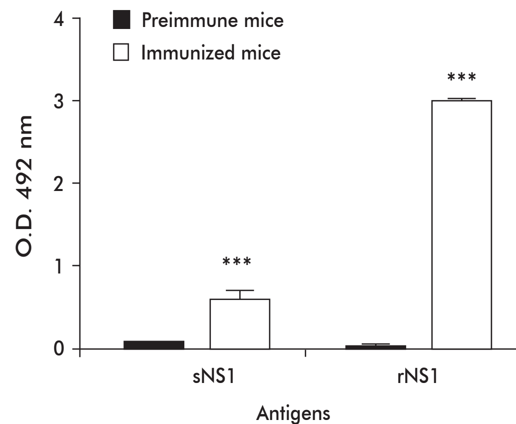

Figure 6. Evaluation of Group 2 sera reactivity by ELISA against the natural NS1 protein (sNS1) from dengue virus serotype 1 (DENV-1), or the recombinant NS1 from DENV-2 (rNS1). Mouse sera were diluted 1:100 in phosphate buffered saline containing 0.5 % skim milk powder and 0.05 % Tween-20. The immunized mouse sera exhibited good reactivity against sNS1 but mouse sera titers were significantly lower compared to those against rNS1 ($p < 0.0001$). *** Very highly statistically significant differences from preimmune mice ($p < 0.0001$). Error bars stand for the standard error of the mean (SEM).

modifications; therefore, refolding protocols must be used to produce rNS1 with a native-like protein conformation, preserving important conformation and antigenic determinants of the natural virus protein, not present in the denatured form of the antigen [14, 18, 30, 31]. However, these protocols are time consuming and sometimes very inefficient.

This study was aimed at obtaining a serotype 2 NS1 recombinant antigen useful for mouse immunizations with the objective of generating hyper-immune mouse sera against the viral antigen. In our work, the strategy was to use the denatured rNS1 protein as immunogen. Antibodies obtained with the denatured recombinant protein will recognize only linear epitopes in the natural secreted antigen, but it has been reported that linear epitopes, present in NS1 protein, are immune-dominant and cross-reactive to all four DENV serotypes [7, 13, 20, 21].

Expression experiments showed similar results using Rosetta and Turner *E. coli* strains (Figure 1) but additional smaller sized bands were observed in immunoblotting experiments, perhaps due to truncated NS1 protein or degradation. Although a moderate expression level (5 %) was obtained, we decided to continue using Rosetta strain, mainly because of the high percentages of rare codons for this host such as AGA, CCA and CAC in the cloned insert (11 %). This 'non-optimal gene' could reduce the efficiency of translation or even disengage the translational machinery. The Rosetta strain enhances expression of proteins having codons rarely used in *E. coli*; by supplying tRNAs for six rare codons: AUA, AGG, AGA, CUA, CCC, and GGA. In the case that higher yield of the protein devoid of degradation products or truncated protein is required, an additional purification step could be used, to discriminate degradation products by molecular size (*e.g.*, size exclusion chromatography), since the affinity process captures all the molecules bearing the histidine tag, and particularly small fragments based on their lower steric hindrance. Additionally, conditions more restrictive for degradation could be also implemented during sample rupture.

For better protein recovery during the purification process by Ni-NTA, 10 mM beta-mercaptoethanol was added to the solubilization and chromatography purification buffers. The viral protein sequence contains 12 cysteine residues and some folded structures could therefore be present even when using denaturants such as 8 M urea, hindering proper exposure of the 6xHis tag.

The purified recombinant antigen was recognized in Western blot analysis using dengue convalescent-phase sera, but results showed several smaller sized bands, as explained above, perhaps due to incomplete protein expression, degradation or perhaps anomalous electrophoretic behavior of the NS1 protein [30].

In the ELISA assay, 6 samples from convalescent-phase disease showed reactivity against the denatured recombinant antigen. The highest O.D. was obtained with a 12 days convalescent severe DHF female case. Some human dengue-infected patient samples exhibited no or poor reactivity against the recombinant antigen, but all of them were samples from the acute stage of the disease, in which antibody titers against the viral antigen might be still low.

The unfolded rNS1 antigen was used as immunogen and hyper-immune mice sera against the viral NS1 antigen were obtained. In this paper, it is shown that the antibodies elicited in immunized mice with the denatured DENV-2 rNS1 protein have good reactivity against the secreted NS1 protein from DENV-1.

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This antigen therefore could be useful for obtaining specific antibodies against the natural viral proteins; possibly to conserved regions of the four serotypes, for example amino acids residues 154-161 which are highly conserved among different DENV serotypes [32].

This approach could be useful for obtaining monoclonal antibodies that could recognize secreted NS1 present in the sera of infected dengue patients, enabling the development of an NS1 antigen capture test for an early and accurate detection of acute infection in suspected dengue cases.

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