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Polyclonal antibodies against properly folded Dengue virus NS1 protein expressed in *E. coli* enable sensitive and early dengue diagnosis

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The non-structural 1 (NS1) protein plays an important role in dengue diagnosis because it has been detected as a soluble serum antigen in both primary and secondary infections. The NS1 protein was expressed in *Escherichia coli* cells, and the efficiency of four different refolding protocols was tested. All of the protocols generated dimeric NS1 in a conformation similar to that of the protein expressed by eukaryotic cells. A polyclonal antibody produced from the properly folded *E. coli* recombinant NS1 (rNS1) protein proved to be a useful tool for the diagnosis of Dengue virus because it detected 100% of the Dengue virus 2 (DENV2) in infected patients' sera and 60% of the DENV IgM-positive sera not detected by commercial NS1-based diagnostic kits. These data suggest a high-efficiency method for correctly folding rNS1 that maintains its structural and immunogenic properties. In addition, a detection method using the polyclonal antibody against correctly folded rNS1 seemed to be more sensitive and efficient for NS1 detection in serum, highlighting its usefulness for developing a high-sensitivity diagnostic kit.

1. Introduction

The NS1 is a glycoprotein of approximately 45 kDa that is conserved among flaviviruses (Falgout et al., 1989; Rice et al., 1985; Zhao et al., 1987). NS1 is naturally found as a plasma membrane-associated, non-covalently linked homodimer (Winkler et al., 1988) and as a soluble multimer in the extracellular medium (Falconar and Young, 1991; Flamand et al., 1999). Data from the literature show that the NS1 protein exhibits complement-fixing activity (Smith and Wright, 1985), that may be used as a protective antigen (Schlesinger et al., 1987) and it seems to be involved in viral RNA replication (Mackenzie et al., 1996), although its function in dengue pathogenesis remains poorly elucidated.

The diagnosis of dengue diseases remains an open question because current methods have limitations, such as high costs and low detection accuracy. Different laboratory diagnostic techniques are available today, including the following: (i) virus isolation; (ii) viral RNA direct detection; and (iii) virus-specific antibody determination. Antibody determination has the advantages of lower costs and more widely available reagents, but it has the disadvantage of poor early detection of infection (Kao et al., 2001). Alcon et al. (2002) showed that the NS1 protein circulates in human serum mostly from days 1–6 after the onset of clinical symptoms, with the peak NS1 antigen detection occurring between days 3 and 5 in both primary and secondary infections. These properties make it an attractive marker for developing a diagnostic detection test for the beginning of symptoms. Currently, there are at least two commercially available kits for DENV NS1 detection: the Platelia™ dengue NS1 kit (Bio-Rad Laboratories, Marmes La Coquette, France) and the Pan-E dengue early ELISA kit (Panbio Diagnostics, Brisbane, Australia).

In the present study, the NS1 protein was expressed in *E. coli* bacterial cells and four different refolding protocols were tested to verify the refolding efficiency for producing polyclonal antibodies against NS1. The investigation of the oligomeric state of NS1 showed dimer formation in almost all of the methods used, consistent with the oligomeric state observed in the native-mammalian NS1 protein. Moreover, the polyclonal antibodies generated from the *E. coli* recombinant NS1 (rNS1) protein were shown to recognize

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positive serum from DENV2-infected patients. In addition, a panel of 43 sera was tested by a capture ELISA assay, resulting in a 100% detection of DENV2-positive serum and a 60% detection of DENV IgM-positive serum, which was not detected either by the PanBio or Platelia diagnostic kits. These results confirmed the utility of the polyclonal antibodies raised from rNS1 for developing a sensitive and low-cost serological test for rapid and early diagnosis of DENV infection, making it ideal for larger population screening.

2. Material and methods

2.1. Ethics statement

The rabbit and mouse antibody production was conducted in compliance with the ethical standards for animal experimentation of the Brazilian College of Animal Experimentation and was approved by the Institute's Animal Use Ethical Committee (CEUA-FIOCRUZ) (number L-067/08). The human samples were collected as part of an ongoing project in the Flavivirus Laboratory and the project was approved by the Ethics Committee on Human Research (CEP: 274/05).

2.2. Construction of the pETNS1his plasmid

The DENV2 *ns1* gene was amplified by PCR and cloned in the pET23b plasmid (Novagen, USA). The total RNA from C6/36 cells infected with DENV2 New Guinea C strain (NGC) was extracted with Trizol (Invitrogen, USA) according to the manufacturer's protocol and was used as a template for cDNA synthesis. The cDNA synthesis was performed with the antisense primer 5′-CATAAGCTTACAGAGGTTCCCCCATG-3′, which hybridizes with the nucleotide sequence 1422–1438 within the *ns3* gene. The cDNA was then used to amplify the *ns1* gene sequence, with sense 5′-GGGGGATATCGATAGTGGTTGCGTTG-3′ and antisense 5′-GGGGCTCGAGGGCTGTGACCAAG-3′ primers containing restriction sites for *Eco*RV and *Xho*I, respectively. The PCR was performed as follows: 2 min at 94 °C followed by 30 cycles of 1 min at 92 °C, 1 min at 55 °C, and 2 min at 72 °C, with an extension step of 5 min at 72 °C. The amplified product was electrophoresed on a 1% agarose gel, recovered with glass beads, purified using a GeneClean kit (Stratagene, USA), digested with *Eco*RV and *Xho*I, and ligated to a pET23b vector that was previously digested with these same enzymes. Chemically competent *E. coli* DH5α cells were transformed with the ligation products, and the recombinants were screened by restriction mapping and sequencing using an ABI PRISM dye terminator cycle sequencing core kit (Applied Biosystems, USA). The recombinant plasmid, named pETNS1his, was isolated by alkaline lysis and purified using a Qiagen Plasmid Mini Kit (Qiagen, Germany).

2.3. Expression of the NS1 protein and purification from inclusion bodies

Chemically competent *E. coli* cells BL21(λDE3)pLyS were transformed with 200 ng of the pETNS1his plasmid, and positive clones were selected in an LB-agar medium containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol at 37 °C overnight. A single positive colony was pre-inoculated in 10 mL of LB medium containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, and this culture was stirred at 200 rpm at 37 °C overnight. The overnight culture was diluted to 1:50 in 1 L of fresh antibiotic-containing medium and grown at 37 °C until an optical density ($O.D._{600\,nm}$) of approximately 0.7–0.8 was reached. The induction of protein expression was conducted with 0.5 mM IPTG followed by 3 h of expression. Then, the cells were harvested by centrifugation at 5000 × *g* for 20 min at 4 °C, and the total-cell lysate was prepared. The pellet was resuspended in 25 mL of Buffer A (Table 1) with 1 mM PMSF. Then, 5 mg/mL of lysozyme, 10 μg/mL of DNase A, and 5 mM of magnesium chloride were added, and the solution was incubated for 30 min at 4 °C with stirring. The total-cell lysate was sonicated using 10 cycles of 15 s on and 1 min off at 40% amplitude. The purification of inclusion bodies was performed by centrifugation at 37,200 × *g* for 20 min at 4 °C. Finally, the inclusion bodies were resuspended in Buffer B (Table 1) overnight at 4 °C.

2.4. NS1 purification by affinity chromatography

Because recombinant NS1 (rNS1) has a 6× histidine tag at its C-terminal end, a Ni^{2+}NTA affinity chromatography (HisTrap HP 5 mL; GE Healthcare, USA) was used to purify the protein from the inclusion bodies. After equilibrating the column with 5 column volumes (CV) of Buffer B, the protein was loaded into the column with a flow of 1 mL/min, followed by a 10-CV wash with Buffer B to remove all the nonspecific binding proteins. The elution was performed using a gradient of Buffers B and BI (Table 1) at a flowrate of 2 mL/min. All the collected samples were analyzed by 12% SDS-PAGE, and the tubes containing the rNS1 were pooled for further refolding.

2.5. rNS1 refolding

Four distinct methods were used to evaluate the refolding efficiency of rNS1. In each protocol, 5 mg of urea-denatured rNS1 were adjusted to 100 μg/mL prior to the refolding step. In Protocol I, the denatured rNS1 was subjected to a slow 20-fold dilution with Buffer ASB (Table 1), followed by a final dialysis in 50 mM Tris–HCl (pH 8), 100 mM NaCl, 1 mM DTT and 0.05% ASB-14. In Protocol II, the protein was subjected to a slow 20-fold dilution with Buffer ASB followed by dialysis in PBS (8.06 mM sodium phosphate, 1.94 mM potassium phosphate, 2.7 mM KCl, and 137 mM NaCl, pH 7.4). Protocol III consisted of dialysis of the protein in Buffer TA (Table 1) for three days with two buffer changes, followed by a final dialysis in PBS as has been previously described (Das et al., 2009). In Protocol IV, denatured rNS1 was subjected to a slow 20-fold dilution with Buffer TNA (Table 1) followed by final dialysis in PBS. All these experiments were performed at 4 °C. The refolded proteins were named $NS1_I$, $NS1_{II}$, $NS1_{III}$ and $NS1_{IV}$, corresponding to protocols I, II, III and IV, respectively. After refolding, the proteins were concentrated using a Centriprep YM-10 system (Millipore Corporation, USA) to a final concentration of approximately 1.5 mg/mL.

2.6. Fluorescence spectroscopy and circular dichroism

The fluorescence spectroscopy measurements were performed in a Varian Cary Eclipse spectrofluorometer (Australia). The excitation wavelength was fixed at 278 nm, and the emission spectrum was recorded from 300 to 420 nm. All the experiments were per-

Table 1
Buffer used for cell resuspension, inclusion bodies purification and protein refolding.

Buffer	Composition
Buffer A	50 mM Tris–HCl pH 8, 100 mM NaCl and 1 mM β-mercaptoethanol
Buffer B	50 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM β-mercaptoethanol and 8 M urea
Buffer BI	50 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM β-mercaptoethanol, 8 M urea and 0.5 M imidazole
Buffer ASB	50 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM β-mercaptoethanol and 0.1% (w/v) ASB-14
Buffer TA	50 mM Tris–HCl pH 8, 0.4 M L-arginine, 1.0 mM GSH (glutathione, reduced) and 0.1 mM GSSG (glutathione, oxidized)
Buffer TNA	50 mM Tris–HCl pH 8, 100 mM NaCl, 0.4 M L-arginine, 1.0 mM GSH (glutathione, reduced) and 0.1 mM GSSG (glutathione, oxidized)

formed at 25 °C. The circular dichroism (CD) experiments were conducted in a Chirascan Circular Dichroism Spectropolarimeter (Applied Photophysics, UK) at 20 °C using a quartz cuvette with a 0.01 cm path length. Spectra from three scans at a 30 nm/min speed were averaged, and the buffer baselines were subtracted from their respective sample spectra. The secondary structure was estimated from fitting the far-UV CD spectra using the CDSSTR method, which is available on the Dichroweb server (Whitmore and Wallace, 2004, 2008).

2.7. Anti-rNS1 antibody production

Five-week-old male Balb/c mice were intraperitoneally immunized with 20 μg of $rNS1_{II}$ per animal in complete Freund's adjuvant (Sigma, USA) followed by four injections of the protein in incomplete Freund's adjuvant (Sigma, USA), which were administered two weeks apart. Two weeks after the final immunization, the animals were euthanized and bled by total cardiac puncture. A three-month-old isogenic male rabbit was first immunized subcutaneously with 1 mg of $rNS1_{II}$ in complete Freund's adjuvant followed by two subcutaneous injections of rNS1 in incomplete Freund's adjuvant, which were administered two weeks apart. One week after the final immunization, approximately 20 mL of blood was obtained by ear bleeding. The serum samples were stored at −20 °C for subsequent analysis.

2.8. Western blot analysis

The rNS1 proteins refolded by the four different protocols were subjected to heat treatment for 10 min in the presence of 2 mM DTT or non-heat treatment in the absence of reducing agents prior to analysis (Costa et al., 2006b). To remove albumin and to enhance the detection of less-abundant proteins of the patient's serum, 30 μL of seven sera were treated with Cibacron blue resin (Sigma, USA) and were further concentrated by adding 5× volumes of cold acetone and incubated for 2 h at −20 °C, centrifuged at 13,400 × *g* for 20 min at 4 °C and then resuspended in half of the initial volume. All the samples were separated by electrophoresis on 12% SDS-PAGE and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, Sweden). The nitrocellulose membrane was blocked with 5% skim milk in TBST (0.1% Tween 20 in TBS [25 mM Tris–HCl pH 7.4, 3 mM KCl, and 140 mM NaCl], pH 7.6) for 2 h followed by overnight incubation with an anti-rNS1 polyclonal serum diluted to 1:10,000 (for the reactions with the refolded NS1, whole cell extracts and culture supernatant) or with purified anti-rNS1 polyclonal antibody diluted to 1:500 (for the reactions with the patient's serum). The membrane was then washed three times with TBST, incubated for 2 h with anti-mouse IgG conjugated to horseradish peroxidase (Promega, USA) diluted to 1:2500 in blocking solution, washed again, developed with a Picotag kit (Pierce, USA) and exposed to Kodak MXG/PLUS film. The anti-NS1 antibody was purified by incubating the rNS1-protein-containing nitrocellulose membrane with anti-NS1 mouse serum for 48 h, eluting the purified antibody with 0.2 M glycine and 1 mM EDTA at pH 4 and neutralizing with 10 mM Tris base.

2.9. Enzyme-linked immunosorbent assay (ELISA) for NS1 detection

The ELISA was performed with a recombinant NS1 glycoprotein expressed in insect cells (Hawaii Biotechnology Group), and the four NS1 proteins were refolded using the protocols described previously. The proteins were used as the solid-phase bound antigen, and the epitope specificity was evaluated using a serum sample collected from mice immunized with a DNA vaccine that elicited antibodies recognizing native DENV2 NS1 protein (Costa et al., 2006a). Wells of MaxiSorp plates (Nalge Nunc, Denmark) were coated with 0.4 μg of rNS1 protein expressed in *E. coli* or with 0.1 μg of the insect-cell NS1, both in 100 μL of PBS at 37 °C for 1 h. The plates were blocked overnight at 4 °C with 2% skim milk in PBST (0.05% Tween 20 in PBS), washed with PBST and incubated with serial dilutions of serum for 1 h at 37 °C. The plates were then washed again and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotechnology, USA) for 1 h at 37 °C. The reactions were visualized after 20 min at room temperature with ortho-phenylenediamine dihydrochloride (OPD) (Sigma, USA) and H_2O_2 as a substrate and 9 N H_2SO_4 as the stopping solution, followed by absorbance measurement at 490 nm. The titers were defined as the reciprocal of serum dilution, which gave an absorbance above that of the corresponding pre-immune serum.

2.10. Capture ELISA with patient's serum

The wells of MaxiSorp plates were coated with 100 μL of mice-produced anti-NS1 polyclonal antibody diluted to 1:10 in PBS at 37 °C for 1 h and blocked overnight at 4 °C with 2% skim milk in PBST. The wells were washed five times with PBST prior to incubation for 30 min at 37 °C with 10 μL of the patient's serum that had been diluted in PBS to a final volume of 50 μL. Afterwards, the wells were incubated with 100 μL of rabbit-produced anti-NS1 polyclonal antibody diluted to 1:20,000 in PBS for 1 h at the same temperature, followed by incubation with anti-rabbit IgG conjugated with horseradish peroxidase (Promega, USA) for 1 h at 37 °C, and it was then washed again with PBST. The reactions were visualized after 20 min at room temperature with OPD, H_2O_2 as the substrate and 9 N H_2SO_4 as the stopping solution followed by absorbance measurement at 490 nm. Negative controls were performed in the absence of the NS1 antigen, and the absorbance values were corrected using the mean value of the negative controls.

3. Results

3.1. Expression and purification of rNS1 from E. coli

The DENV2 *ns1* gene was cloned into the pET23b plasmid in the correct open reading frame. The transformed BL21(λDE3)pLyS bacterial cells expressed a high level of the recombinant NS1 protein as inclusion bodies. After solubilization with urea, the rNS1 was successfully purified using a HisTrap Ni^{2+}NTA affinity column, with a final protein yield of approximately 130 mg/L bacterial culture.

3.2. rNS1 refolding

Four different protocols were used to evaluate the best refolding strategy for rNS1. Protocol I used slow dilution of the urea-denatured rNS1 in ASB buffer composed of a zwitterionic detergent that stabilizes protein interactions at the critical point of refolding. About 90% of the rNS1 protein was recovered using this method (Table 2). In Protocol II, the rNS1 was also subjected to slow dilution with an ASB buffer, but the final dialysis was performed in PBS instead of a refolding buffer, resulting in an 82% protein refolding yield (Table 2). Both of these protocols were successfully used to obtain properly refolded rNS1 at high protein concentrations (above 1 mg/mL) (data not shown). Protocol III used dialysis in a TA buffer but was limited to a protein concentration below 100 μg/mL due to protein aggregation (Das et al., 2009). This protocol yielded a protein recovery of about 67% (Table 2). Protocol IV consisted of slow dilution of the urea-denatured rNS1 in TNA buffer; 82% of the refolded protein was obtained after the final dialysis in PBS (Table 2). The significant differences between Protocols III and IV include the presence of NaCl, which stabilizes the refolded protein,

Table 2
Protein recovery of the refolding protocols.

Protocol	rNS1	Refolding buffer	Refolding method	Initial mass (mg)	Final mass (mg)	Protein recovery (%)
I	$NS1_I$	Buffer ASB	Dilution	5	4.4	90
II	$NS1_{II}$	Buffer ASB with final dialysis in PBS	Dilution	5	4.0	82
III	$NS1_{III}$	Buffer TA with final dialysis in PBS	Dialysis	5	3.3	67
IV	$NS1_{IV}$	Buffer TNA with final dialysis in PBS	Dilution	5	4.0	82

and the choice of dilution for protocol IV, which allowed the use of larger amounts of protein without aggregation. Notably, the rNS1 from Protocol II was chosen to produce the anti-NS1 antibodies because this protocol is the most cost-effective and generated the highest protein-recovery yield.

3.3. Spectroscopic analysis of refolded rNS1

The four refolding protocols used for the urea-denatured rNS1 were analyzed by fluorescence spectroscopy of the Tryptophan (Trp) residue to verify whether the protein obtained in each protocol was properly folded. Trp is an excellent intrinsic fluorescence probe because its fluorescence spectrum shifts to higher wavelengths as it becomes more exposed to solvent (Melo et al., 2009). The DENV2 NS1 protein contains 14 tryptophan residues; as expected, a peak at approximately 340 nm was observed in the emission spectra of the refolded rNS1 obtained from all of the refolding protocols, and a peak at 355 nm was observed in the denatured rNS1 emission spectrum (Fig. 1A). These results confirm that the four protocols used in the present work were able to refold the rNS1 protein correctly because a peak of 340 nm is usually observed in proteins with an intact tertiary structure, whereas unfolded proteins present a maximum single peak at approximately 350 nm.

The refolded rNS1 proteins were also analyzed by CD to investigate their secondary structure. An analysis of their far-UV CD spectra showed two negative peaks, one at 208 nm and another subtle peak of lower intensity at 225 nm, indicating a mixture of secondary-structure components (Fig. 1B). According to the CDSSTR decomposition algorithm, the rNS1 protein obtained from all the refolding protocols was composed of 27% α-helices, 43% β-sheets and 30% random coils/turns.

3.4. Investigation of rNS1 oligomeric state

Prior to their analysis in SDS-PAGE and western blot, the refolded rNS1 proteins were subjected to either heat treatment in the presence of reducing agent or non-heat treatment in the absence of reducing agent to verify their oligomeric state. A band of 45 kDa corresponding to the rNS1 protein was visualized in all of the heated samples (Fig. 2A). Another band of approximately 38 kDa was also observed in those lanes, which may have corresponded to an NS1 degradation product (Fig. 2A). These bands were also confirmed to be DENV2 NS1 by mass spectrometry (data not shown). In addition, bands at 40 kDa, 80 kDa and above 200 kDa were clearly observed in the unheated $NS1_{II}$, $NS1_{III}$ and $NS1_{IV}$ samples; these three bands corresponded to the monomers, dimers and (probably) hexamers of rNS1, respectively (Fig. 2A). A difference in apparent molecular weight between the monomers of the heated and unheated samples was probably observed because the heated NS1 was not as compact as the unheated protein, resulting in altered migration through SDS-PAGE. A western blot analysis of these samples confirmed the SDS-PAGE bands (Fig. 2B). Interestingly, while no 80 kDa band was observed in the unheated $NS1_I$ sample in SDS-PAGE (Fig. 2A), the western blot showed a low-intensity band in that position (Fig. 2B). This finding confirmed the presence of dimeric rNS1 under this refolding condition, although a lower quantity was observed than in other refolding protocols.

To determine whether the rNS1 proteins assumed the correct native structure, an ELISA assay was performed using a polyclonal antibody that recognized the conformational epitopes of the NS1 protein produced in the NS1-DNA vaccinated mice (Costa et al., 2006b). The results indicated that all of the proteins were recognized by conformational-specific antibodies (Fig. 2C). Nevertheless, the antibody titers varied significantly depending on the refolding protocol, with values of 4500, 22,000, 28,000 and 130,000 for $NS1_I$, $NS1_{II}$, $NS1_{IV}$ and $NS1_{III}$, respectively (Fig. 2C). These titration results correlate well with the amount of dimeric rNS1 detected by the western blot analysis (Fig. 2B), in which the highest titer (130,000) was achieved by $NS1_{III}$, the refolding condition that also produced the greatest amount of dimeric rNS1 protein (Fig. 2C). The glycosylated NS1 protein produced in the insect cells was used as a positive control. Taken together, these results suggest that the NS1 obtained from the *E. coli* adopted a conformation similar to that of the NS1 obtained in

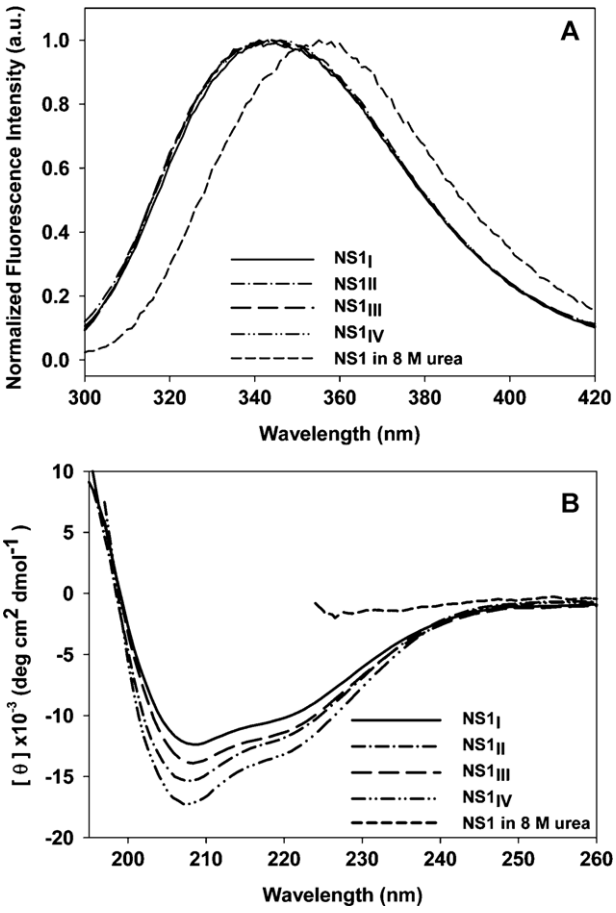

Fig. 1. Analysis of tertiary and secondary structures of refolded proteins. (A) All refolded proteins were analyzed using fluorescence spectroscopy in order to verify tertiary structure content. The excitation wavelength was fixed at 278 nm and the emission spectrum was recorded from 300 nm to 420 nm. (B) Circular dichroism spectra of all refolded NS1 proteins. Experiments were performed at 20 °C. Spectra were averaged from three scans at a 30 nm/min speed, and the buffer baselines were subtracted from their respective sample spectra.

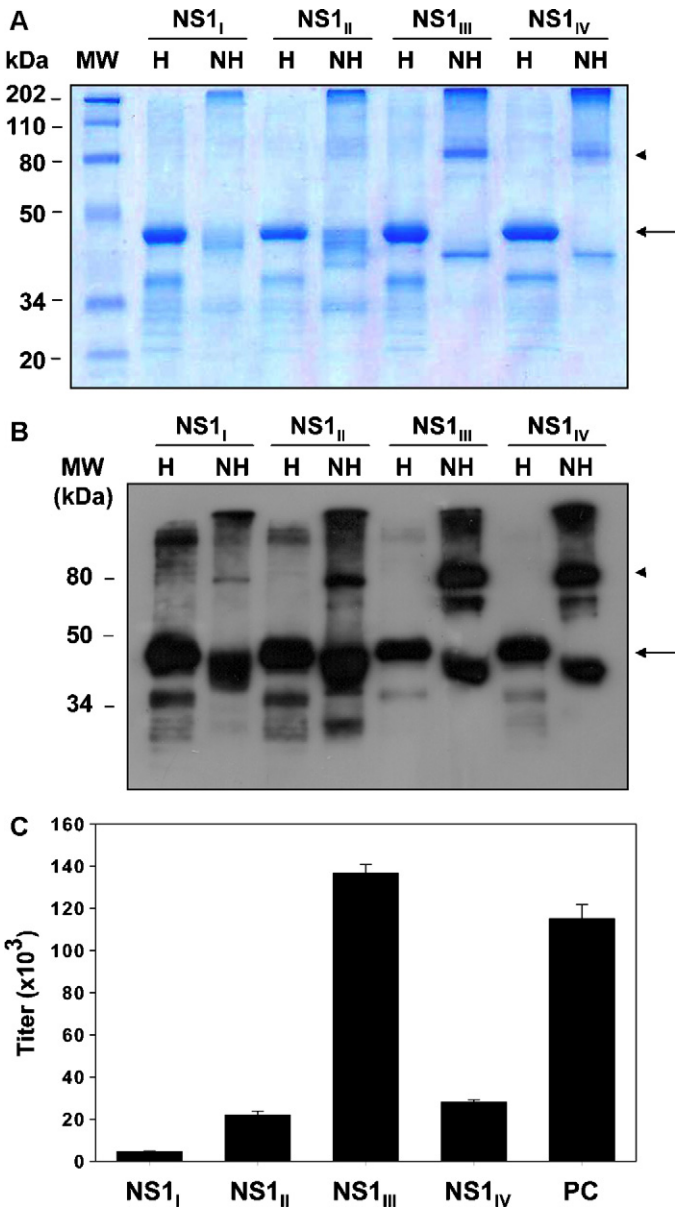

Fig. 2. Analysis of the oligomeric state of rNS1. The four refolded rNS1 samples were subjected to a heated and non-heated treatment in the presence or absence of 2 mM DTT, respectively, followed by analysis in 12% SDS-PAGE (A) and western blot using an anti-NS1 polyclonal antibody (B). H, heating treatment; NH, non-heating treatment. (C) Evaluation of the tertiary structure integrity of rNS1. Four different refolded rNS1 samples were tested by ELISA using a polyclonal antibody that recognizes conformational epitopes against native NS1 glycoprotein produced in mice immunized with a DNA vaccine based on the NS1 gene. Purified recombinant NS1 glycoprotein expressed in insect cells was used as a positive control. Titers were established as the reciprocal serum dilutions that gave an absorbance above that of preimmune sera. Data represent the mean of duplicate values for each sample and bars show the standard deviation of the mean. PC, positive control.

the native, glycosylated form and it maintained its immunogenic properties.

3.5. Analysis of the serum samples of dengue-infected patients

The mouse hyperimmune serum raised against the bacterial rNS1 was able to detect both the linear and conformational epitopes of the NS1 glycoprotein produced during viral infection (Suppl. Figs. S1 and S2). Based on these results, four serum samples were collected from acute-phase (5–7 days after symptom onset) DENV2-infected patients, and three negative control samples were

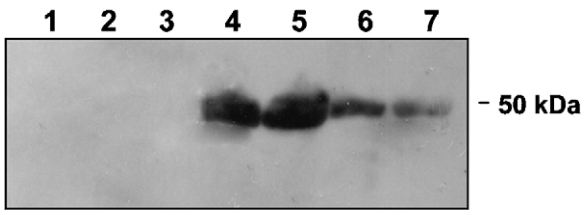

Fig. 3. Detection of the NS1 antigen in serum samples of dengue patients by western blot using mouse purified anti-NS1 polyclonal antibody. Lanes 1–3, negative sera; 4–7, different serum samples from acute-phase DENV2-infected patients. All samples were first subjected to albumin depletion using Cibacron Blue resin.

analyzed by western blot assay using purified anti-rNS1 polyclonal antibody. The results showed a single band of approximately 50 kDa, corresponding to the glycosylated NS1 in its monomeric form, in all the infected patients' sera. However, no such band was observed in the negative controls' (healthy patients') sera (Fig. 3). These observations confirm that the polyclonal antibody produced from the rNS1 protein recognized the NS1 antigen secreted in the dengue-infected patients. These results also indicate that this antibody can be an important tool for detecting dengue infection from patient's sera.

3.6. The capture ELISA for NS1 detection in different patient's serum samples

To assess the usefulness of the polyclonal antibodies generated by the rNS1 expressed in *E. coli* for the early detection of dengue infection, a capture ELISA was performed using purified anti-rNS1 mouse polyclonal antibodies to capture the NS1 antigen and an anti-rNS1 rabbit polyclonal antibody as the secondary detector. The cutoff value was calculated as twice the value of the negative control. A panel of 43 serum samples collected from the patients on different days of the infection's course (with day 0 defined as the first day of fever) was analyzed. The panel included samples from acute-phase patients infected with DENV1, DENV2, and DENV3 and from DENV-infected patients in the beginning of the clearance phase. The dengue infection in these patients was not detected by any of the commercial NS1-based diagnostic kits, such as the Platelia™ dengue NS1 and Pan-E dengue early ELISA kits (Table 3). Three negative samples from patients not infected with DENV were also analyzed to validate the experiment. As expected, the $O.D._{490\,nm}$ values measured from the non-infected patients' sera were below the cutoff line, while all of the DENV2 sera's values were above this line (Fig. 4 and Table 3 – S1 to S13), including the sample that was not detected by the PanBio kit (Fig. 4 and Table 3 – S5), resulting in an 100% sensitivity for the DENV2 samples. The differences observed in the $O.D._{490\,nm}$ values were related to the differences in the amount of plasma-circulating NS1, which varied from 1 to 10 μg/mL depending on the patient's susceptibility, the virus virulence (Alcon et al., 2002) and the levels of free antigen after the eventual formation of immune-complexes with anti-NS1 IgM/IgG.

Interestingly, this method successfully detected the DENV1 NS1 in 6 of the 10 samples (Fig. 4 and Table 3 – S14, S16, S17, S18, S20 and S21). Surprisingly, 60% (6/10) of the DENV-positive samples collected from patients at the beginning of the clearance phase, which were not detected by any of the commercial kits, were detected by the method used here (Fig. 4 and Table 3 – S34, S35, S40, S41, S42 and S43). This result suggests that the polyclonal antibodies produced against the correctly refolded NS1 expressed by the bacteria could recognize the circulating serum NS1 protein at low levels and when immune complexes were already present. However, no NS1 was detected in the DENV3-infected patients' sera (Fig. 4). Altogether, these results suggest the potential utility of this method for

Table 3
NS1 detection of the patients' sera by Capture ELISA.

Sample	Days post-infection	IgM 1	IgG 1	Virus type	NS1 PanBio	NS1 Platelia	This work
N1	N.D.	N.D.	N.D.	Neg	Neg	Neg	Neg
N2	N.D.	N.D.	N.D.	Neg	Neg	Neg	Neg
N3	N.D.	N.D.	N.D.	Neg	Neg	Neg	Neg
S1	03	N.D.	N.D.	DENV2	Pos	Pos	Pos
S2	04	N.D.	N.D.	DENV2	Pos	Pos	Pos
S3	01	N.D.	N.D.	DENV2	Pos	Pos	Pos
S4	03	N.D.	N.D.	DENV2	Pos	Pos	Pos
S5	06	Pos	Pos	DENV2	Neg	Pos	Pos
S6	09	Pos	Pos	DENV2	Pos	Pos	Pos
S7	09	Pos	Pos	DENV2	Pos	Pos	Pos
S8	09	Pos	Pos	DENV2	Pos	Pos	Pos
S9	N.D.	N.D.	N.D.	DENV2	N.D.	N.D.	Pos
S10	N.D.	N.D.	N.D.	DENV2	N.D.	N.D.	Pos
S11	N.D.	N.D.	N.D.	DENV2	N.D.	N.D.	Pos
S12	N.D.	N.D.	N.D.	DENV2	N.D.	N.D.	Pos
S13	N.D.	N.D.	N.D.	DENV2	N.D.	N.D.	Pos
S14	03	Neg	N.D.	DENV1	Pos	Pos	Pos
S15	04	Neg	Pos	DENV1	Pos	Pos	Neg
S16	03	Neg	N.D.	DENV1	Pos	Pos	Pos
S17	03	Neg	N.D.	DENV1	Pos	Pos	Pos
S18	0	Pos	N.D.	DENV1	Neg	Pos	Pos
S19	0	Neg	N.D.	DENV1	Neg	Pos	Neg
S20	03	Neg	N.D.	DENV1	Pos	Pos	Pos
S21	02	Neg	Pos	DENV1	Pos	Pos	Pos
S22	04	Neg	N.D.	DENV1	Pos	Pos	Neg
S23	05	Neg	N.D.	DENV1	Pos	Pos	Neg
S24	01	N.D.	N.D.	DENV3	Pos	Pos	Neg
S25	0	Neg	N.D.	DENV3	Pos	Pos	Neg
S26	08	Neg	N.D.	DENV3	Neg	Pos	Neg
S27	03	N.D.	N.D.	DENV3	Pos	Pos	Neg
S28	03	N.D.	N.D.	DENV3	Pos	Pos	Neg
S29	05	Neg	N.D.	DENV3	Pos	Pos	Neg
S30	02	N.D.	N.D.	DENV3	Pos	Pos	Neg
S31	02	N.D.	N.D.	DENV3	Neg	Pos	Neg
S32	0	N.D.	N.D.	DENV3	Pos	Pos	Neg
S33	02	N.D.	N.D.	DENV3	Pos	Pos	Neg
S34	04	Pos	N.D.	N.D.[a]	Neg	Neg	Pos
S35	04	Pos	N.D.	N.D.[a]	Neg	Neg	Pos
S36	05	Pos	N.D.	N.D.[a]	Neg	Neg	Neg
S37	07	Pos	N.D.	N.D.[a]	Neg	Neg	Neg
S38	06	Pos	N.D.	N.D.[a]	Neg	Neg	Neg
S39	07	Pos	N.D.	N.D.[a]	Neg	Neg	Neg
S40	04	Pos	N.D.	N.D.[a]	Neg	Neg	Pos
S41	06	Pos	N.D.	N.D.[a]	Neg	Neg	Pos
S42	03	Pos	N.D.	N.D.[a]	Neg	Neg	Pos
S43	06	Pos	N.D.	N.D.[a]	Neg	Neg	Pos

N.D., not determined.
[a] Virus detected but not determined.

developing a sensitive, low-cost, and early detection test for DENV infection.

4. Discussion

Different heterologous protein expression systems, such as yeast expression systems, vaccinia virus, baculovirus and other insect cells, have been proposed for producing recombinant DENV NS1 protein (Falgout et al., 1989; Falgout and Markoff, 1995; Pryor and Wright, 1993, 1994; Wallis et al., 2004; Wu et al., 2003; Zhao et al., 1987; Zhou et al., 2006), and these methods are suitable protocols for generating glycosylated protein. Because these are complex systems, however, manipulation and protocol optimization are expensive and time consuming. By contrast, prokaryotic systems are easy to manipulate, allow rapid protocol optimization and express high levels of recombinant proteins but lack post-translational modifications. Several reports have also proposed achieving NS1 expression using bacterial cells (Das et al., 2009; Mason et al., 1990; Qiu et al., 2009; Xu et al., 2006), but none of these studies were able to produce a high yield of functional and correctly folded NS1 protein.

Four different refolding strategies were evaluated to determine which one generated the best refolding efficiency and restored the structural properties of the NS1 protein expressed by *E. coli* BL21(λDE3)pLyS cells. All the protocols were able to recover the rNS1 efficiently, and their integrity was confirmed by fluorescence and CD spectroscopy. Another protocol for recovering rNS1 protein has been published recently, but it had an efficiency yield of 2.6% (Amorim et al., 2010). Unfortunately, it was not possible to reproduce this finding because all of the proteins aggregated immediately under the buffer conditions used in the study.

The NS1 protein exists predominantly as a heat-sensitive homodimer, which may be its functional form (Winkler et al., 1988, 1989). The expression by COS cells of mutant NS1 that lacks glycan chains reveals that this protein is able to form dimers, although they are less stable than dimers of the glycosylated protein (Pryor and Wright, 1994). It has been previously reported that dimers of rNS1 failed to form when the protein was expressed in *E. coli* (Lemes et al., 2005). The rNS1 obtained from all of the four refolding protocols was able to form dimers, although in differing amounts. These results clearly indicate that *E. coli* rNS1 is able to form

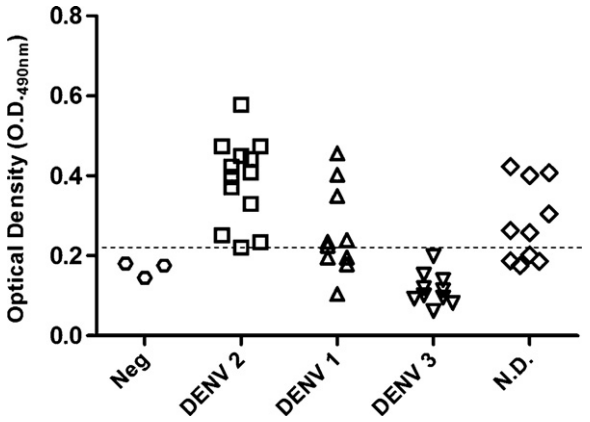

Fig. 4. Capture ELISA using purified mouse anti-NS1 polyclonal antibody to capture the NS1 antigen from serum samples of dengue patients and rabbit anti-NS1 antiserum as the secondary detector. The cut-off value was calculated as twice the mean value of the negative control ($O.D._{490\,nm}$ = 0.203). Neg, negative serum samples; DENV2, serum samples collected from DENV2-infected patients during the acute phase; DENV1, serum samples collected from DENV1-infected patients during the acute phase; DENV3, serum samples collected from DENV3-infected patients during the acute phase; N.D., serum samples from DENV IgM-positive patients at the beginning of the clearance-phase but not determined by either PanBio or Platelia dengue diagnosis kits.

dimers, depending on the protocol used to recover it. It is worth mentioning that the rNS1 generated by all of the methods was in the correct conformation. Because all the samples were recognized by a conformational-specific antibody and recognition was dependent on the amount of dimeric rNS1 formed, this result reinforces the proposal that the oligomeric (dimer) state is important for NS1 immunogenicity. The analysis of the anti-NS1 polyclonal antibodies raised against the $rNS1_{II}$ indicates that they are able to recognize not only conformational but also linear epitopes that are maintained in both folded and unfolded proteins, which highlights their utility as tools for dengue laboratory research and diagnosis.

Because rapid and accurate diagnosis of dengue is critical for appropriate treatment, different approaches have been developed to generate a sensitive acute-phase diagnostic assay (dos Santos et al., 2004, 2007). It is known that the NS1 protein is secreted into the patient's plasma at the beginning of clinical symptoms, mostly from days 1 to 6 after the onset of fever (Alcon et al., 2002), which makes NS1 an important marker for detecting DENV infection in the first few days after symptoms appear. In this scenario, the anti-NS1 antibody is an attractive tool for the early diagnosis of DENV-infected patients. Although there are commercially available diagnostic kits that detect the NS1 antigen, developing new approaches is still relevant to improving sensitivity and specificity. This study examined whether an anti-NS1 polyclonal antibody raised from a correctly refolded protein expressed by *E. coli* is able to recognize the NS1 found in human serum. A western blot analysis confirmed that the purified anti-rNS1 polyclonal antibody was only able to recognize a single and specific band corresponding to the NS1 protein in infected patients. Using a serum volume five-fold smaller than that used in the commercial kits, the approach proposed here was able to identify the NS1 antigen in all the sera from the DENV2-infected patients (i.e., there was 100% sensitivity for DENV2). In addition, the NS1 antigen was identified in 60% of the DENV-positive sera from the beginning of the clearance-phase, which the commercial NS1-based diagnostic kits were not able to do. This result highlights the sensitivity of polyclonal antibodies for developing alternative diagnostic approaches to dengue detection. Interestingly, the method also successfully detected 60% of the DENV1-infected samples. By contrast, no DENV3-infected serum was detected. This result was not entirely unexpected because a band corresponding to the NS1 protein from some of the DENV3-infected sera was detected with much lower intensity than that of the DENV-2 infected sera in the western blotting assay (data not shown). Therefore, it seems that antibodies to DENV2 NS1 cross-react with DENV3, albeit at a low level, as has been reported previously (Falconar, 1997). In fact, the sensitivities of the Platelia™ dengue NS1 and Pan-E dengue early ELISA kits vary with the DENV serotype (Ramirez et al., 2009), and both kits poorly detected DENV3-infected samples (Lima et al., 2010). When considering all the samples together (excluding the S9 to S13 samples, which were not analyzed by the abovementioned kits), the method described here was able to detect 52.8% (20/38) compared to 60.5% (23/38) for the PanBio and 73.3% (28/38) for the Platelia kits. Although the proposed method seems to be less sensitive, both of the commercial kits are based on monoclonal antibodies that recognize a conserved epitope present in all four DENV serotypes, whereas the method suggested above is based on a polyclonal antibody raised against DENV2 rNS1 protein that recognizes multiple epitopes of the protein. Notably, although the polyclonal antibody production is prone to batch-to-batch variability, this problem can be overcome by immunoaffinity purification and/or by establishing a standard titer for different antibody pools. These results highlight the high sensitivity of the polyclonal antibodies raised against the correctly folded rNS1 protein for detecting circulating NS1 in serum. Moreover, the production of such antibodies, using a low-cost, high-yield efficiency rNS1 refolding protocol, makes it significant for developing a sensitive and cost-effective early detection kit, which is important for countries suffering from dengue outbreaks.

In conclusion, this work describes four efficient refolding protocols for producing rNS1 from *E. coli* that are able to form dimers in a native-like protein conformation. Based on these data, Protocol II combined a high yield of the recovered protein with preserved biochemical and immunogenic properties and low-cost production, making it the best method for refolding the rNS1 protein. This study also demonstrated that the NS1 protein is composed of a mixture of α-helices, β-sheets and random coils. Finally, a method for DENV detection using the anti-NS1 polyclonal antibodies raised from correctly refolded rNS1 was able to detect IgM-positive samples using a five-fold lower sample quantity than the commercial Platelia™ dengue NS1 and Pan-E dengue early ELISA kits. Taken together, these results demonstrate the utility of the correctly refolded rNS1 protein as a laboratory tool for dengue research and detection and for developing a serological test for sensitive, rapid, early and reliable diagnosis of DENV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2011.04.029.

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