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Expression of recombinant dengue virus type 1 non-structural protein 1 in mammalian cells and preliminary assessment of its suitability to detect human IgG antibodies elicited by viral infection

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A B S T R A C T

In recent years dengue has become a rapidly growing public health problem worldwide, however, the availability of accurate and affordable diagnostic immunoassays is limited, partly due to the difficulty of producing large quantities of purified antigen. Non-structural protein 1 (NS1) has shown to be a good candidate for inclusion in diagnostic assays and for serosurveys, particularly in endemic countries as a prerequisite for vaccination. In this work the NS1 antigen derived from dengue virus type-1 (DENV1) was expressed in HEK293-T cells and purified by affinity chromatography. The recombinant protein was recovered properly folded as dimers, highly purified and with good yield (1.5 mg/L). It was applied as a serological probe in an indirect ELISA developed in this work to detect human IgG antibodies. Preliminary comparative performance values of 81.1% sensitivity and 83.0% specificity of the developed and preliminary validated iELISA, relative to a commercial kit were obtained, suggesting that the purified recombinant DENV1 NS1 antigen is suitable to detect IgG antibodies, indicative of past DENV infection.

1. Introduction

Dengue is a mosquito-borne disease caused by dengue virus (DENV). The virus circulation is endemic in >120 countries, and it has become a rapidly growing public health problem in the world (Brady et al., 2012; Shepard et al., 2016). It is estimated that around 390 million people are infected each year, of which approximately 96 million manifest clinical symptoms (Bhatt et al., 2013). Even though most infected people are asymptomatic or develop an illness with flu-like symptoms, some individuals can progress to severe forms of the disease characterized by vascular leakage and coagulation dysfunctions with or without bleeding, historically known as dengue hemorrhagic fever and dengue shock syndrome (Guzman et al., 2016). The current World Health Organization (WHO) classification system establishes three levels of severity: dengue without warning signs, dengue with warning signs, and severe dengue (Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control, 2009).

DENV comprises four different serotypes (DENV1, DENV2, DENV3 and DENV4) belonging to the *Flaviviridae* family (genus *Flavivirus*), which includes other relevant pathogens such as japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV) and Zika virus (ZIKV). Natural DENV infection induces long-lasting protective immunity against homotypic viruses, but only short-term protection from infection with other serotypes (Montoya et al., 2013; Snow et al., 2014). Secondary heterotypic infections are associated with an increased risk of developing severe dengue due to the phenomenon of

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antibody-dependent enhancement (ADE) (Guzman et al., 2013; Katzelnick et al., 2017).

There are no effective antiviral drugs against DENV. The only licensed vaccine, CYD-TDV (Dengvaxia®, Sanofi Pasteur Inc., Lyon, Francia), is manufactured using four recombinant viruses constructed by replacing pre-membrane (PrM) and envelope (E) coding regions of each DENV serotype into the live-attenuated YFV 17D strain backbone. Trials in Latin America and Asia found low overall efficacy of CYD-TDV (around 60%) and its use is only recommended for individuals aged 9 to 45 years with confirmed previous DENV infection or living in hyperendemic regions (Villar et al., 2015; Capeding et al., 2014; *Vaccine*, 2019). In this context, early diagnosis and epidemiological surveillance are essential to reduce morbidity and mortality. Virus isolation, nucleic acid amplification tests and viral non-structural protein 1 (NS1) detection immunoassays are employed to diagnose DENV infection during the acute phase of the disease (up to 5 days after the onset of illness) (Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control, 2009). Following this period, diagnosis relies on the screening for virus-specific antibodies (Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control, 2009). Serology is also the most adequate tool to conduct prevalence surveys since it can be used to reveal past infections in asymptomatic individuals (Chatchen et al., 2017). Moreover, for countries that consider vaccination as part of their dengue control program, the recommended strategy is the screening for antibodies indicative of previous DENV infection prior to vaccination (*Vaccine*, 2019).

Seroprevalence data are limited in regions considered at high risk of DENV transmission (Fritzell et al., 2018; Li et al., 2021). This may be due to the high costs of laboratory tests, together with the lack of public policies for the implementation of in-depth epidemiological studies. In fact, according to the Pan American Health Organization (PAHO), $<45\%$ of dengue cases reported in the Americas between 2016 and 2020 were laboratory confirmed (Pan American Health Organization, n.d.). Enzyme linked immunosorbent assays (ELISA) for the detection of immunoglobulin M (IgM) or immunoglobulin G (IgG) antibodies are the most widely used dengue tests, since they are easy to perform and require less complex equipment than molecular or virological methods. IgM/IgG ratio and IgG level determined by ELISA can be used to classify primary and secondary DENV infections (Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control, 2009; Nguyen et al., 2018). Nevertheless, published sensitivities and specificities of the available commercial ELISAs are dissimilar and the high cross-reactivity with other flaviviruses may produce false positive results (Blacksell et al., 2012; Pal et al., 2015; Lee et al., 2019; Schwartz et al., 2000; Bonaparte et al., 2019; Bonaparte et al., 2020). These limitations lead to uncertainty in the number of reported cases and seroprevalence estimates, hence the urgent need to develop accurate and affordable laboratory tests in Latin America.

NS1 is a glycoprotein of approximately 46–55 KDa highly conserved within the four DENV serotypes. It can be found as a membrane-associated dimer or as a lipid-associated hexamer that is secreted into the bloodstream (Gutsche et al., 2011). Intracellular NS1 plays a key role in viral RNA replication and in infectious particle assembly through its interaction with other viral and host proteins (Scaturro et al., 2015; Płaszczyca et al., 2019) whereas secreted NS1 contributes to immune evasion by inhibiting the complement system (Avirutnan et al., 2011; Conde et al., 2016; Thiemmeca et al., 2016). In addition, it may be involved in triggering vascular leakage, coagulopathy and thrombocytopenia. It has been demonstrated that this viral protein stimulates the release of pro-inflammatory cytokines from immune cells (Adikari et al., 2016; Alayli and Scholle, 2016; Modhiran et al., 2017), induces disruption of the vascular endothelium (Puerta-Guardo et al., 2016; Chen et al., 2016) and can elicit antibodies that cross-react with endothelial cells, platelets or coagulation factors (Falconar, 1997; Lin et al., 2003; Sun et al., 2007; Chuang et al., 2014).

During DENV infection, a strong humoral immune response is generated against NS1. Antibodies targeting this viral glycoprotein have been proposed as biomarkers of long-term and recent DENV infections (Shu et al., 2002; Shu et al., 2003; Nascimento et al., 2018a; Mora-Cárdenas et al., 2020). Furthermore, the quantity and repertoire of anti-NS1 antibodies have been correlated with disease severity (Jayathilaka et al., 2018).

The present work describes the production of recombinant DENV1 NS1 employing a mammalian expression system and its potential use as a serologic probe in an indirect ELISA for the detection of IgG antibodies elicited by DENV infection.

2. Materials and methods

2.1. Amplification of the NS1 coding sequence by reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was purified from C6/36 cells infected with DENV1 Hawaii prototype strain using the QIAamp® Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands), following the manufacturer's instructions. Complementary DNA (cDNA) was obtained by reverse transcription with SuperScript™ III First-Strand Synthesis system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using random primers, according to the manufacturer's instructions. The complete NS1 coding sequence was amplified by polymerase chain reaction (PCR) with Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using forward primer D1NS1-F (5′-AAAGTTAACGACTCGGGATGTGTAATTAAC-3′) and reverse primer D1NS1-R (5′-TTTGCTAGCCTAGTGATGGTGATGGTGGTGTGCAGAGACCAATGACTTAAC-3′), containing a hexahistidine tag (*His tag*) coding sequence. Cycling conditions were: initial denaturation at 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s – 60 °C for 30 s – 72 °C for 30 s and a final elongation of 5 min at 72 °C. The amplified product was purified from a 1% *w/v* agarose gel with the Wizard® SV Gel and PCR Clean-Up system (Promega Corporation, Madison, Wisconsin, USA).

2.2. Construction of the recombinant NS1 expression plasmid

The strategy implemented for the construction of the NS1 expression plasmid, pCAGGS-SS-DENV1-NS1, is illustrated in Supplementary Fig. 1. The DENV1-NS1 sequence, amplified by RT-PCR (Supplementary Fig. 2.A), was cloned in the pCAGGS-SS vector, a modified version of the pCAGGS vector containing a protein secretion signal upstream of the cloning site. The PCR amplicon was digested with *Hpa*I and *Nhe*I enzymes while the pCAGGS-SS plasmid was digested with *Pvu*II and NheI enzymes. Digestion products were purified from the agarose gel with the Wizard® SV Gel and PCR Clean-Up system (Promega Corporation, Madison, Wisconsin, USA) and ligated with T4 DNA Ligase (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Chemically competent *E. coli* DH5α bacteria were transformed with ligation mixtures by heat shock method. Transformants were selected on Luria-Bertani agar supplemented with 100 μg/mL of ampicillin. The recombinant construct was verified by restriction pattern analysis after digestion with *Xho*I and *Hin*dIII (Supplementary Fig. 2.B). Insert identity was confirmed by DNA sequencing (Macrogen Inc., Seoul, Republic of Korea) using forward primer pCAGGS-F (5′-GTTCGGCTTCTGGCGTGTGA-3′) and reverse primer pCAGGS-R (5′-GCCAGAAGTCAGATGCTCAAGG-3′). The cloned sequence exhibited 99.9% nucleotide identity with NS1 coding region from DENV1 Hawaii strain (GeneBank number: KM204119).

2.3. Recombinant protein expression in HEK293T cells

Expression of recombinant DENV1 NS1 was carried out in HEK293T cells grown in 145/20 mm cell culture dishes (CELLSTAR®, Greiner Bio-One, Kremsmünster, Austria). This procedure was performed in a BSL-2 facility. Cells were grown for 24 h at 37 °C and 5% CO_2 in DMEM supplemented with 10% *v*/v fetal bovine serum (FBS), 2 mM L-glutamine, 1× penicillin-streptomycin solution (Gibco™, Thermo Fisher Scientific,

Waltham, Massachusetts, USA) 1× MEM Non-Essential Amino Acids Solution (Gibco™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1 mM sodium pyruvate, until 80–90% confluence. Next, the growth medium was replaced with 10 mL of fresh DMEM (without FBS and antibiotics), and transfection was performed by slowly dripping 2 mL per plate of a mixture containing 18.5 μg/mL of NS1 expression plasmid (pCAGGS-SS-DENV1-NS1) and 55.5 μg/mL of branched polyethylenimine (Sigma-Aldrich, Saint Louis, Missouri, USA), in DMEM (PEI to DNA ratio of 3:1). The culture dishes were filled at 3–5 h post transfection with 10 mL of DMEM supplemented with 2% FBS. Transfected cells were maintained at 37 °C and 5% CO_2. After 72 h, the cell culture supernatant was collected and the cells were lysed by addition of lysis buffer (25 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5% *v*/v Triton X-100, 10% v/v glycerol, pH: 7.6). Recombinant protein expression was analyzed both in the supernatant and in the cellular lysate by Western Blot, with HRP Anti-6× His tag® antibody (Abcam, Cambridge, UK). The identity of the recombinant antigen was confirmed by Western Blot with the monoclonal antibody (mAb) 15F3–1 (Biodefense and Emerging Infections Research Resources Repository, Manassas, Virginia, USA), specific to DENV1 NS1, followed by incubation with HRP-conjugated anti-mouse IgG (H + L) secondary antibody (Promega Corporation, Madison, Wisconsin, USA).

2.4. Purification and quantification of the recombinant antigen

Purification of the recombinant antigen was performed through immobilized metal ion affinity chromatography (IMAC) using a Ni-NTA Agarose matrix (Qiagen, Venlo, The Netherlands). Culture supernatants and cell lysates, from multiple transfection plates, were pooled. The suspension was centrifuged at 10,000 x*g* for 20 min. Supernatant was diluted by the addition of binding buffer 2× (100 mM NaH_2PO_4, 600 mM NaCl, 20 mM Imidazole, pH: 7.4), mixed with 200 μL of chromatographic matrix and incubated overnight at 4 °C with gentle shaking. The matrix was packed into a chromatographic column and washed twice with at least 10 mL of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM Imidazole, pH: 7.4). Recombinant protein was eluted twice with 0.7–1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM Imidazole, pH: 7.4). Aliquots of all steps of the purification process were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

The purified recombinant protein was quantified by gel densitometry. The polyacrylamide gel stained with Coomassie brilliant blue R-250 was digitalized and analyzed with GelAnalyzer 2010a software. Antigen concentration was calculated by interpolating the optical density value of the 50 KDa band (NS1 monomer) or 100 KDa band (NS1 dimer) into a calibration curve generated with different concentrations of bovine serum albumin (BSA) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The purified protein was analyzed by Western Blot with HRP Anti-6× His tag® antibody (Abcam, Cambridge, UK).

2.5. Serum samples

This study was approved by the Ethics Committee of Clementino Fraga Filho University Hospital (HUCFF/UFRJ) (number: 80709). Patients were admitted to different hospitals in Rio de Janeiro, and blood was collected to confirm clinical diagnosis by Rio de Janeiro Public Health Reference Laboratory – LACEN-RJ. Sera samples were used after the conclusion of the diagnostic investigation. All patients' personal information was anonymized. Therefore, the ethics committee waived the requirement for informed consent from patients.

Two control sera were used for ELISA standardization: Positive control serum originated from a convalescent individual with infection status confirmed by RT-PCR, collected at 14 days post symptoms onset, and Negative control serum was derived from a healthy individual living in an area with no record of Dengue cases.

Serum specimens used to assess the performance of the DENV-NS1 IgG iELISA for detection of past infection were obtained from healthy individuals in a dengue endemic area and were characterized as DENV IgG positive ($n = 106$) or negative (n = 106) by the Panbio™ Dengue IgG Indirect ELISA (Abbott Laboratories, North Chicago, Illinois, USA).

A second set of samples used for assessment of anti-NS1 IgG antibody levels in sera from symptomatic individuals (suspect Dengue cases) were collected in the State of Rio de Janeiro between 2012 and 2016. Specimens had been tested for DENV infection according to their collection date: 0–4 days after symptoms onset (acute phase) by RT-PCR following the protocol previously described (Lanciotti et al., 1992); 5 or more days after symptoms onset (convalescent phase) by Dengue IgM Capture ELISA (Panbio™ from Abbott Laboratories, North Chicago, Illinois, USA or DxSelect™ from Focus Diagnostics, Cypress, California, USA). According to the test results and collection date, sera were classified into four categories: acute dengue (RT-PCR positive samples, $n = 24$), early convalescent dengue (IgM Capture ELISA positive samples collected 5–8 days after symptoms onset, $n = 14$), convalescent dengue (IgM Capture ELISA positive samples collected >9 days after symptoms onset, $n = 10$) and not detected dengue (RT-PCR negative, $n = 5$, plus IgM Capture ELISA negative, $n = 22$).

2.6. DENV NS1 specific IgG indirect ELISA (DENV-NS1 IgG iELISA)

The optimum concentration of the recombinant antigen and test serum dilution in the indirect ELISA protocol were established after conducting a checkerboard titration (data not shown).

Immulon™ 1B plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated overnight at 4 °C with 100 ng/well of the recombinant NS1 antigen diluted in 100 μL of coating buffer (40 mM Na_2CO_3, 60 mM $NaHCO_3$, pH: 9.6). Next, wells were washed with PBS (phosphate buffer saline) and non-specific binding sites were blocked with blocking buffer (3% *w/v* skim milk in PBS with 0.05% *v*/v Tween-20) for 1 h at 37 °C. After discarding the blocking solution, human serum samples were diluted 1:200 in dilution buffer (1.5% w/v skim milk in PBS with 0.05% *v*/v Tween-20) and added at 100 μL volume per well. Plates were incubated for 1 h at 37 °C and washed four times with PBS-T (PBS with 0.05% v/v Tween-20). Then wells were incubated for 1 h at 37 °C with 100 μL of Peroxidase AffiniPure Goat Anti-Human IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) diluted 1:8000 in dilution buffer. Following four washes with PBS-T, plates were incubated at room temperature for 15 min with 100 μL volume per well of TMB (3,3′,5,5′-Tetramethylbenzidine) substrate reagent (BD OptEIA™,BD Biosciences, San Jose, California, USA). Color development reaction was stopped by the addition of 1 M H_2SO_4 and the absorbance at 450 nm (Abs_{450}) of each well was measured using a microplate reader.

Two internal controls, an IgG positive serum with strong reactivity against recombinant NS1 (C_{pos}) and an IgG negative serum with weak reactivity against this antigen (C_{neg}), were used for normalization of results by transforming the raw data into a reactivity index (R.I.) according to the following equation: $R.I. = 100 \text{ x } [(\text{Sample } Abs_{450} - C_{neg.} \, Abs_{450}) / (C_{pos.} \, Abs_{450} - C_{neg.} \, Abs_{450})]$. Absorbance values of the positive and negative controls were calculated as the average of two replicates assayed in each plate.

2.7. Data analysis

Inter-assay repeatability of the developed immunoassay was studied by calculating the coefficient of variation (CV) between results recorded for three serum samples, which exhibited high, moderate and low degree of reactivity against recombinant NS1, respectively, in three independent experiments conducted on different dates, by distinct operators and varying reagent preparations. A CV < 10.0% was considered acceptable.

Data derived from processing IgG positive and negative samples (classified by Panbio™ Dengue IgG Indirect ELISA) with the DENV-NS1

IgG iELISA were used to construct a receiver operating characteristic (ROC) curve. The test performance was estimated through the determination of the area under the curve (AUC) and its 95% confidence interval (95% CI). The optimal decision threshold, the one that maximized the Youden's index (J = sensitivity + specificity – 1), was established as the cut-off value for the assay. Sensitivity and specificity, with their respective 95% CI, were then computed at the chosen cut-off value. Concordance between Panbio™ Dengue IgG Indirect ELISA and DENV-NS1 IgG iELISA was assessed by the calculation of the Cohen's kappa index (κ) and its 95% CI. The analyses described in this paragraph were performed with the pROC and vcd packages of the Rstudio statistical software.

Kruskal-Wallis test followed by Dunn's *post hoc* test was carried out (using GraphPad Prism software) to evaluate differences in DENV-NS1 IgG iELISA normalized reactivity values among sera categories: acute, early convalescent, convalescent and not detected dengue. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of the recombinant NS1

The expression of the recombinant NS1 in HEK293T cells was analyzed 72 h post transfection both in the cellular fraction and in the supernatant by Western Blot using the HRP Anti-6× His tag® antibody as well as monoclonal antibody 15F-3, specific against DENV1 NS1 (Fig. 1). Two bands of approximately 45–50 Kda were detected with both antibodies, likely reflecting glycosylation variants of recombinant NS1. These two variants were also detected after recombinant NS1 purification (Fig. 2). Approximately half the total amount of recombinant protein was secreted to the culture media.

Purification was accomplished using IMAC after pooling transfected cells lysates and supernatants from multiple plates. The SDS-PAGE analysis revealed a recombinant protein with a high degree of purity considering the complex nature of the starting material (Fig. 2.A). To analyze the oligomeric state of the protein, IMAC elutions were not subjected to heat and reduction treatment prior to SDS-PAGE. In these conditions, the presence of a 100 KDa band indicated that the purified antigen is assembled forming the characteristic dimeric structure of the native protein (Fig. 2.A). This was also observed by Western Blot with HRP Anti-6× His tag® antibody (Fig. 2.B).

Quantification of the antigen carried out by gel densitometry demonstrated an adequate final yield of the entire process (Fig. 2.C), rendering up to 30 μg of purified recombinant antigen per culture plate (1.5 mg antigen per liter of culture medium). Moreover, it evidences that the mass estimated for both bands corresponding to NS1 monomer glycosylation variants (E1) equals that estimated for the NS1 protein dimer (E1*).

3.2. Implementation of the recombinant NS1 antigen in an indirect ELISA to detect IgG antibodies

An indirect ELISA for detection of DENV NS1 specific IgG antibodies was developed using the NS1 antigen expressed in mammalian cells (DENV-NS1 IgG iELISA).

The operating range of the assay was evaluated through serial dilutions of positive and negative control sera. As shown in Fig. 3.A, the positive serum showed a maximum Abs_{450} of approximately 2.000 obtained up to dilution 1:200, which dropped subsequently along with the higher serum dilutions, although they were not extensive enough to allow endpoint titration. A background color was obtained at 1:50 and 1:100 dilutions of the negative sera, reflecting the nonspecific attachment of the serum to the antigen coated wells. This nonspecific signal is slightly higher than the plate background (Abs_{450} around 0.030, registered for the negative sera at the highest dilution). The Abs_{450} obtained for the negative serum at dilution 1:200 was 0.126. The results of titration curves for the positive and negative sera were used to select the working dilution (1:200) for the present indirect ELISA to analyze sera in single wells, allowing for proper discrimination of positive and negative results (adequate reactivity/noise ratio).

Subsequently, the reactivity against recombinant NS1 of serum samples with IgG positive ($n = 6$) and negative ($n = 5$) serostatus, according to Panbio™ Dengue IgG Indirect ELISA, was analyzed by the developed immunoassay (Fig. 3.B). As expected, high Abs_{450} values were found for IgG positive samples, all above 3× standard deviations (SD) of the negative sera mean value. These results suggest that the DENV-NS1 IgG iELISA can be applied for the detection of past DENV infection.

Reproducibility studies of independent experiments carried out on different days with three sera corresponding to strong, moderate and low positive Abs_{450} values indicated that the DENV-NS1 IgG iELISA has good inter-assay repeatability with coefficients of variation ranging

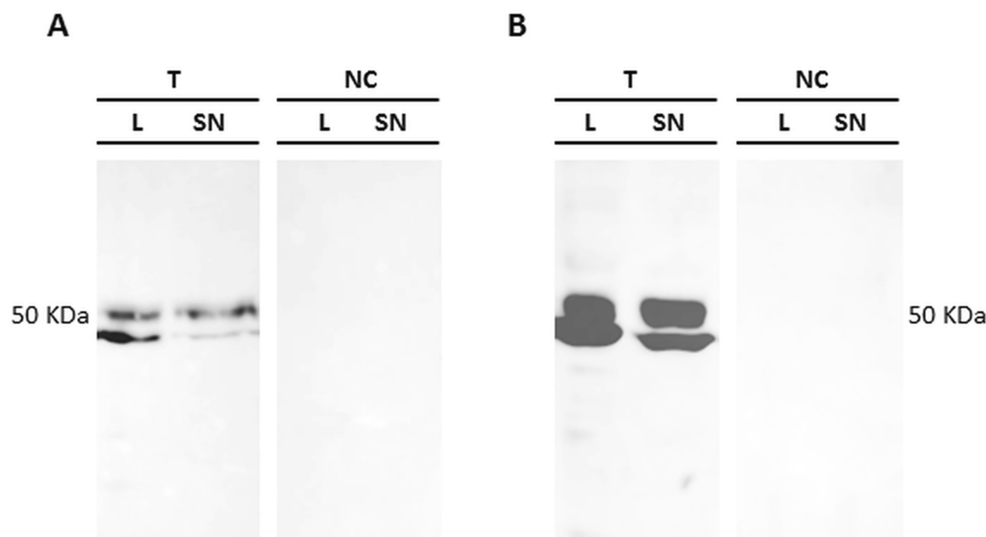
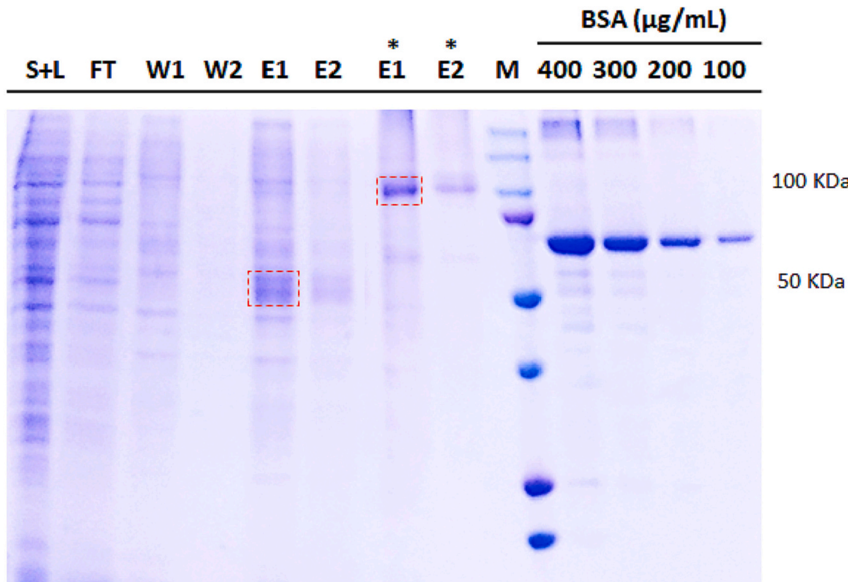
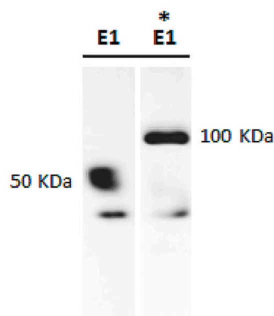


Fig. 1. Detection of recombinant DENV1 NS1 expression in HEK293T cells by Western Blot. A) Assay performed using HRP Anti-6× His tag® antibody. B) Assay performed using 15F3–1 monoclonal antibody (specific for DENV1 NS1). T: cells transfected with pCAGGS-SS-DENV1-NS1, NC: not transfected cells, L: cellular lysate, SN: cell culture supernatant.





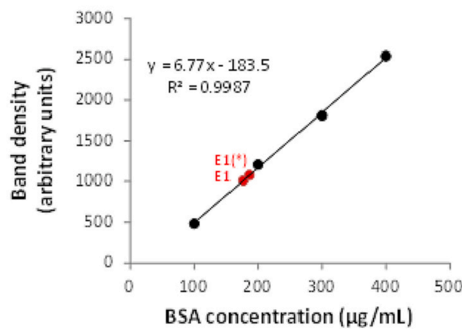
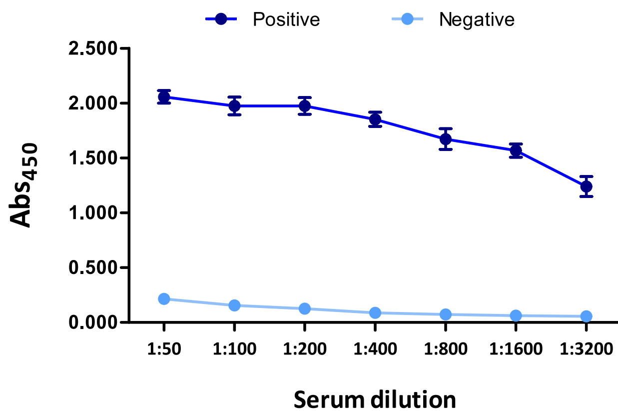


Fig. 2. Purification and quantification of recombinant antigen. A) SDS-PAGE analysis of protein samples obtained during the purification process. Boxes indicate bands corresponding to the recombinant proteins that were quantified. M: Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-rad), S + L: supernatant plus cell lysate from cells transfected with pCAGGS-SS-DENV1-NS1, FT: flow-through, W1–2: washes, E1: 1st elution, E2: 2nd elution, *: Sample not subjected to heat and reduction treatments. B) Western Blot of the eluted fraction using HRP Anti-6× His tag® antibody. C) Determination of recombinant protein concentration by interpolating SDS-PAGE band density (expressed in arbitrary units) over a calibration curve generated with different concentrations of BSA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



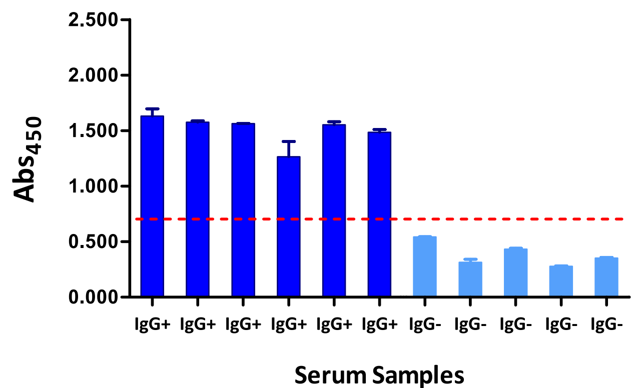


Fig. 3. Development of an indirect ELISA based on the recombinant NS1 for detection of IgG antibodies elicited by a DENV infection. A) Dilution curves for positive and negative control sera. B) Analysis of IgG positive (IgG+, $n = 6$) and negative (IgG-, $n = 5$) samples (serostatus assigned by the Panbio™ Dengue IgG Indirect ELISA) under optimized conditions. Data is shown as the mean Abs_{450} (absorbance at 450 nm) ± standard deviation (SD) of duplicates. The dashed line represents the mean Abs_{450} + 3× SD of the IgG- samples.

Table 1
Inter-assay repeatability of DENV-NS1 IgG iELISA.

Serum	Reactivity against recombinant NS1	Abs_{450} #1	#2	#3	Mean	SD	CV (%)
A	High	2.176	2.257	1.912	2.115	0.180	8.5
B	Moderate	1.473	1.361	1.225	1.353	0.124	9.2
C	Low	0.598	0.545	0.541	0.561	0.032	5.7

Abs_{450}: Absorbance at 450 nm.
SD: standard deviation.
CV: coefficient of variation.

Table 2
Performance parameters of DENV-NS1 IgG ELISA taking as reference the serological status of samples assigned by Panbio™ Dengue IgG Indirect ELISA.

		Panbio™ Dengue IgG Indirect ELISA		
	N° of samples	Positive	Negative	Total
DENV-NS1 IgG iELISA	Positive	86	18	104
	Negative	20	88	108
	Total	106	106	212
Comparative sensitivity		81.1% (95% CI: 73.6–87.7%)		
Comparative specificity		83.0% (95% CI: 75.5–89.6%)		
Cohen's kappa index (κ)		0.642 (95% CI: 0.538–0.745)		

between 5.7% and 9.2% for the samples studied (Table 1). The intra-plate repeatability gave values below 4.0% in all cases (data not shown).

3.3. Assessment of DENV-NS1 IgG iELISA for detection of past DENV infection

The DENV-NS1 IgG iELISA was used to test serum samples derived from healthy individuals with positive ($n = 106$) and negative ($n = 106$) results in the Panbio™ Dengue IgG Indirect ELISA (Fig. 4.A). Most IgG positive or negative samples were equally classified by the immunoassay developed in this work (absorbance results were normalized using two internal controls run in each plate, as described in Materials and Methods). The ROC curve derived from the normalized data displayed an AUC of 0.855 (95% CI: 0.803–0.908) indicating that the DENV-NS1 IgG iELISA has comparable performance to the Panbio™ assay for detecting past DENV infection (Fig. 4.B). In fact, using a decision threshold of 20.1 R.I. (that produced the highest Youden's Index value) as the assay cut-off, comparative sensitivity and specificity were 81.1% (95% CI: 73.6–87.7%) and 83.0% (95% CI: 75.5–89.6%), respectively. Concordance between the test developed in this study and the commercial Panbio™ ELISA was moderate to good with a Cohen's kappa index (κ) of 0.642 (95% CI: 0.538–0.745). Results obtained and estimated performance parameters are summarized in Table 2.

3.4. Anti-NS1 IgG antibody levels in sera from symptomatic individuals

The DENV-NS1 IgG iELISA was also employed to determine the NS1 specific antibody levels in sera from symptomatic individuals from an endemic area. As referred in Materials and Methods, four categories were established according to previous results in either RT-PCR or IgM Capture ELISA and collection date: acute (n = 24); early convalescent (n = 14), convalescent (n = 10) and not detected ($n = 27$) dengue.

The results obtained with the DENV-NS1 IgG iELISA for samples in each category are shown in Fig. 5. Positive results (R.I. > 20.1) were found in 51.8% of samples in the not detected dengue category, probably reflecting past exposure to the virus. In the dengue acute category, 54.2% of the sera exhibited IgG anti-NS1 antibodies, also indicating previous DENV infection (samples in this category were collected up to 4 days after symptoms onset), and therefore, a current secondary infection. Even though the positivity rate was similar between both categories, positive acute samples had only moderate R.I. values whereas many samples in the not detected category showed high R.I. values. This suggests that high anti-NS1 antibody levels may correlate with protection against disease. Sera in the early convalescent dengue category registered 64.3% positivity rate. On the other hand, the convalescent dengue category presented 90.0% positivity and statistically higher R.I. values than the acute and early-convalescent dengue categories (Kruskal-Wallis test followed by Dunn's *post hoc* test, $p < 0.05$), as expected considering that IgG antibodies are elicited after the acute phase of the disease. These findings pointed to differences in the distribution of anti-NS1 IgG antibody levels between acutely infected and convalescent patients in endemic regions.

4. Discussion and conclusions

The main obstacle in manufacturing low-cost dengue serological tests is the difficulty of producing viral antigens on a large scale. Several studies have reported the recombinant production of the NS1 employing prokaryotic expression systems (Das et al., 2009; Amorim et al., 2010; Allonso et al., 2011). Such approaches resulted in insoluble or aggregated products that require a refolding process to be immunologically active. Bacteria-derived NS1 lacks glycosylation and therefore does not exhibit complete functional, structural, and antigenic properties found in the native protein. This drawback of the prokaryotic systems led to attempts to express NS1 in yeast (Zhou et al., 2006; Allonso et al., 2019; Xisto et al., 2020a), insect (Rozen-Gagnon et al., 2012), plant (Marques

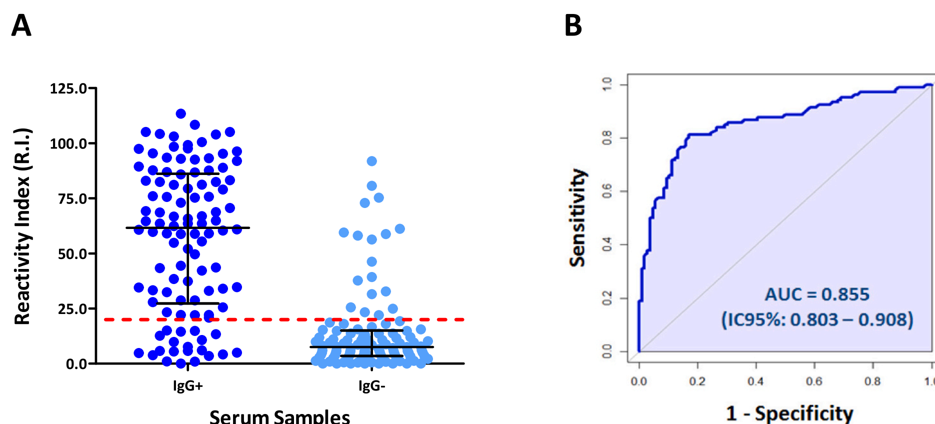


Fig. 4. Performance evaluation of DENV-NS1 IgG iELISA for detecting past DENV infection. A) Normalized reactivity values registered for DENV IgG positive (IgG+) and negative (IgG-) sera. Reference serostatus of samples was assigned by the Panbio™ Dengue IgG Indirect ELISA. The solid line and bars show the median and interquartile range of reactivity indexes (R.I.). The dashed line indicates the chosen cut-off value. B) ROC curve.

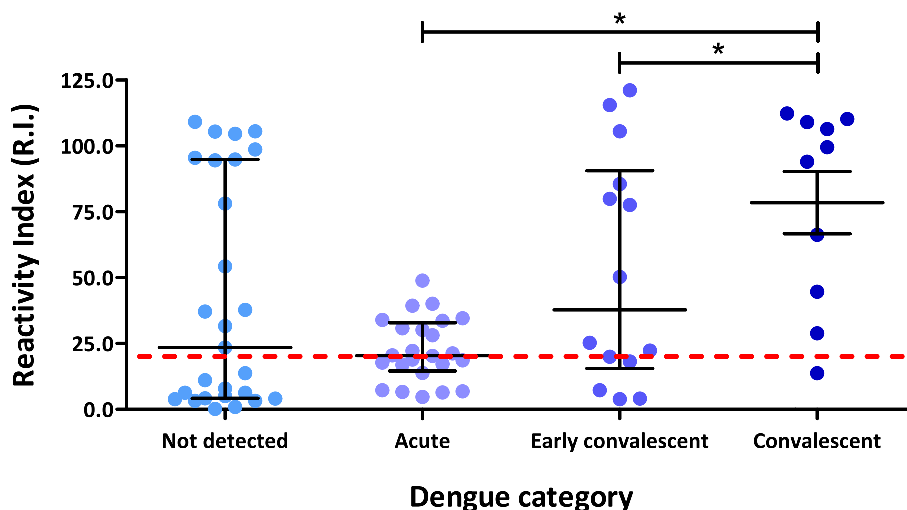


Fig. 5. Analysis of anti-NS1 IgG antibody levels in sera from symptomatic individuals. Comparison of reactivity indexes (R.I.) among categories was performed with the Kruskal-Wallis test followed by Dunn's *post hoc* test (*$p < 0.05$). The solid line and bars show the median and interquartile range of R.I. in each sera category. The dashed line indicates the cut-off value established for DENV-NS1 IgG iELISA (R.I. = 20.1).

et al., 2020; Xisto et al., 2020b), or mammalian cells (Mora-Cárdenas et al., 2020; Noisakran et al., 2007; Chao et al., 2015; Galula et al., 2021).

In this work, a recombinant antigen corresponding to the NS1 of dengue virus serotype 1 was obtained by transient transfection of HEK293T cells followed by IMAC purification. The purified protein was correctly folded forming dimers and was detected with a mAb specifically directed against the DENV1 NS1. The methodology presented herein allows to achieve quite good yields of purified NS1 (1.5 mg/L), rendering similar levels to those previously reported in *Pichia pastoris* (1–5 mg/L) (Allonso et al., 2019; Xisto et al., 2020a) and baculovirus (1.6 mg/L) (Rozen-Gagnon et al., 2012) expression systems, at a cost per milligram of antigen substantially lower than purchasing an equal quantity of commercially available recombinant NS1, expressed in insect or mammalian cells. In fact, it represents about one-third of the price of recombinant NS1 antigens marketed by the Native Antigen Company (Oxfordshire, UK), commonly used in basic and applied research projects.

Most research has focused on the application of immunoassays for the diagnosis of acute dengue, and thus little information is available about their performance in serological screenings of healthy population. Bonaparte et al. assessed commercially available ELISAs and rapid diagnostic tests (RDTs) for the determination of prior DENV infection (Bonaparte et al., 2019). High sensitivity and specificity (>90%) were reported for Panbio™ Dengue IgG Indirect ELISA and Dengue Virus IgG DxSelect™ (Focus Diagnostics, Cypress, California, USA). Dengue rapid diagnostic tests (RDTs) exhibited high specificity but lower sensitivity than ELISAs (<70%). It is relevant to point out that these sensitivity estimations were biased towards performance in individuals with documented past symptomatic DENV infection whereas, in an endemic area, it is expected that most seropositive individuals have experienced undocumented asymptomatic infection. It can be calculated from the published data that sensitivities of both ELISAs decline below 75% if only samples without documented past infection are considered (Table 3), as those included in the current study. This might be due to lower levels of DENV specific antibodies after asymptomatic infections. A similar behavior was observed for Dengue IgG ELISA (SciMedx Corporation, Denville, New Jersey, USA) (Bonaparte et al., 2020) (Table 3). According to manufacturers, Panbio™ and DxSelect™ indirect ELISAs exhibit 62.0% (95% CI: 51.8–71.5%) and 39.4% (95% CI: 22.7–57.9%) sensitivity in detecting seropositive individuals from endemic areas, respectively. Another study registered low sensitivity of Panbio™ Dengue IgG Indirect ELISA compared to focus reduction neutralization test (FRNT), reaching a value of 77.1% (95% CI: 72.2–81.9), in healthy children with monotypic dengue profile (Lopez et al., 2021). Furthermore, all aforementioned ELISAs, based on the detection of antibodies against structural proteins, showed considerable extent of cross-reactivity with JEV, WNV, YFV and ZIKV seropositive sera (Schwartz et al., 2000; Bonaparte et al., 2019; Bonaparte et al., 2020). This could be partly explained considering that these kits are based on detection of antibodies against the structural proteins of DENV (either recombinant proteins or purified whole virus particle), which show an important degree of conservation with those of other flaviviruses.

Table 3
Sensitivity and specificity of commercial ELISAs to detect prior DENV infection.

Test	Sensitivity (95% CI)	Specificity (95% CI)	Reference test
Panbio™ Dengue IgG Indirect ELISA	90.0% (85.8–93.3)[a] 71.1% (60.6–80.2)[b]	99.6% (98.7–100.0)	PRNT
Dengue Virus IgG DxSelect™	90.7% (86.6–93.9)[a] 73.3% (63.0–82.1)[b]	94.6% (92.3–96.3)	PRNT
SciMedx Dengue IgG ELISA	97.8% (94.4–99.4)[c] 76.0% (69.5–81.7)[b]	100.0% (98.9–100.0)	PRNT

PRNT: plaque reduction neutralization test.
Performance data derived from previous studies (Bonaparte et al., 2019; Bonaparte et al., 2020).
[a] Overall sensitivity.
[b] Sensitivity in samples with undocumented past DENV infection.
[c] Sensitivity in samples with symptomatic past DENV infection.

An indirect ELISA based on the recombinant NS1 antigen obtained in this work proved suitable for the detection of IgG antibodies elicited after DENV infection. Comparative performance of the DENV-NS1 IgG iELISA with respect to Panbio™ Dengue IgG Indirect ELISA to detect past DENV exposure was satisfactory, rendering 81.1% sensitivity and 83.0% specificity. These values should not be considered absolute parameters since the assay used for comparison is not a gold standard, such as the plaque reduction neutralization test (PRNT). Considering that the samples analyzed in this work come from a dengue endemic region where a substantial proportion of the population has been vaccinated against YFV (Shearer et al., 2017) together with the low sensitivity and

specificity of the Panbio™ test, particularly due to cross-reactivity to other flaviviruses, it is highly probable that performance of the developed assay has been underestimated.

Antibodies targeting NS1 are highly cross-reactive within the DENV serocomplex but poorly cross-reactive to other closely related flavivirus (Shu et al., 2002; Nascimento et al., 2018a; Mora-Cárdenas et al., 2020; Chao et al., 2015; Nascimento et al., 2018b; Tyson et al., 2019a). This property is of great relevance for the establishment of tests that can be used for surveillance of DENV prevalence or pre-vaccination screening, mainly in regions with circulation of multiple flavivirus or vaccination against JEV or YFV. It also allows for differentiation of individuals that were naturally exposed to DENV from those that were vaccinated with CYD-TDV vaccine. Well-characterized ZIKV positive/DENV negative sera will be needed to further analyze the putative cross-reactivity of the DENV-NS1 IgG iELISA.

There are currently no commercially available tests for the detection of NS1 specific antibodies, but several in-house methods have been developed (Table 4). IgG indirect or capture ELISAs based on the recombinant NS1 have proven more sensitive than commercial tests in individuals with undocumented past DENV exposure.

In endemic regions, the presence of IgG antibodies against DENV in sera from symptomatic individuals before day 9 of illness is indicative of past infections (Shu et al., 2003). Therefore, the DENV-NS1 IgG iELISA could be used together with RT-PCR or NS1 antigen detection assays to differentiate primary from secondary infection in acutely infected individuals. This information would be valuable for patient management. Conversely, IgG positive results in convalescent symptomatic individuals do not allow distinguishing between current or past infection. The differences in serum anti-NS1 IgG levels observed in this study between acute and convalescent symptomatic individuals suggest that testing paired-samples with this assay could be an alternative to confirm or discard current infections.

Previous studies demonstrated that antibodies against NS1 can provide protection in mouse models through complement-dependent cytotoxicity of infected cells and blocking NS1-induced toxicity, without inducing ADE (Costa et al., 2006; Wan et al., 2014; Beatty et al., 2015; Lai et al., 2017; Wan et al., 2017). Interestingly, higher R.I. values were observed for anti-NS1 IgG positive sera in the not detected and convalescent dengue categories when compared with the R.I. values of anti-NS1 IgG positive sera in the acute category. This observation supports the hypothesis that high levels of NS1 specific antibodies could prevent disease outcome in humans.

In summary, the present work reports the affordable production of the recombinant NS1 antigen in human cells, showing adequate purity, and its implementation into an indirect ELISA for the detection of IgG antibodies with putative application in epidemiological surveillance, pre-vaccination screening, and eventually as a complementary diagnostic test. Further studies using sera with different flavivirus infection/vaccination status should be carried out to attain thorough validation and to establish the scope of the assay. Additionally, the recombinant NS1 could be used for the development and characterization of monoclonal antibodies or single domain antibodies with potential diagnostic or therapeutic applications, as well as for functional protein studies.

CRediT authorship contribution statement

Cristian Miguel Malnero: Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Renata Campos Azevedo:** Investigation, Resources, Writing – review & editing. **Ingrid Evelyn Bergmann:** Conceptualization, Writing – review & editing. **Marcelo Damião Ferreira de Meneses:** Data curation, Writing – review & editing. **Andrea Cony Cavalcanti:** Resources, Data curation, Writing – review & editing. **Lorena Itatí Ibañez:** Investigation, Conceptualization, Resources, Writing – review & editing. **Viviana Malirat:** Conceptualization, Supervision, Project administration, Writing – review & editing.

Table 4
Performance of NS1-based immunoassays in determining DENV serostatus.

Assay	NS1 Antigen	Sensitivity (95% CI)	Specificity (95% CI)	Reference test
Indirect IgG ELISA (Nascimento et al., 2018b)	DENV1–4*	91.9 (89.4–93.9)[a]	95.1 (93.2–96.7)	PRNT
Indirect IgG ELISA (Tyson et al., 2019a)	DENV1*	94.5 (89.8–96.9)[b]	91.9 (87.1–94.4)	RT-PCR
	DENV1–4*	95.6 (91.4–97.8)[b]	89.5 (84.1–92.3)	
IgG MIA (Tyson et al., 2019b)	DENV1*	89.7 (83.3–92.9)[b]	99.1 (97.2–100.0)	RT-PCR
	DENV1–4*	94.3 (89.4–96.8)[b]	97.2 (94.1–98.8)	
Indirect IgG ELISA (Galula et al., 2021)	DENV1–4	91.7 (81.6–97.2)[c]	82.4 (69.1–91.6)	FRNT
GAC-ELISA (Galula et al., 2021)	DENV2 + 3	95.0 (86.1–98.1)[c]	98.0 (89.6–100.0)	FRNT

PRNT: plaque reduction neutralization test.
RT-PCR: reverse transcription-polymerase chain reaction.
FRNT: focus reduction neutralization test.
* Purchased from the Native Antigen Company (Oxfordshire, UK).
[a] Overall sensitivity.
[b] Sensitivity in samples with symptomatic past DENV infection.
[c] Sensitivity in samples with undocumented past DENV infection.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2023.113503

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